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INTERNATIONAL CENTRE FOR GENETIC ENGINEERING AND BIOTECHNOLOGY

ACTIVITY REPORT - 1993

INTERNATIONAL CENTRE FOR GENETIC ENGINEERING AND BIOTECHNOLOGY

ACTIVITY REPORT 1993

under the aegis of the
United Nations Industrial Development Organization (UNIDO)

That this will be the last Activity Report of the International Centre for Genetic Engineering and Biotechnology (ICGEB) with a preface by the Director General of UNIDO is a source of some satisfaction — for the Centre, for UNIDO and for me personally. The Centre, promoted and nurtured by UNIDO since the early 1980s, came of age in February 1994 when the 24th State notified entry into force of the Statutes. Today the terms genetic engineering and biotechnology are household words. The multidisciplinary fields they refer to will remain among the frontier technologies of the 1990s and beyond. The Centre's autonomy is a tribute to all those who have dedicated themselves over the last seven years to the UNIDO project that furthered the objectives for which the ICGEB was established. No one should doubt that the goals of creating an instrument for strengthening developing countries through research, development and training in this key feature oriented field have been realized in a large measure.

Looking back on the emergence of ICGEB, it was UNIDO which initiated in 1981, under the leadership of Dr. Abdel Rahman Khane, the concept of an international centre in genetic engineering and biotechnology. Events moved quickly thereafter and one must compliment the decision of the representatives of 28 nations who favoured the establishment of the Centre at their meeting in Belgrade in 1982. The Statutes of the Centre were adopted by the Plenipotentiary Meeting in Madrid the following year. Thanks to the generous contributions by the Governments of Italy and India, the interim operations of the Centre started within the next three years.

The Chairman of the Preparatory Committee for the Establishment of ICGEB, Ambassador Adolfo R. Taylhardat, should be particularly complimented for his sustained efforts during the preparatory years in successfully steering the Centre towards autonomy. The UNIDO Secretariat also devoted considerable time and effort to the promotion of ICGEB and is proud to see its autonomy achieved.

The 1993 Activity Report shows how the main research programmes relevant to developing countries have been actively pursued during the last six years at the two Components of the Centre. Significant progress has been achieved in research towards development of stress resistant food crops, diagnostics against AIDS, hepatitis and malaria, in understanding the molecular biology of Papilloma virus and its relation to cervical cancer, and in microbial degradation of lignin to useful products. The Centre has trained over 1,200 scientists from some 35 developing countries during this period. Excellent research publications have appeared in peer reviewed journals.

There are promising prospects of commercializing the research results. Industrial firms are already taking advantage of ICGEB's expertise through contracts with the Centre to develop products or technology acquired through licences. The ICGEB's Director, Prof. Arturo Falaschi, can indeed take pride in the Centre's achievements. Thanks are due also to the distinguished Panel of Scientific Advisors for advising the Director and the Heads of Components in maintaining a high level of scientific excellence at the Centre.

With 47 signatories to the Statutes, 29 of which have ratified them, ICGEB is attracting increasing international attention. A number of other countries are considering joining. The Rockefeller Foundation and the World Health Organization awarded grants to the Centre for specific projects. UNEP uses the Centre's expertise in biosafety training programmes. ICGEB expertise was also evident in the Preparatory Committee meetings of the United Nations Conference for Environment and Development (UNCED) of the Biodiversity Convention of UNEP and at the Third Review Conference on Biological and Toxin Weapons Convention.

Biotechnology is one of the key technologies that contribute to environmentally sustainable industrial development. With its programmes in strengthening human resources development of developing countries in this area, ICGEB complements with the new priorities of the restructured UNIDO. I am therefore confident that the close links which existed between the Organization and the Centre will be maintained during the transitional period and thereafter and further strengthened. This is also in line with UNIDO's own work as the Task Force Manager, designated by the Interagency Commission for Sustainable Development in Biotechnology to help implement Agenda 21 decisions. UNIDO is part of the UNIDO UNEP WHO FAO Working Group which developed a Voluntary Code of Conduct for Release of Genetically Modified Organisms. It has also established a Biosafety Information Network and Advisory Service (BINAS) to advise developing countries on biosafety issues. Hopefully, ICGEB will continue to cooperate with UNIDO in these and other activities.

UNIDO wishes ICGEB every success in its future endeavours.

Mauricio de Maria y Campos
Director-General of UNIDO

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Introduction

The year 1993 can be seen, fundamentally, as the time when ICGEB gained its status as a fully autonomous international organization. There is no doubt that, although the official calendar date of the entry into force of the Statutes is 3 February 1994, ICGEB's autonomy was the result of the important work which was realized during 1993. Without the solid base of the research activities carried out by the Centre, the international recognition necessary for its establishment as an autonomous entity could not have been attained. Furthermore, significant undertakings which included the establishing and nurturing of contacts with Member Countries gained ICGEB the number of notifications (deposited with the Secretary General of the United Nations) required for the entry into force of the Statutes.

Moreover, 1993 was the year in which the permanent premises of the Trieste Component were completed and during which the scientific groups started to relocate to the new, state-of-the-art "W" Building. At present, the Component and Directorate occupy 5,700 square metres of laboratory and office space and during the course of 1995, when the AREA Science Park — the authority that administers the science park where ICGEB Trieste is located — has completed the restructuring of the temporarily released "F1" Building, they will occupy a total area measuring 7,000 square metres.

The construction of the permanent premises of the New Delhi Component (measuring more than 10,000 square metres of laboratory, greenhouse, animal house and office space) advanced considerably during 1993 and it is expected that the premises will be completed by the middle of 1994.

The training and research activities carried out in both Components continued to expand in 1993, though they will stabilize during 1994 in accordance with the current availability of funds.

Positive research results of various scientific projects implemented in the ICGEB laboratories are now starting to emerge, and patents were filed during 1993 by UNIDO, on behalf of ICGEB. Research being carried out at the Affiliated Centres in the framework of the Collaborative Research Programme is also leading to very interesting results; as the number of projects continues to increase, once again, an entire section in this Activity Report has been dedicated to the findings of these research projects.

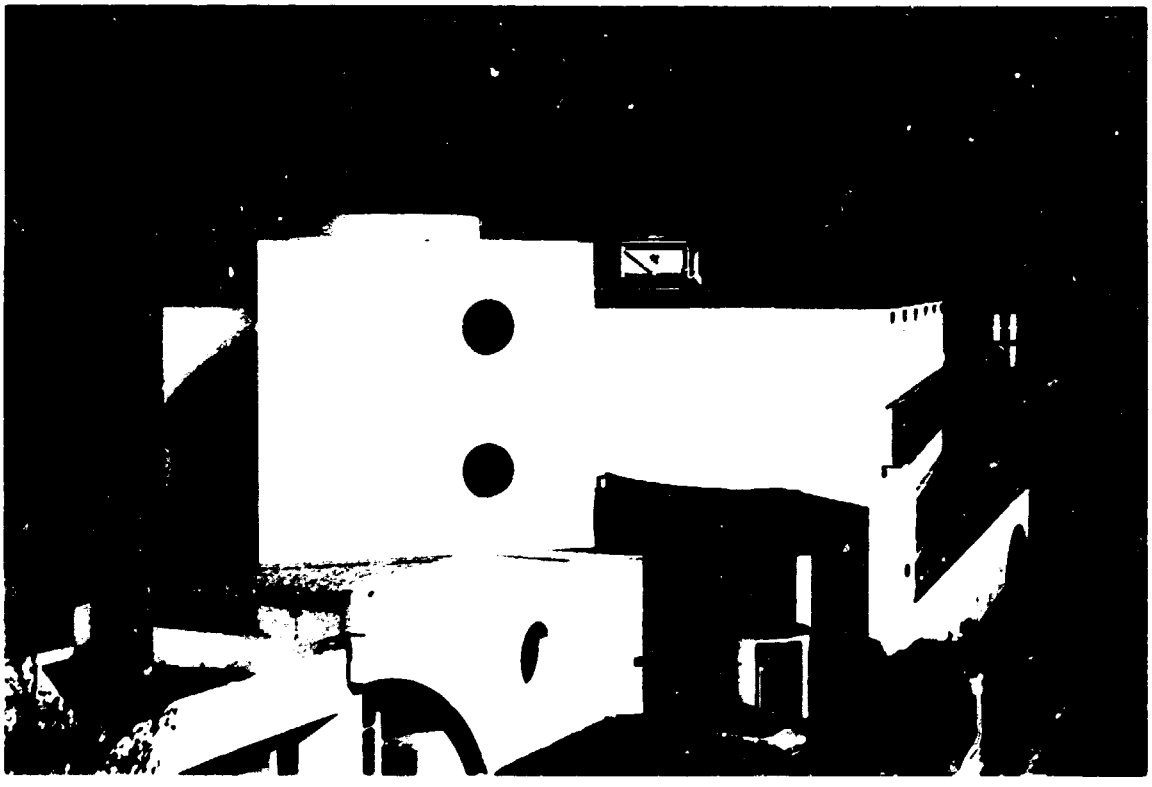
Other scientific services offered by ICGEB to its Member Countries remain operational and it is with great pleasure that we witness the success of the most popular ICGEBnet: the ICGEB computer resource for molecular biology. The number of individuals and/or laboratories with user accounts on the mainframe computer in Trieste (which provides free access to scientific data bases and to the software necessary for their utilization) has increased from 40 in 1990 to over 600 in 1993.

The information contained in this Activity Report shows, without any doubt, that ICGEB is becoming a mature, viable and internationally recognized organization. The entry into force of the Statutes is a tangible sign of this recognition. Further acknowledgement is expected in the future, together with the welcome expansion of the ICGEB constituency to still more members - countries which will require the services of the Centre or countries which believe in its mandate and its ideals.

The ICGEB Directorate

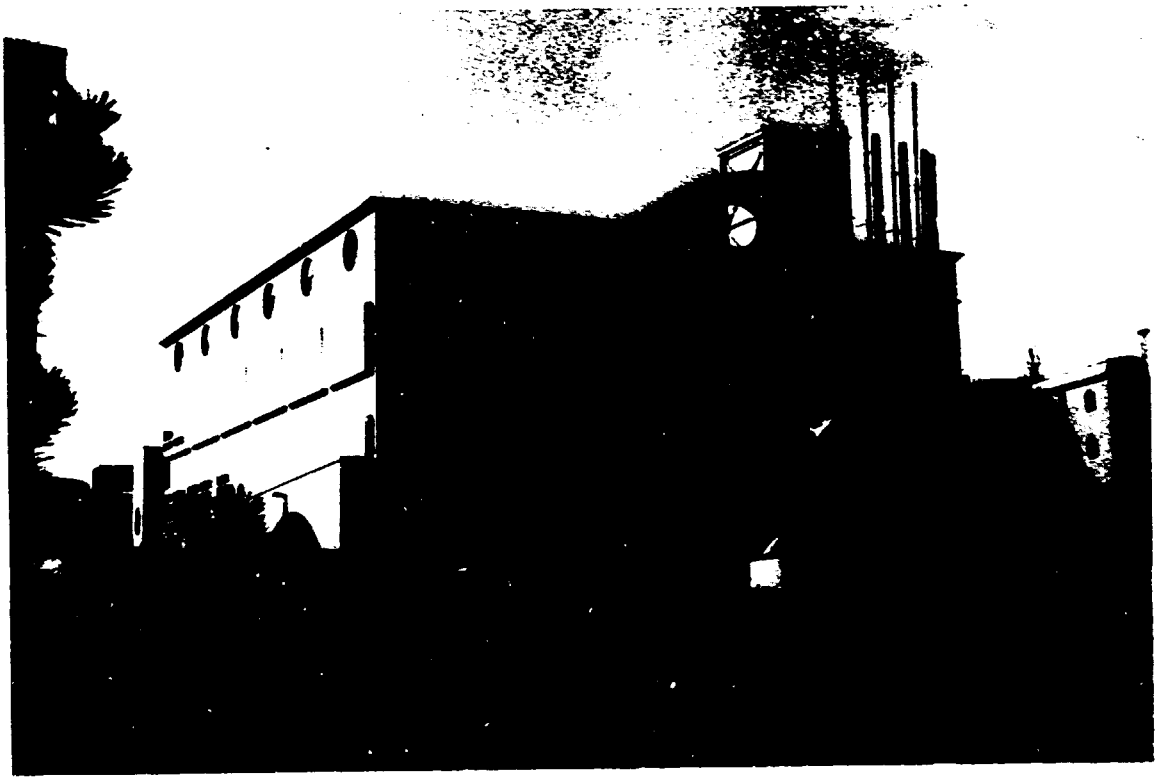
Director	*Arturo FALASCHI*
Personal Assistant to the Director	*Elena STUBEL*
Programme and Administrative Coordinator	*Decio RIPANDELLI**
Junior Administrative Officer	*Sanjay PAHWA**
Secretary to the Progr. and Admin. Coord.	*Suzanne KERBAVCIC**
Science Coordinator (Vienna Office)	*George TZOTZOS*
Programme and Training Unit	
Programme Assistant (Fellowships)	*Susan VINCENT*
Programme Assistant (Meetings/Courses)	*Diana VITI VANDEYAR*
Programme Assistant (CRPs)	*Loredana LEDOVICH EZE*
Personnel Unit	
Administrative Assistant (Personnel)	*Françoise MISITI**
Finance and Accounts Unit	
Senior Accountant	*Giulia BON CASSELEK**
Accountant	*Elisabetta LIPPOLIS CALCI**

*Servicing also the Trieste Component, **Left ICGEB during 1993
Names in *italic* correspond to ICGEB appointments



The main entrance of the permanent premises of the Trieste Component, the recently completed "W" Building located at the AREA Science Park of Trieste.

The Trieste Component



The north-eastern face of the ICGEB Trieste permanent premises.

Head of Component	*Francisco BARALLE*
Personal Assistant to the Head of Component	*Ann CRUM*
Secretary to the Head of Component	*Mariella SANTORO*
General Services	
Librarian	*Eleonora MILLO*
Storekeeper	*Giorgio PATTAVINA*
Driver	*Carlo FRATNIK*
Receptionist/Switchboard Operator	*Ivana BOCCI*
Building Management and Safety Services	
Safety Assistant	*Bodil HOLLE*
Precision Mechanic	*Walter SCHMITT*
Technician	*Carlo GREGORI*
Technician	*Pietro TERCON*
Technician (Animal House)	*Mauro STURNEGA*
Procurement Unit	
Responsible Officer	*Francisco BARALLE*
Purchasing Officer	*Sergio TISMINETZKY*
Purchasing Assistant	*Ann CRUM*
Administrative Assistant (Procurement)	*Grazia DENARDO SPINA*

Tissue Culture and Kitchen Services

Responsible Officer	*Georgine FAULKNER VALLE*
Technician	*Maria Elena LOPEZ*
Technician	*Miranda THOMAS*
Technician	*Sulena POLEZ*
Technician	*Mirella DELL'OSTE*
Technician	*Fabiana LEUZ*
Technician	*Nadia CENDACH*

Computer Unit

Responsible Officer	*Sándor PONGOR*
Computer System Manager	*György SIMON***
Computer Technician	*Spartaco H. BARTH*

Left to Right: Members of the Panel of Scientific Advisors: Prof. Arthur Kornberg, Prof. Pierre Chambon, Prof. Robert Haselkorn, Prof. Luigi Luca Cavalli-Sforza (Chairman), Head of Trieste Component of ICGEB: Prof. Francisco E. Baralle, Head of New Delhi Component of ICGEB: Prof. Krishna K. Tewari, Director of ICGEB: Prof. Arturo Falaschi, Programme and Administrative Coodinator: Mr. Decio Ripandelli

Research Groups

Molecular and Cellular Biology

Senior Scientist (Director ICGEB)	*Arturo FALASCHI*
Assistant Research Scientist	*Mauro GIACCA*
Assistant Research Scientist	*Narendra TUTEJA*
Scientific Assistant	*Luisa MESTRONI*
Trainee	*Dimiter DEMIROV***
Trainee	*Daniela DIMITROVA*
Trainee	*Ning Wu HUANG*
Trainee	*Poonam TANEJA*
Pre-doctoral Trainee	*Sanjeev KUMAR*
Pre-doctoral Trainee	*Doris SKOPAC*
Technician	*Maria Ines GUTIERREZ*
Technician	*Alexander OCHEM*

Guest Scientist (Univ. of Trieste, Burlo Hospital)	Lorena ZENTILIN
Consultant (AREA/ICGEB)	Daniela PEDACCHIA**
Research Fellow (Santini Hospital)	Beatrice CHIAROTTO
Research Fellow (Univ. of Trieste, Burlo Hospital)	Manola COMAR
Research Fellow (Italian Institute of Health)	Francesca DEMARCHI
Research Fellow ("Amici del Cuore" Foundation)	Maja KRAJINOVIC
Research Fellow (Univ. of Trieste, Burlo Hospital)	Daniela LEDER
Research Fellow ("Buzzati-Traverso" Found.)	Giovanni Maria SEVERINI
Graduate Student (ISAS)	Fabrizio D'ADDA DI FAGAGNA
Graduate Student (ISAS)	Silvia DIVIACCO
Graduate Student (ISAS)	Gabriele GRASSI
Graduate Student (ISAS)	Lidija MARUSIC**
Graduate Student (ISAS)	Giuseppe MARZIO
Graduate Student (ISAS)	Paolo NORIO**
Graduate Student (ISAS)	Cristina PELIZON
Graduate Student (ISAS)	Sabrina TAFURO
Undergraduate Student	Sara HRVATIC
Undergraduate Student	Francesco RUSSO

Genome Studies

Senior Scientist (Consultant)	*Claudio SCHNEIDER*
Junior Scientist (Consultant)	*Elisabetta RUARO*
Technician	*Stefano BOTTEGA*
Technician	*Stefania MARZINOTTO*
Technician	*Federico VOLPATTI*

Research Fellow (AIRC)	Claudio BRANCOLINI
Research Fellow (Univ. of Trieste)	Giannino DEL SAL
Research Fellow (CNR)	Sandro GORUPPI
Research Fellow	Dejan LAZAREVIC
Research Fellow	Paolo VATTA
Graduate Student (ISAS)	Licio COLLAVIN
Graduate Student (Univ. of Trieste)	Elsa FABRETTI
Graduate Student (ISAS)	René UTRERA
Undergraduate Student (Univ. of Trieste)	Mauro BENEDETTI
Undergraduate Student (Univ. of Milan)	Paolo MARCANDALLI

Virology

Senior Scientist	*Lawrence BANKS*
Associate Research Scientist	*Georgine FAULKNER VALLE*
Junior Scientist	*Véronique BOUVARD*
Junior Scientist	*David PIM*
Junior Scientist	*Alan STOREY*
Trainee	*Rengang WU***
Technician	*Paola MASSIMI*
Graduate Student	Antonella PICCINI
Undergraduate Student (Univ. of Trieste)	Marta MOLINARI**

Microbiology

Senior Scientist	*Carlo BRUSCHI*
Junior Scientist	*Jacques OBERTO*
Trainee	*Aleksandra COMINO*
Trainee	*Vijayalakshmi HINDUPUR NAGARAJ*
Trainee	*Binh LE THANH*
Trainee	*Goran LJUBIJANKIC*
Trainee	*Zhiping WENG***
Trainee	*Vladimir YONG GONZALEZ*
Pre-doctoral Trainee	*Solomon NWAKA***
Technician	*Maristella COGLIEVINA*
Technician	*Giuliano DEGRASSI*
Technician	*Simone UGOLINI*
Research Fellow (AREA)	Paolo ZACCARIA
Graduate Student	Iris BERTANI
Graduate Student	Giorgia Maria DANEK**
Graduate Student (AREA)	Daniela DELNERI
Graduate Student (Univ. of Torino)	Monica GOTTA**
Graduate Student	Francesca STORICI
Graduate Student (ISAS)	Anna ZAGO
Undergraduate Student (Univ. of Trieste)	Raffaella KLIMA
Student	Aleksandra JANOUSEK
Student	Daniella SANTORO

Protein Structure and Function

Senior Scientist	*Sándor PONGOR*
Associate Research Scientist	*András SIMONCSITS*
Trainee (from April 1993 Technician)	*Sotir ZAHARIEV*
Trainee	*Valeria BEVILACQUA*
Trainee	*Ivan BRUKNER*
Trainee	*Alicia CHAGOLLA LOPEZ*
Trainee	*Miklós CSERZÖ***
Trainee	*Péter FÁBIÁN*
Trainee	*Zsolt HÁTSÁGI*
Trainee	*Roberto SANCHEZ*
Trainee	*Vesna SKERL***
Trainee	*Slavoljub SUSIC*
Trainee	*Sheng Lun WANG*
Trainee	*Jianwen ZHANG*
Technician	*Corrado GUARNACCIA*
Graduate Student (ISAS)	Giorgio PERCIPALLE

Molecular Pathology

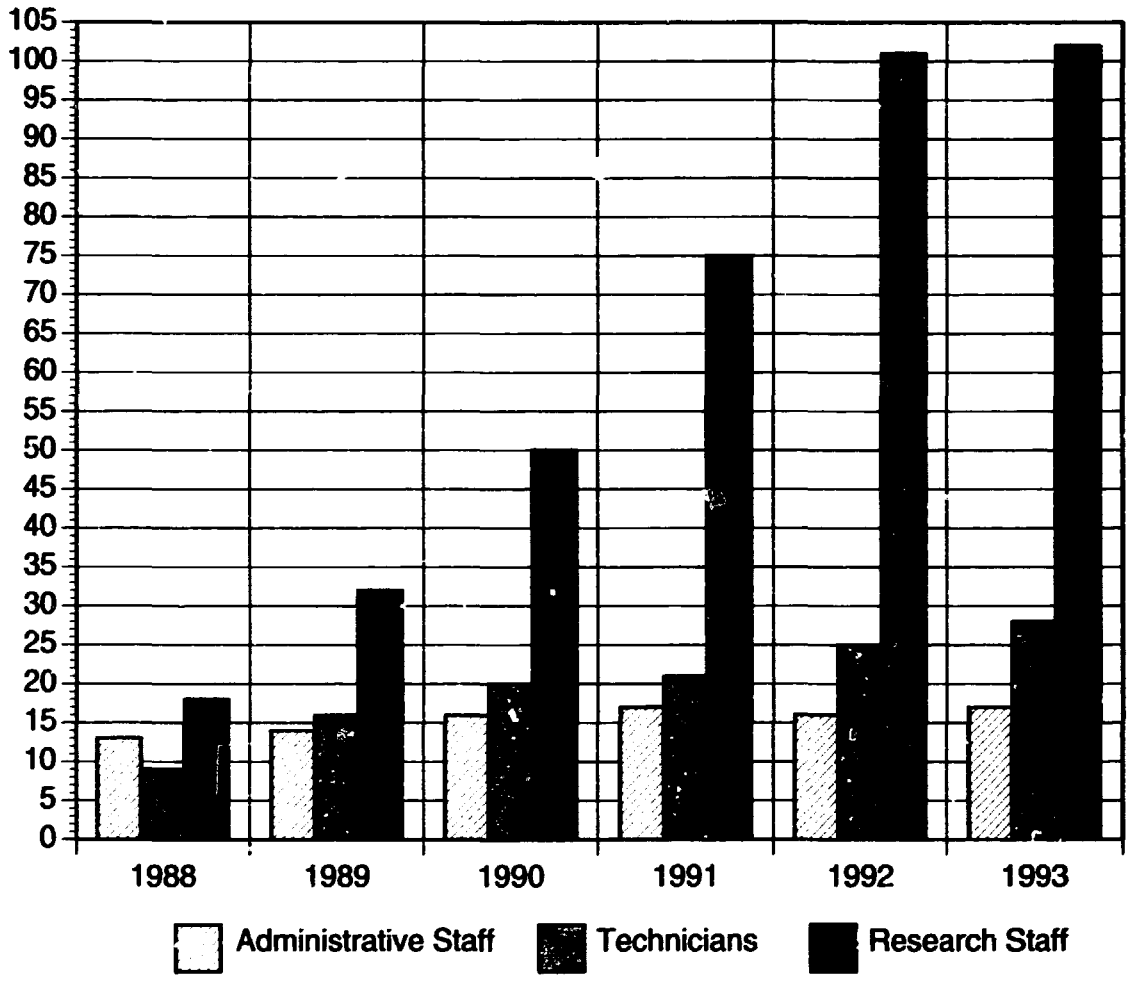
Senior Scientist (Head of Component)	*Francisco BARALLE*
Associate Research Scientist	*Eduardo SCODELLER*
Junior Scientist	*Rodolfo GARCIA*
Junior Scientist	*Sergio TISMINETZKY*
Junior Scientist	*Renu TUTEJA*
Trainee	*John AGUIYI***
Trainee	*Yuanding CHEN*
Trainee	*Andres Fernando MURO*
Trainee	*Monica SCHIAPPACASSI*
Trainee	*Violeta STOYANOVA*
Trainee	*Ali Fazil YENIDUNYA***
Pre-doctoral Trainee	*Rajalakshmi PARIYARATHUPARAMBIL*
Technician	*Giulia DEVESCOVI*
Technician	*Alessandra IACONCIG*
Technician	*Fabiola PORRO*
Guest Scientist (Univ. of Trieste, Burlo Hospital)	Donatella KOBAL
Guest Scientist (Univ. of Trieste, Burlo Hospital)	Cristina SERRA
Technician (CNR)	Bianca Rosa GUERRA
Visiting Scientist	Mathilde Adriana BONDER
Research Fellow (Univ. of Trieste)	Marco BARALLE
Research Fellow (Univ. of Padova)	Martina GEROTTO
Research Fellow (Univ. of Trieste)	Maurizio ROMANO
Research Fellow (Univ. of Trieste)	Marina VALENTI
Graduate Student	Emanuele BURATTI
Graduate Student (ISAS)	Massimo CAPUTI
Undergraduate Student (Univ. of Trieste)	Elena DONADEL
Undergraduate Student (Univ. of Trieste)	Elisabetta FALASCA
Undergraduate Student (Univ. of Trieste)	Valentina POLONIO

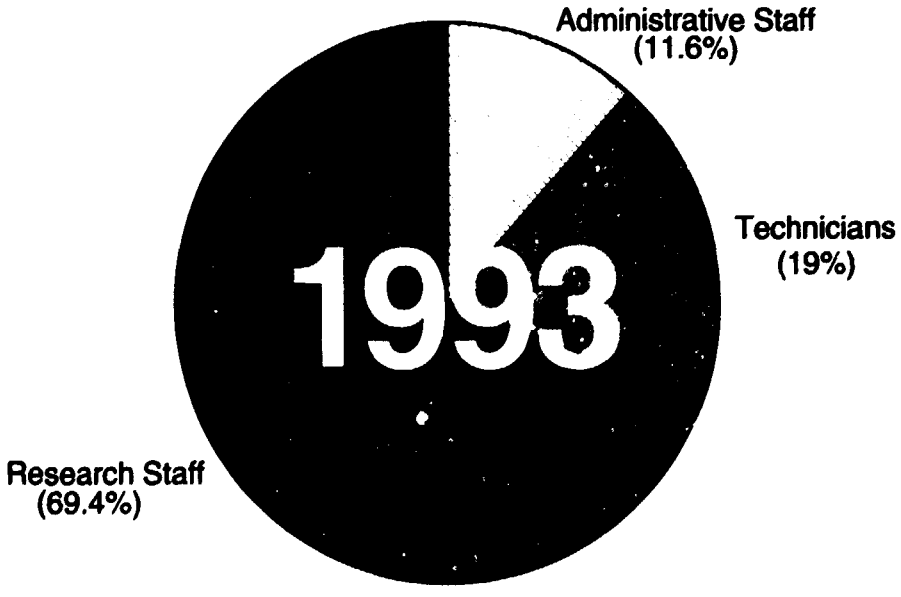
Molecular Immunology

Senior Scientist	*Oscar BURRONE*
Junior Scientist	*Marco BESTAGNO*
Trainee	*Dimitar EFREMOV*
Trainee	*Susana GIAMBIAGI*
Trainee	*Ileana GONZALEZ-RODRIGUEZ***
Trainee	*Erqiu LI*
Trainee	*Alicia PEDRAZA*
Trainee	*Mao Sheng SUN*
Trainee	*Tatiana TKACH*
Pre-doctoral Trainee	*Ivka AFRIKANOVA*
Technician	*Sabrina MANCARDI*
Research Fellow (from July 1993 Trainee)	Facundo BATISTA
Research Fellow (Univ. of Trieste)	Sabrina PERISSUTI

ON DUTY UNTIL DECEMBER 1993
Research Staff: 102
Laboratory Technicians: 28
Administrative Staff: 17
TOTAL: 147

Staff and Guests Located at the ICGEB Directorate and the Trieste Component





The New Delhi Component

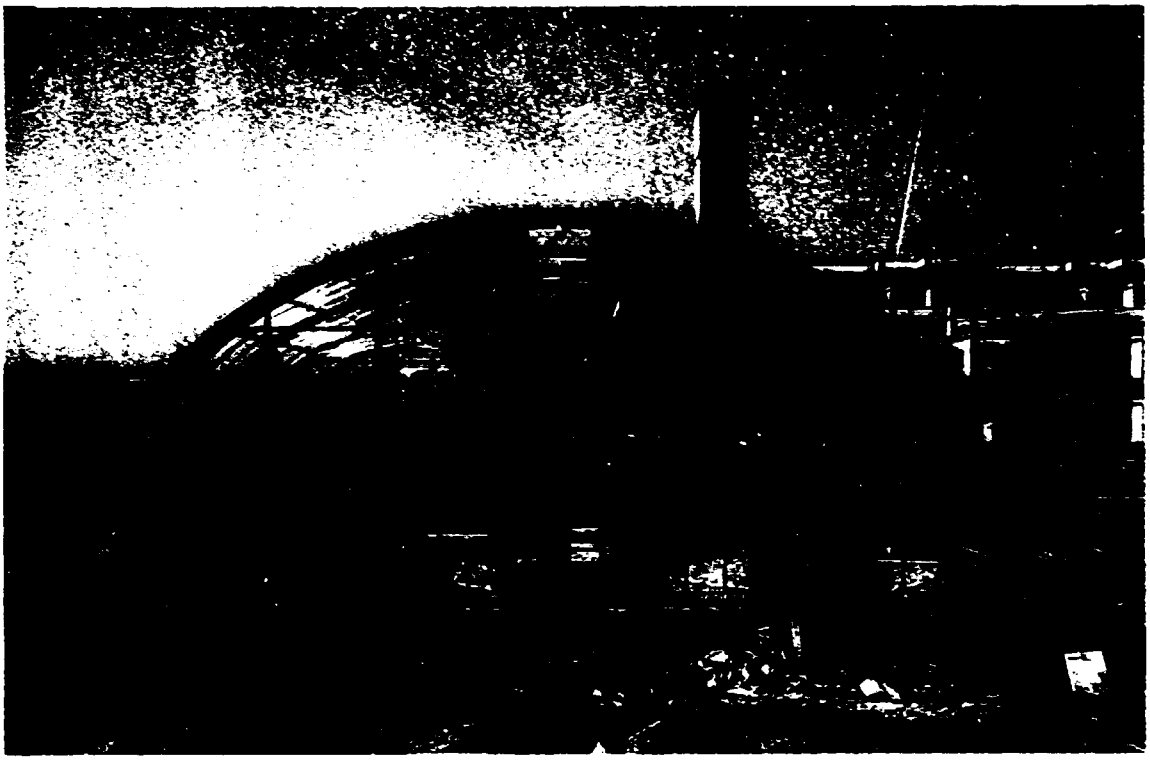
Head of Component	*Krishna Kumar TEWARI*
Executive Secretary	*Gita SRINIVASAN*
Administration	
Administrative Officer	*Gurudas CHATTERJEE*
Secretary to the Administrative Officer	*Tina DIAS*
Research Management Officer	*Yashpal Roy SAXENA*
Maintenance Officer	*Alok Kumar SHARMA*
Procurement	
Purchase Officer	*Vikas DWIVEDI*
Procurement	*Ashok KUMAR*
General Services	
Secretary	*Ramchandra RADHA*
Typist	*Pratibha CHATURVEDI*
Senior Driver	*Madan Lal YADAV*
Plumber	*Umaid Singh RANA*
Electrician	*Bhagwan DAS*
Messenger	*Ramakant PAL*

** Left ICGEB during 1993
Names in *italic* correspond to ICGEB appointments

View of the almost completed permanent premises of the New Delhi Component located at the Jawaharlal Nehru University Campus in New Delhi.

Research Groups

Mammalian Biology: Virology	Assistant Scientist	*Shahid JAMEEL*
	Assistant Scientist	*Kanury V.S. RAO*
	Research Scientist	*Vijay KUMAR*
	Senior Research Fellow	*Vankatasamy MANIVEL*
	Trainee	*Hakan OZDENER*
	Trainee	*Juan ROCA*
	Trainee	*Kezban Tuly YALCINKAYA***
	Research Associate	*Masood AHMAD*
	Laboratory Technician	*Lalita VIJAYAKRISHNAN*
	Laboratory Technician	*Mohammad ZAFRULLAH*
	Laboratory Technical Assistant	*Ganesan BALASUNDARAM*
	Laboratory Technical Assistant	*Ravinder KUMAR*
Mammalian Biology: Malaria	Assistant Scientist	*Virander Singh CHAUHAN*
	Assistant Scientist	*Fred Alexander S. KIRONDE*
	Research Scientist	*Pawan SHARMA*
	Research Fellow	*Aruna SETH*
	Research Fellow	*Pratima RAY*
	Trainee	*Cemil CELIK***
	Trainee	Fakhreidin OMER
	Laboratory Technician	*Ashima BHARADWAJ*
	Laboratory Technician	*Balwan SINGH*
	Laboratory Technician	*Archana MATHUR*
	Laboratory Technician	*Sailaja Naga VEERANKI*
	Laboratory Technician	*Tapas MAL*
	Laboratory Technician	*Vidhu BANSAL***
	Laboratory Technician	*Komal KAPUR **
	Laboratory Technical Assistant	*Naresh SAHOO*
	Laboratory Attendant	*Narinder SINGH*
	Laboratory Attendant	*Ashok DAS*
Mammalian Biology: Recombinant Gene Products	Senior Scientist	*Qi Song WANG***
	Assistant Scientist	*Navin KHANNA*
	Senior Research Fellow	*Dinkar SAHAL*
	Research Fellow	*Narayana JAYSURYAN*
	Trainee	*Yong Jie MIN***
	Trainee	*Hanying YUAN*
	Laboratory Technician	*Manisha MALHOTRA*
	Laboratory Technician	*Ganesh RAO*
	Laboratory Attendant	*Joginder SINGH*
Plant Biology: Chloroplast Genome	Senior Scientist (Head of Component)	*Krishna Kumar TEWARI*
	Assistant Scientist	*Sunil MUKHERJEE*
	Senior Research Fellow	*Sujata LAKHANI*
	Research Fellow	*M.K. REDDY*
	Research Fellow	*Nyaya Swaroop KELKAR*
	Laboratory Technician	*Dhirendra KUMAR*
	Laboratory Attendant	*Kedar SINGH*
	Laboratory Attendant	*Chandan SINGH*

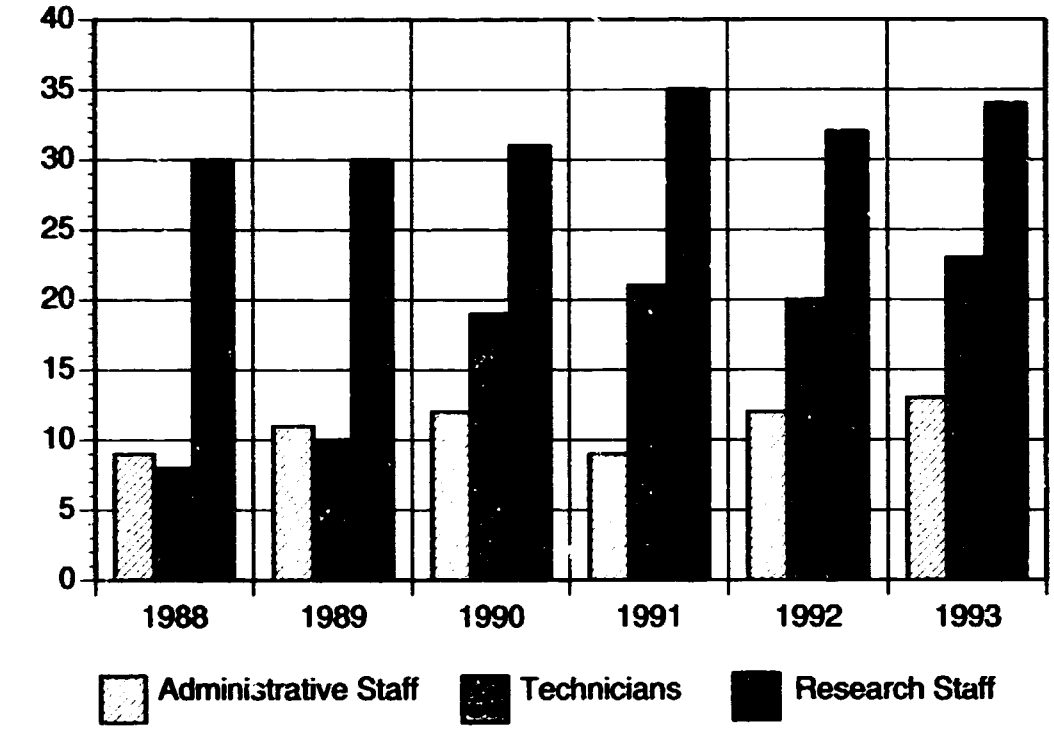


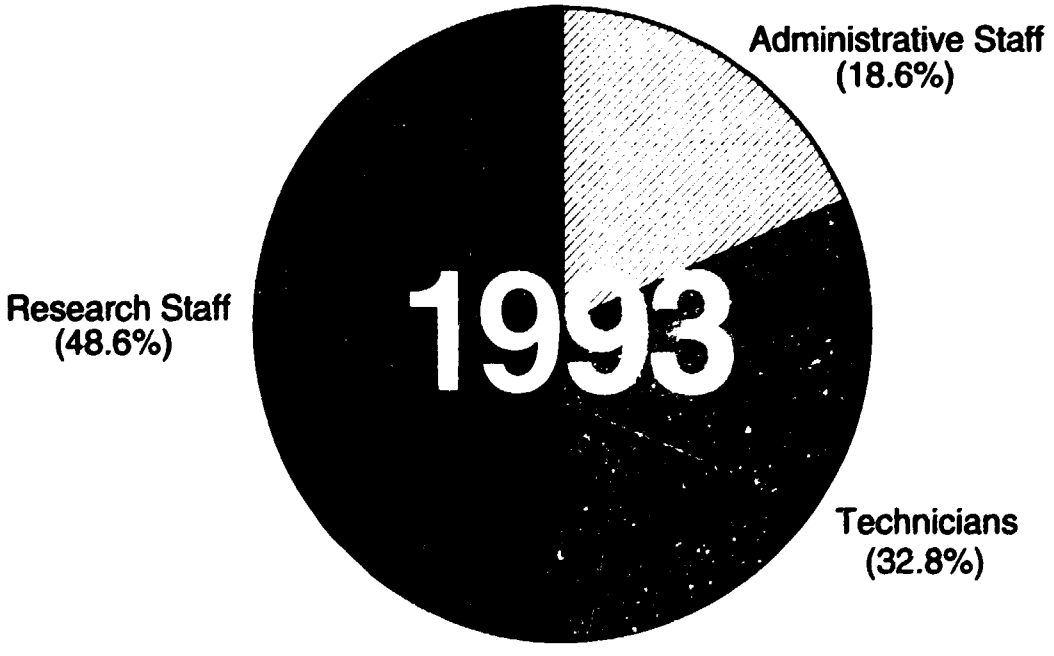
A view of the ICGEB New Delhi greenhouse facility in an advanced stage of construction.

Plant Biology: Plant Resistance	Research Scientist	*Raj K. BHATNAGAR*
	Research Scientist	*Suhail AHMAD*
	Senior Research Fellow	*Madan MOHAN*
	Research Fellow	*Angamuthu SELVAPANDIYAN*
	Research Fellow	*Suresh NAIR*
	Trainee	*Le Thi Lan OANH*
	Trainee	*Vo Thi THU*
	Research Associate	*Nasreen EHTESHAM*
	Laboratory Technical Assistant	*Naresh ARORA*
	Laboratory Attendant	*Prakash Singh PATWAL*
	Attendant (Greenhouse)	*Ramesh SHARMA*
	Laboratory Technician	*K. MAJUNDER*
	Trainee	*Farkad A. FATTAH***
	Research Associate	*Anurag GOEL***
	Trainee	*Pedro MOLINA-GUEVARA***
Plant Biology: Plant Transformation	Senior Research Fellow	*Vanga Siva REDDY*
	Senior Research Fellow	*Sudhir JAISWAL*
	Laboratory Technician	*Sangeeta CHOUDHARY*
	Laboratory Technician	*Girish CHANDEL*

ON DUTY UNTIL DECEMBER 1993
Research Staff: 34
Laboratory Technicians: 23
Administrative Staff: 13
TOTAL: 70

Staff and Guests Located at the New Delhi Component





Research Programme: The Trieste Component

Molecular and Cellular Biology

A. Falaschi, M. Giacca, N. Tuteja, M. Comar, F. d'Adda di Fagagna, F. Demarchi, D. Demirov, D. Dimitrova, S. Diviacco, G. Grassi, M. I. Gutierrez, S. Hrvatic, N. W. Huang, M. Krajinovic, S. Kumar, D. Leder, L. Marusic, G. Marzio, L. Mestroni, P. Norio, A. Ochem, D. Pedacchia, C. Pelizon, F. Russo, G. M. Severini, D. Skopac, S. Tafuro, P. Taneja, L. Zentilin.

Molecular Characterization of Replication Origins of Mammalian DNA

The structure and organization of mammalian chromosomal replicons and the molecular mechanisms underlying the activation of new rounds of DNA replication during the S-phase are still largely unknown. In particular, the identification of mammalian DNA replication origins (i.e. the chromosomal DNA sequences which direct the initiation of DNA replication) is still an elusive problem, largely due to the lack of a suitable functional assay.

Over the last few years, we have concentrated our efforts on the identification of the start site for DNA replication in a human region mapping in the subtelomeric area of the short arm of chromosome 19 (band p13.3). This region was selected because of its property of being synthesized immediately at the beginning of the S-phase in HL-60 myeloid cells synchronized with aphidicolin. The region (which was originally cloned from a DNA library obtained from synchronized cells) contains several transcripts with a complex pattern of tissue-specific and proliferation-dependent regulation. The data obtained are consistent with two tandemly-arranged transcription units, the 3' end of one separated from the 5' end of the other by a sequence of about 600 bp containing an active promoter. The isolation and sequencing of specific cDNAs permitted the identification of two genes, encoding, respectively, for a B-type human lamin (homologous to mouse lamin B2) and for an unknown protein.

In order to precisely identify the start site of DNA replication within this region, we studied the actual movement of the replication fork in living synchronized cells. The method employed entails the purification of newly synthesized, BrdUrd-substituted DNA from cultured cells by size-fractionation on sucrose gradients and affinity chromatography purification with anti-BrdU monoclonal antibodies. The abundance of selected sequences of the lamin B2 chromosomal region within this population of short (1,000-3,000 bp), newly synthesized DNA fragments was evaluated through a newly developed technique of quantitative PCR in cells synchronized at the G1/S border. The technique entails the utilization of short DNA fragments containing the same sequence as target genomic DNA plus a 20 bp insertion in the middle as competitors in PCR experiments (competitive PCR). The competitors were obtained directly from the genomic amplification products through application of the recombinant PCR technology. With the use of 9 different competitors scattered along the chromosomal area, we could obtain evidence of a bi-directional replication start point corresponding to the region spanning the end of the lamin B2 transcript and the beginning of the other transcription unit.

The power of the technique is such that it could also be successfully applied

to non-synchronized cells, where nascent short single strands in brief pulses should be highly enriched in (or even contain exclusively) sequences corresponding to origins. In fact, the application of this technique to asynchronous HL-60 cultures confirmed enrichment in the short BrdUrd-containing DNA for the same sequences detected in synchronized cells. The data obtained allowed for the narrowing down of the site of bi-directional initiation to the 600 bp area located between the two transcripts.

The second part of the project (which is currently being actively carried out) concerns the identification and characterization of the protein-DNA interactions occurring in the origin area. For this purpose, we have started both *in vivo* and *in vitro* studies. The *in vivo* studies were performed by an *in vivo* footprinting technique based on ligation-mediated PCR. This technique entails treatment of living cells with dimethylsulfate, DNA extraction and specific breakage in correspondence of methylated guanines with piperidine (or, alternatively, DNase I treatment of isolated nuclei followed by DNA

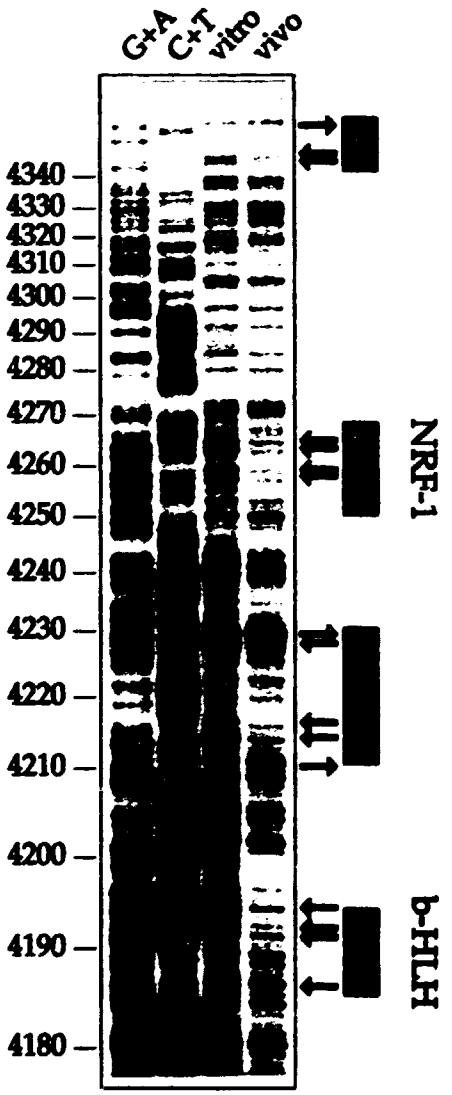


Figure 1. *In vivo* footprinting by ligation-mediated PCR of the lamin B2 replication origin region in human cells.
lane 1: G+A sequencing reaction; lane 2: C+T sequencing reaction; lane 3: *in vitro* DMS-treated naked DNA; lane 4: *in vivo* DMS-treated cell DNA. Protein binding sites are shown by rectangles on the right side: hypersensitive sites are indicated by arrows pointing to the right and protected nucleotides by arrows pointing to the left.
The areas corresponding to the sites recognized by b-HLH proteins and by transcription factor NRF1 are indicated.

extraction and denaturation), primer extension with an oligonucleotide specific for the region of interest, ligation of a linker to the blunt-ended fragments generated, and, finally, PCR amplification of the fragment ladders (Figure 1). The resulting patterns allow for the identification of the nucleotides specifically involved in protein interactions. With this technique, we have identified several protein binding sites occurring at the origin area. These sites include a target sequence for the basic-Helix-Loop-Helix family of proteins (including the USF/MLTF transcription factor and the Myc-Max heterodimeric complex) and for the NRF-1 transcription factor. These interactions have also been studied by *in vitro* binding experiments both with purified and recombinant proteins. Furthermore, other sites, whose nucleotide sequence is not homologous to the consensus binding sequence for any known factor and whose relevance for the origin activation process is now under experimental evaluation have been identified.

Study of the Molecular Mechanisms Regulating HIV-1 Expression

At least three types of experimental evidence point out that the mechanisms that control HIV-1 expression are involved in AIDS pathogenesis. First, although detectable viremia levels can be detected during all the phases of natural infection, the transcriptional activity, evaluated as proviral DNA/messenger RNA ratio, is directly related to disease progression. Second, a large number of CD4 lymphocytes and macrophages are infected within the lymph nodes, several of which in a latent manner. Third, the HIV-1 LTR is an evolutionary example of a promoter which is finely tuned by, and highly sensitive to, the extracellular stimuli which trigger cellular proliferation or activation.

In order to understand which LTR sites are responsible for transcriptional activation in infected cells, we developed an *in vivo* footprinting technique. In the T-lymphoblastoid cell line H9, which is productively infected, several LTR sites interact with nuclear proteins; in the monocytic cell line U1, considered a valuable experimental model for latency since viral production is inducible in basal conditions, the two Sp1 sites close to the enhancer, the proximal enhancer sequence, the NFAT site and a purine-rich box within the Negative Regulatory Element are occupied by nuclear proteins. Upon stimulation with PMA, the increase in transcriptional activity is correlated with increased site occupancy at the downstream enhancer repeat. However, the kinetics of the accumulation of viral transcripts is remarkably different from that of the appearance of enhancer binding factors (NF-κB like), suggesting that, after a rapid activation step, further mechanisms are responsible for the maintenance of the induced state. Recent experimental evidence suggests that viral protein Tat is a likely candidate for this role. We are now in the process of confirming these observations by using recombinant Tat protein to trigger transcriptional activation and by studying of the interactions between Tat and cellular transcriptional factors. Altogether, these results suggest that the model for HIV-1 activation by different stimuli acting on the infected cell, converge to the following cascade of events: rapid induction of NF-κB-like factors ➡ transcriptional activation ➡ Tat production from multi-spliced mRNAs ➡ disappearance of NF-κB-like factors from the nucleus ➡ maintenance of high transcriptional levels as the consequence of the direct or indirect interaction of Tat with residual amounts of NF-κB or of other factors which constitutively bind to the enhancer. Studies on the transcriptional activation in other inducible systems (T cells ACH-2, U1 cells upon stimulation with TNF-α, etc.) will be used to further confirm this model.

We identified some novel binding sites on HIV-1 LTR which interact with human proteins. Between nucleotides -152/-174 upstream of transcription start site, we characterized a binding site for members of the basic-Helix-Loop-Helix (bHLH) family, to which transcription factor USF/MLTF

belongs; the activity of these proteins downregulates HIV-1 transcription. Recently, we purified USF/MLTF to homogeneity from HeLa cells and constructed a recombinant fusion protein GST-USF[43]. We are going to perform *in vitro* experiments to study the interactions between the purified and the recombinant protein with the LTR. Moreover, we are testing the ability of these proteins to bend DNA upon binding to the LTR (preliminary results show that the bending degree is different in the two cases); in particular we intend to investigate the role of bending in the control of transcription using *in vitro* transcription assays.

Finally, we are investigating the role of post-transcriptional control in the maintenance of latency state. In HIV-1 infected cells, more than twenty different viral mRNAs are synthesized, deriving from multiple alternative splicing processes. Either during the infection of susceptible cell cultures, or in cellular systems in which transcription is inducible, a temporal regulation of the synthesis of these transcripts is detectable: initially, short transcripts are prevalent, encoding for regulatory proteins; subsequently, intermediate mRNAs (*env* gene) and unspliced mRNA (*gag/pol* and viral genome) accumulate. These observations suggest that the pattern of production of viral transcripts is linked to the development of infection and could represent an interesting marker for disease progression. We are quantitatively studying the different types of viral mRNAs within cellular samples from patients infected by HIV-1 using the competitive RT-PCR technique. This assay is based on retrotranscription and coamplification of the RNA of the sample with a competitor RNA bearing the same sequences recognized by the primers. For this purpose, we have constructed an RNA competitor containing the recognition sites for four primers which correspond to regions that are conserved in the most common clinical strains of HIV-1. One of these primers has the same polarity of the viral mRNA, and its sequence is present in all viral transcripts; the remaining three primers are complementary to the viral mRNA and are localized within the *gag* gene, the *env* gene, and downstream of the splicing acceptor site, respectively. Using the first primer and each one of the complementary ones, it is possible to detect unspliced, single-spliced and multi-spliced transcripts, respectively.

DNA Helicases from Human Cells

DNA in the double-stranded form is a rather stable structure that assures the integrity of the biological inheritance. However, for the most important DNA transactions such as replication, repair, recombination and transcription the DNA has to become partially unwound, at least transiently, to form the single-stranded intermediate template. DNA helicases are the enzymes which catalyze the unwinding of the double-stranded DNA to provide single-stranded templates for most of such processes. Helicases generally bind to single-stranded DNA and translocate along the DNA into a duplex region disrupting the hydrogen bonds between the two strands. These enzymes contain an intrinsic DNA dependent ATPase activity which hydrolyses the gamma-phosphate of ATP and provides the energy to move the helicase along the DNA strands.

There are multiple DNA helicases within a single cell and they have been isolated both from prokaryotes and eukaryotes. To date, at least eleven different DNA helicases from *E. coli*, five from bacteriophage, seven from virus, ten from yeast, eleven from calf thymus, two each from mouse and plant and one from *Xenopus laevis* have been isolated. So far little is known about the DNA helicases present in human cells. We have started a systematic study of the human DNA helicases with the objective of purifying and characterizing them and eventually cloning their genes and defining their role in different aspects of nucleic acid metabolism.

Previously, we have reported the purification and characterization of four

different DNA helicases from HeLa cells, namely human DNA helicase (HDH) I, III, IV and V. Recently, we were able to purify the HDH II which is a heterodimer of 72 and 87 kDa protein. It prefers a fork-like structure of the substrate and proceeds in the 3' to 5' direction along the bound strand. HDH II is also a dsDNA binding protein which binds through the ends of the DNA. The microsequencing data of the HDH II protein, its DNA binding activity, native Mr and recognition by specific antibodies showed that it is identical to Ku, an autoantigen recognized by the sera of scleroderma and lupus erythematosus patients; this molecule binds specifically to duplex

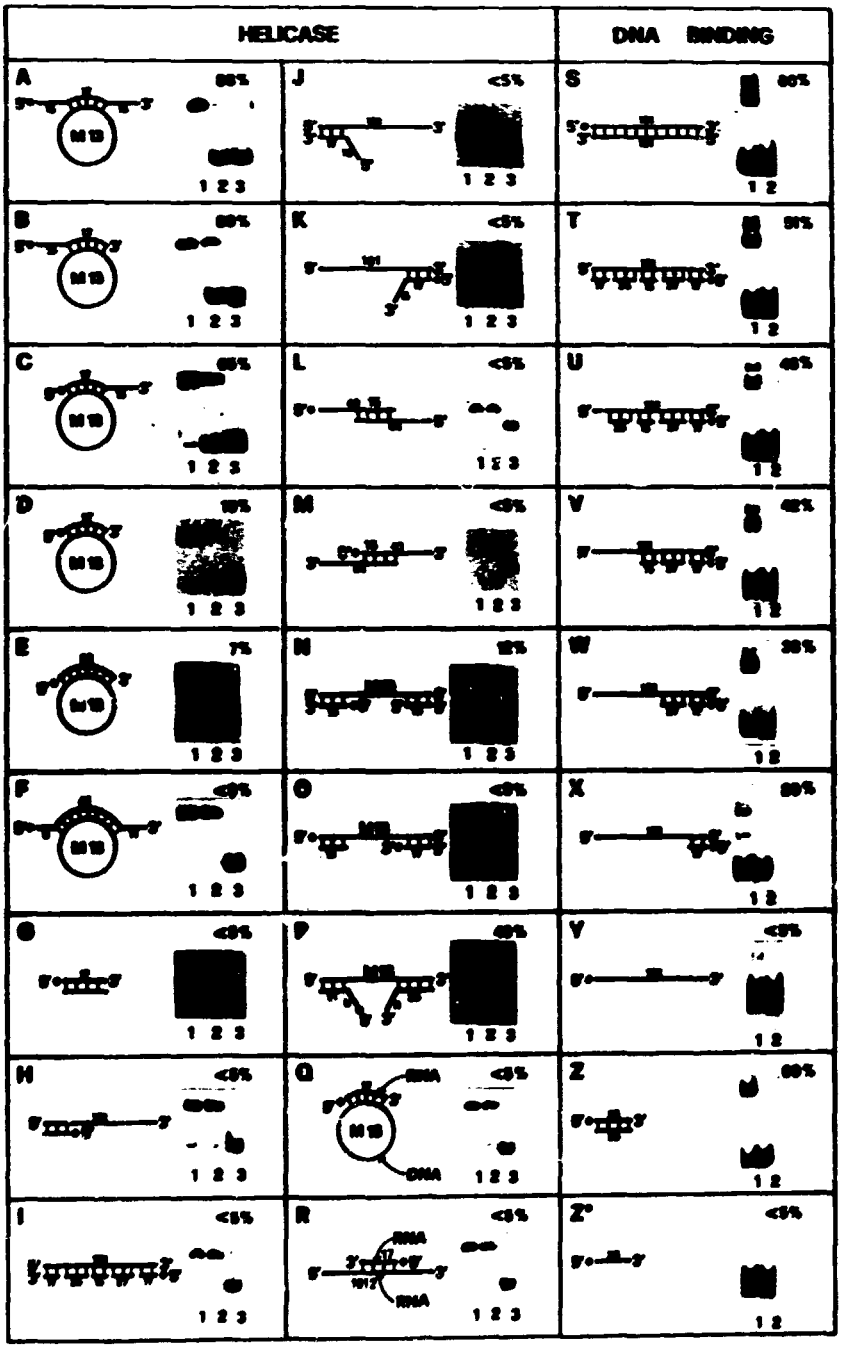


Figure 2.
Substrate specificity of HDH II/Ku as helicase (panels A to R) and DNA binding protein (panels S to Z*). For the helicase assay: lane 1, control without enzyme; lane 2, with enzyme; lane 3, denatured substrate. For the binding assay: lane 1, with protein; lane 2, substrate alone.

DNA ends, regardless of their chemical structure, and then slides along the duplex in multiple copies with a 25 bp periodicity. The high abundance of this molecule (5×10^5 copies exclusively in the nucleus), its high specificity for DNA and its newly demonstrated ability to unwind exclusively DNA duplexes point to a significant role of this molecule in DNA metabolism.

We have also raised polyclonal antibodies in rabbits against HDH I, II, III and IV. After screening the cDNA expression library with anti HDH IV antibody we have recently cloned a gene for HDH IV. The nucleotide sequencing of the cDNA showed that it is homologous to the human nucleolin, a major nucleolar phosphoprotein involved in the early stages of ribosome assembly and transcriptional regulation. We have also confirmed that, as expected, HDH IV can also unwind RNA-RNA hybrids. To further confirm this identity we have also started collaborating with Prof. F. Amalric of Toulouse, France, whose group is working on human nucleolin.

We have also found that HDH IV (like nucleolin) can be phosphorylated by cdc2 kinase and casein kinase II (kindly provided by Drs. S. Pongor and L. A. Pinna). The phosphorylation enhances significantly, and in an additive way for the two kinases, the unwinding activity of HDH IV. We are still investigating the significance and the possible functional role of this phosphorylation activation.

A collaborative project with Drs. C. Schneider and E. Ruaro of the Genome Studies programme is in progress to determine the *in vivo* role of different helicases by microinjecting the antibodies in S-phase cells and studying their possible effect on replicative synthesis.

In collaboration with Prof. Dr. D. Bootsma and Dr. J. H. J. Hoeijmakers of the Erasmus University of Rotterdam, we are exploring the possible role of human helicases in the DNA repair process by microinjecting them in DNA deficient cell lines from Xeroderma Pigmentosum and Cockayne syndrome patients. We have thus found that HDH I and IV cannot correct the defect of XP-B, XP-G and XP-D complementation groups, whereas crude fractions of HDH III, containing also HDH V, can correct the repair defect only in XP-B. This observation is particularly interesting since the gene for this complementation group called ERCC-3 encodes a presumed DNA helicase. We are still continuing this study to check whether the correcting protein corresponds to the pure HDH III or HDH V.

Furthermore, we have also started collaborating with Dr. D. Lane of the University of Dundee, Scotland, U.K., to determine whether p53 protein can down regulate the human DNA helicase activity and also to study the interactions between the p53 and helicases.

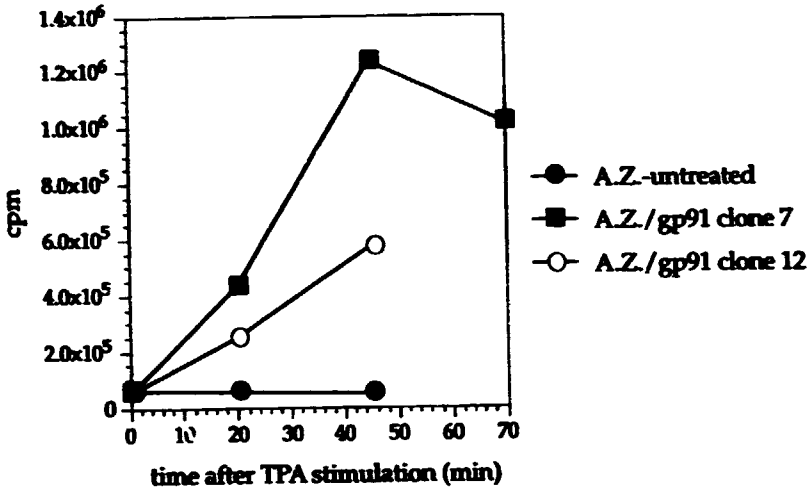
The cloning of the genes for human DNA helicases, a more detailed characterization of their molecular and functional properties as well as the purification of other novel DNA helicases are in progress. Raising antibodies against other DNA helicases are also in progress.

Medical Genetic Studies

Cardiology. For the last two years, we have implemented a collaborative research project with the Division of Cardiology, Hospital and University of Trieste, to study the pathogenesis of idiopathic dilated cardiomyopathy (IDC), a heart muscle disease with an incidence of about 8 new cases/100,000 persons per year, which represents the first indication for heart transplantation in the world.

Several long-lasting clinical and experimental observations suggested that enteroviral persistence (in particular coxsackieviruses type B) may underlie idiopathic myocarditis and dilated cardiomyopathy. We have developed a method for the detection of enterovirus-specific RNAs in myocardial tissue based on reverse transcription and nested polymerase chain reaction. Our findings, however, in agreement with the results of other very recent studies,

Patient A.Z.



Patient A.G.

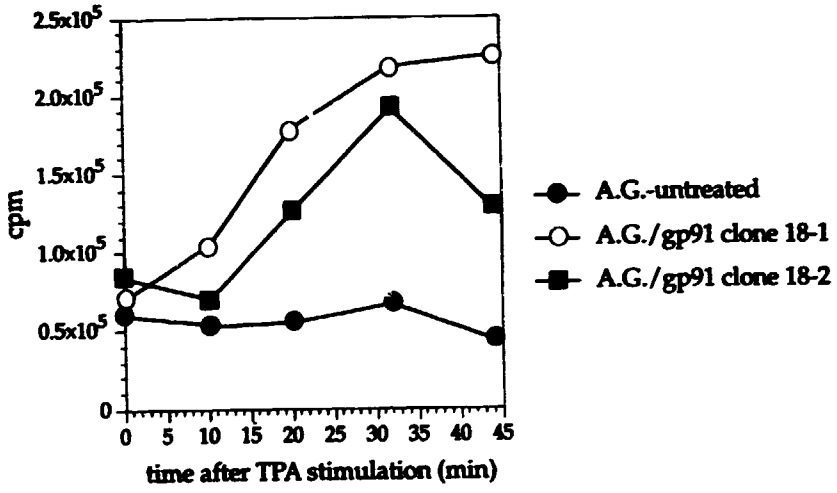


Figure 3.
Correction of X-linked Chronic Granulomatous Disease defect by retroviral-mediated gene transfer in EBV-transformed B cell lines of two different patients. Results of a luminol-based chemiluminescent assay after PMA stimulation.

suggest that the persistence of enteroviral RNA is not a major cause of the disease.

We have also focused our attention on the familial form of IDC, which is estimated to concern more than 20% of the patients, with a mainly autosomal dominant pattern of transmission. So far, 24 families with familial IDC, including 146 family members, have been examined. DNA samples from these families are used for a linkage analysis study in order to identify the gene responsible for the disease, by utilizing highly informative microsatellite markers. Preliminary data allow us to rule out linkage of the disease to a series of candidate genes, including genes coding for contractile and regulatory proteins of the myocardium, genes involved in the regulation of the immune system (including the HLA region) and genes coding for receptors or intermediate messengers of metabolic pathways relevant for the myocardial function.

Paediatrics. Chronic Granulomatous Disease (CGD) is a rare inherited disease characterized by the inability of phagocytic cells (granulocytes and macrophages) to produce superoxide anion (O_2^-) due to a defect of a membrane NADPH oxidase complex. As a consequence, affected children develop fatal chronic bacterial and fungal infection of several organs. We are currently studying patients affected by the X-linked form of the disease, caused by a defect of the large subunit (gp91-*phox*) of the oxidase, in order to develop a gene therapy protocol based on transduction of the correct gene in bone marrow cells.

We have established EBV-transformed lymphoblastoid cell lines from three patients as a source of DNA and RNA to study the molecular defect underlying the disease. No gross DNA abnormalities were detected by hybridization studies, while the level of transcription is decreased in two patients both in B-cell lines and in peripheral blood granulocytes. Point mutations of the carboxy-terminal portion of the protein appear to be responsible for the defect; one such mutation falls in a intron-exon boundary and is likely to be responsible for decreased mRNA stability.

Contrary to EBV-transformed B-cell lines from normal individuals, which, upon stimulation, produce superoxide anion similarly to phagocytes (although 100 times less efficiently) B-cells from the patients are completely inactive; therefore, these cells represent an excellent model to test the functional efficiency of gene transfer protocols. We have cloned the coding portion of the gp91-*phox* cDNA in a retroviral vector, selected productive clones of packaging cell lines and used them to infect the lymphoblastoid cells of the patients. The transduced lymphoblasts show from 20 to 30% of the normal activity of superoxide anion production. We are now analyzing the ability of the recombinant retrovirus to infect bone marrow stem cells.

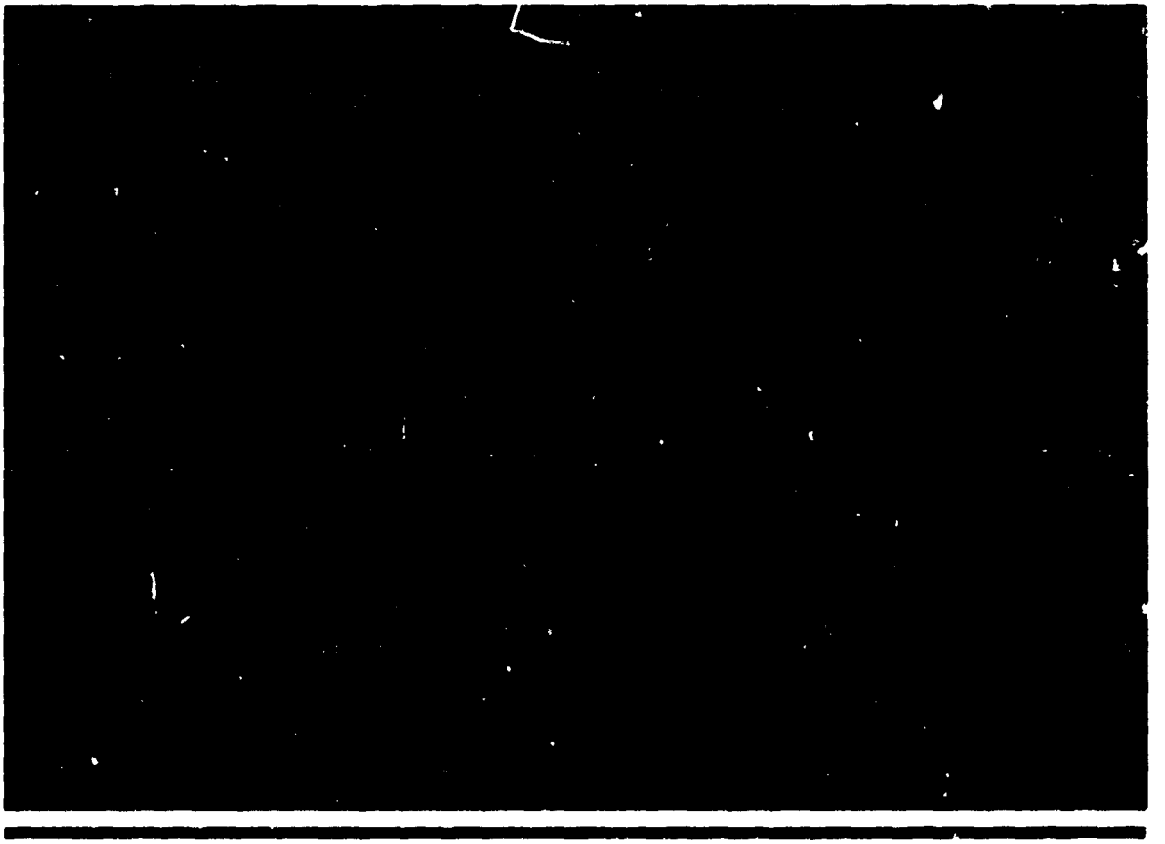
Genome Studies

C.Schneider.M.E.Ruaro.S.Bottega.L.Collavin.D.Lazarevic.S. Marzinotto. R. Utrera.

The aim of the research of this group is the dissection of the molecular events that induce and maintain growth arrest in mammalian cells. Through the study of a set of genes that are highly expressed in arrested cells and downregulated in exponentially growing cells (gas - growth arrest specific genes) we are contributing towards this aim. The localization of the gas gene products analyzed up to now is quite diverse.

Gas1 codes for a plasma membrane protein that is linked covalently to the lipid glicophosphatiolylinositol (GPI-anchored). When ectopically expressed in exponentially growing cultured cells gas1 is able to block cell cycle progression. (Del Sal, *et al.*, Cell, **70**, 595-607,1992). We have recently shown that the gas1 induced growth suppression requires the presence of a functional p53, this represents the first evidence of a growth inhibitory pathway that originates from the plasma membrane and is transduced via p53.

Gas3 codes for an integral membrane protein with four transmembrane domains. It is highly expressed in myelinating Schwann cells where it seems to be required both for establishing growth arrest and for coordinate myelin formation. When gas3 is ectopically expressed in fibroblastoid cells it is able



to induce apoptosis. The hypothesis has thus been advanced that gas3 plays a role in the induction of programmed cell death, this cellular response being strictly dependent on the specific differentiative cellular background.

Gas6 codes for a secreted protein that shows homologies with Protein S, a vitamin K dependent protein (Manfioletti *et al.*, Molecular and Cellular Biology, **13**, 4976-4985, 1993). Its function however is completely different from its relative: testing the activity of the recombinant protein expressed mammalian cells has posed the possibility that it acts as a powerful survival factor.

We are establishing new techniques for efficient subtraction cloning protocols with the aim of identifying genes that are differentially or universally induced und · different growth arrest conditions.

One of the .e genes is sdr (Gustincich and Schneider. Cell Growth and Differentiation, **4**, 753-760, 1993) that is induced only by serum starvation but not by contact inhibition.

Virology

L.Banks, V.Bouvard, G.Faulkner Valle, P.Massimi, M. Molinari, A. Piccini, D. Pim, A. Storey, M. Thomas, R. Wu.

The virology laboratory focuses its attention on the Human Papillomaviruses (HPVs). These viruses have been implicated as causative agents in a variety of human cancers, especially uterine cervical cancers. Cervical cancer is particularly devastating in developing countries, with this being the second major cause of cancer related death in women worldwide after breast cancer. The work of the group is primarily aimed at elucidating the mechanisms by which HPV brings about cell transformation. The studies are divided into two broad areas concerning either the mechanism of action of the viral oncogenes or regulation of viral oncogene expression. The results of these studies will provide the means for designing strategies aimed at the prevention and treatment of HPV associated disease.

Characterization of the HPV E6 Oncogene

A key function of the E6 proteins from oncogenic HPVs appears to be their ability to bind to and induce p53 degradation via the ubiquitin pathway. Mutations in the p53 gene are very common in many forms of human cancer, but are extremely rare in the early stages of cervical cancer. The normal function of p53 appears to be to induce growth arrest of the cell in late G1 following DNA damage thus preventing incorporation of damaging mutations into the genome. This function of p53 appears to be abolished by the oncogenic HPV E6 proteins, thus permitting uncontrolled cell proliferation.

Other laboratories have shown the binding of HPV E6 to p53 *in vitro* and subsequent p53 degradation. However, the exact importance of this interaction *in vivo* has not been investigated. We have carried out an extensive site-directed mutagenesis of the HPV-18 E6 protein in order to locate regions of the molecule required for: a) binding/degradation of p53 *in vitro*; b) abolition of p53 transcriptional activation *in vivo*; c) ability of E6 to cooperate with an activated *ras* oncogene to transform primary mouse cells.

A total of 15 mutants were constructed throughout the HPV-18 E6 molecule. Most consist of short deletions of 3-4 amino acids; single point mutations were found at an early stage to be resistant to this type of analysis. This probably reflects a high degree of intrinsic stability within the E6 molecule. All the mutants produced to date retain the ability to transactivate the Adenovirus E2 promoter confirming that stable protein is produced *in vivo*.

a) The first set of analyses concerned the ability of these mutant proteins to induce p53 degradation *in vitro*. These studies identified 3 broad domains of the E6 molecule which are necessary for inducing p53 degradation.

b) We then proceeded to investigate the activity of these mutants *in vivo*. A central activity of p53 in its role as a tumour suppressor is its ability to stimulate gene expression from promoters which contain a p53 responsive element. Cotransfecting HPV-18 E6 and p53 together with a CAT reporter plasmid containing a p53 responsive element results in an E6, dose dependent abolition of p53 transcriptional activation. The biological relevance of this assay is amply demonstrated by studies where endogenous p53 was activated

by UV irradiation. Again, transfecting HPV-18 E6 into these cells abolished UV induced p53 activity (Gu *et al.*, Oncogene, **9**, 629-633, 1994). Most of the HPV-18 E6 mutants which failed to degrade p53 *in vitro* also failed to abolish p53 transcriptional activation. One mutant was found, however, which failed to degrade p53 *in vitro* but was wild type in the *in vivo* transcriptional assay. This result indicated that degradation of p53 was not essential for the abolition of p53 function. We investigated this further with a series of microinjection experiments. Plasmids expressing p53 and E6 plus a p53 responsive CAT plasmid were coinjected into human cells and stained for p53 and CAT proteins. The results obtained are shown in Figure 1 and demonstrate that cells coinjected with p53 and HPV-18 E6 can continue to express p53 protein but have lost p53 transcriptional activation as determined by the level of CAT expression. These results confirm that the *in vitro* degradation is not relevant for *in vivo* function.

c) We had previously observed that HPV-18 E6 could cooperate with an activated *ras* oncogene to transform primary rodent cells (Storey and Banks, Oncogene, **8**, 919-924, 1993). We then proceeded to investigate the activity of these mutants in this assay. Most surprisingly, all retained some ability to cooperate with the *ras* oncogene in this assay. Since we had shown that only oncogenic associated E6 proteins could cooperate with *ras*, we then analysed some chimaeric E6 proteins (half oncogenic, half non oncogenic) and these demonstrated that the carboxy terminal half of the protein was essential for this activity. These results indicate that p53 inactivation is not necessary for

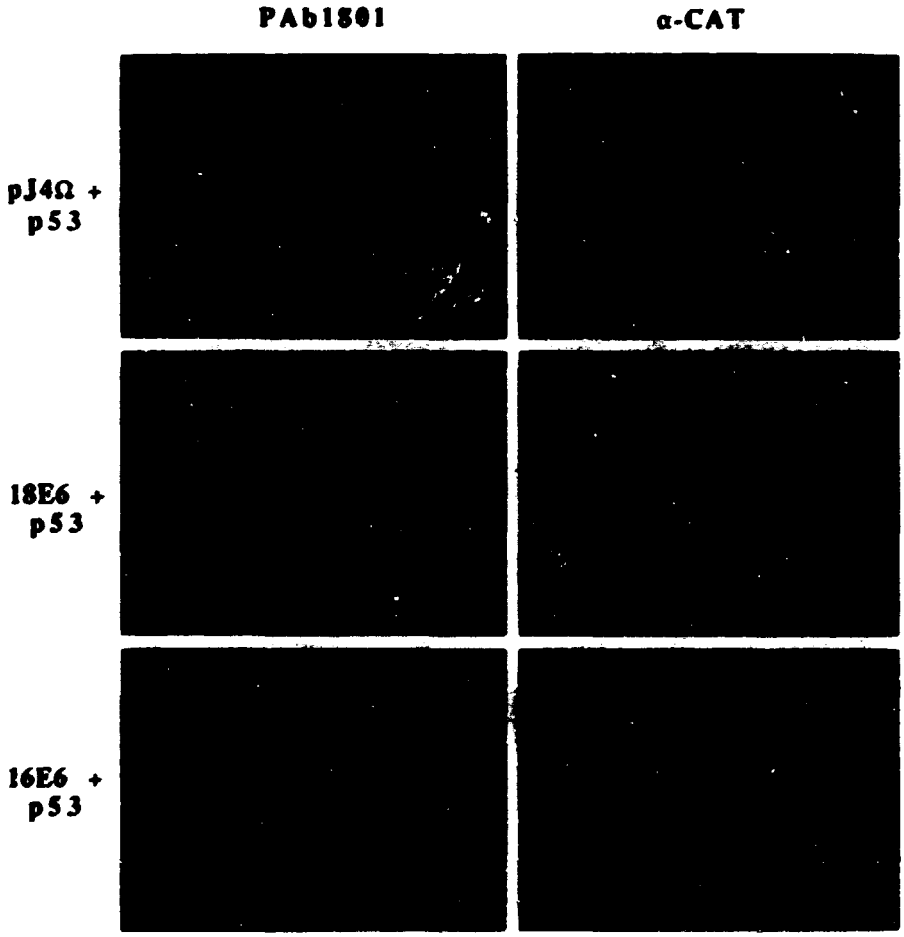


Figure 1.
Abolition of p53 transcriptional activation by co-microinjection of HPV E6 and p53. Saos-2 cells were microinjected with the p53 responsive CAT reporter plasmid (pG13CAT) plus indicated expression plasmids. After 24 hrs. the cells were fixed, and p53 and CAT proteins detected with pAb1801 and anti CAT antibody respectively.

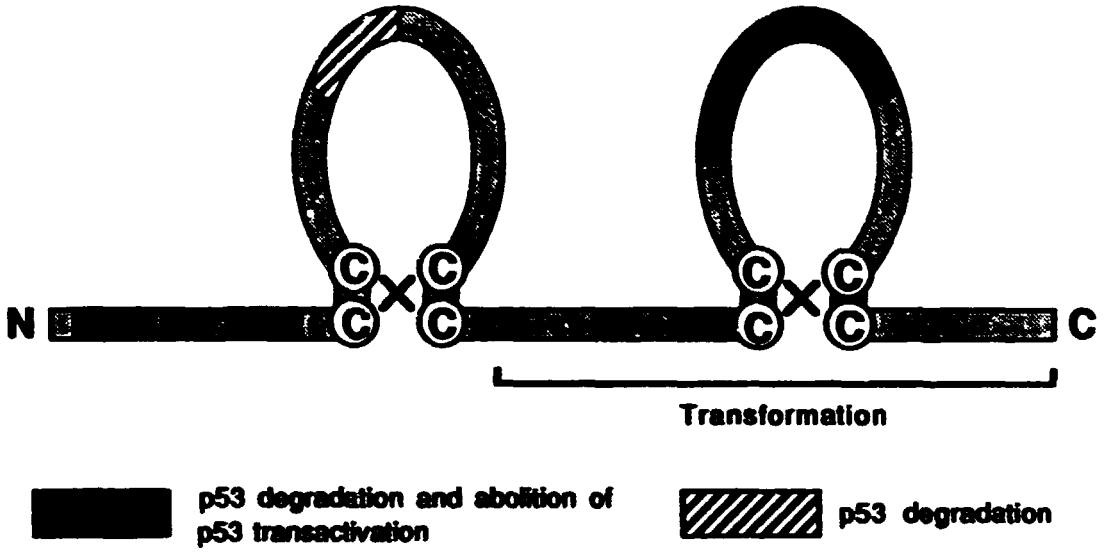


Figure 2.
Schematic representation of the HPV-18 E6 functional domains. Boxes represent regions of E6 which if mutated result in loss of indicated functions. Transformation refers to cooperation with EJ-*ras* in primary rodent cells.

ras cooperation and that an additional, as yet unidentified function of E6 is responsible for this activity. Current studies are aimed towards identifying this function and assaying the mutant E6 proteins for immortalizing activity in human cells.

A summary of the functional domains of E6 based on the mutational analysis is shown in Figure 2 (Pim *et al.*, Oncogene, in press).

Mechanism of E6 Abolition of p53 Transcriptional Activation

Wild type p53 binds in a sequence-specific manner to DNA and this characteristic is related to the ability of wild type p53 to transactivate genes adjacent to the p53 recognition sequence. Using a 20 bp oligomer containing a consensus p53 recognition sequence we have demonstrated the ability of *in vitro* translated p53 to bind this oligo in a gel retardation assay. We have demonstrated that *in vitro* translated wild type HPV 18E6 is able to disrupt p53 DNA binding under conditions which do not cause the degradation of p53. In addition, this disruption of the complex is probably due to the ability of 18E6 to displace p53 from DNA to which it is already bound. This implies that E6's binding to p53 may be sufficient to prevent the transactivation of genes containing the p53 recognition sequence and that the degradation of p53 may not be necessary in this context. We are now investigating the exact nature of this interaction using the 18E6 mutants discussed above and we are extending these studies to examine the effect of wild type and mutant 18E6s upon the interaction of p53 with other transcription factors.

Analysis of E6 Mutants for Dominant Negative Effects

The above analysis indicates that the E6-p53 association is very complex and probably involves multimeric E6 and p53 molecules. With this in mind we have started to assay the HPV 18 E6 mutants for dominant negative effects. Cotransfection of certain E6 mutants together with the wild type protein results in clear inhibition of wild type function in terms of abolition of p53 transcriptional activation. Similar results are obtained when *in vitro* degradation experiments are performed. Studies are now currently underway

to determine whether these dominant negative E6 mutants can abolish the function of wild type HPV E6 in cells derived from human cervical tumours.

Regulation of Viral Gene Expression

An aspect critical to viral induced tumourigenesis is the regulation of viral oncogene expression. The major regulator of viral gene expression is encoded by the viral E2 protein. We had previously observed that alternative splicing of this gene resulted in two forms of the protein, one being a transcriptional activator, the other a transcriptional repressor. Further, the shorter repressor form of the molecule is capable of inhibiting the activator function of the full length form of E2. Using gel retardation analysis we have been able to show that the repressor form of the protein complexes directly with the long form. Hence abolition of E2 transcriptional activation by the repressor E2 is brought about through formation of heteromeric molecules and under normal circumstances activation is only obtained if the E2 molecule exists as a homodimer.

Mutational Analysis of the E2 Protein

Work continues on the set of E2 mutants which we have produced. Within the transactivation domain of the E2 protein we now know of five distinct regions which are essential for the transactivation function. One of these functional domains is responsible for synergy with the glucocorticoid receptor. We are now in the process of identifying other cellular transcription factors with which E2 interacts. One of the E2 domains has homology with a known amino acid sequence for interacting with TATA box binding protein and this association is currently under investigation. An additional domain of E2 also has limited homology with a common transactivation domain on c-*fos* and c-*jun*. Work currently in progress is aimed at addressing whether this homology is functionally conserved.

Dissection of the Viral E1-E2 Association

A critical activity for viral DNA replication is the association between the viral E1 and E2 proteins. Using the mutant E2 proteins described above we have proceeded to map the domain of E2 essential for this association. E1 and E2 have been expressed both *in vitro* and as GST fusion proteins. A strong complex was obtained when the two proteins were mixed together. In addition, this complex could exist even when E2 was bound to DNA. Using the mutant E2 proteins, one short stretch in the activation domain was found to be essential for the association with E1. Interestingly, this mutant E2 protein is also defective for transcriptional activation. This suggests the possibility of a competition between E1 and an as yet unidentified transcription factor for E2 binding. Current studies are aimed at assaying the effect of E2 binding on E1 enzymic activities. In addition, we are attempting to block the E1-E2 complex formation using synthetic peptides which correspond to the domain of E2 which binds E1. It is hoped that a viral DNA replication assay can be developed in the near future and this could then be used to assay the domains of E2 which are essential for the replication function. Such an assay would also provide the basis for a screen for any antiviral compounds which may block viral DNA replication.

Microbiology

C. V. Bruschi, J. Oberto, I. Bertani, M. Coglievina, A. Comino, G. M. Danek, D. Delneri, G. Degrassi, V. Hindupur Nagaraj, A. Janousek, M. Gotta, R. Klima, B. Le Thanh, G. Ljubijankic, S. Nwaka, D. Santoro, F. Storici, S. Ugolini, Z. Weng, V. Yong Gonzales, P. Zaccaria, A. Zago.

Lignin Biodegradation

Work has progressed towards the identification and characterization of enzymatic activities involved in degradation of lignocellulose, with particular focus upon the degradation of ferulic and p-coumaric acid. These two cinnamic acids form the structural bridge between hemicellulose and phenolic components of core lignin and their degradation is an essential step in the resolution of the whole lignocellulose structure.

A ferulic acid and p-coumaric acid decarboxylase has been characterized and purified from *Bacillus pumilus* which was found associated with the rumen fluid. SDS-PAGE analysis of purified protein showed a single band of 23 kDa, while the molecular weight of the native protein evaluated by gel filtration has been estimated to be approximately 45 kDa, therefore, the protein should be a dimer. The isoelectric point of the protein is 4.6 and the optimal pH range of activity between 5.5 and 6.5. The 23 kDa band from SDS-PAGE has been blotted on PVDF membrane and the amino-terminal sequence determined by microsequencing at the CRIBI Centre of the University of Padova. The eighteen amino acid sequence obtained has been used to synthesize a DNA probe on the basis of a *Bacillus subtilis* frequency codon usage programme. Since DNA probe hybridizes with *Pst*I and *Hind*III genomic DNA fragments, it will be used to screen the genomic library of *Bacillus pumilus* which is being constructed. The same probe has been used to screen *Bacillus subtilis* and *Bacillus licheniformis* genome since they are also able to decarboxylate the two phenolic compounds. The probe showed significant homology only with *B. licheniformis* and a comparison of the hybridization bands suggested the presence of a DNA polymorphism with respect to *B. pumilus*. *B. licheniformis*, a regular inhabitant of the rumen, has been successfully transformed by electroporation with plasmid pC194 and is currently tested for transformation with larger cloning vectors. This bacterium will be used as a host for cloned genes whose high expression is desired to improve utilization of lignocellulose.

Acinetobacter calcoaceticus DSM 586 has been found to be able to completely degrade ferulic acid and p-coumaric acid up to the fission of the aromatic ring, thereby representing an interesting candidate for new enzymatic activities. HPLC and NMR analysis (the latter conducted in collaboration with the Technological Biopolymer Laboratory of the AREA Science Park of Trieste) showed that the first stable intermediates in the degradative pathway are respectively vanillic acid and p-hydroxybenzoic acid, while the last intermediate before ring fission is protocatechuic acid. Biochemical characterization of the degradative pathway is in progress: transformation of ferulic acid to vanillic acid has been reproduced *in vitro* using the crude extract of an induced culture with the addition of CoA, ATP, NAD^+ and formaldehyde. This is a key-step in the conversion of cinnamic acids to benzoic acids and their further degradation.

Plasmid Stability

The expression of at least 35 genes encoding enzymes in 12 different amino acid biosynthetic pathways is co-regulated in *S. cerevisiae*. Typically, starvation for a given amino acid not only induces an increase in the level of the enzymes necessary for its biosynthesis, but results also in elevated levels of enzymes for the biosynthesis of other amino acids. However, many amino acid biosynthetic genes are also under pathway-specific control, which can override the general control. Thus, starvation for one or more amino acids or nucleotides affects the cellular metabolism, with possible effects on the maintenance in the cell of plasmid species carrying genes encoding amino acids, nucleotides or other markers.

To investigate this possibility we constructed a series of 2 μm-based plasmids carrying genes for the synthesis of either amino acids (histidine, leucine, tryptophan) or nucleotides (adenine, uracil). These plasmids were individually introduced in a recipient yeast strain and parallel cultures were grown in selective media to determine the growth rate. With this approach we could not detect appreciable differences in the growth rate of the transformed cultures, while other parameters such as plasmid stability and plasmid copy number are under investigation.

The cell-division-cycle gene *CDC6*, important for yeast centromere (YCp) and yeast episomal (YEp) plasmid stability, seems to influence the endogenous 2 μm DNA plasmid transmission as well. We performed our experiments in two temperature-sensitive *cdc6* [*Cir*$^+$] strains from which we

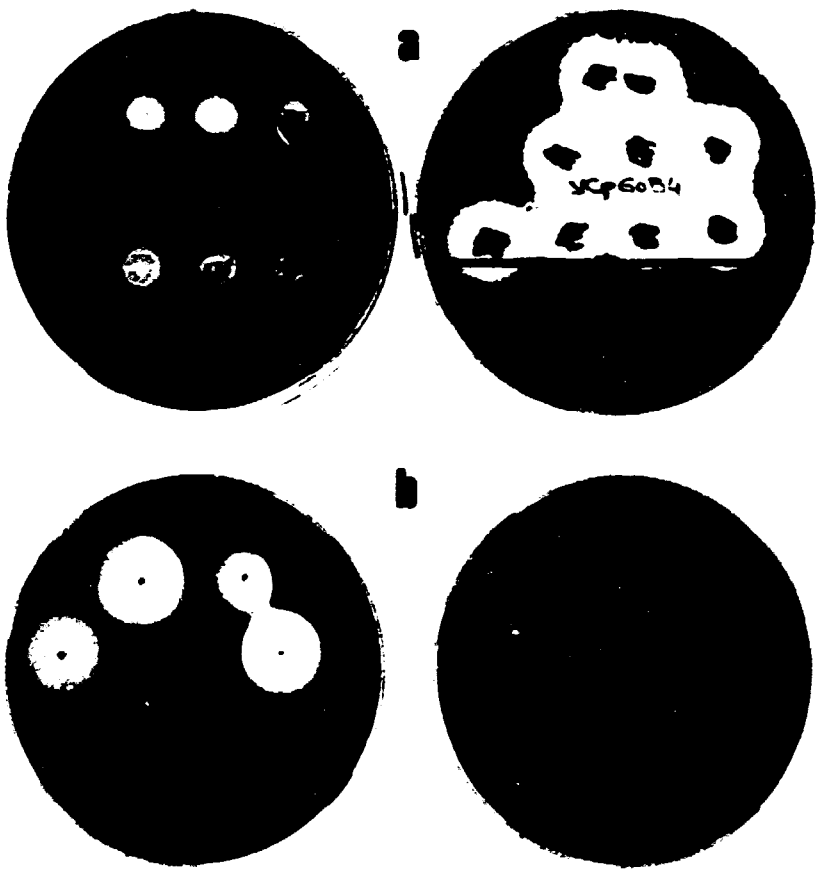


Figure 1. Detection of PA-positive colonies using the 6-APA-sensitive bacterium *Serratia marcescens* (red background).
a) Comparison of halos of growth inhibition due to 6-APA produced by clones of *PA* genes in *E. coli* (left) and *S. cerevisiae* (right). b) Comparison of halos of growth inhibition around *S. cerevisiae* strain CBL1-30/pGoB2 colonies after growth under induction of *PA* gene by galactose (left) and repression by glucose (right).

obtained revertant colonies able to grow at the restrictive temperature of 37° C. We observed that in these colonies grown at the restrictive temperature, 2 μm DNA plasmid is lost in 40 to 60% of the cases, while it is 100% stable in the *CDC6* wild-type strain grown at the same temperature. These results, together with double-shift experiments carried out with the drug nocodazole, which affects function and structure of the mitotic spindle, tend to confirm a possible second role of the *CDC6* gene in the segregation of genetic elements.

Expression of Bacterial Penicillin G Amidase Genes

Penicillin G amidases (PA) of gram negative bacteria are industrially important enzymes which catalyse deacylation of the acyl group of penicillin G, yielding 6-aminopenicillanic acid (6-APA), a key intermediate in production of semisynthetic β-lactam antibiotics. These gene products have features that render them quite unique among prokaryotes: the proteolytic release of the functional enzyme from a polypeptide precursor, a characteristic of eukaryotic regulation of gene expression.

To improve present biotechnological methods for production of 6-APA and to better understand the origin of *PA*'s post-translational regulation, we decided to express this gene in the yeast *Saccharomyces cerevisiae*. Using different kinds of yeast expression vectors in combination with differently modified *PA* genes from *E. coli* (*GoB4*) and *Providencia rettgeri* (*GoB2*), we successfully obtained expression of functional bacterial enzymes in yeast. An expression cassette composed of *UAS(Gal 10)*, α-factor promoter, α-factor leader sequence and bacterial gene with deletion of bacterial leader sequence, even when present as single copy per cell (on centromeric plasmid YCpGoB), gives a level of recombinant enzyme which is comparable to or higher than the level obtained by expression of the cloned *PA* genes in *E. coli* (Figure 1a). The same expression cassette containing the gene *GoB4* was cloned into the 2μ-based high copy number vector pBLAST, constructed in our laboratory, which does not require selection for its maintenance. The very high level of expression of the *PA* gene under galactose induction is shown in Figure 1b. The enzymatic activity is localized either in the culture medium (YCpGoB2 and pGoB2) or in the periplasmic space (YCpGoB4 and pGoB4). This pattern of distribution of active enzymes can only be correlated with differences in coding sequences between *PA* from *P. rettgeri* and *PA* from *E. coli*.

Yeast Genome Sequencing Project

As part of the BIOTECH project of the European Community aimed at sequencing *Saccharomyces cerevisiae's* genome, we are involved in the sequence analysis of the cosmid clone pBi210, containing a partial *Sau3*A fragment of approximately 37 Kb from the left arm of chromosome *VII*. This fragment spans the *DST2* locus and, as we demonstrated by Southern blot analysis, includes also the *PMR1*, *SUA5* and *RAD54* genes, previously mapped in this region (Mortimer, R. K., Contopoulou, C. R. and King, J. S., 1992, Genetic map of *Saccharomyces cerevisiae*, edition 11, Yeast 8, 817).

DNA of cosmid pBi210 was double-digested with *Sal*I-*Not*I obtaining three fragments of about 13.0 (*Sal*I-*Not*I), 16.0 (*Sal*I-*Sal*I) and 8.0 kbp (*Sal*I-*Not*I), which were inserted into the shuttle vector pRS426 to give three subclones called pBi13, pBi16 and pBi8, respectively. Further subcloning of the 13-kb fragment was performed to generate subfragments, both ordinated and random, suitable for sequencing. All these fragments were directly subcloned into M13mp18 and/or M13mp19 sequencing vectors. The strategy of chromosome walking with synthetic primers was adopted to sequence the entire clone pBi13. All the primers used, including those used to locate the random clones generated by shotgun and to cover the junctions, were designed with the computer program DNAsis (Pharmacia Biotech) and

synthesized at ICGEB Trieste. So far, a total of 56,996 nucleotides have been read to give a final assembled sequence of 12,680 bp, which is fully determined on both strands (Figure 2). Sequence analysis of the entire 13-kbp fragment is in progress. At present, 6 open reading frames (ORFs), which represent polypeptide sequences longer than 100 aa, were found. Computer analysis (PROSEARCH against the Prosite database) identified some conserved patterns of Ser/Thr protein kinases in two of these ORFs, called **a** and **d**. ORF-**a** lacks the 5' portion, being truncated by the cloning into pWE15 cosmid vector. A search for intron-splicing signals revealed the presence of a putative intron of 634 nucleotides of length in ORF-**e**. This ORF is translated into a putative protein characterized by a high content of serine, and is homologous to the yeast YGL023 putative regulatory protein and *Drosophila melanogaster* maternal pumilio protein. A search for protein patterns revealed a characteristic sevenfold internal repeat motif in the protein encoded by ORF-**e**. This pattern is similar to that reported for YGL023 and pumilio proteins. The analysis of the other ORFs found revealed no significant homology with known genes. The sequence of subclone pBi13 is now complete.

We are currently working on subclone pBi16 contiguous to pBi13. This fragment contains already known genes such as *DST2*, *NSP49*, *tRNALys*, *SUA5* and a portion of *PMR1*, the other portion of which is on subclone pBi8.

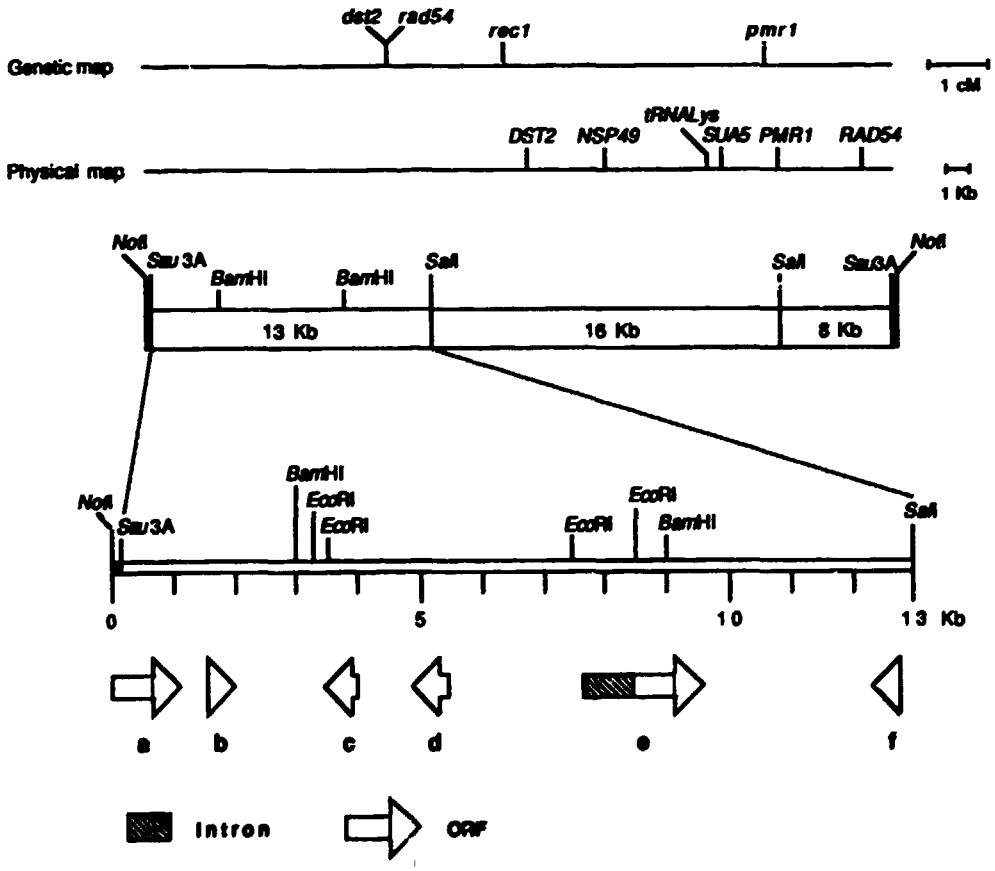


Figure 2. **Genetic, physical and ORF maps of the region of chromosome *VII* of *S. cerevisiae* encompassed by cosmid 210.**
The *Sau*3A open bar represents the yeast genomic fragment cloned into cosmid vector pWE15. The ORFs longer than 100 bp found by sequencing the subfragment of 13 kb are indicated. The location of the genes *DST2*, *NSP49*, *tRNALys*, *SUA5*, *PMR1*, and *RAD54* on the physical map is deduced by restriction and Southern blot analysis. The genetic map is taken from edition 11 of the genetic map of *Saccharomyces cerevisiae* (Mortimer, *et al.*, 1992).

Protein Structure and Function

S. Pongor, A. Simoncsits, S. Zahariev, V. Bevilacqua, I. Brukner, A. Chagolla Lopez, M. Cserzö, P. Fábián, C. Guarnaccia, Z. Hátsági, G. Percipalle, R. Sanchez, G. Simon, V. Skerl, S. Susic, S. Wang, J. Zhang.

The understanding of structural principles that underlie specific functions of proteins and their genes is the fundamental problem of protein engineering. The knowledge gained in this area is central in understanding how newly discovered proteins function and in designing new proteins for practical uses, such as vaccines and other therapeutic agents. Our interest is centred around structural domains and motifs that convey a specific binding function to groups of proteins which can thus be used to add a desired function to a new protein product. Such domains are required for binding proteins to receptors, to nucleic acids or to other ligands and the interactions involved play important roles in many natural and pathological processes. Interaction domains often evolve autonomously and many of them appear to be independent folding units, making them a good target for engineering studies. Difficulties stem from the fact that ligand interactions may induce changes in both the binding domain and in the ligand, so the process of interaction may be more than a simple key-lock mechanism. In nature, interaction domains are the product of molecular evolution, a process of trial and error by which successful domain structures are selected and maintained. On the other hand, conscious engineering of such structures requires a rational understanding of the underlying principles. *In vitro* selection techniques based on combinatorial shape libraries (peptides, oligonucleotides or phage display systems) are a complementary, powerful method for obtaining binding structures. In practice, knowledge-based design and non rational selection methods can be combined. For example, rational design is used to select a useful protein scaffold, and randomization/selection techniques can be used to develop new binding specificities on the molecule.

We are using four main experimental approaches: i) biocomputing analysis of sequence databases; ii) molecular modelling and molecular mechanics to design and test 3-D models of the peptides and proteins; iii) peptides are produced by methods of protein chemistry and molecular biology and their activity is tested by *in vitro* biochemical methods; iv) random peptide and oligonucleotide libraries to study ligand-ligate and protein-DNA interactions by employing affinity selection and binding techniques *in vitro* and *in vivo*.

Biocomputing

The long term goal is the application of computational methods to the rational design of proteins and their genes. Our efforts centre around methods for building rational consensus models from existing structures, this being a fundamental problem of molecular modelling. The programs are developed in C and run on UNIX computers. The programming environment includes the sequence databases EMBL, Genbank, Swiss-Prot and PIR, the University of Wisconsin GCG package for sequence analysis, as well as public domain software such as FASTA and the PHYLIP program package for phylogenies. Part of this research is carried out in collaboration with the Institute of Protein Research, Agricultural Biotechnology Center, Gödöllö, Hungary.

Molecular Modelling

Techniques of molecular graphics and molecular mechanics are used to model peptides and to study their interaction with other macromolecules. The Insight and Discover of BIOSYM, the Sibyl package of Tripos, along with a host of other software are used for most of the modelling studies that are carried out on a Silicon Graphics IRIS Indigo XS24 workstation.

Protein Chemistry

This laboratory employs the techniques of solid phase peptide synthesis using the Fmoc strategy and a MilliGen 9050 automated peptide synthesizer. We adapted methodologies to synthesize phospho- and thiophospho-peptides and to produce multiple antigen polypeptides (MAP) that can be used directly for immunization. Progress has been made in the production of long peptides with continuous flow solid phase technology. Peptides of up to 130 amino acid residues and analogues of the insulin A chain were produced with a high yield. In 1993, a total of 91 peptides were synthesized, predominantly as a service to other ICGEB researchers. All peptides were purified by HPLC and most of them were characterized by amino acid analysis.

Combinatorial Shape Libraries

Combinatorial shape libraries are a population of randomized biopolymers from which suitable ligands that bind to a specific target can be selected. Combinatorial shape libraries can be prepared by peptide synthesis and oligonucleotide synthesis, and the latter can be cloned into suitable vectors and expressed, e.g. on the surface of filamentous phages.

Semisynthetic combinatorial antibody libraries with human framework sequences were used to isolate human anti-hapten antibodies expressed on the surface of filamentous phages by biopanning techniques. Previously, similar libraries led to the selection of consensus motifs mainly in the H-chain CDR3. Our studies indicate that the L-chain CDR3 could also substantially contribute to the affinity selection.

An *in vivo* selection method was developed for the selection of cognate sequences that bind to DNA-binding proteins or *vice versa*. This approach allows for the simple selection of specific protein/cognate pairs and thereby random oligonucleotide libraries can be used for altering the specificity of these interactions.

Our research topics, described below, focus on two main areas: a) extraction of information from sequence databases; b) modelling of protein/ligand interactions (protein/DNA interactions, enzyme/substrate and enzyme/inhibitor interactions, etc.)

Functional Domains and Structural Motifs in Protein Sequences

Identification of new structural motifs is a crucial task both in the interpretation of newly determined sequences and in the design of novel proteins. Current sequence alignment algorithms are sufficiently fast in searching large sequence databases for homologous sequences. The interpretation of the results becomes increasingly difficult in the range of 15-25% identities and homologies which are functionally interesting very often fall in this range. The goal of this project is to develop search tools for the detection of distant similarities in protein sequences. Our working hypothesis is that the information required is implicit in the database search results, only the appropriate tools of interpretation, or eventually, the modified databases have to be developed in order to find it. Conceptually, database searches return a group of homologous sequences and the experimenter has to intuitively determine whether or not this is a biologically meaningful homology group. The solution is straightforward if the identified group is identical to one of the known homology groups and is characterized by a known sequence pattern. On the other hand, identification of new homology groups and new domain types is, by-and-large, an intuitive process and our efforts concentrate on tools that facilitate this task.

We have developed SBASE, a protein domain library that contains the primary sequence of over thirty thousand protein segments of known structure/function that facilitates the identification of domains and potential functions in a newly determined sequence. The domain sequences are grouped by cluster analysis methods that assist in detecting distant similarities between them. This database was added to the ICGEBnet computer resource and was also released for public use through the network. In 1993 we started an electronic mail server (sbase@icgeb.trieste.it) that automatically analyses domain homologies in query sequences which are sent by electronic mail.

In a parallel effort we have developed tools that detect recurrent patterns in database search results. Starting from an unknown query sequence, the programs produce a set of "interesting" sequence patterns along with domain descriptions which are potentially associated with them.

Central to this approach is a generalized entity-relationship data model that represents molecular structure as a set of substructures and relationships with assigned quantitative and qualitative properties (Pongor, Nature, **323**, 24, 1987). Our future plans include the development of a logically coherent protein sequence database in which not only the sequences but also the textual information can be processed by computational methods.

Protein/Nucleic Acid Interactions

Sequence-specific interactions of proteins with DNA play a central role in gene regulation. 3D structures of protein-DNA complexes are now available for a number of repressors and it has been experimentally shown that short peptides derived from the 'leucine-zipper' domain bind to DNA *in vitro*. We are studying the helix-turn-helix motif of the *E. coli* phage 434 repressor protein and computer modelling is being used to design peptides that can mimic the DNA-binding units of these proteins. In 1993 we produced a series of peptides that contain DNA binding motifs in various arrangements and that

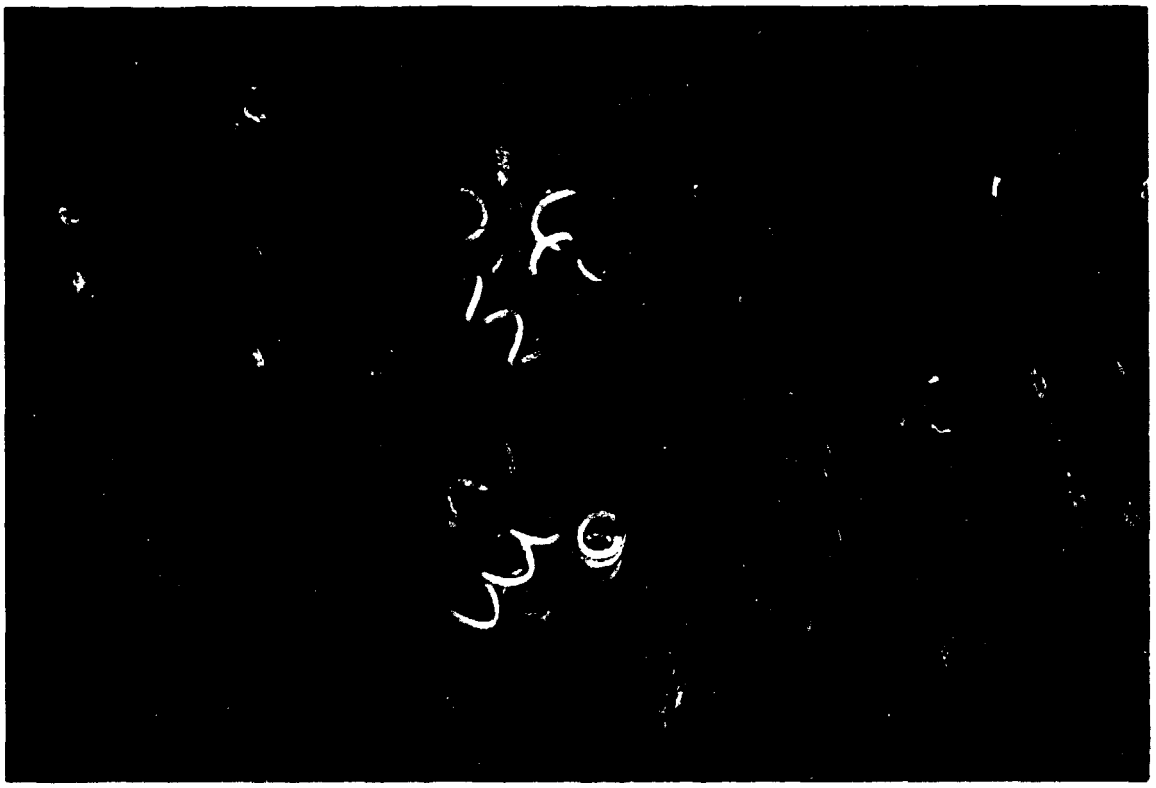


Figure 1.
Binding of a *de novo* designed, fully synthetic, 130 residue long bipartite DNA-binding protein to its cognate operator site in DNA (blue). The helical regions of the protein are yellow, the rest is green. The synthetic linker region is shown in atomic detail.

show sequence specific DNA binding activity both *in vitro* and *in vivo* by using peptide synthesis and protein engineering techniques. In particular, different chimaeric and functionally active DNA binding proteins have been designed and constructed. Random and rationally designed libraries of such single-chain molecules with bipartite DNA-binding motifs are being studied using both *in vitro* and *in vivo* selection. This is being done in conjunction with randomized DNA regions, in order to discover new specificities in DNA/protein interactions. Chimaeric DNA binding proteins based on the helix-turn-helix motif have been designed and expressed in *E. coli*. An *in vivo* selection system for studying specific protein-DNA interactions has been developed. In this system, a single expression vector carries all the elements required for selection: the regulated gene for the DNA binding protein; the possible binding site (cognate DNA) for this protein; and, a marker gene which is placed under control of this binding site. A rational redesigning of both the binding motif and its cognate DNA sequence has been experimentally tested showing that the selection system is capable of detecting binding interactions with new specificities. This opens the way to a random approach in which the binding motifs and the cognate DNA sequence can be independently or simultaneously randomized.

Sequence-dependent folding and flexibility of DNA seems to be an important feature in many regulatory mechanisms. There is growing evidence that local curvature and flexibility strongly influence the mode of protein-DNA interactions, therefore affecting important biological events such as DNA replication, recombination and transcription. We have recently shown that GGGCCC elements in ds DNA are curved. We have also found that physiological concentrations of divalent cations drastically influence the magnitude of local DNA flexure. This finding has important implications for predictive models of sequence-dependent and protein-induced DNA flexure, since all experimental values used for establishing such models were hitherto determined under non-ionic conditions.

Currently, we are developing a model for sequence-dependent DNA bendability. DNA bendability is a potential of DNA to adopt particular conformation imposed by interacting protein. This "potential" is determined by the sequence context of DNA. It is well documented that such conformational/topological changes of DNA are coupled with replication, recombination and transcription of DNA. By using DNase I as an indicator for bendable regions in DNA and subjecting a large number of DNA molecules to digestion, we were able to deduce the bendability potential for each sequence motif. Currently, we are working on numeric models that can predict DNA bendability from sequence data by combining contributions calculated for the constituent di-, tri- or tetra-nucleotides. Our preliminary results show that origins of replication and upstream regulatory regions invariably show bendability profiles that are very different from those of bulk DNA or of coding regions.

Substrate Specificity of Protein Phosphorylation

Protein kinases phosphorylate oncogene products, transcription and replication factors in a specific and regulated manner. We have synthesized substrates of CDC2 kinase that were used to purify this enzyme from natural sources. A systematic study was undertaken in order to assess the substrate specificity of cyclin-B/CDC2 isolated from human HeLa cells, using 13-15 residue peptides with a central histone-like KKSPKK motif as a model. Replacement of the proline residue by any of the other 19 amino acids or D-proline drastically reduces or abolishes phosphorylation by CDC2. Changing the basic residues to Ala on either side the of -SP- structure differentially reduces phosphorylation. Molecular modelling and dynamics simulation indicated that the phosphorylation site may have to adopt a

turn-like conformation that allows interaction with postulated binding surfaces within the CDC2 kinase active site. The substrate/active site models are used in the rational design of specific inhibitors to CDC2 and related kinases, in collaboration with Prof. J. Allende, University of Santiago, Chile.

Antibody/ Antigen Interactions

Semisynthetic combinatorial antibody libraries and phage display technology offer a route for producing high affinity human monoclonal antibodies without the need for immunization. In this approach, molecular diversity is generated in the complementary determining regions (CDRs), at the DNA level using chemical DNA synthesis. In the reported cases, the heavy chain (H) CDR3 was randomized in order that antibodies with altered specificity could be selected. Using a new strategy based on iterative randomization and chain-shuffling, we have isolated several human anti-hapten antibodies with moderate affinity, which differ from the original antibody in the light chain (L) CDR3 only. In this manner, the specificity of the original antibody molecule could be changed so that it now binds a small molecular hapten, fluorescein. Molecular modelling is being used to study the LCDR3 contribution to the hapten binding and to predict further structural modifications to obtain high affinity antibody combining sites. It appears however, that the binding of fluorescein to this altered antibody may be very similar to that found in a completely different antibody/fluorescein complex studied by X-ray crystallography, i.e. the *in vitro* selection strategy successfully reproduces antibody/hapten interactions. The success of the iterative randomization and chain-shuffling strategy shows that binding specificities can be built up in a successive application of randomization steps.

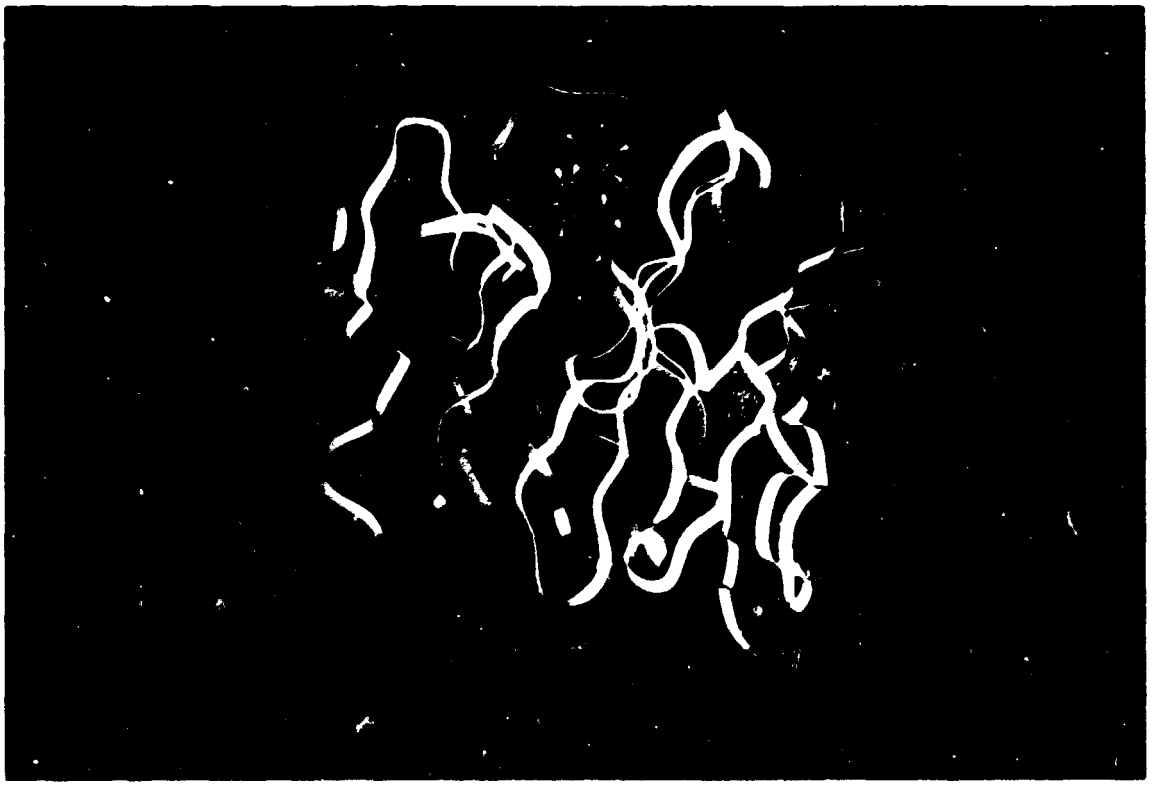


Figure 2.
Binding of an *in vitro* developed antibody variable region to a small molecular hapten, fluorescein (structural model developed by homology modelling and molecular dynamics refinement). The antigen binding loops of the light chain (L) and heavy chain (H) are coloured in yellow and green, respectively.

Figure 3.
Structure of a new type of α-amylase inhibitor (structural model developed by homology modelling based on sequence and disulphide connectivity data). The β-sheets are represented as arrows, the disulphide bridges are yellow.

The Structure of a New α-amylase Inhibitor

Enzyme inhibitors are important tools of nature used in regulating the activity of certain enzymes. Peptide inhibitors are often quite short in sequence and still have a well defined 3D structure, so they provide optimal targets for folding studies as well as for substrate/active site investigations. The best studied group of peptides are the protease inhibitors, relatively little is known about amylase inhibitors. In collaboration with Dr. A. Patthy, Agricultural Biotechnology Center, Gödöllö, Hungary and Dr. A. Blanco-Labra, CINVESTAV, Mexico, we have recently determined the sequence and the disulphide bridge topology of a new α-amylase inhibitor isolated from the Mexican crop plant, *Amaranthus hypochondriacus*. This small protein of 32 amino acids has a weak homology to members of the squash family protease inhibitors. Its 3 disulphide bridges determine a chain topology that is characteristic of the knottin family of small proteins, which makes it possible to build a knowledge based model of the peptide for future engineering experiments.

Solid-phase Peptide Synthesis of Insulin and its Analogues

Insulin is clearly the most important macromolecular drug in use today. Its complex mode of action is a challenge both to molecular biologists and synthetic chemists. The key to this process is the interaction of the hormone with its surface receptor which appears to be a dynamic mutually adaptive conformational change rather than a simple lock-and-key mechanism. Insulin/receptor interactions can be best studied with insulin analogues labelled with noncoded amino acids. Presently, the preparation of such analogues is hampered by the comparatively low yield of chemical insulin synthesis. In collaboration with Prof. Dietrich Brandenburg's group (Deutsches Wollforschunginstitute, Aachen, Germany), we have started the design and synthesis of insulin A-chain analogues for receptor binding studies. The preliminary results show that an optimization of the synthesis strategy

(selection of protecting groups and solvents) can substantially improve the yield of synthesis which allows the production of new insulin analogues for receptor interaction studies.

Structural Principles of Conformationally Restricted Peptides

Repetitive sequences are found in many non-globular proteins of interesting biological and physicochemical properties such as the immunoactive circumsporozoite protein of the malaria parasite or the adhesive and nucleic acid binding proteins of various organisms. A common property of these quite wide-spread repeats is that they contain a conformationally restricted amino acid, proline, at regular intervals. While in search of a general strategy for modelling of repeat sequences, in collaboration with Dr. V. Chauhan, ICGEB-New Delhi, we started to build repetitive model peptides containing various conformationally restricted amino acids such as dehydro alanine, dehydro-phenylalanine, etc. The strategy consists in generating a large number of repetitive conformations under various dielectric conditions and selecting those that correspond to specific chain symmetries and H-bonding patterns. In the case of poly-dehydroalanine we found two major conformer families, one corresponding to a regular 3/10 helix or type III turn, the other to an irregular conformation, $\Phi = -157$ to $-170°$, $\Psi = -1$ to $15°$ which however can be found in the i+2 position of gamma-turns. These data confirm that ΔAla may induce turn-like structures in peptides and also indicate that it may confer flexibility to the peptide chain.

Molecular Pathology

F. E. Baralle, E. Scodeller, R. Garcia, S. Tisminetzky, R. Tuteja, J. Aguiyi, M. Baralle, M. A. Bonder, E. Buratti, M. Caputi, Y. Chen, G. Devescovi, E. Donadel, E. Falasca, M. Gerotto, B. R. Guerra, A. Iaconcig, D. Kobal, A. F. Muro, V. Polonio, F. Porro, R. Pariyarathu, M. Romano, M. Schiappacassi, C. Serra, V. Stoyanova, A. F. Yenidunya, M. Valenti.

Human Molecular Genetics

Cystic Fibrosis (CF): We have continued our studies on the alternative spliced form of CFTR mRNA described in the 1992 Activity Report. In fact, CFTR36 is produced by the insertion of a differentially spliced exon carrying an in frame stop codon. This is a novel cellular mechanism alternative to RNA editing for the production of a protein that shares common sequences with another but has different properties and functions. The first step in establishing whether CFTR36 is a functional protein was to obtain cells which expressed it independently of other CFTR forms. We took advantage of the C127 cell system. The CFTR- C127, a mouse mammary epithelial cell line, was transfected with the CFTR36 cDNA (Melo, *et al.*, 1993) creating a cell line producing CFTR36 under the control of the SV40 promoter. Similar cell lines were obtained from Dr. A. Smith (Nature, 358, 761-764, 1992) producing CFTR100 and CFTR100 ΔF508. We have carried out immunofluorescence studies which show CFTR36 behaving very differently from CFTR100, in particular, accumulating in large intracytoplasmic vesicles and not reaching the cell membrane in significant amounts. We are now studying the ion channels present in the C127, C127 CFTR36 and C127 CFTR100 to see if we can detect differences due to CFTR36. A cell producing both CFTR 100 and CFTR 36 simultaneously is also being constructed.

We have continued to study the genetic defects in the CF patients of the Friuli-Venezia Giulia region and have set up, in collaboration with Dr. D. Faraguna of the Burlo Garofolo Istitute, a simplified screening method for the early diagnosis of CF. During this study a new polymorphism of the CFTR gene was found in normal subjects. It consists of an A to G transition in exon 19 at position 3662 that results in a conservative Lys to Arg substitution.

Gaucher Disease: We have continued our research on the molecular basis of this glycolipid storage disease and have found a new mutation caused by an A to G transition at position 5985 of the glucocerebrosidase gene (Tuteja, *et al.*, 1994). This mutation produces the replacement of a tyrosine to cysteine at position 418 of the protein. The Y418C mutation adds one more cysteine to the protein in this critical region and this change is likely to alter the folding of the protein and adversely affect its stability and/or enzymatic activity. It may also be possible that the Y418C mutant enzyme is poorly stimulated by a natural activator saposin C, as has been shown for another mutated enzyme R463C.

Eosinophile Peroxidase Deficiency: Eosinolphile peroxidase (EPO) is a highly basic haemoprotein, one of the most abundant proteins of the secondary granules of eosinophil granulocytes. In combination with H_2O_2 and a halide, EPO catalyzes the formation of hypohalous ions with strong cytotoxic activity against bacteria, parasites and eukaryotic cells and has the

ability to inactivate inflammatory mediators such as leukotriens. EPO can also act independently of its peroxidase activity by modulating the functions of other inflammatory cells such as mastcells, monocytes and neutrophils. The study of an EPO-deficient subject and his family has yielded interesting results. The subject lacks the enzymatic and immunochemical characteristic of EPO. Analysis of cDNA obtained from eosinophil precursors derived from blood progenitor cells reveals a compound heterozygosity for the defect with mutations consisting in a base transition leading to an amino acid substitution (Arg 286 → His) and an insertion that, by shifting the reading frame, generates a stop codon resulting in a truncated protein. The Arg 28 His

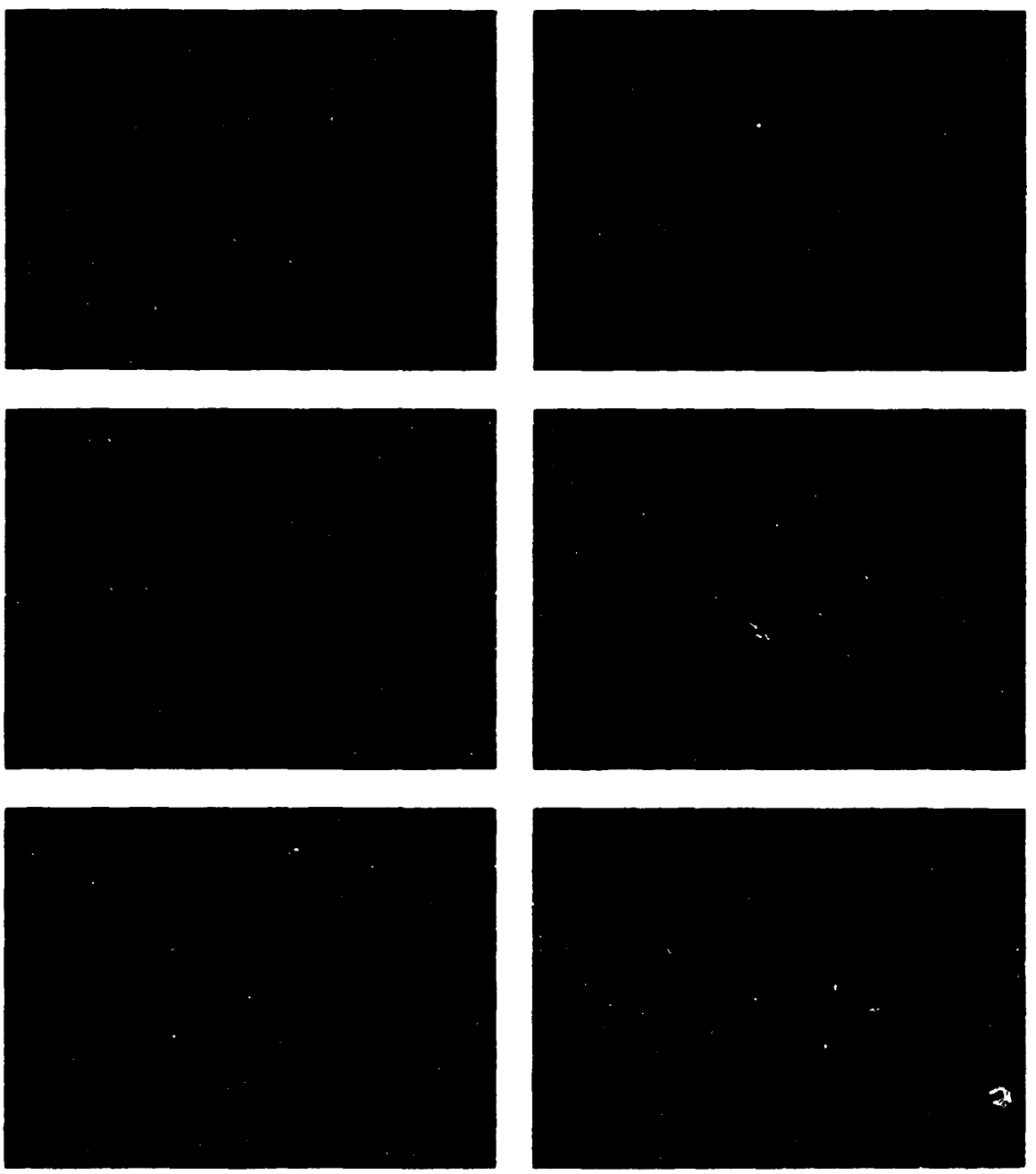


Figure 1.
The peroxidase cytochemistry of eosinophile precursors at different times of culture. The precursors from the normal subject (left column) were homogeneously reactive for peroxidase with a high degree of staining throughout the observation time (from top to bottom days 13, 21 and 27). The precursors from the EPO-deficient subject (right column) were initially less positive than their normal counterparts and displayed some heterogeneity in cell staining. This heterogeneity increased from day 13 to 21 and was particularly evident at day 27 of culture (right, bottom) when cells almost totally negative for peroxidase cytochemistry could be observed.

mutation alters the heme environment and affects the function and stability of the enzyme. This explains the presence of EPO activity in the early precursors of the subject and its total absence upon maturation (Figure 1). Biochemical and genetic studies in the family members are compatible with an autosomal recessive mode of transmission of the defect.

Dyslipidemia: We are proceeding with our genetic studies on families with derangement in the lipoprotein metabolism (ICGEB Activity Reports 1991-1992). A large prospective study in collaboration with the local hospitals is under way. The results of this study are expected towards the end of 1994.

We have also proceeded with the construction of transgenic mice and rabbits bearing Apolipoprotein gene mutations. These animal models will be useful for studing the complex interactions involved in maintaining normal plasma lipid levels.

Molecular Biology of Hypertension

The Milan hypertensive strain of rats (MHS) develops a genetic form of renal hypertension which, when compared to its normotensive control (MNS), shows renal dysfunction similar to that of a subset of human patients with primary hypertension. MHS and MNS were shown to be homozygous by multilocus minisatellite analysis and monolocus microsatellite markers. We have now definitively shown (Bianchi, *et al.*, 1994) that one point mutations in each of two genes coding for the membrane skeleton protein adducin are associated with blood pressure in the Milan strain of rats. Adducin is a heterodimer formed by α and β subunits which promotes the assembly of actin with spectrin (ICGEB Activity Reports 1991-1992). MHS and MNS differ respectively with the amino acids tyrosine (Y) and phenylalanine (F) at position 316 of the α subunit. In the β adducin locus, MHS is always homozygous for arginine (R) at position 529, whilst in MNS either R or Q occur in that position. The R/Q heterozygotes showed lower blood pressure than any of the homozygotes. *In vitro* phosphorylation studies suggest that both of these amino acid substitutions occur within protein kinase recognition sites. Analysis of an F_2 generation demonstrated that tyrosine (Y) alleles segregated with a significant increment in blood pressure. This effect is modulated by the presence of the arginine (R) allele of the β subunit. Taken together, these findings strongly support a role for Adducin polymorphisms in causing variation of blood pressure between the MNS and MHS strains.

We are currently studying the mechanisms by which the lack of phosphorylation in both the α and β adducin chains affect cellular functions such as Na/K cotransport or cytoskeleton organization.

Molecular Biology of the Extracellular Matrix

Fibronectin (FN): This important component of the plasma and extracellular matrix has multiple functions in cell-cell and cell-matrix interactions. Some of its functions are modulated by specific FN isoform synthesis and these polymorphisms are in turn generated by cell specific alternative splicing of a primary transcript derived from a single gene. We have previously investigated the splicing patterns of these regions during development and aging in rats (ICGEB Activity Reports 1991-1992). We have now addressed the question of the mechanism involved in this regulation, particularly in the EDA exon.

EDA is a facultative type III homology of human fibronectin encoded by an alternative spliced exon. The EDA+ and EDA- mRNA forms show a cell type specific distribution with their relative proportion varying during development, aging and oncogenic transformation. We have previously demonstrated that an 81 bp nucleotide sequence within the exon itself is essential for differential RNA processing. Fine mapping of *cis*-acting elements within this region has been carried out to identify possible target sites for the

modulation of alternative splicing. There are at least two short nucleotide sequences involved. Element A (GAAGAAGA) is a positive modulator for the recognition of the exon, its deletion results in constitutive exclusion of the EDA exon. Element B (CAAGG) is a negative modulator for exon recognition, its deletion results in constitutive inclusion of the EDA exon. This bipartite structure of the splicing enhancer is a novel feature of the mammalian exons (Caputi, Melo and Baralle, 1994).

Infectious Diseases

Vaccine Development Programme: Production of recombinant proteins carrying Human Immunodeficiency Virus-type I (HIV-1) specific sequences: The principal neutralizing determinant (PND) of HIV-1 lies within the loop forming the third hypervariable region (V3 loop) of the glycoprotein gp120. The relationship between titer of anti V3 loop in immunized animals or human vaccinees and virus neutralization capacity is already well established. This region has been originally defined as hypervariable. However, recently, further analysis of a large number of isolates found two PND consensus (IGPGRA and GPGRAF) that appeared to be present in approximately 60% of the viruses isolated in North America. This sequence seems to be extremely important in the antigenic structure of the V3 loop. In fact, human monoclonal antibodies (HumAb) displaying a broad neutralization activity against several divergent strains of HIV-1 bind to this region. A seven amino acid sequence (IGPGRAF) from this region was genetically inserted in to two loops of the protein that make up the capsid shell of the insect Flock House Virus (FHV). This is a carrier system which we have recently developed aimed at locating immunogenic epitopes on the surface of viruses or virus-like particles. The positions for the insertions were selected based on the known 3-D structure of the viral particles and they are expected to expose the foreign sequences at the protein surface. The hybrid proteins were expressed in insect cells by recombinant baculoviruses. Three different hybrids were used as immunogens; two of these presented a single copy of the insert, each at different positions of the carrier; the third hybrid carried two copies of the insert in the same molecule, at the positions used for the single ones. All hybrid proteins induced a strong specific response in immunized animals, as evaluated in Western blot against recombinant HIV-1 gp160. The sera of animals immunized with either antigen also showed a broad specificity for different V3 loop sequences and they were able to recognize peptides representing the PND of very divergent HIV-1 isolates by ELISA. However, only one of these immunogens was able to induce a strong neutralizing response against HIV-1 (MN and IIIB isolates) in immunized guinea pigs. Our results show that a very small sequence of HIV-1 can constitute a valuable immunogen able to induce a strong neutralizing response if properly presented to the immune system.

Production of FHV-rotavirus hybrids: FHV chimeric proteins carrying 20 amino acid long epitopes of the rotavirus protein VP4 (trypsine cleavage site) are also being evaluated as immunogens. Hybrids were produced in bacteria as well as in insect cells by recombinant baculoviruses and were used to inoculate guinea pigs. In all cases the immunized animals showed a strong response specific for VP4.

Production of FHV-HCV hybrids: FHV chimeric proteins carrying HCV sequences were also produced. We inserted, the sequence encompassing amino acid 1 to 20 and 21 to 40 of the HCV core protein, in several positions of our carrier system. It is known that in this region the strong immunodominant epitopes responsible for the seroconvertion in most infected humans reside. These proteins, produced and purified from *E. coli*, are now being tested for their capacity to recognize specifical sera from infected patients in Western and ELISA assays.

Genotypes of hepatitis C virus in Italian patients with chronic hepatitis C and response to Interferon therapy: Hepatitis C virus (HCV) was clearly established as the major etiological agent of post-transfusion non-A non-B hepatitis. HCV is a positive stranded RNA virus with a linear genome comprising approximately 9,400 nucleotides. A 5' noncoding region (NC) precedes the large coding sequence and represents the region of choice for the detection of HCV-RNA by polymerase chain reaction (PCR). Analyses of different isolates have led to the identification of significant nucleotide variation, thus establishing the existence of distinct genotypes of HCV. To assess the prevalence of different HCV genotypes in our patients with chronic hepatitis C, we amplified and sequenced part of the 5'NC of the virus in 21 patients. On the basis of the data obtained, oligonucleotide probes were designed to be used in a more rapid dot blot genotyping test. Using this method, 79 consecutive patients were studied, 42% of them were found to be infected by HCV type 1, 45% by HCV type 2 and 4% by HCV type 3. Patients infected by HCV type 3 were significantly younger and had a milder form of liver disease, compared to those infected by HCV type 1 or type 2. Regarding the response to interferon therapy, complete transaminase normalization during treatment was observed in 26% of patients with HCV type 1, 80% of those with HCV type 2 and in 100% of those with HCV type 3, thus confirming that the genotype of HCV influences the response, as well as, identifying in HCV type 3 a strain of HCV which is highly sensitive to interferon. Our results also provide information on the prevalence of different HCV genotypes in Italian patients and indicate the usefulness of the dot blot hybridization procedure for a rapid screening of HCV genotypes (Tisminetzky, *et al.*, 1994).

Host defence and inflammatory activity of human phagocytes: The involvement of cytosolic proteins in the exocytosis of the different granule populations of human neutrophils has been studied using a cell-free system. Proteins that could participate in this process have been shown to translocate from cytosol to isolated primary and secondary granules of different density in a calcium-dependent manner. Three of these proteins, of molecular weights 32, 35 and 68 kDa, have been immunochemically characterized as annexin IV, I and VI, respectively. They belong to a family of calcium- and phospholipid-binding proteins whose specificity of action is determined by their amino-terminal end. We have found that these proteins have different affinities for primary and secondary granules, as well as different calcium dose responses regarding their translocation. These findings could have implications concerning the well known differences in the response of primary and secondary granules to cell stimulation.

Molecular Immunology

O.Burrone, M.Bestagno, I.Afrikanova, F.Batista, D.Efremov, S. Giambiagi, I. Gonzalez-Rodriguez, E. Q. Li, S. Mancardi, A. Pedraza, S. Perissutti, M. S. Sun, T. Tkach.

Recombinant Monoclonal Antibodies

Monoclonal antibodies specifically directed against human tumours are powerful tools to study and characterize relevant antigens on cancer cells and they could be extensively applied in diagnosis and therapy. Their use in humans is, however, limited by the rapid raising of human anti-mouse antibodies (HAMA) in treated patients, because of their murine origin.

This project is aimed at developing chimeric antibodies (Figure 1) which combine the mouse antigen-specific variable regions (V_H and V_L) with the human constant regions of both heavy and light chains (C_H and C_L).

New recombinant proteins are at present also under investigation, utilizing various arrangements of the immunoglobulin domains, which can lead to the development of new molecular tools of defined specificities for clinical use.

In collaboration with Dr. José Mordoh, from the Campomar Foundation, Buenos Aires, Argentina, we obtained the complete chimeric genes for both heavy and light chains from the monoclonal antibody FC-2.15 , specific for a tumour membrane antigen. A different monoclonal antibody whose

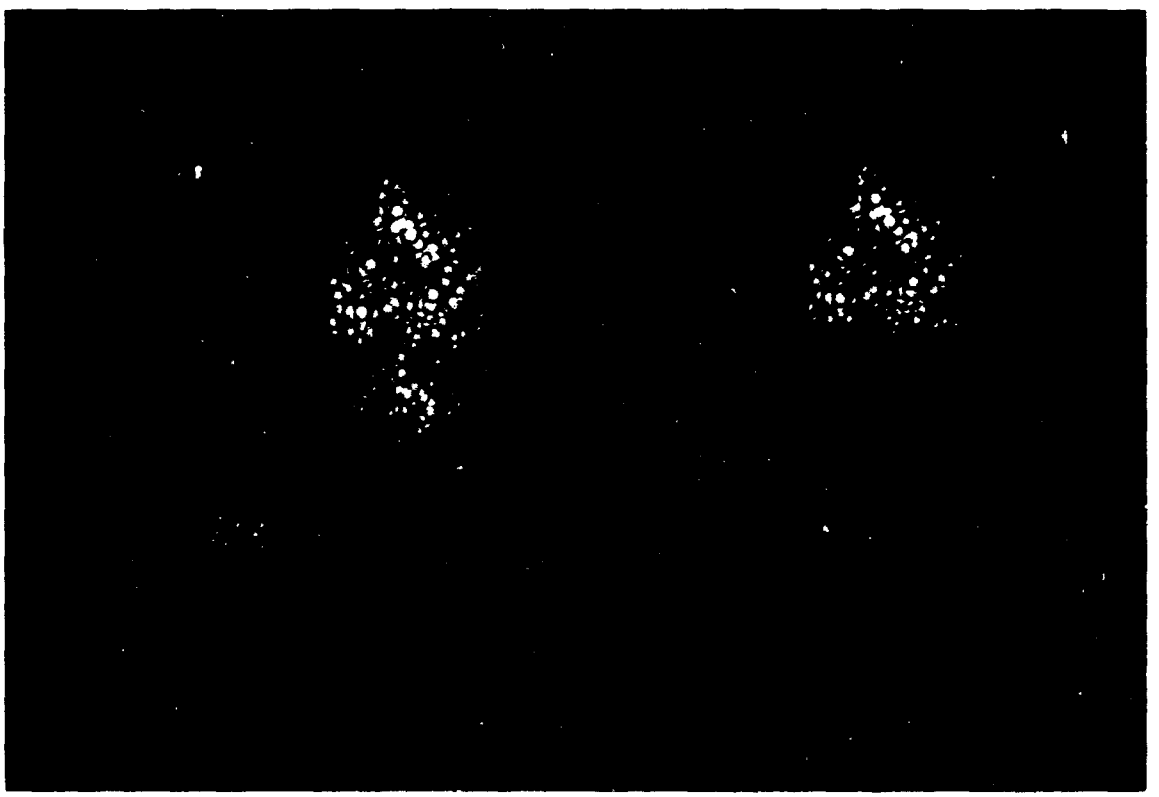


Figure 1.
Three dimensional representation of a mouse Immunoglobulin Fab fragment (left) and the corresponding chimeric V mouse/C human Fab molecule (right). Dark colours correspond to H chains and light colours to L chains. In both cases the mouse sequences are in green and the human sequences in blue.

chimerization has been successfully achieved is the 6C6 antibody, which is specific for a human breast cancer surface antigen, originally developed by Dr. Er Qiu Li from Peking University, China.

Reliable protocols were developed for the expression of functional chimeric antibody molecules in both CHO and SP2/0 cells. We have established conditions to obtain transfectants of antibody producing clones which are able to secrete up to 90 µg/ml of the chimeric antibody protein.

Immunofluorescence studies in different positive and negative cell lines and Western blotting analysis on membrane extracts performed with these two antitumour specific antibodies showed that the pattern of reactivity of the chimeric hFC-2.15 and h6C6 corresponded in both cases to that of the original murine antibody. At present, their *in vivo* activity is being investigated

A third monoclonal antibody (IORT-1), directed against the CD6 T cell surface marker (MW ~90-100 kDa) has also been humanized. This antibody, originally developed by Dr. Jorge Gavilondo (Centre for Genetic Engineering, Havana, Cuba), will be tested for its activity in the treatment of cutaneous T cell lymphoma.

The high specificity showed by the 6C6 antibody for a 30 kd protein, found in membrane extracts of several cell lines, allowed us to successfully screen a λgt11 cDNA expression library and to isolate clones in order to identify the 6C6 antigen; such clones are at present being characterized.

Analysis of Human IgE Expression

Allergy is the most common environmental disease affecting more than 20% of the world population. It is mediated by antibodies of the Immunoglobulin E (IgE) class which bind to the high affinity receptors (FcεRI) on mast cells and trigger them to release mediators of the acute inflammatory response upon contact with allergen. Despite the significant biological effect of IgE, its serum levels are three to four orders of magnitude lower than the other Ig families. Elevated levels can be encountered in atopic individuals and during certain parasitic infections in which IgE antibodies play a major role in the host defence. IgE-secreting B cells are abundant in the main sites of parasitic invasion, such as the skin, lungs and gut, but can also be detected at extremely low frequencies among peripheral blood lymphocytes (PBL).

We have recently developed a sensitive RT/PCR procedure with which we investigated the IgE heavy chain repertoire expressed by PBL in several normal and atopic individuals. Nucleotide sequence analysis of ε VH and CDR3/FW4 regions showed that the IgE-producing B cell clones undergo somatic mutation, as expected for lymphocytes involved in secondary immune responses. Furthermore, particular ε CDR3/FW4 regions were found to be shared by µ and γ4 transcripts in the same individual, suggesting that a common IgM expressing B cell precursor gives rise to B cell populations that express IgE and IgG4. This last finding could point to a possible mechanism for modulating the allergic response since antibodies of the IgG4 subclass have recently been shown to act as blocking antibodies in certain patients with chronic helminthic infections which, despite high levels of parasite-specific serum IgE, do not manifest allergic reactions.

Further work in our laboratory focused on the possible differences in the effector functions of the various ε transcripts. A number of alternatively spliced ε transcripts, differing in their 3' ends, have been identified in an IgE secreting myeloma cell line (U266) by several investigators. Our analysis of the ε transcripts expressed by PBL and also the U266 cell line identified most of these forms, together with the classical secreted and membrane RNA molecules and several yet undescribed forms, lacking different parts or the complete ε exon 4 (CH4). The different forms identified in the PBL were further investigated by constructing mouse/human chimeric antibodies

containing a mouse VH segment with anti-NIP affinity and the different human ε constant regions. The constructs were expressed in a mouse myeloma cell line (J558L) which produces only the light chain of the anti-NIP antibody. Pulse-chase labelling experiments and immunofluorescence analysis showed that the alternatively spliced forms are retained and degraded in the endoplasmatic reticulum while only the classical secreted form was compartmentalized in the Golgi and subsequently secreted. The forms with partial and total deletions of the CH4 domain were unable to form dimers confirming an important role for this domain in establishing the tertiary structure of the IgE molecule. These data demonstrated that the classical secreted and membrane-bound IgE are the only completely processed IgE molecules, while the other isoforms appear to be products of aberrant splicing which are elminated by post-transcriptional events.

Future studies will attempt to characterize the human IgE repertoire affinity by constructing combinatorial libraries with IgE specificity from PBL of normal and atopic individuals. The Fv portion of the IgE antibodies will be expressed on the surface of filamentous phages to allow for the characterization of the specific V region gene usage and to the precise mapping of the dominant epitopes of recombinant allergens.

Molecular Analysis of Ig V-Gene Usage in B-cell Neoplasms

Molecular analysis of the immunoglobulin (Ig) variable region gene segments has provided important insights into the normal B cell repertoire and in its malignant counterparts. The restricted usage of particular heavy chain variable region (V_H) gene segments has been documented for the human fetal repertoire among human autoantibodies, and for certain lymphoid neoplasms such as chronic lymphocytic leukaemia (CLL). The recent sequence characterization of a large number of human germ-line V_H gene segments has enabled the analysis of the level of somatic mutation in particular antibodies. Studies pertaining to the immunoglobulin expressed by different B-cell malignancies have shown an absence of somatic mutation in B-cell acute lymphoblastic leukaemia (B-ALL) and CLL. On the other hand, somatic mutation has been identified to different extents in follicular lymphoma, hairy cell leukaemia and multiple myeloma. Furthermore, intraclonal variation was observed among the sequences of V_H gene segments obtained from follicular lymphoma and hairy cell leukaemia samples. Together these data have given further insight to the stage of B-lymphoid development in which the malignant conversion has taken place and, in the case of follicular lymphoma, have suggested a role for antigen stimulation in the clonal evolution of the disease.

The V_H and V_L segments of the Ig expressed by the malignant B-cell clone in patients with Waldenstrom's macroglobulinemia (a rare B-lymphoplasmacytic neoplasm manifested by a monoclonal IgM in the plasma) are currently being investigated in our laboratory. This study should provide information regarding clonality, possible somatic mutation and intraclonal variation and address the question of antigen stimulation in the pathogenesis of the disease. Comparable data will also be obtained for mixed cryoglobulinemia, another lymphoproliferative disorder also characterized by the production of a monoclonal rheumatoid factor. A high frequency of hepatitis C virus infection has recently been documented for both diseases. Studies designed to investigate a possible etiopathogenetic role for the hepatitis C virus in these disorders are in progress.

Metastatic Genes

The transition of a tumour cell from non-metastatic to metastatic is a complex phenomenon that involves a variety of genes. We have focused our studies on the characterization of those genes that participate in this transformation, using mouse tumour cell lines with characteristic phenotypes

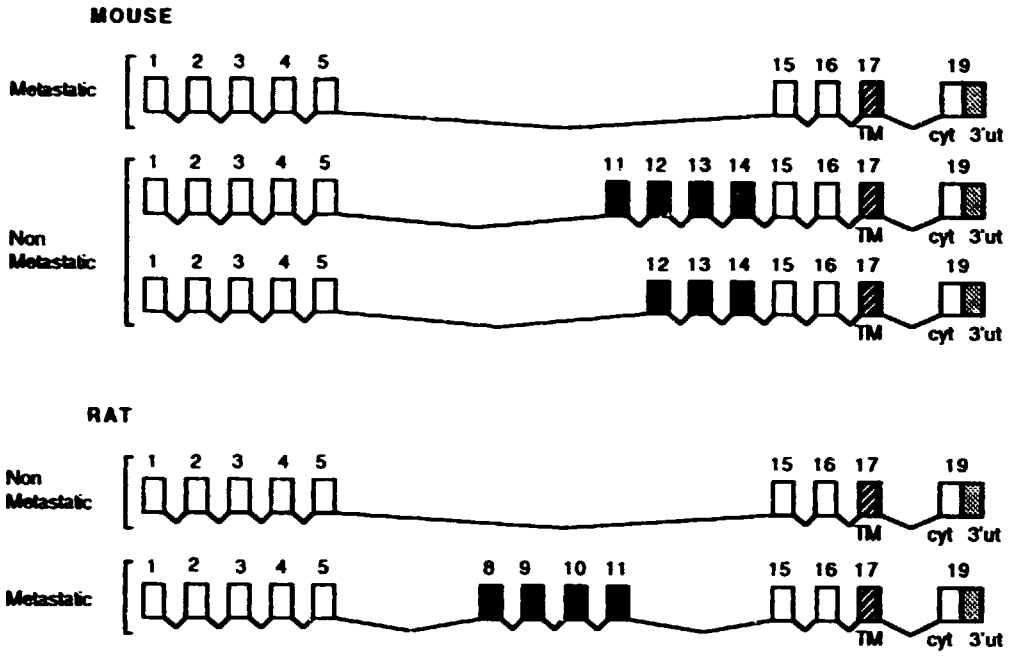


Figure 2.
Different alternative spliced mRNAs of the CD44 gene are associated to the metastatic and non-metastatic phenotypes in mouse and rat tumours. The isoforms are schematically represented with the exons that constitute the mature transcripts; alternative exons are shown as shadowed boxes.

of metastatic behaviour.

Recently we have found that the CD44 gene (which codes for a surface transmembrane protein) is differentially spliced in metastatic and non-metastatic cells. Variants of CD44 which include exons 11 to 14 and 12 to 14 were found to be associated to the non-metastatic phenotype (Figure 2), thus suggesting that some of the domains associated to those exons play an important role as retention elements when interacting with specific components of the extracellular matrix and when signalling cells not to migrate.

Other genes also described as been involved in the metastatic phenotype, such as nm23 and TIMP1, were found in our studies not to correlate with the metastatic characteristics of many different cell lines.

The metastasin gene (mts1) has also been associated to this process. Its expression is found to greatly increase in metastatic cells. We have recently found a cell line with extremely low levels of mts that is, nevertheless, metastatic. However, since cells derived from these metastatic foci were positive for mts1, we suggest that activation of this gene can be essential for the establishment and development of metastatic foci in the lung.

Molecular Biology of Rotavirus

Among the infectious agents that produce diarrhoea, rotavirus represents the most extended viral infection affecting millions of children every year. Rotaviruses are widely spread and can infect a number of species of mammals and birds. They are non-enveloped viruses that belong to the reoviridiae family and have a segmented genome composed of 11 segments of double stranded RNA. Some of the viral non structural proteins, which are only found in virus infected cells, are not yet fully characterized and their functions are still unknown.

The rotavirus project focuses on two main aspects: a) the biology of rotavirus replication; and, b) the development of experimental rotavirus vaccines.

a) With the aim of obtaining recombinant rotaviral particles carrying

exogenous genes, we have constructed a recombinant plasmid that transcribed *in vitro* with T3 polymerase, yields a positive sense RNA that, when contains the rotavirus gene 11 5' and 3' untranslated regions, which flank the coding sequence of the CAT reporter gene. It was found that this RNA was greatly amplified, only when transfected into the cytoplasm of rotavirus infected cells. In accordance with the conservative mode of replication of rotavirus gene segments, we have demonstrated that only the RNA positive strand - and not the negative strand nor the double stranded - could enter into the virus replicative complex and become amplified. It was also possible to establish that the last 25 nucleotides of the 3' end sequence of the transfecting RNA were essential to obtain amplification of the CAT activity.

Further studies on the packaging of the foreign gene are at present being carried out. This will open the possibility of investigating the effect of specific modifications to the different viral proteins and the relation to their functions as well as performing detailed analysis of the different signals for transcription, replication and packaging and a better understanding of the mechanisms underlying such processes. These studies may also prove to be useful for the development of new and safe rotavirus vaccines.

b) The two main components of rotavirus outer shell, VP7 and VP4, are the proteins against which neutralizing antibodies can be raised. Several studies have shown that VP4 is an efficient target for neutralizing antibodies. We have initiated a project for the development of rotavirus recombinant vaccines through different approaches. One of our strategies is concerned with the expression of either the complete VP4 molecule or of its most exposed amino terminus in Adenovirus expression vectors which direct intramuscular immunization with plasmid DNA. Since membrane expression of the outer shell protein VP7 has proven to be very efficient in augmenting its immunogenicity, we are at present investigating the expression of different soluble and membrane forms of the VP4 protein, with an aim to obtaining high expression levels and to compare their capacity to induce neutralizing immunity. For this purpose, different genes have been constructed, in which appropriate signal secretion and transmembrane domains have been introduced next to the rotavirus SA11 VP4 gene in order to investigate the feasibility of its expression as an integral membrane protein. The different VP4 chimeric genes were used to immunize guinea pigs by DNA injection. One particular construct containing almost 80% of the normal VP4 upstream of a transmembrane domain resulted in the efficient presentation and induction of anti VP4 antibodies, which were detected by ELISA and by virus neutralization tests. Further studies on the characteristic of the immune response obtained by DNA immunization are at present in progress. The adenovirus expression vectors will also allow to coexpress VP4 and VP7 in the same cell, thus enlarging the repertoire of possible virus neutralizing epitopes.

Other Collaborations

Vibrio Cholerae: The argentine outbreak of cholera infection which started in 1992 was studied in collaboration with Drs. Rodolfo Ugalde and Carlos Frasch, from the Campomar Foundation, Buenos Aires, Argentina. A rapid and reliable PCR method for the characterization of *V. Cholerae* toxigenic strains was established. The development of the detection test of both *ctx* A1 and *ctx* A2-B regions allowed for a clear characterization of particular strains carrying toxigenic genes. The two region amplification procedure was found to be necessary to obtain complete correlation with the current immunological GM1-ELISA test. These studies should prove useful in controlling the incidence and spread of toxigenic variants.

Complement Component C8: The complete C8 molecule is composed of three polypeptides α, β and γ of which α and γ are covalently linked through

a disulfide bridge. In collaboration with Dr. F. Tedesco from the Department of Pathology, University of Trieste, Italy we have initiated biochemical studies regarding the mechanism of assembly and secretion of the individual chains and the molecular analysis of C8β deficiency in affected patients. Deficiency of the C8 component of complement is associated to the high incidence of repetitive meningitis. Two types of C8 deficiency have been described: the C8α-γ and the C8β deficiencies. We have been able to detect, in both normal and affected individuals a splice variant of the C8b mRNA leading to the coding of a truncated protein of a yet unknown function. At present we are attempting to characterize a particular mutated C8β allele associated with an Italian family.

β-Thalassemia: A study of the factors affecting the Hb F levels in β-thalassemia was undertaken in collaboration with the Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, GA, USA and the Research Center for New Technologies, Macedonian Academy of Sciences and Arts, Skopje, F.Y.R. Macedonia. β-Thalassemia is the most common single gene disorder worldwide, especially frequent in developing countries, where it causes a major health problem due to the continuous blood transfusion requirements of most patients. In a subset of patients, elevated levels of HbF which can substantially ameliorate the severity of the disease are present. To further investigate the factors that can lead to increased HbF production, we developed a competitive RT/PCR procedure with which we determined the relative amounts of the different globin mRNAs in β-thalassemia patients and in other individuals with elevated HbF levels. This procedure could also prove useful in monitoring the effects of the pharmacological manipulation of Hb F production in patients with β-thalassemia and sickle cell disease.

Research Programme: The New Delhi Component

Mammalian Biology: Virology

S. Jameel, M. Ahmad, B. Ganesh, R. Kumar, V. Kumar, V. Manivel, M. H. Ozdener, K. V. S. Rao, J. Roca, L. Vijayakrishnan, K. T. Yildirimkaya, M. Zafrullah.

Viral hepatitis represents one of the most common infectious diseases in man. The family of viruses that cause acute and chronic hepatitis in humans now includes five members: hepatitis A, B, C, D and E viruses (HAV, HBV, HCV, HDV and HEV). While HAV and HEV are transmitted feco-orally as water-borne infections and lead to self-limited disease, HBV and HCV are blood-borne and lead to chronic carrier states in a significant proportion of infected individuals. For HBV alone, there are an estimated 300 million carriers and 300,000 infections annually around the world. On the other hand, HEV causes rampant sporadic and epidemic disease in countries with a low socio-economic status and, in terms of numbers, globally represents the most common form of viral hepatitis.

The problems of viral hepatitis are not just limited to the infections as fairly serious post-infective sequelae are known to occur resulting in significant mortality rates. Examples of these are liver cancers in HBV and HCV carriers, fulminant liver failure during HBV and HEV infections, etc. The inability to culture hepatitis viruses has precluded detailed virological studies but the application of molecular biological methods has circumvented some of these problems. The Virology Programme at ICGEB, New Delhi is focused on studying the molecular biology and immunology of these viruses.

Hepatitis B Virus

Here, our aim is to understand the molecular basis of HBV-mediated disease and to develop a molecular vaccine against HBV infection.

We are studying the role of HBV protein X in disease pathogenesis. Protein X has been implicated in HBV-related liver injury and primary liver cancers but the details of its action and its interactions with the cellular network are poorly understood.

In order to study the role of HBV protein X in the transcriptional activation of viral and cellular genes and to identify the cellular proteins with which it interacts, X fusions were generated with the glutathione-S-transferase gene. These proteins expressed in *E. coli* have now been purified to homogeneity. The transcription-activating domain of X is being mapped by means of transfection experiments employing a battery of X mutants.

A strain of *Saccharomyces cerevisiae* expressing the HBV major envelope protein, the hepatitis B surface antigen (HBsAg), has been developed. This recombinant HBsAg, the basis for a hepatitis B vaccine, is being pursued as one of the commercial products of ICGEB.

Studies in Polyvalent Vaccine Design

Our aim is to examine strategies for the development of epitope-based vaccines. Taking the hepatitis B virus as a model, initial studies focused on generating a synthetic peptide mimetic of the group-specific conformational epitope present on the surface antigen (HBsAg). We were successful in

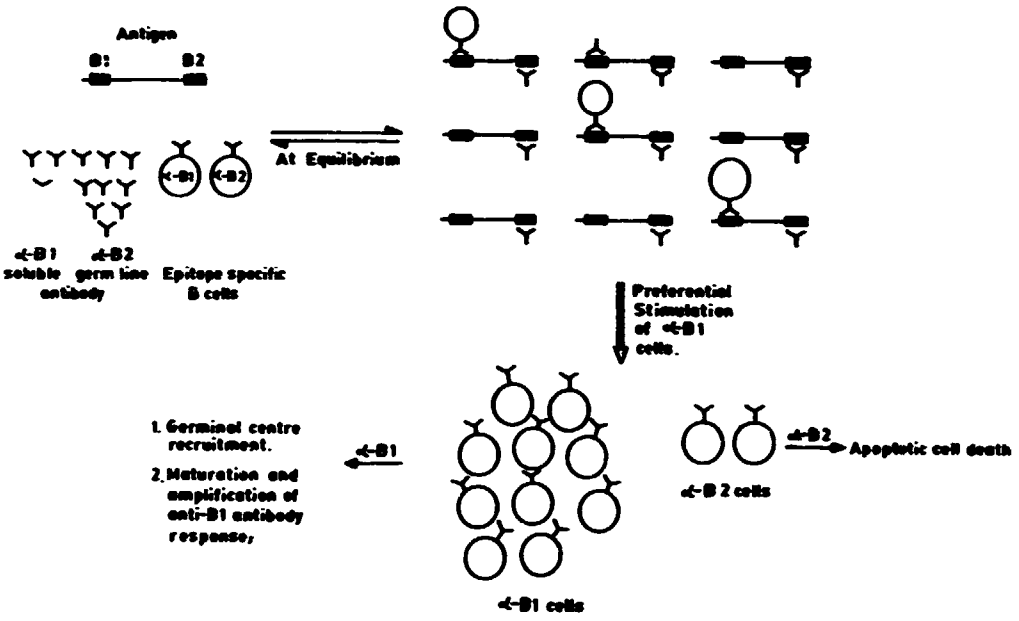


Figure 1. **Etiology of relative intramolecular immunodominance.**
A scheme for germ-line antibody mediated regulation of immunodominance between two B cell epitopes (B1 and B2) on an antigen is presented. Epitope B2 elicits high affinity (and therefore higher levels) of antibodies which subsequently compete successfully with B2 epitope-specific B cell for antigen recognition. This leads to an attenuation of anti-B2 antibody responses. In contrast, germ-line antibodies to B1 are of lower affinity as a result of which free epitope on antigen is always available for B1-specific B cell recognition, antigen capture, processing and presentation to Th cells. The outcome of these interactions is successful maturation and amplification of anti-B1 humoral responses.

achieving this objective and, in subsequent studies, we obtained a lipidated derivative that was highly immunogenic. Next we considered the possibility of linking this sequence with other functionally relevant domains of HBsAg. We anticipated that this would prove a useful model to evaluate the various structural and immunological parameters that need to be considered in the design of poly-epitope vaccines. For this a gene coding for a multiple epitope polypeptide (MEP-1) that included the above sequence, in addition to other selected immunoprotective regions of HBsAg, was synthesized and expressed in *E. coli*. With chemically induced disulfide-rearrangement we were able to regenerate the above described conformational epitope in the context of MEP-1. A preliminary analysis indicated that MEP-1 possessed a number of characteristics requisite for a potential vaccine. It was highly immunogenic and immunogenicity was genetically non-restricted. Antibodies were of the desired fine-specificities and did not include sub-populations that were directed against inter-epitope junctions. Finally the HBV-derived T helper cell epitopes were all found to be functional within the context of MEP-1 in the murine model.

However, in spite of the positive features described above, a major drawback became evident when we quantitated the relative proportion of murine antibodies against the various HBV-derived determinants on MEP-1. A hierarchy of immunodominance within the different sub-regions was observed as pS1>S>pS2. This deviated from the idealized situation where one would expect co-dominant antibody responses. We therefore proceeded to examine the etiology of this relative intramolecular immunodominance. A detailed investigation revealed that selective immunodominance was established as a consequence of maturation of the primary humoral response. This eventuality correlated with the inability of B cells specific for some of these epitopes to interact productively with T helper cells, thereby receiving reduced help. This interaction between epitope-specific B cells and antigen-

experienced T helper cells was attenuated in the presence of early primary anti-MEP-1 antiserum and the extent of inhibition was directly proportional to level and affinity of epitope-specific immunoglobulins. These studies identified the germ-line humoral response to an antigen as a major player in the etiology of relative intramolecular immunodominance of B cell epitopes (Figure 1). Subsequently we were able to resolve this problem by altering the mode of antigen delivery. A panel of nine mutants of MEP-1 was generated where the immunodominant epitope was selectively modified. The influence of these modifications on the hierarchy of B cell immunodominance is currently being investigated.

More recently we have also been examining the "naive" murine B cell repertoire against the immunodominant epitope on MEP-1. The outcome of these studies indicate that the virgin B cells are relatively degenerate with respect to epitope-recognition. This degeneracy appears to be due to the presence of B cell subpopulations which recognize antigen through contact with a single amino acid side-chain.

An immunogen vector is being developed to direct the bacterial expression of a small peptide, enabling high-titer antibody production without the need for chemical coupling to a carrier protein. Two forms of the vector, for intracellular or periplasmic localization of the expressed protein, have been developed. As a test case, one B cell epitope from the HBV pre-S1 region has been expressed. Immunological characterization of this is in progress.

Hepatitis E Virus

Hepatitis E virus (HEV) has been identified as the likely cause of enteric non-A, non-B hepatitis. It is maintained in the population as sporadic infections, to break out occasionally into large-scale water-borne epidemics. Such epidemics have been reported from all parts of the world. In India, for

Figure 2. **The rhesus monkey model for HEV transmission.**
Hepatitis E virus (A) present in the stools of an enteric non-A, non-B hepatitis patient was inoculated into rhesus monkeys (B) to produce a characteristic rise in serum aminotransferases. This model was validated by demonstration of HEV replication in monkey liver (C). A strand-specific PCR strategy was used to demonstrate the presence of HEV negative-stranded RNA, the replicative intermediate for the viral positive-stranded genomic RNA. Part C: lanes 1-4, uninfected control animal; lanes 5-8, infected animal; detection of positive-stranded RNA (lanes 1, 3, 5, 7) or negative-stranded RNA (lanes 2, 4, 6, 8) was carried out in the presence (lanes 1, 2, 5, 6) or absence (3, 4, 7, 8) of reverse transcriptase.

example, HEV infections contribute to about 50% of the total acute viral hepatitis cases.

Our aim is to clone the genome of the Indian isolate of HEV for the development of recombinant diagnostics and vaccines and to study disease pathogenesis in humans and model systems.

To aid in the cloning of HEV, an animal model was set up in rhesus monkeys (Figure 2). This has now been validated with respect to the replication of HEV in monkey liver as well as with the development of an IgM anti-HEV response.

The HEV genome is being cloned by means of multiple overlapping PCR. Using this strategy the entire genome has been amplified. The cloning, sequencing and assembly of the clones is currently in progress. Parts of the genome, especially the structural region, have also been expressed in *E. coli* to aid in the development of diagnostic systems.

A synthetic peptide-based IgM anti-HEV assay that faithfully detects an acute HEV infection has been developed. Based on the testing of nearly 2,000 samples of infected and normal sera from India and other parts of the world, the test shows a specificity of 98% and a sensitivity of 92%. Attempts are being made to commercialize this test.

In a human volunteer study of HEV transmission (ICGEB Activity Report 1992) we had observed HEV serum viremia early in infection and had also suggested possible parenteral transmission, as well as the feco-oral transmission, in endemic areas. As an extension of that study, we have now correlated HEV serum viremia and the anti-HEV response in a number of patients with sporadic and epidemic HEV infections. Besides confirming our earlier observations, this study shows, for the first time, the presence of a protracted viremia in about 10% of the patients. Such patients may represent the reservoir of HEV in endemic areas.

Prior to the development of a vaccine against HEV, the correlates of immune protection must be defined. Our studies involving patients from a large HEV epidemic in 1978 suggest that the IgG anti-HEV response is long-lasting. In experiments with the rhesus monkey model, we have also observed complete protection from challenge up to 6 months after recovery. Longer-term protection experiments are in progress.

Genetic Variation in Viral Populations

Genetic variation among viruses has important implications for the development of diagnostics, therapeutics and vaccines against these agents. It is being increasingly realized that local strains and field isolates have to be evaluated prior to embarking on a control programme. We have chosen two viruses; the human immunodeficiency virus (HIV) and the hepatitis C virus (HCV), to address a study of their genetic variation in India.

Countries in South-East Asia, especially India, have begun to show the steepest rise in HIV seropositivity. Yet, very little information is available on the strain(s) of HIV-1 infecting this population. Based on sequence analysis of a portion of the gp160 gene encoding the V3 hypervariable region, the predominant Indian strain is divergent from the major North American/ European and African strains. This difference is also reflected in the anti-V3 antibodies, which are type-specific.

Based on the nucleotide sequencing of HCV isolates from around the world, a number of viral genotypes have been identified. These correlate with different states of disease pathogenesis and response to antiviral therapy. Since only limited information on HCV genotype distribution is available from developing countries, we have started mapping these in HCV-positive sera from Cuba, India and Turkey. The cloning, sequencing and expression of the core and envelope regions of HCV is also being pursued.

Mammalian Biology: Malaria

V. S. Chauhan, V. Bansal, A. Bharadwaj, C. Celik, A. Das, K. Kapoor, F. A. S. Kironde, T. K. Mal, A. Mathur, F. Omer, P. Ray, N. Sahoo, A. Seth, P. Sharma, B. Singh, N. Singh, S. N. Veeranki.

Malaria is acknowledged to be by far the most serious tropical parasitic disease, resulting in the infection of some 350 million people annually. Over the past decades, insecticide resistant mosquitoes and drug resistant parasite strains have rendered disease control increasingly difficult. It is believed that the epidemiological situation is likely to continue to deteriorate over the next few years.

Malaria in humans is caused by four species of the parasite *Plasmodium*: *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. Of the four species, *P. falciparum*, the only one which causes death, accounts for most infections in Africa and for over one third of infections in other tropical countries. Over two billion people, nearly half of the world's population, is at risk. The estimated direct and indirect cost due to malaria is expected to be more than US $ 1,700 million per year by the end of 1994. Vaccines represent an attractive, cost effective strategy for reducing the burden of infectious diseases. With the rapid emergence of multi-drug resistant parasites, vaccines are considered promising and, therefore, considerable efforts are being applied in many research institutions worldwide to malaria vaccine development.

At ICGEB, our work on malaria is focused on: 1) identification of novel protective malaria antigens; 2) understanding effector mechanisms in malaria immunity and; 3) developing synthetic subunit vaccine constructs.

Novel Antigens of *Plasmodium*

The results of laboratory experiments with several purified antigens of *Plasmodium* and recent trials of synthetic and recombinant immunogens encourage the expectation that an effective vaccine against malaria will be developed. Moreover, several recently described vaccine-candidate molecules are yet to be tested and only a fraction of potentially protective antigens have been cloned and expressed. At ICGEB, we have continued to conduct research towards the identification of novel antigens of *Plasmodium falciparum*. We are applying a new approach where we probe *λ-gt11* expression library of *P. falciparum* with antibodies raised against the rodent malaria parasite *P. yoelii*. The rationale is that *P. falciparum* proteins recognized by binding with anti-*P. yoelii* antibodies (interspecies probing) will contain peptide sequences that have been conserved in different species of *Plasmodium*. The interspecies conserved antigens are likely to be critically important for the survival of the parasite, hence they may be very good targets for developing not only a widely effective antimalarial vaccine but also potent curative drugs.

Interspecies Probing Approach

Development of the interspecies probing approach was based on the following experimental observations. When we incubated mouse anti-*P. yoelii* serum with *P. falciparum* parasitized erythrocytes, it was noticed that the serum antibodies bind to all three bloodstages of *P. falciparum*.

Immunoblot assays and metabolic labelling experiments identified at least 15 polypetides of *P. falciparum* (15 to 150 kDa) which cross reacted with anti-*P. yoelii* antibodies. Many of the polypetides were bound with high affinity and at high dilution of the anti-*P. yoelii* serum. Eight of these polypetides appeared to be integral-membrane proteins potentially involved in merozoite invasion and formation of the parasitophorous vesicle. As observed by others for malaria antigens, we have found that some of the interspecies conserved antigens were not synthesized throughout the bloodstage cycle of the parasite. Significantly, anti-*P. yoelii* serum IgG potently inhibited *P. falciparum* growth and erythrocyte invasion, indicating that some of the interspecies cross-reactive antigens are critically important for the parasite survival in erythrocytes.

Novel *P. falciparum* Antigens

From 10^7 recombinant clones of a *P. falciparum* DNA expression library, three clones (pcO, pcT and pc7) were identified which express parasite antigens recognized by anti-*P. yoelii* serum antibodies. Two of the clones (pcO and pcT containing DNA inserts of 3.1 kb and 2.8 kb, respectively) were also recognized by human anti-*P. falciparum* sera. Human antibodies affinity purified for pcO and pcT expression products bind to parasite bloodstages and specifically recognize distinct polypetides in *P. falciparum* cell lysates. Both pcO and pcT have been subcloned in the plasmid vector pBS. Partial nucleotide sequences of the clones suggest that pcO and pcT express novel antigens that have not been reported before. Immunoaffinity purified antibodies specific to pcT bind to schizonts. The two cloned inserts have also been subcloned in the pET vector and have been expressed in *Escherichia coli*. The expressed product will be used in immunization as well as affinity purification of antibodies required for inhibition assays and other immunochemical studies of the novel antigens.

Malaria Immunity and Development of Synthetic Subunit Peptide Vaccine Constructs

Immunity to the developmental stages of malaria parasites is stage-specific and, for those living in endemic areas, this immunity is very slow to develop. The exact nature and specificity of immunity in humans is not well understood, being generally incomplete and transient. It is thought that both cell-mediated and humoral immune functions contribute to acquired immunity although there is no direct evidence to clearly support this view.

Most efforts in sporozoite stage vaccine development have focused on the circumsporozoite (CS) protein, although recently a few new target antigens have been recognized. In comparison, a large number of asexual erythrocytic stage antigens are being explored as potential vaccine candidates. Merozoite surface antigens (MSA-1 and MSA-2), SERP (serine rich protein), HRP II (histidine and alanine rich protein II), RESA (ring-infected erythrocyte surface antigen) and AMA-1 (apical membrane antigen) are some of the major blood stage antigens that have shown promise for protective immunization in animals and humans. However, immunization with the recombinant antigens has not provided the expected protection. The main reason for this appears to be the inability to present appropriate B and T-cell epitopes to the immune system when recombinant antigens are used. Synthetic peptides comprising immunodominant epitopes offer an attractive alternative strategy to develop suitable vaccine candidates. Although, on one hand, synthetic peptides offer advantages in terms of easy, large scale synthesis, high purity, enhanced stability at ambient temperature, they are, on the other hand, mostly poor immunogens. Other major concerns in their possible use as vaccines are genetic restriction of the immune response, specificity of the immune response and possible generation of new epitopes. We are attempting to address these questions, with a long term goal to develop multiple epitope peptides as possible malaria vaccine candidates.

Our initial studies were focused on major blood stage antigens, MSA-1 and RESA. These two conserved major merozoite antigens share invariant regions in their structures, among different strains of *P. falciparum* and even in different species of plasmodia. It is believed that these regions may be crucial for the parasite's survival, and for this reason we have focused our attention on such conserved sequences. A multiple epitope peptide (52-mer) based on these two antigens was highly immunogenic in animals and showed good reactivity with blood samples collected from individuals living in malaria endemic areas in India. The immune response in mice was not genetically restricted and, further, the hybrid peptide was highly immunogenic when inoculated with alum as the adjuvant. Protective immunization with this peptide in mice against a heterologous challenge has provided us with a rationale for using this approach to develop peptide vaccine constructs.

During the past year, along with our ongoing work with the blood-stage antigens, we have focused on identifying peptide motifs from the conserved region of another major malaria protein called thrombospondin related anonymous protein (TRAP), first described to be a blood stage antigen. It has recently been shown that this protein is also expressed on the sporozoite surface. It is of great interest that a highly conserved peptide motif from the TRAP sequence is also seen in the region II of the CS protein of all the *Plasmodium* species sequenced so far. We have shown earlier that the antibodies raised against the motif peptide (W-S-P-C-S-V-T-C-G) completely inhibited the merozoite invasion of the erythrocyte. Others have shown that the same motif is also involved in the sporozoite invasion of the hepatocyte. It appears that this motif represents a critical sequence involved in the malaria parasite invasion of the host cells at both the liver as well as the erythrocyte stages.

The 18 residue peptide P-18, which includes the above motif, itself inhibited the merozoite invasion of the RBC's. This also may suggest that the conserved motif has a role to play in the merozoite interaction with the erythrocytes. Rabbit anti P-18 antibodies, also inhibitory to the merozoite invasion, recognized a 78 kDa protein in *P. falciparum* blood stage lysate. Although it was first described as a merozoite antigen, there has been a

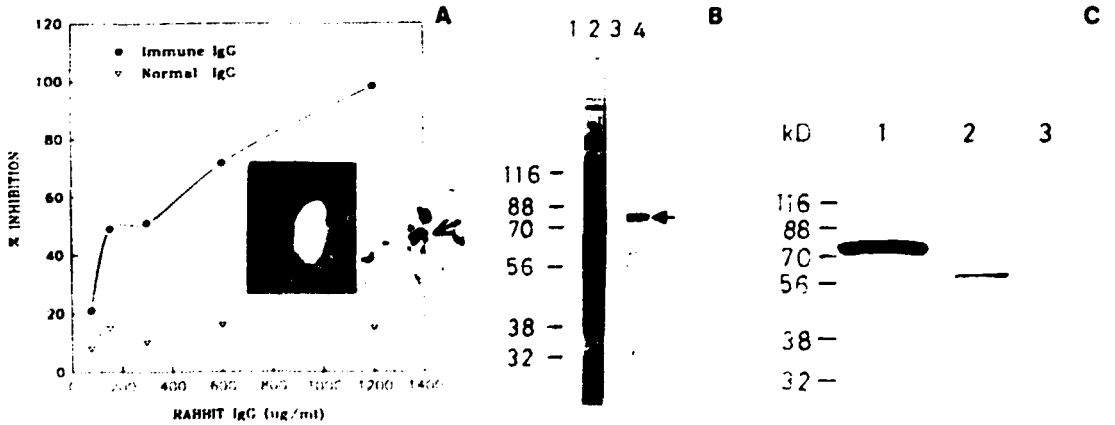
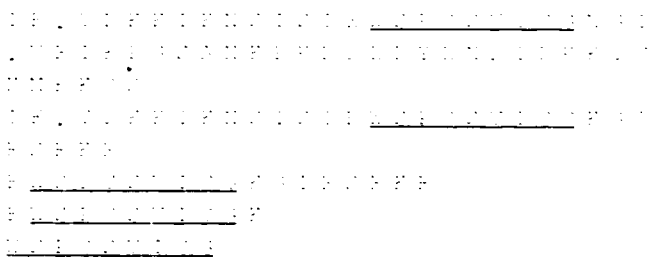


Figure 1
A thrombospondin related anonymous protein (TRAP) contains motifs which are conserved in the CS protein of all *Plasmodium* spp. sequenced so far. Its presence in the asexual erythrocytic stages of the parasite has remained controversial.
Antibodies to the most conserved non-apeptide stretch of this protein (peptide **P-9**) exerted a strong growth-inhibitory effect on the asexual blood-stages of *P. falciparum* in a dose dependent manner (**A**) and stained the parasites specifically in an immunofluorescence assay (**A**, inset). Antibodies to an 18 mer (**P-18**) recognised a 78 kDa parasite protein in immunoblots [**B**: total parasite lysate was probed with rabbit preimmune sera (lanes 1 & 3), anti parasite serum (lane 2) and anti peptide serum (lane 4)]. The 18-mer antibody reacted specifically with only those recombinant TRAP gene products (courtesy of Dr. A. Crisanti, Rome, Italy) which contained the conserved motif of TRAP and not with those which lacked it [**C**: total parasite lysate (lane 1), truncated TRAP constructs containing conserved motif (lane 2) or without it (lane 3) probed with anti-peptide serum]. These results clearly establish the expression of TRAP in the asexual blood stages of *P. falciparum*.

controversy on the exact location of this antigen. Our results clearly show that this antigen is expressed during the blood stages (Figure 1). Furthermore, support for this was provided by specific recognition of recombinant TRAP constructs, with and without the conserved motif sequences, by antipeptide antibodies (Figure 1). Based on these results we synthesized a 60-mer peptide (P-60; residues 331-390 of CS protein) which contained this motif sequence, flanked by at least two T-cell determinants and several smaller fragments to assess its potential as a vaccine candidate.

Synthetic Peptides Containing the Conserved Motif Sequence

1. (**P-60**, CSP)
2. (**P-32**, TRAP)
3. (**P-18**, TRAP)
4. (**P-11**, TRAP)
5. (**P-9** TRAP)



Upon studying the immunological properties of P-60, we found that it is able to induce high titre antibody (10^5 to 10^6) in several different strains of mice without the use of a carrier protein. If these mice are challenged with a lethal strain of *P. yoelii*, P-60 is able to protect up to 80% of the mice. We have also looked for a T cell response to this peptide and find that it is able to induce the proliferation of T cells in different strains of mice. These T cells appear to be CD4+ T helper cells which secrete IL-2 and IFNγ (namely Th1 type). Studies are currently underway to determine whether Th2 cells which secrete IL-4, IL-6 etc. are also involved in this response. The role of Th1 and Th2 cells in malaria is not yet clear.

To determine the fine specificity of the response to P-60, we synthesized two more peptides: P-32 and P-18 as shown above. While P-32 was immunogenic, P18 was not. 60% of mice immunized with P-32 were also protected upon challenge with the lethal *P. yoelii* strain. A cross-reactivity of 30-50% was observed when sera of P-60 immunized mice were tested against P-32 and P-18. In T cell proliferation assays, however, cells from P-60 immunized mice reacted only minimally with P-32 and P-18. Studies are in progress to delineate other regions of P-60 in response to which the proliferative response might be occurring. We also analyzed the capacity of P-60 to induce cytotoxic CD8+ T cells in mice but have not obtained any cytotoxic T cell activity using spleen cells from mice immunized with P-60. This probably indicates a problem in the processing of this peptide in an appropriate fashion required to expose the CTL epitope to the immune system of the mice. These issues will have to be considered in designing multiple epitope synthetic peptides as potential vaccine constructs.

We have also analyzed responses of sera from malaria infected humans to P-60. 70% of *P. falciparum* positive sera and 55-70% of *P. vivax* positive sera recognize the P-60 sequence. Studies on cellular responses from human peripheral blood lymphocytes to these various constructs are in progress. Based on the results from the two constructs, described here, we now propose to design a single peptide containing immunodominant epitopes from the sporozoite as well as the merozoite stage antigens to study its immunological properties and its potential in providing protection against malaria infection.

A putative synthetic peptide vaccine must contain B cell determinant(s) as well as Th epitopes so as to obviate the need for an extraneous "carrier" protein with its attendant complications such as epitope-specific immunosuppression. In this context, we (as well as others) have previously shown that "universal" Th cell epitopes derived from various proteins not

only enhance the immunogenicity of B cell determinants but also overcome genetic restriction of immune response and remain free from immunosuppressive activity. In particular, we have demonstrated that two "universal" Th epitopes, one derived from tetanus toxin (tt830-844) and the other from CS protein of a human malaria parasite, *P. falciparum* (CS.T3, residues 378-398), enhanced the immunogenicity of the parasite merozoite surface antigen peptide in several inbred strains of mice bearing different H-2 haplotypes. We have also shown that epitopic sequences in a hybrid construct may provide reciprocal helper activity for antibody production. Furthermore, antibodies to a Th epitope need not downregulate its helper activity. However, novel junctional B cell determinants may be formed in such constructs. We have now observed that the two "universal" Th epitopes above elicit similar levels of T cell proliferation responses from mice primed with tetanus toxoid. A similar pattern of response with high stimulation indices was obtained in three different strains of mice, namely, C57BL/6, DBA/2 and B10.BR. Thus, the two "universal" Th epitopes, which do not share any primary structural homology, generated strikingly similar responses suggesting the possible role of secondary conformational preferences in this behaviour. The circular dichroism spectroscopy studies indicated that both of these peptides display a tendency to acquire a common secondary structure motif. This may perhaps be important in some way in their being "universal" Th epitopes. Our findings suggest that it may be possible to design immunogens which will generate immunological cross-stimulation between tetanus toxoid and other heterologous (as in our case CS.T3) immunogens and *vice versa*.

Conformationally Restricted Peptides

Short peptides display a wide array of biological activities and can also elicit specific humoral immune response. Their role in cellular immune response has dramatically influenced the research in this field and has raised hopes for peptide based immunotherapy in different disease conditions. Contrary to earlier beliefs, secondary structural motifs such as, β-turns, 3_{10} or α helices and amphipathic structures in these peptides are often associated with their functional and/or immunological responses. Stabilization of secondary structures in short peptide sequence could give rise to highly specific immune responses, and in the context of bioactive peptides, may produce highly stable and active peptide analogues.

Following an ongoing programme of designing peptides with preferred secondary structures, by introducing α,β-dehydroamino acids (Δaa), we have successfully synthesized peptide models for β-turn and γ-turn structures. Using more than one ΔPhe we were able to stabilize 3_{10} and α-helical motifs. Crystal structure and solution studies (NMR, IR and CD) have revealed that the designed peptides acquire the same structures in solution as seen in the solid state. Our recent results have shown that inclusion of a single ΔPhe residue is sufficient to nucleate a helical structure in short peptides. Detailed NMR studies including ROESY have provided clear evidence that Boc-Ala-Leu-ΔPhe-Phe-Ala-Leu-OCH_3 adopts α-helical structure in apolar solvents. In an exciting finding we have noticed that the peptide, Boc-Pro-ΔPhe-Ala-ΔPhe-Ala-OCH_3, adopts a β-bend ribbon conformation, wherein the peptide backbone assumes a nearly planar 'S' shape, maintaining the hydrogen bonding features of a β-bend ribbon (Figure 2). This structure is novel because in the β-bend ribbon conformation reported so far alternating positions are occupied by residues devoid of hydrogen bond donating N-H group (e.g. proline), whereas in this structure consecutive 4-->1 hydrogen bonds are formed. It is noteworthy that in all other model peptides studied so far we have found that -ΔPhe-X-ΔPhe- (X is any amino acid residue) sequence stabilized 3_{10}-helical structures. We are now investigating the design elements required for stabilizing β-bend ribbon structures. Furthermore,

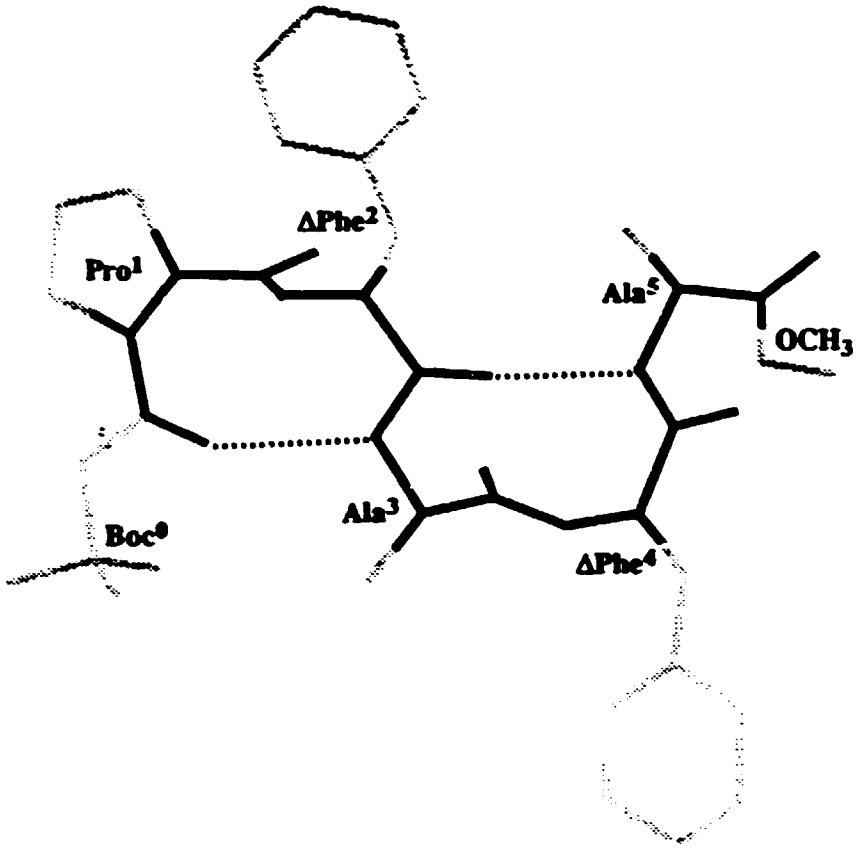


Figure 2.
Molecular structure of Boc - Pro - ΔPhe - Ala - ΔPhe - Ala - OMe, showing flat β-bend ribbon conformation. The dotted lines indicate intramolecular 4-->1 hydrogen bonds.

in the present peptide molecule the torsion angles of some residues (e.g. Ala3) deviate from helical values, providing a novel structure which may be of value for peptidomimetics with restricted backbone mobility.

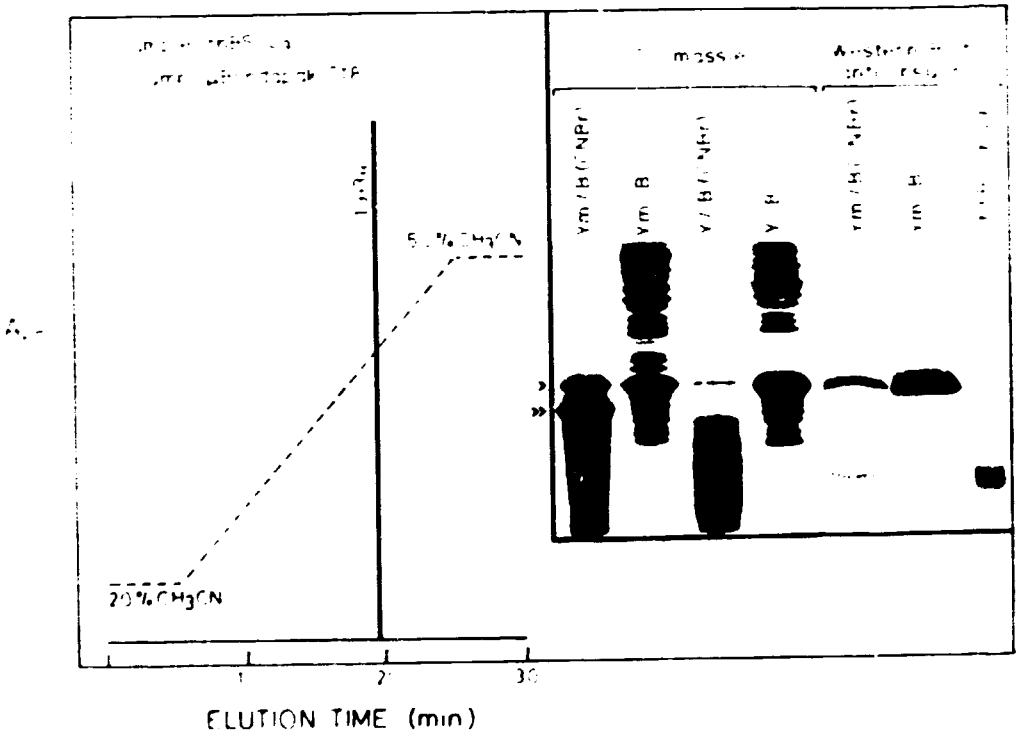
Using isoaminobutyric acid (Aib) and dehydrophenylalanine (ΔPhe) residue as inducers of helical structures we have designed and synthesized two eleven residue peptides which contain tripeptide sequence motifs such as KEK, KEL and LEK, known to be crucial for malaria parasite interactions with erythrocyte. Antibodies raised against these "designed" peptides strongly recognize the linear original sequences containing the KEK motif. Further work on the specificity and efficacy of such peptides is in progress.

Mammalian Biology: Recombinant Gene Products

N. Khanna, N. Jayaswal, M. Malhotra, G. Rao, D. Sahal, J. Singh

Our programme involves the production of therapeutically important proteins using recombinant DNA technology. As described earlier (ICGEB Activity Report 1992), we have successfully produced recombinant human γ-interferon. This "know-how" is now being transferred to a commercial partner for large scale production. Presently, the group is involved in the production of recombinant human insulin.

The magnitude of the need for insulin can be assessed from the fact that close to 0.7% of the world population suffers from insulin dependent diabetes. Assuming a mean daily intake of 40 units (1.6 mg) of insulin per person per day, every diabetic requires around half a gram of insulin every year. The *per annum* requirements for insulin are 10-20 kg for Japan, >100 kg for Germany, >200 kg for Italy and close to 4000 kg for India. Therefore, we have embarked on a project of making recombinant human insulin.



HPLC profile of recombinant S-sulfonated B-chain of human insulin isolated from inclusion bodies

This project has progressed from gene design, cloning and miniprep expression to a phase of large scale expression, isolation of A and B chains and their assembly into chromatographically, immunologically and biologically proven human insulin. The fusion partner ("Y") has been successfully mutagenized to replace all but the N-terminal and Y-A/B junctional methionines by alanine. These site directed mutageneses were carried out to design a fusion protein which on site specific cleavage by CNBr, would give a much larger fusion partner and the much smaller fragments of A or B chain. This has obvious advantages in facilitating purification of chains.

Direct proof of the successful mutagenesis is shown in Figure 1 (inset, Coomassie) where the CNBr cleavage patterns of Y-B (wild type) have been compared with the mutated protein designated as Ym-B. As predicted, the cleavage patterns are characteristic: a cluster of small molecular weight peptides in Y/B versus a marginally (~3 kDa) shifted prominent protein band in Ym/B. The Western blot in Figure 1 (inset) has confirmed the appearance of B chain as a 3.5 kDa band. This band is seen only in the lane where CNBr cleaved protein was loaded. A trace of uncleaved fusion protein is also visible in the CNBr lane. The sulfitolyzed B chain obtained from CNBr cleaved Ym-B has been purified to apparent homogeneity as is evident in the figure showing the reverse phase HPLC profile. In the HPLC system used, a sample of standard B chain of sulfitolysed porcine insulin (BSSO3) could not be separated from the recombinant human insulin BSSO3 purified from the inclusion bodies. It may be noted that the porcine and human B chains differ merely in that the carboxy termini of the B chain in man and pig are threonine and alanine, respectively. The recombinant chain was subjected to solid phase protein sequencing. Twenty one cycles which could be monitored confirmed the predicted sequence as: F-V-N-Q-H-L-C-G-S-H-L-V-E-A-L-Y-L-V-C-G-E. Amino acid analysis of the recombinant B chain confirmed its purity both by absence of methionine and isoleucine and by the occurrence of other amino acids in the expected ratios.

A mass spectrum of the recombinant human B chain di-s-sulfonate showed the predominant molecular ion peak at 3588.5 daltons indicating that this peptide has the expected molecular weight of 3588 daltons. This has confirmed both the integrity of the B chain and also the presence of two S-fulfonate moieties introduced by post translational chemical modification. Highly specific radioimmunoassays for insulin and its chains have also been developed for monitoring the assembly of recombinant human insulin.

Plant Biology: Chloroplast Genome

K. K. Tewari, V. Khanna, D. Kumar, S. Lakhani, S. Mukherjee, M. K. Reddy, V. S. Reddy.

Transgenic plants are widely used to study nuclear gene function and regulation. Production of transgenic plants is achieved primarily through methods developed for the transformation of the nuclear genome of higher plants. However, there has been great concern about the transmission of these foreign genes from transgenic crops to their wild relatives and other weeds, following cross-pollination. This problem might be solved if the integration of foreign genes can be directed to the chloroplast. In the majority of crop plants, chloroplast DNA is maternally inherited, i.e. not transmitted through pollen. Moreover, due to various similarities shared with prokaryotes with respect to the nature of transcriptional-translational signals and codon-bias, the chloroplasts offer a more suitable compartment for expressing agronomically important genes of bacterial origin (e.g. C-P lyase from *Pseudomonas* for glyphosate (herbicide) degradation, δ-endotoxin from *Bacillus thuringiensis* for insect resistance). Owing to the availability of extensive sequence data, including the complete nucleotide sequence of three plant chloroplast genomes (tobacco, rice and liverwort), there is also a possibility of targeting genes to a particular site or of replacing an existing gene with an engineered alternative on the chloroplast genome by homologous recombination. However, the stable and reproducible expression of foreign genes in chloroplasts is yet to become a reality. This is partly due to inadequate knowledge of the replication and transcriptional machinery of the chloroplast DNA. This project aims to understand the fundamental mechanisms involved in the replication and transcription of chloroplast genes.

Transcription

The transcription of genes encoded by the chloroplast relies on the activity of a chloroplast specific DNA-dependent RNA polymerase. Since the polypeptides co-purifying with RNA polymerase activity in chloroplasts have not yet been functionally characterized, the subunit composition of the RNA polymerase remains largely unknown.

Chloroplast RNA Polymerases

We are interested in the identification of proteins involved in the transcription of chloroplast genes. Earlier, two transcriptional activities from pea chloroplasts had been identified which showed specificity for ribosomal and messenger RNA promoters. Efforts are being made to investigate whether the two transcriptional activities are due to two RNA polymerases, or whether these are conferred by the interaction of different "specificity" factors with a single RNA polymerase. The two transcriptional activities share at least five common functional polypeptides including the one responsible for promoter recognition. Using deletion mutants and a homologous *in vitro* pea chloroplast transcription system, a 66 base pair sequence (-54 to +12) was identified as a minimal promoter for accurate expression of 16S rRNA *in vitro*. This promoter has prokaryotic promoter elements (-10 and -35). Chloroplast *in vitro* transcription systems from spinach, maize and mustard have also been

shown to contain prokaryotic promoter sequences which direct transcription and which probably function *in vivo* as chloroplast promoters.

Template and Transcript Binding of Chloroplast Polypeptides

The use of a functional approach through photoaffinity labelling of the chloroplast transcriptional complex has identified the template and nascent RNA-binding proteins. The template-binding protein (150 kDa) binds exclusively to chloroplast DNA containing promoter sequences and does not discriminate between ribosomal or messenger promoters. Through photoaffinity labelling, efforts are being made to explore the way in which the template-binding polypeptide interacts with the two strands of the DNA. Using dimethyl sulfate protection assay, contact points made between RNA polymerase and ribosomal and messenger RNA promoters are also being identified and sequenced.

Chloroplast Transcript-Binding Protein is Encoded by the Nuclear Genome

The nascent RNA-binding protein (nRbp) has been identified to be a 48 kDa protein. This protein was distinguished from other RNA-binding proteins by its ability to bind only nascent transcripts. The protein "tagged" by UV-crosslinking with radiolabelled nascent RNA was separated from other chloroplast proteins by 2-dimensional polyacrylamide gel electrophoresis. The radiolabelled photoconjugate was sequenced and the N-terminal amino acid residues appeared in the following order: Met-Asn-His-Ile-Asn-Gly-Thr-Ile-Asn-Lys-Val-Glu-Ala-Asn-Leu. After partial chemical cleavage an internal sequence Asn-Asn-Ile-Leu-Val-Val-X-Asp-Ala-Tyr-Thr-Lys-Ala-Glu-Pro was obtained. These sequences were used to

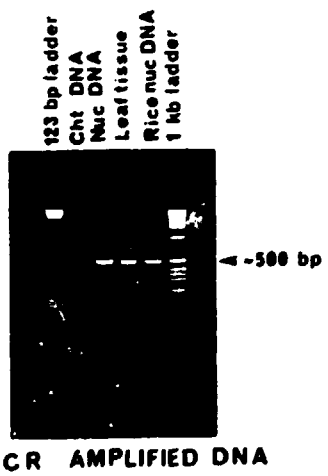
Sequences of Primers

5′ - ATG AAC CAC ATI AAC GGI ACI ATI AAC ACI GTI GG - 3′
T T T TG

Forward Primer

5′ - GGC TCC AGG GCC TTG GTG TTG GCG TC - 3′
G A G

Reverse Primer



PCR AMPLIFIED DNA

Figure 1. **The chloroplast nascent RNA-binding protein is encoded by the nucleus.**
PCR amplification of the partial gene of transcript-binding polypeptide of chloroplast transcription complex. The sequence of forward and reverse primers is shown. The inset shows amplification of a 500 bp fragment from various DNA

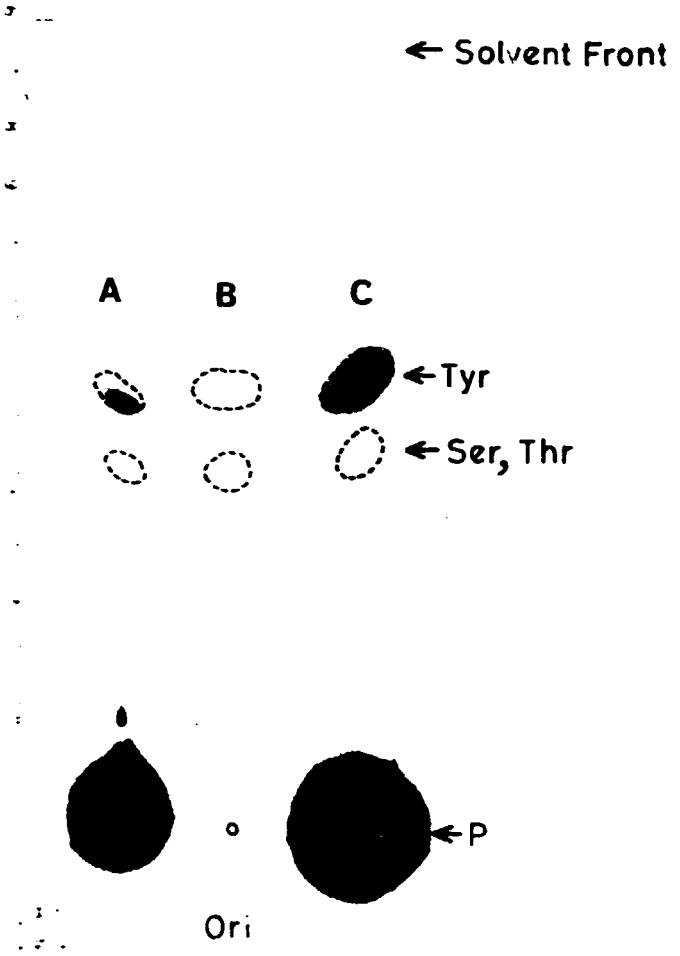


Figure 2
Thin Layer Chromatography. TLC analysis of the tryptic digest of the complex formed between the radiolabelled DNA and 69 kDa topoisomerase I. The enzyme cleaves the covalent DNA through the tyrosine (Tyr) residues.
Origin, Ori; application point of the tryptic digest sample; TLC-P, inorganic phosphate; Phosphotyrosine, Tyr-P; Phosphothreonine, Thr-P were used as markers.

design oligonucleotide primers for the PCR amplification of a part of the nascent RNA-binding protein gene from pea genomic DNA. The sequences of the primers are shown in Figure 1. The N-terminal amino acid sequence yielded the forward primer and the internal sequence was used for the reverse primer in the 3'-5' orientation. Amplification from pea nuclear DNA, pea leaf tissue and rice nuclear DNA yielded a fragment of about 502 bp (Figure 1). No amplification was observed when chloroplast DNA was used as template. This indicated that the chloroplast nRbp is encoded by the nuclear genome.

The expression of the nRbp gene was examined in several tissues by Northern slot blot analysis. The data showed that the nRbp mRNA is present in green buds, mature leaf, etiolated dark-grown buds and in seedling stems as well as in the roots. The nRbp mRNA was not found to be present in chloroplasts, as expected for a nuclear-encoded chloroplast protein.

Replication

We have identified many of the protein components required for pea-chloroplast DNA replication, such as DNA-polymerase, primase, helicase, topoisomerases etc. We have also established the *in vitro* replication of pea chloroplast DNA with the partially purified proteins from the chloroplasts.

Polymerization by the *in vitro* replicative system was at the rate of 650 nucleotide/sec and was insensitive to chain-terminators like

dideoxynucleotides, ara -CTP and cordycepintriphosphate. Both strands of template DNA were synthesized and single stranded DNA templates underwent more than one round of replication. When sequences of either of the two chloroplast origins of replication (OriA or OriB) were used as templates, the replicative intermediates were found to have sigma structures However, with the template DNA containing both OriA and OriB sequences together in cis conformation, the *in vitro* replication proceeded in the theta mode; the mode of replication usually observed *in vivo*.

At present, it is difficult to study the effects of the individual components in the various stages and processes of *in vitro* replication because of the limited availability of the components. Similarly, reconstitution of the defined *in vitro* replication system seems untenable due, primarily, to the low level of the proteins required for pea chloroplast DNA replication. In order to overcome these problems, the present approach aims to identify and clone the relevant genes and to express the products in the bacterial system to enable *in* vitro assembly of chloroplast DNA replication.

Eukaryotic Type I Topoisomerase

In an attempt to identify proteins necessary for the replication of chloroplast DNA, we have purified a 69 kDa eukaryotic topoisomerase to homegeneity. The type I property was clearly identified following the pattern of relaxation of supercoiled DNA both in solution and *in situ*. The monomeric nature of the catalytic entity was established by glycerol gradient sedimentation chromatography. The enzyme was sensitive to topoI-specific inhibitors like camptothecin and berenil, but unaffected by novobiocin and doxorubicin at the topoII-inhibitory dosage. In the presence of the enzyme, supercoiled DNA was nicked and the 3' phosphoryl end of the nick became covalently linked with the enzyme (Figure 2). A tyrosine residue of the enzyme was responsible for the covalent linkage. Rabbit antisera have been raised against this protein. Since the N-terminal amino acid of this protein is blocked, we are attempting to generate data on internal amino acid sequences through partial proteolysis.

Search for Genes Coding for Replication Proteins

Eukaryotic type I topoisomerase is coded by nuclear gene(s) and transported to the chloroplast. Pea nuclear DNA has been used to generate an expression library in λgt 11 phage-system and a genomic library in λgt10 vectors. Using the purified antisera, we are, at present, screening the expression library at present. Using internal sequences, when available, DNA hybridization probes could be made. With these probes or other probes from heterologous sources (topo I genes from *Aradibdopsin thaliana* or/and human DNA, etc.), the pea libraries will be screened. Using these multi-pronged approaches, we hope to clone the gene for the 69 kDa topoisomerase of pea chloroplasts.

Other Topoisomerases

We have also detected at least one additional Mg^{++}-dependent type I topoisomerase activity within the pea-chloroplasts which is antigenically distinct from the 69 kDa enzyme. Attempts are being made to purify this enzyme to near homogeneity and to clone the appropriate gene.

Search for Single-Stranded DNA Binding (SSB) Activities

Proteins with SSB activities are essential for the initiation and elongation of DNA replication. In a preliminary search for SSB's of pea-chloroplasts, southwestern analyses of various fractions of crude chloroplast-proteins (as recovered from DEAE-chromatography) were performed using denatured and intact nick-translated DNA-probes. Strong signals were obtained with the denatured probes at various ranges of molecular sizes. An array of proteins of the molecular size 28-35 kDa reacted strongly but these had been identified as RNA-binders in previous studies. Other polypeptides that bound DNA included proteins of 17, 40-45, 65 kDa. These proteins eluted from

DEAE celluiose columns at 600-700 mM NaCl after the recovery of DNA-polymerase, primase and topoisomerases. Proteins of 40 and 65 kDa size were hardly recognizable by Coomassie staining but bound to the denatured probes very strongly. Attempts are being made to characterize these proteins.

Puzzling DNA-Binder

Pea chloroplasts contained a DNA-binding activity which recognized only the negatively supercoiled form of duplex DNA. The bound DNA (complex) migrated with the mobility equivalent to that of nicked or/and linear DNA species in agarose-gel-electrophoresis. The formation of the complex was reversed by denaturants or inactivators of protein, namely sodium dodecyl sulfate, high temperature, ethidium bromide and proteases. The binding activity was enhanced with zinc or cupric ions and the enhancement was abolished in the presence of high concentrations of EDTA. The binding was cooperative in nature and altered the topological status of DNA as evidenced in biochemical and physical analyses. The protein also acted on single stranded DNA and compacted the substrate as revealed by agarose gel electrophoresis of the bound complex. Hence, the target site of action on supercoiled DNA seems to be the open or unwound regions. The potential for the formation of DNA-protein complex remained the same irrespective of the size of the starting DNA.

From glycerol gradient sedimentation studies, the native protein appeared as 50 kDa. The chloroplast extract containing the protein has been subjected successively to chromatography on DEAE cellulose, heparin sepharose, blue sepharose, QAE- and phenyl-sepharose columns. The active material from the last column still showed the presence of 5-6 bands in the silver-stained SDS gel. Attempts are being made to purify the protein to homogeneity. The partially pure protein was successfully crosslinked with the substrate DNA with a combination of formaldehyde and gluteraldehyde. Using the *in vivo* labelled DNA, we hope to transfer the radiolabel from DNA in the crosslinked complex to the specific active protein. These experiments are expected to identify the subunit molecular weight of the DNA-binding moiety.

Plant Biology: Plant Resistance

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Glyphosate Resistance

Glyphosate is a broad spectrum herbicide which kills 76 out of 78 of the most damaging weeds. Its action is mediated by inhibiting one of the key enzymes of aromatic amino acid biosynthesis, 5-enolpyruvyl shikimate-3 phosphate synthase (EPSPs). Since the effect of glyphosate is non-discriminatory between crops and weeds, the successful use of the herbicide necessitates the conferring upon the desired crop plant, of the ability to escape herbicidal inhibition. Through genetic manipulation the target enzyme can be manipulated to reduce the binding affinity of glyphosate. The genetically altered EPSPs could be introduced in the crop plant by established protocols.

To manipulate EPSPs, the gene coding for EPSPs, *aro*A, was cloned from *Bacillus subtilis* by complementing growth of *aro*A deficient mutant of *E. coli*. Clustal alignment of amino acid sequences of EPSPs from *B. subtilis* with different bacteria and plants revealed several conserved homologous regions. Certain conserved residues which were candidates for substrate binding were identified and substituted by site directed mutagenesis. The following substitutions were generated: K19E, R24D, R104K, R104Q, P105S and H382K. To investigate various critical kinetic parameters relevant for glyphosate binding, mutant genes were cloned on pBSK vector and expressed by providing T7 RNA polymerase in trans- on a compatible plasmid pGP1-2. Using a heat inducible lambda promoter, EPSPs was expressed to about 30% soluble protein content. Six mutants as well as the wild type genes were expressed to comparable levels. EPSPs was purified in one step to near homogeneity from heat induced cells. Kinetic analysis of purified EPSPs of wild type and mutant genes revealed that the enzyme is strongly activated by ammonia and that the nonactivated form of native enzyme exhibits allostery. Hysteresis of wild type EPSPs is abolished by ammonia. Both of the substrates, phosphoenol pyruvate and shikimate-3-phosphate, have multiple interaction sites. There are two sites for PEP binding, primary and secondary. Glyphosate competes for binding at the primary site and does not interact at the secondary site. Glyphosate in the absence of ammonia increases co-operativity of PEP binding and favours dimerization of the enzyme. Dimerization probably occurs through an interaction between the sites of PEP binding. The H382K and R24D substituted EPSPs exist as dimer in the absence of NH_4^+ and glyphosate and acquire tetrameric structure in their presence. Analysis of the mutants R104K and R104Q suggests that the guanidinium side chain of the conserved arginine is critical in discriminating between phosphoenol pyruvate and glyphosate. Conserved residue K19 is proximal to the shikimate-3-phosphate binding site. Mutation at this residue increases affinity for shikimate-3-phosphate with a concomitant hypersensitization to glyphosate. Thus the binding of shikimate-3-phosphate is directly related to the binding of either phosphoenol pyruvate or glyphosate. Credence to this mechanism is augmented

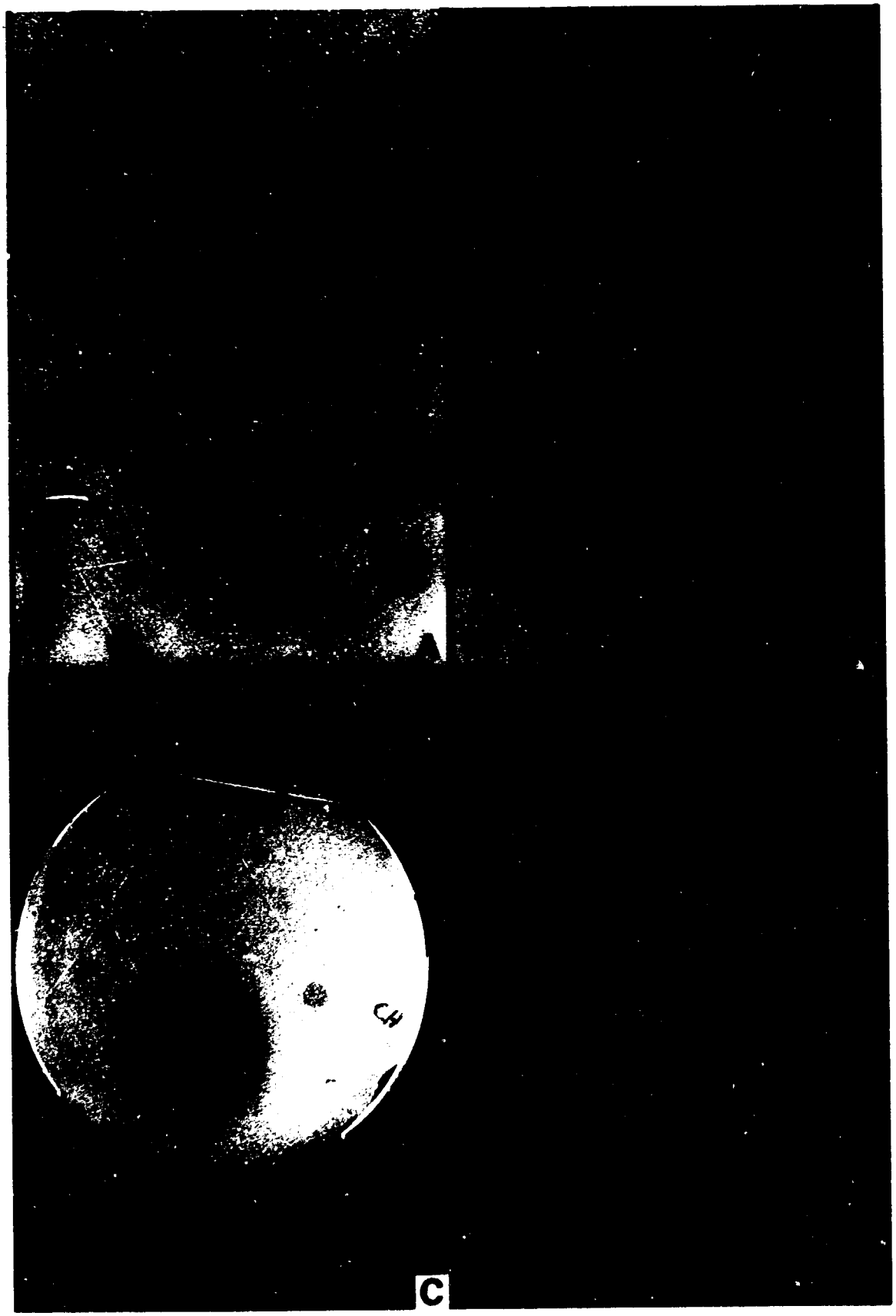

Figure 1. **Isolates of *Bacillus thuringiensis* and *B. sphaericus*.**
A. Cells, spores and crystals of *B. thuringiensis* (1000 x); B. Parasporal crystals of *B. thuringiensis* purified by renografin gradient (1000 x); C. Identification of *B. sphaericus* by antibiotic sensitivity profile. Mosquitocidal isolates are resistant to tetracycline, chloramphenicol and streptomycin and sensitive to erythromycin; D. Spores and crystals of *B. sphaericus* (1000 x).

by the observed increase in Km for shikimate-3-phosphate and by a consequent increase in Ki for glyphosate in the mutant R104Q. Based on the above observations a novel approach for conferring glyphosate tolerance to crop plants is being pursued.

Insect Resistance

Each crop plant is a host for several different types of insects. The pests for each crop plant are specific and may belong to different taxonomic classes e.g., *Lepidoptera*, *Diptera* and *Coleoptera*. Through natural selection, crop plants have evolved resistance mechanisms to insects but the modes of action are complex and poorly understood. However, a common biopesticidal mechanism of insect control with parasporal crystals of *Bacillus thuringiensis* has been described and analyzed in great detail. Insects of different classes are susceptible to toxic polypeptides of parasporal crystals. Toxins produced by different isolates vary in potency and specificity for different insects. The Plant Stress Resistance Group has initiated a programme on "Insecticidal Endotoxins". Strains of *Bacillus thuringiensis* have been isolated from terrestrial and aquatic habitats.

Soil and water samples were collected from several sites at Manesar (Haryana), 60 km south west of Delhi. Strains of *Bacillus thuringiensis* were isolated through selective enrichment procedure.

Following enrichment 31 isolates of *B. thuringiensis* were isolated and typed in relevant morphological and biochemical tests. Parasporal crystals were purified in renografin density gradient centrifugation and polypeptide profile was analyzed by resolving solubilized crystals on SDS-PAGE gels. In addition, plasmid profiles of a few isolates have been established. Using standard toxin coding gene as a probe, the presence of homologous DNA segments was demonstrated on plasmids of different isolates. We are now carrying out toxicity tests of parasporal endotoxins on these isolates on larvae of *Heliothus sp.*

Local strains of *B. sphaericus* have also been isolated from aquatic habitats. Based on the near absolute correlation between resistance to chloramphenicol, tetracycline, streptomycin and adenine (N) utilization and the production of mosquitocidal proteins two isolates have been obtained through enrichment. The toxicity of parasporal crystals produced by these isolates is being screened against *Anopheles* and *Culex* larvae.

Mapping and Tagging of Insect Resistance Genes in Rice

One-fifth of yield losses in the area of rice production can be attributed to insects. Gall Midge (*Orseolia oryzae*), a dipteran, is one of the most important of these pests especially in India, South-east Asia, China and parts of Africa. The problem is compounded by the fact that there are at least 4-6 different biotypes of this insect in India and 4 more are known to occur in China. Genes for resistance against Gall Midge are found in nature and many of the rice improvement programmes, especially in India, are centred around breeding resistance to Gall Midge into elite susceptible varieties. Little is known about the exact process leading to resistance but it is known that resistance is due to a single major gene (at least in the case of resistance to biotype 1 of Gall Midge).

We have mapped the *Gm2* gene on chromosome 4 with the intention of speeding the selection of resistant phenotypes in a breeding programme and ultimately helping in the faster production of new resistant rice varieties. Restriction Fragment Length Polymorphism (RFLP) and Random Amplified Polymorphic DNA (RAPD) were used in conjunction with bulked segregant analysis on a set of 40 recombinant inbred (RI) lines obtained from a cross between two indica rice varieties, ARC6650 and Phalguna. (The two rice varieties, RI lines and the phenotyping data on these lines were obtained from our collaborators at The Directorate of Rice Research, Hyderabad, India.) Of

Of 520 primers, only 65 amplified bands were characteristic of either the resistant parent and resistant pool or the susceptible parent and the susceptible pool. These primers were expected to be linked to the *Gm2* gene. However, when these markers were used to amplify the DNAs from individual susceptible lines and individual resistant lines, which constituted the susceptible pool and the resistant pool respectively only two markers amplified either in all susceptible RI lines ($F08_{1700}$) or in all resistant RI lines ($F10_{600}$), indicating that these two markers were very closely linked to *Gm2* gene. This was confirmed by using the amplified products as RFLP markers and with subsequent cosegregation analysis with the disease phenotype. With other markers the picture was not as clear.

Based on the sequence information of pool specific bands, a PCR-based assay was developed to select resistant and susceptible plants. The $F08_{1700}$ specific primers selectively amplified a 1.7 kb fragment in the susceptible plants while $F10_{600}$ amplified a 0.6 kb fragment in the resistant plants (Figure 2). This allows for accelerated screening, selection and subsequent faster breeding for Gall Midge resistance by utilizing marker-aided selection. Currently, we are in the process of determining whether these primer sets are population specific.

Once a gene has been mapped between two very closely linked flanking DNA markers, the next logical step would be to utilize the linkage data for map based gene cloning. In collaboration with the Rice Genome Group (RGP) of the National Institute of Agrobiological Resources, Tsukuba, Japan we are establishing a cloning system for the isolation of resistant genes against Gall Midge. The availabiiity of a saturated genetic map of the rice genome makes it possible to map genes of interest and this information in turn can be utilized to map based gene cloning. This will require: 1) a large mapping population to facilitate the marking of more recombination events between the resistance genes and markers cosegregating with them;

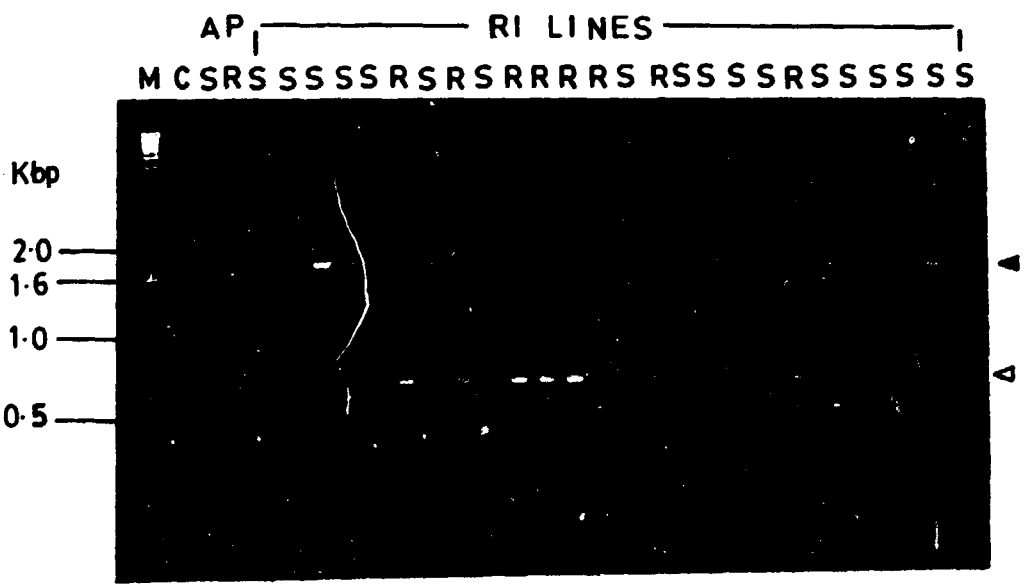

Figure 2. **PCR-based Screening for Gall Midge Resistant and Susceptible Lines in Rice.**
Ethidium bromide stained agarose gel showing DNA fragments after a multiplexed allele-specific PCR performed on genomic DNAs of the two parents (ARC6650 and Phalguna) and recombinant inbred (RI) lines derived from a cross between the two parents. In the multiplexed PCR two pairs of primers, each specific for one of the alleles (Resistant or Susceptible), were used in a single reaction. The lanes M: Molecular weight marker (1 Kb ladder); R: Resistance allele; S: Susceptible allele; A: Susceptible parent ARC6650; P: Resistant parent Phalguna. The closed arrowhead represents the susceptible allele and the open arrowhead represents the resistant allele.

Figure 3. **pNZE25 is organized as a 123 bp repeat element.**
*Hinf*I digested genomic DNA of *O. oryzae* was fractionated on 1.2% agarose gel, transferred to nylon membrane and hybridized to the 300 bp *Hinf*I fragment of pNZE25 or pNZE25 as described. 123 bp ladder (BRL, USA) was used as DNA size marker (lane 2).

2) additional DNA markers around the gene; and 3) a very good Yeast Artificial Chromosome (YAC) or cosmid library with an extensive coverage of the genome.

DNA-typing of Gall Midge

Asian rice Gall Midge (*Orseolia oryzae*) is a major pest of rice which causes enormous economic losses. There are at least 5 different biotypes of Gall Midge reported from India in addition to a few which we reported from other Asian countries. Different biotypes differ in their host range.

During the last 15 years, international screening programmes have identified a number of distinct sources of resistance to Gall Midge and some of these have already been used in breeding programmes. Host plant resistance against insects is an effective and environmentally safe alternative to the use of pesticide. However, the durability of host resistance against a given species of insect is always threatened by the emergence of new biotypes. Unequivocally, identification of Gall Midge biotypes is, therefore, crucial. We have been investigating genetic differences between different biotypes based on polymorphism with respect to repetitive DNA sequences. Probes detecting changes in the structure of repeated sequence among individual biotypes and sympatric species will, therefore, have immediate application. With these objectives, studies were initiated to isolate, characterize and document repetitive DNA sequence clones for potential use in DNA typing of different biotypes and for distinguishing between sympatric species such

as *O. fluvialis* (paspalum Gall Midge).

Gall Midge biotypes were collected from different geographical locations within India. High molecular weight genomic DNA was isolated and a partial genomic library in pUC18 was constructed. Clones representing repetitive sequences were isolated from this library based on differential hybridization screening.

Two classes of repetitive DNA probes were identified from the genomic library of *O. oryzae*. One class, represented by clone pNZE25 has been isolated and characterized. DNA hybridization analyses demonstrated its ability to reveal RFLPs between different *O. oryzae* individuals. It detects genomic sequences whose organization within the genome varies markedly between individuals and biotypes. In a glasshouse population this probe shows genetic uniformity within one biotype. Therefore, this probe can be used to assess the genetic uniformity of field isolates employed to create or maintain a greenhouse population of specific biotypes and for monitoring the genetic diversity of a biotype population.

The other class of probe is represented by the clones pNZE16, pNZE22 and pNZE29. These clones detect sequences that are monomorphic between different biotypes of *O. oryzae* but polymorphic between *O. oryzae* and *O. fluvialis*. Further analysis is indicative of pNZE22 being genetically conserved as "primordial" sequence.

We are currently evaluating the utility of these probes to identify a true alternative host of *O. oryzae*.

Plant Biology: Plant Transformation

V. S. Reddy, G. Cyhandel, S. Choudhury, S. K. Jaiswal.

Tissue Culture of Rice and Cotton

This group concentrates on two plants of economic importance for plant transformation studies: rice and cotton. The regeneration of rice plants from tissue culture has been reported. Since we want to transform rice that is widely planted, four indica rice varieties have been selected; Phalguna, ARC 6650, Ruchi, and, Mashuri. All four varieties have been inducted into callus from mature/immature embryos on MS basal medium containing 2 mg/L of 2,4-D. Regeneration of plantlets was achieved from Ruchi and Mashuri on MS medium containing kinetin and NAA (1 mg/L each). Similar methods will be applied to Phalguna and ARC 6650. We are now in the process of generating suspension cultures from callus for eventual transformation.

In cotton, there is only one variety which has been cultured and from which plants have been regenerated from callus. We have started with the Pusa 8-6 genotype. Several growth regulators, e.g., 2,4-D, kinetin, IAA, NAA, Zeatin riboside were tried in varying concentrations and combinations. Zeatin riboside and 2,4-D were effective for callus induction from cotyledon and hypocotyl explants, respectively. Approximately a concentration of 2,4-D eighty fold higher than the conventionally used amount was optimal for callus induction. Callus originating from meristematic zones close to vascular bundles is being used for regeneration.

Chloroplast Transformation

In higher plants, DNA, the genetic material is present in three organelles: the nucleus, the plastid and the mitochondria. So far the introduction of foreign DNA into the nucleus has been the most common practice in improving the genetic traits of crop plants.

There are several advantages of plastid transformation over the conventional nuclear transformation as described earlier. Insertion of a foreign gene into a plastid genome may result in the amplification of 1,000-10,000 copies of the foreign gene per cell because of the known ploidy of chloroplast DNA. The codon usage of chloroplast genes are more like that of prokaryotic genes and are, therefore, a suitable place in which to express useful bacterial genes.

Our extensive studies on the transcription of chloroplast DNA have shown that 16S ribosomal RNA promoter was more than ten times active *in vitro* compared to psbA or rbcL promoters which have been used in transformation. In order to identify promoters for the optimal expression of foreign genes in chloroplasts, we have investigated the strength of 16S ribosomal RNA promoter in transient expression and compared it with the psbA promoter. Transformation vectors were constructed containing 400 bp rRNA promoter or 412 bp psbA promoter coupled with β-glucoronidase (GUS) coding sequences and ribosome binding sites. Experimental conditions were optimized for the introduction of DNA into tobacco suspension cells and in young leaves using microprojectile bombardment (Figure 1). Histochemical and spectrofluorimetric assays were carried out to follow transient expression of GUS activity for 12 days after bombardment. The maximum expression of GUS using 16S or psbA promoter was found to be around 72 hrs of bombardment. The 16S rRNA promoter consistently showed a three fold

increase in activity compared to the psbA promoter. These recombinants have been modified to contain flanking DNA sequences of chloroplast DNA for site directed integration of GUS into plastid genome and aminoglycoside adenine transferase (aadA) gene in order to confer spectinomycin and streptomycin resistance for the selection of stable transformants. Using these recombinants, transgenic plants are being generated.

Electron microscopic studies have led to the identification of displacement loops (D-loops) on pea chloroplast DNA. The recombinant plasmids containing such D-loop sequences have been shown to replicate in an *in vitro* replication system. We introduced these D-loop containing sequences (2-4 kb) into transformation vectors described above in order to investigate their role in the transformation of plastids. The preliminary results indicate that the expression of GUS is continued up to 2 weeks of transformation when D-loop containing sequences are present in the transformation vectors, wheras no such activity could be detected in the control vectors lacking D-loop sequences. In order to produce transgenic plants, an EPSP synthase from *Bacillus* is being introduced into transformation vectors for selecting stable transplastomic lines.

Figure 1.
Tobacco leaf showing GUS activity after microprojectile bombardment with plasmid construct containing chimeric GUS gene. Conditions were optimized for delivering DNA into the leaf/suspension cells. Each blue spot represents an independent transformation event. Using this method the strength of 16S ribosomal RNA promoter was compared with psbA promoter from pea chloroplast DNA.

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The ICGEB Collaborative Research Programme

The Collaborative Research Programme of ICGEB is now entering its "mature" phase. With several projects completed and many more in full progress, the Programme has begun to show its viability, through an ever increasing number of publications by the responsible Principal Investigators. It is also hoped that it will lead to some patents for the relevant Affiliated Centres.

Following an analysis of its first years of operation, and upon specific recommendation by the Panel of Scientific Advisers, Affiliated Centres were requested to increase regional cooperation among different Member Countries. The response to this has been very positive.

During 1993, from a total of 37 research proposals submitted, 22 were approved for funding by ICGEB (Figure 1), for a total commitment of US$ 1,198,000. Three of these proposals, originating from Member Countries which have not yet ratified the ICGEB Statutes, are still pending the decision by the Board of Governors of the eligibility of Affiliated Centres located in countries which are not full members of ICGEB.

The progress of the Collaborative Research Programme from the time of its inception is presented in Figure 2, while a listing of projects approved in 1993 is given in Table 1. A total of 35 Principal Investigators, working on projects financed with ICGEB funds, have submitted scientific reports which are presented in the following pages.

Figure 1: Collaborative Research Grants Awarded in 1993

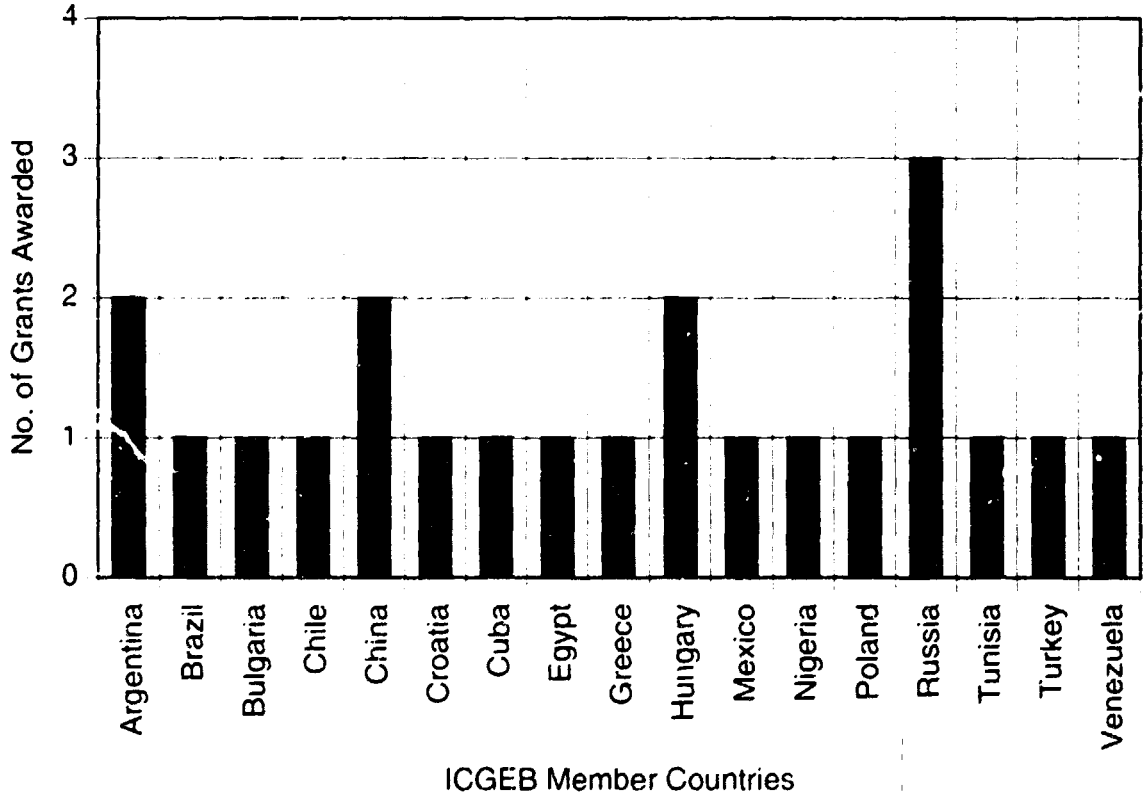


Table 1: Collaborative Research Projects Approved in 1993

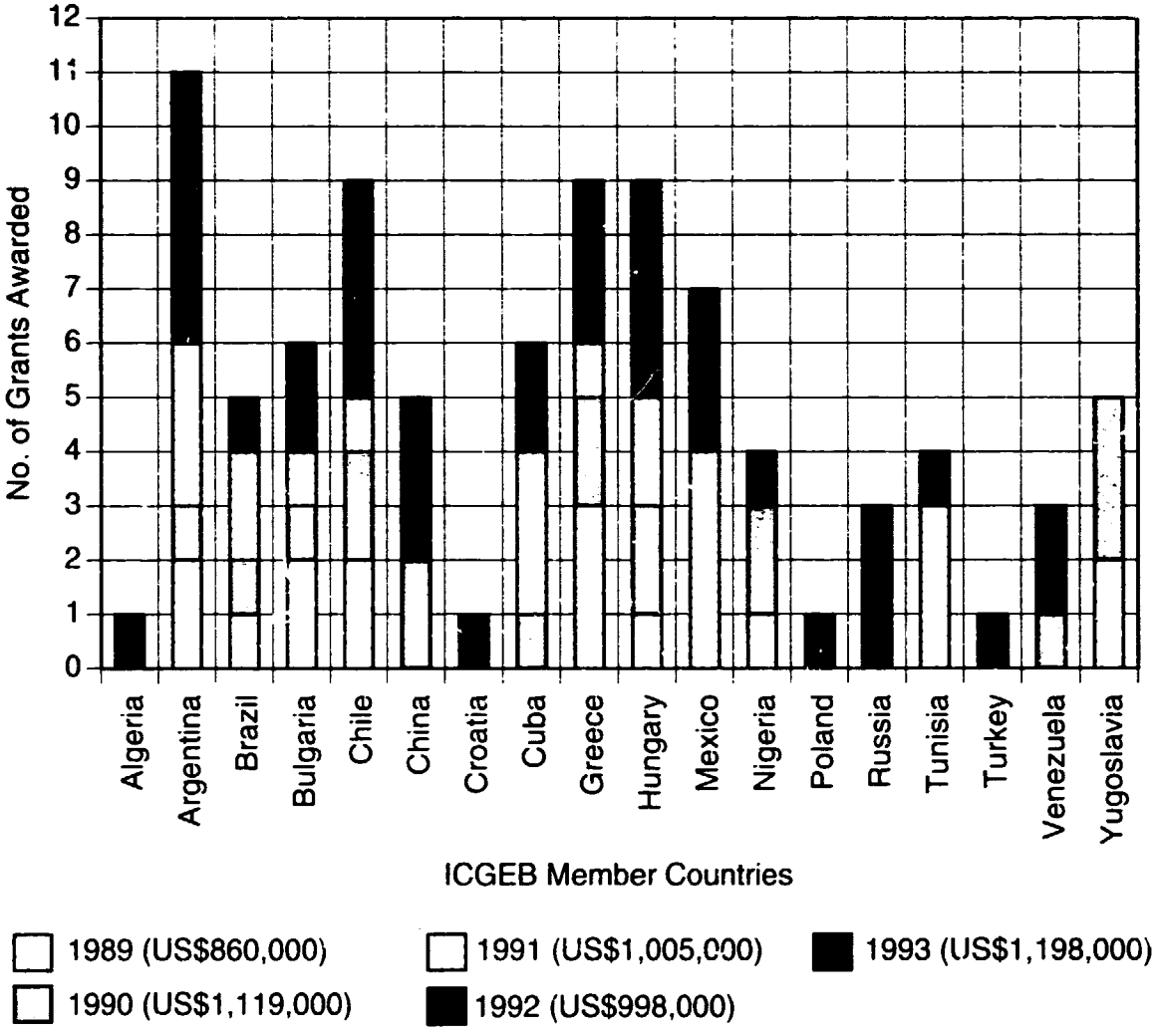
COUNTRY	PRINCIPAL INVESTIGATOR	TITLE OF RESEARCH PROJECT
Argentina	Eduardo H. CHARREAU *Instituto de Biologia y Medicina Experimental. Buenos Aires*	Molecular mechanisms of progestins induction of murine mammary cancer.
	Susana PASSERON *Departamento de Quimica Biologica Universidad de Buenos Aires Buenos Aires*	Molecular basis of dimorphism in the pathogenic fungus *Candida albicans* the causative agent of candidosis.
Brazil	Marlinda Lobo de Souza PINHEIRO *Area de Controle Biologico, Brasilia*	Molecular biology of virus-host interactions for AgMNPV, an important biocontrol agent for a major pest of soybean.
Bulgaria	George RUSSEV *Institute of Molecular Biology, Bulgarian Academy of Sciences. Sofia*	Protein factors controlling DNA replication in mammalian cells.
Chile	Manuel KRAUSKOPF *Institute of Biochemistry. Universidad Austral de Chile, Valdivia*	Studies of growth control in fish. Effect of environmental factors.
China	Ming-Guo ZHOU *Department of Plant Protection. Nanjing Agricultural University, Nanjing*	Resistance of fudaria on rice and wheat to fungicides.
	Bing-quan WU *School of Basic Medical Sciences, Cancer Research Center. Beijing Medical University, Beijing*	Modulation of tumor growth and metastasis by IL-6 and IL-8 gene transfer.
Croatia	Drago PETRANOVIC *Department of Molecular Genetics, Ruder Boskovic Institute. Zagreb*	Genes and enzymes involved in the inhibition of recombination.
Cuba	Jose DE LA FUENTE GARCIA *Mammalian Cell Genetics Division. La Habana*	Studies of growth control in fish. Transgenesis for manipulating growth in fish.
Egypt	Samia ALI TEMTAMY *National Research Centre. Cairo*	New DNA techniques for identification of mutations in the beta globin gene in Egyptian beta thalassemia patients.
Greece	Penelope MAVROMARA-NAZOS *Hellenic Pasteur Institut. Athens*	Human papillomavirus (HPV) and cervical cancer in Greece: Epidemiological studies HPV typing, development of immunoreagents.
Hungary	Istvan SIMON *Biological Research Centre. Institute of Enzymology, Budapest*	Structural principles of repetitive sequence motifs in proteins.

Table 1 continues on the next page

Table 1: Collaborative Research Projects Approved in 1993 *continued*

COUNTRY	PRINCIPAL INVESTIGATOR	TITLE OF RESEARCH PROJECT
Hungary	Laszlo HORNOK *Agricultural Biotechnology Center. Godollo*	Strain specification, genome analysis and isolation of genes encoding lysing enzymes in the genus *Trichoderma*.
Mexico	Edmundo LOZOYA GLORIA *CINVESTAV - Unidad Irapuato. Guanajuato*	Molecular factors involved in a plant-defence response.
Nigeria	Michael O. EZE *Tropical Diseases Research Group. Department of Biochemistry, University of Nigeria. Nsukka. Enugu State*	Antioxidants, red cell atpase, malaria and sickle cell.
Poland	Wojciech NIEWIAROWSKI *Polish Academy of Sciences. Centre of Molecular and Macromolecular Studies. Lodz*	Biological activity of CFTR (Cystic Fibrosis Transmembrane Regulator) protein mutants.
Russia	Georgii P. GEORGIEV *Institute of Gene Biology, Russian Academy of Sciences. Moscow*	The role of the metastasin 1 gene and its product in cancer metastasis.
	Mikhail P. KIRPICHNIKOV *Institute of Molecular Biology. Russian Academy of Sciences. Moscow*	Engineering of proteins with pre-determined structure and biological functions based on 'de novo' protein albebetin structure.
	Sergei RAZIN and Anderj LUCHNIK *Institute of Gene Biology. Russian Academy of Sciences. Moscow*	Characterization of the domain organisation of the eukaryotic genome.
Tunisia	Samir JAOUA *Centre de Biotechnologie. Laboratory Biopesticides. Sfax*	*Bacillus thuringiensis* in crop protection: strains isolation and molecular characterization and production of the entomocidal toxins.
Turkey	Huseyin BAGCI *Akdeniz University, School of Medicine. Department of Genetics, Antalya*	Determination of the spectrum of β-thalassemia genes by use of Southern and Dot-Blot analysis of amplified β-globin DNA from heterozygous subjects in Antalya-Turkey.
Venezuela	Maria Gloria DOMINGUEZ BELLO *Lab. de Fisiologia Gastrointestinal, Centro de Biofisica y Bioquimica, Caracas*	Study of the digestive microflora as a tool for overcoming nutritional constraints of tropical legumes.

Figure 2: Progress of the Collaborative Research Programme 1988-1993



For 1994, 48 collaborative research proposals were submitted for consideration; the number of proposals per Affiliated Centre is given below:

• Argentina	4	• Greece	1
• Bulgaria	3	• Hungary	4
• Chile	4	• Mexico	5
• Colombia	1	• Nigeria	2
• Costa Rica	2	• Russia	2
• Croatia	5	• Tunisia	1
• Cuba	4	• Turkey	3
• Egypt	3	• Venezuela	4

Peer reviewing of these proposals has commenced and a final evaluation will be made in the third quarter of 1994.

Collaborative Research Programme Activity Reports

ARGENTINA

IMPROVEMENT OF CHAGAS DISEASE DIAGNOSIS AND PROGNOSIS OF CARDIAC INVOLVEMENT

Principal Investigator [illegible] INGEBI-CONICET [illegible] ARGENTINA [illegible]

Keywords [illegible]

Abstract [illegible]

Background [illegible]

Objectives [illegible]

1. A specific diagnostic test [illegible] Chagas disease; and

2. [illegible]

Work Progress

[illegible]

Leishmaniasis. The sera from chagasic patients reacted with the R-13 peptide but reacted weakly with the L-13 peptide. Sera from six Bolivian patients with the mixed infection show a chagasic pattern of anti-P reactivity, recognizing R-13, and weakly L-13. The results demonstrate the extreme importance of the Glu residue located 11 residues from the C-terminus in the generation of the anti-ribosomal P protein reactivity, and non antigenic nature of the C-terminal portion of the Leishmania P proteins in the human infection.

3) Characterization of a *T. cruzi* peptide sequence that cross reacts with the second intracellular loop of the b-1 adrenoreceptor.

A five amino acid peptide of the C-terminal portion of the *T. cruzi* ribosomal PO protein seems to generate a cross reactive autoimmune response. In fact, antibodies to this region seem to be markers of severe heart complaint (see above). The sequence AESEE, is homologous to the AESDE sequence present in the second extracellular loop of the b-1 adrenoreceptor (b-1 loop peptide: HWWR AESDA ARRCYNDPKCCDFVTNR). We demonstrate that the anti-PO reactivity of chagasic sera is partially inhibited by the b-1 peptide, whereas the anti-b-1 reactivity of the sera is partially inhibited by the AESEE peptide. These inhibitions were confirmed by Western blotting. The results suggest a general mechanism in Chagas disease (molecular mimicry), by which antibodies generated against parasite intracellular proteins may have a direct effect on membrane proteins of the host cells, affecting their function and/or viability.

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Kaplan, D., Vazquez, M., Saunal, H., Muller, S., Levitus, G., Van Regenmortel, M.H.V. and Levin, M.J. (1993) Differences in the immune response of patients with Chagas disease or systemic lupus erythematosus to human ribosomal P peptides. Mem. Inst. Oswaldo Cruz 88, 51-52.

Rosenbaum, M.B., Chiale, P.A., Schejtman, D., Levin, M.J., Elizari, M.V. Antibodies to b-adrenergic receptors disclosing agonist-like properties in idiopathic dilated cardiomyopathy and Chagas heart disease. J. Cardiovascular Electrophysiology. In press, 1994.

Networking: Laboratories that were in contact with our group in the period covered by this report: M.S. FIOCRUZ, Centro de Pesquisas René Rachou, Belo Horizonte, Brazil; Instituto de Higiene, School of Medicine, Universidad de la Republica, Montevideo, Uruguay; Institato de Medicina Tropical Alexander Von Humboldt, Lima, Peru.

In accordance with the participants of the net, we agreed to perform the assay of the recombinants and synthetic peptides with Dr. Luquetti (Institute of Tropical Medicine, Goiana, Brazil). Furthermore, contacts were made with the Instituto Boliviano de Biologia de la Altura (Dr. Mireille Hontebeyrie's laboratory, IBBA, La Paz, Bolivia). In fact, the work concerning the antibodies reacting with the antib-1 receptors is performed in close collaboration with IBBA, the Centro de Pesquisas René Rachou together with Dr. Johan Hoebeke from the University François Rabelais, Tours, France. The work performed on the antigenicity of P proteins in Leishmaniasis was carried out by INGEBI, the Instituto de Medicina Tropical Alexander Von Humboldt, and the laboratory of Dr. Carlos Alonso (Centro de Biologia Molecular, Universidad Autonoma de Madrid, Spain).

HUMANIZATION OF MONOCLONAL ANTIBODIES FOR CANCER TREATMENT

Principal Investigator: Jose Mordoh, Instituto de Investigaciones Bioquimicas, "Fundacion Campomar", Patricias Argentinas 435, 1405 Buenos Aires, ARGENTINA. Tel.: +54-1-884015, Fax: +54-1-8652246

UNIDO Contract No. 92/057

ICGEB Reference No. CRP/ARG91-02

Keywords: monoclonal antibodies, humanized, cancer treatment

Abstract: The work plan involves several interrelated projects:

1) In our laboratory several mouse (m) monoclonal antibodies (MAbs) directed against tumor proliferating cells have already been produced, some of which have the ability to produce clinical remissions in cancer patients (4,8). To circumvent the generation in patients of human anti-mouse antibodies (HAMA), the humanization of mMAbs has been undertaken. For this purpose the nucleotide sequences and the corresponding DNA fragments of the V-L and V-H fragments will be obtained, amplified and cloned into suitable vectors. As a first step, the replacement of the mouse Ig C regions will be performed, once the chimaeric MAbs are obtained, their expression in eukaryotic systems will be attempted, and the selection of Ig + producing transfectants will be carried out. Once this step is achieved, the specificity and biological activity of the humanized (h) MAbs will be analyzed by comparison to the original mMAbs.

2) Once the previous steps are satisfactorily completed, the chimaeric antibodies will be produced in a larger scale. After adequate purification and controls, their use in Phase I Clinical Protocols similar to those already being conducted will be considered. The main point at this step will be to determine if the chimaeric MAbs have lost the ability to induce a human immune response. Their efficacy to induce clinical remissions will also be estimated.

3) The construction of chimaeric MAbs molecules only containing the mouse CDR regions will be attempted at a later stage, and a similar sequence of assays to those already described will be performed.

4) The antigens recognized by the MAbs have yet to be characterized. The recognition and isolation of these antigens would enable us to obtain second-generation mMAbs that could have greater affinity and mediate more efficiently complement and cell-mediated toxicities.

5) The antigenic characteristics of the tumor stem cells will continue to be studied. Such knowledge will enable us to obtain a greater range of MAbs directed against the tumor stem cells. The MAbs that prove more interesting will be also submitted to the humanization procedure.

Background: The use in cancer therapy of MAbs directed against tumor associated antigens (Ags) offer the theoretical advantage of greater specificity than that obtained with conventional cytotoxic agents. However, most of the clinical results performed so far yielded only modest results (see i.e. Saleh M.N. *et al.*, 1993, Cancer Res. 53, 4555-4562). A priori, the lack of success of MAbs in therapy could be due to several reasons: i) heterogeneity in Ag expression within tumors; ii) binding to inappropriate antigens; iii) shortage of effector mechanisms; iv) poor MAb permeation into tumors; v) short interval of exposure of tumor cells due to the clearance kinetics of murine MAbs and vi) human anti-mouse immunoglobulin (HAMA) formation. One of the causes of cellular heterogeneity in tumors is the coexistence of cellular subpopulations with different grades of differentiation, i.e. stem cells, semi-differentiated cells and differentiated cells (see i.e. Ballare C. *et al.*, 1989, Cancer, 65, 842-848). Since an aim of any curative intent is the elimination of the tumor stem cells, we have developed MAbs directed against proliferating cells. Specially interesting is FC-2.15, since in the presence of complement it diminishes the clonogenic potential of tumor cells but not of normal bone marrow. Since FC-2.15 has shown clinical efficacy in Phase I clinical trails, we are attempting its humanization to circumvent HAMA formation.

Objectives:

1) Obtention and characterization in our laboratory of first

and second - generation mouse monoclonal antibodies.

2) Humanization of these MAbs which exhibit interesting clinical characteristics.

3) Characterization of stem cells from human solid tumors.

Work Progress:

1) An extensive description of one of the MAbs developed in our laboratory, FC-2.15, has been performed (3,7). Basically, FC-2.15 is an IgM obtained by immunizing Balb/c mice with tumor epithelial cells from a human undifferentiated carcinoma. Its main features are: 1. It reacts with more than 90% of breast primary tumors, independently of their histology and hormone receptors content; 2. It reacts with 80% of total breast malignant cells and with more than 90% proliferating cells; 3. It recognizes other neoplasia such as colon cancer, squamous carcinoma and melanoma; 4. By Western blots it detects three major bands of Mr 160, 130 and 115 kDa in membrane extracts; 5. It has strong cross-reactivity with kidney proximal convolute tubules, large bowel epithelium, bone marrow myeloid progeny and peripheral granulocytes.

2) Since this MAb has the ability to produce clinical remissions in cancer patients (8) and has apparently better therapeutic properties than other MAbs previously studied (4), it was the first selected to be humanized. For this purpose the first step performed was the obtention of total RNA from the selected hybridoma. Afterwards, in Dr. Burrone's laboratory (ICGEB - Trieste) the cDNA fragments corresponding to the light (V-L) and heavy (V-H) variable regions from mouse FC-2.15 MAb were obtained by reverse transcriptase reaction. Once these fragments were amplified by PCR techniques with oligonucleotides designed in Dr. Burrone's laboratory, they were sequenced and cloned into suitable vectors. The expression of the chimaeric MAb obtained is being attempted in eukaryotic systems. Several transfectants producing human IgG were selected from myeloma and CHO cell lines. In Dr. Burrone's laboratory they are currently optimizing the antibody production from these cell lines.

3) Since previous studies from our laboratory have supported the presence of a stem cell hierarchy in human breast cancer, we have further investigated the correlation between the expression of several markers and differentiation in this disease.

a. We have established several new cancer cell line from breast (5) and melanoma (manuscript in preparation). We have analyzed in these cell lines several genetic and antigenic profiles that will help to understand the nature of stem cells and differentiation.

b. We have studied the expression of several antigens in human breast cancer and melanoma that will contribute to define a pattern of antigenic expression (1,2,9).

Publications:

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Mordoh, J., Leis, S., Bravo, A.I., Podhajcer, O.L., Ballare C., Capurro, M., Kairiynma, C. and Bouver, L. Description of a new monoclonal antibody, FC-2.15, reactive with human breast cancer and other human neoplasia. Int. J. of Biological Markers, in press.

Networking: This project is being carried out in collaboration with Oscar Burrone Ph.D., Head, Molecular Immunology Group, International Centre for Genetic Engineering and Biotechnology.

REGULATION OF THE FIBRONECTIN GENE TRANSCRIPTION IN LIVER

Principal Investigator: Alberto R. Kornblihtt, Instituto de Investigaciones en Ingenieria Genetica y Biologia Molecular (INGEBI-CONICET), Vuelta de Obligado 2490 2 Piso, 1428 Buenos Aires, ARGENTINA. Tel.: +54-1-7845516/7832871, Fax: +54-1-7868578

UNIDO Contract No. 92/059

ICGEB Reference No. CRP ARG91-03

Keywords: transcriptional regulation, fibronectin

Abstract: The long term objective of this proposal is to gain understanding in the molecular mechanisms that control the expression of the fibronectin (FN) gene in liver, in normal and altered conditions. The project focuses specifically on the protein-protein interaction between two transcription factors that bind to two cis elements closely located within the promoter region of the human FN gene: the cyclic AMP responsive element and the CAAT box. Competitions with sequence-specific oligodeoxynucleotides, the use of specific antibodies and partial protein purification will permit the identification of the actual members of the CRE and CAAT binding proteins families that take part in the interaction. *In vitro* transcription experiments with nuclear extracts from normal adult, fetal, aged or regenerating liver, using the powerful G-free cassette system, will allow us to assess the role of this protein-protein interaction in the transcriptional activity of the gene in different cell conditions. Finally, a functional association between the particular occupancy of the FN promoter in liver and the liver-specific pattern of alternative splicing of FN pre-mRNA will be investigated. The latter constitutes a completely unexplored field.

Background: The gene for fibronectin is a 'housekeeping' gene, being expressed in a wide variety of cell types within the organism. In cells in culture, its expression can be dramatically regulated both positively and negatively by hormones, viral and oncogenic transformation and growth factors. In the whole organism, acute phase (sepsis after burning or trauma, healing, organ regeneration) as well as cancer and ageing are conditions in which the transcription and alternative splicing of the FN gene are deeply altered. The metastic and invasive capacities of cancer cells are also critically dependent on the expression of fibronectin. Cancer, ageing or regenerating cells suffer a re-programming of their genetic systems that, among other effects, alters their adhesive and migratory behaviours. This project intends to elucidate the molecular mechanisms that control the expression of the FN gene in normal and affected liver cells. Some of the transcription factors that control FN gene expression are oncogene products (CREB, Jun and Fos families), and it is conceivable that their altered expression in cancer cells lead to changes in the regulation of FN gene transcription. In this sense, (FN and oncogenes) this project is tightly related to the molecular biology of cancer. On the other hand, from a basic molecular biology point of view, our project includes a section which investigates the totally unexplored relationship between transcriptional control and alternative splicing.

Objectives:

1) Characterization of the CREB and CAAT binding protein that interact with the FN promoter.

2) Perturbation experiments to abolish the CREB/CAAT binding protein interaction.

3) *In vitro* transcription of the FN gene promoter. To determine if the observed interaction between these two transcription factors that bind to the FN promoter plays any role in the transcriptional activity of the FN gene promoter in liver.

4) Variations with the cell type and status. To investigate the effect of the cell type, oncogenic transformation, liver regeneration and ageing on the transcriptional regulation of the FN promoter.

5) Purification of the transcription factors. The CREB/ATF and the CAAT binding protein from liver that bind to the FN promoter will be purified.

6) Correlation between the FN promoter activity and the alternative splicing of the FN primary transcript.

Work Progress: In the fibronectin (FN) gene promoter the cAMP response element (CRE) and the CCAAT box are separated by only twenty base pairs, i.e. two turns of double helix. Binding of nuclear proteins to these elements, assessed by DNase I footprinting, differs in the different cell types: while in a variety of cells tested (HeLa, granulosa, brain and adenocarcinoma) only CRE-binding activity is observed, liver extracts show both CRE and CCAAT binding activities. Competitions with CRE oligonucleotides were able to prevent the binding of both liver factors, while competitions with CCAAT oligonucleotides only abolished the binding to the CCAAT box. Consistently, the occupation of the CAAT box was reduced when the distance between the CRE and CCAAT elements was increased in a series of spacing mutants in which DNA fragments of 20, 28 or 44bp were inserted; and in a construct where the CRE sequence was deleted. Furthermore, the mutants are less efficient than the wild type as templates for *in vitro* transcription elicited by liver

nuclear extracts. Transcriptional activity decreases with the 20 and 28 bp insertions but is partially recovered with the 44 bp insertion. Partial purification of liver CRE- and CCAAT-binding proteins by HPLC on a mono Q column and recombination of column fractions showed that a novel 73 kDa CRE-binding protein facilitates the association of the CCAAT-binding protein to the CCAAT site of the FN gene.

We showed that the 73 kDa CRE-binding proteins is ATF-2 (also named CRE-BP1), a protein implicated in recruiting transcriptional activators to promoters, able to form heterodimers with Jun and for which a sequence-deduced MW of 55 kDa had been reported before.

We are currently preparing FN promoter clones mutagenized *in vitro* in the CRE, CCAAT and TATA sites in order to perform transfections and CAT assays with different liver and non liver cell lines.

Publications:

Muro, A.F., Bernath, V.A. and Kornblihtt, A.R. (1992) Interaction of the -170 cyclic AMP response element with the adjacent CCAAT box in the human fibronectin gene promoter. J. Biol. Chem. 267, 12767-12774.

Srebrow, A., Muro, A.F., Werbajh, S., Sharp, P.A. and Kornblihtt, A.R. (1993) The CRE-binding factor ATF-2 facilities the occupation of the CCAAT box in the fibronectin gene promoter. FEBS Lett. 327, 25-28.

Muro, A.F., Pesce, C.G. and Kornblihtt, A.R. (1993) DNA Sequencing by the chemical method: a 10 minute procedure for the G + A reaction. Trends in Genetics 9, 337-338.

As it was mentioned in previous reports our work with transcription factors that regulate the expression of the fibronectin gene led to a side line of research in which we investigated the binding of transcription factors to a satellite DNA present in certain species of South American rodents. Since the techniques, experimental methodologies and lines of evidence are congruent with the research related to the fibronectin project, the following publications are included in this report:

Rossi, M.S., Pesce, C.G., Rieg, O.A., Kornblihtt, A.R. and Zorzopulos, J. (1993) Retroviral-like features in the monomer of the major satellite DNA from the South American rodents of the genus *Ctenomys*. DAN Seq. 3, 379-382.

Pesce, C.G., Rossi, M.G., Muro, A.F., Zorzopulos, J. and Kornblihtt, A.R. Binding of transcription factors to the major satellite DNA from the South American rodents of the genus *Ctenomys*. Nucleic Acids Res., in press.

In addition, the following was published in a local journal:

Satz, M.L. and Kornblihtt, A.R. (1993) La reaccion en cadena de la polimerasa (PCR) y sus aplicaciones. Ciencia Hoy 4, 53-59.

Networking: ICGEB - Trieste Prof. F. E. Baralle's group

MOLECULAR GENETICS OF PYRUVATE DECARBOXYLASE IN FUNGI

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ICGEB Reference No. CRP ARG92-02

Keywords: fungi, cellular matrix, fermentation, ethanol, recombinant DNA, chromosome walking, RFLP mapping, gene regulation.

Abstract & Work Progress:

The structural gene encoding *N. crassa* pyruvate decarboxylase was cloned from a genomic library constructed in the cosmid vector pMOcosX. The 5' region of the gene was defined by both Southern blot analysis and partial nucleotide sequencing. DNA fragments containing the putative promoter activity were fused to the bacterial gene hygromycin phosphotransferase (*hph*) to measure gene expression *in vivo*. Cells transformed with these constructs were either resistant or sensitive to hygromycin in media containing glucose or ethanol, respectively, as unique carbon sources. The level of antibiotic resistance was dependent on glucose and ethanol concentration in the culture media, and correlated positively with the amount of PDC-mRNA determined in Northern blot experiments. The experiments indicate that the analysed region contains all the information required in *cis* for normal expression of the *N. crassa* PDC gene. Initial gel shift analyses of the promoter was performed by using total protein mycelial extracts. Only unspecific DNA-protein complexes were obtained in these experiments.

Mutant alleles of the *N. crassa* morphological locus *snowflake* (*sn*) alter the assembly state of the *N. crassa* PDC filaments. To clone *snowflake*, which is on the centromere region of Linkage Group I (CEN), we are performing a convergent chromosome walk directed to the centromere. At present we have cloned about 500 Kb corresponding to flanking regions of CEN, distributed in three medium size contigs. Overlapping clones were obtained by using two ordered genomic cosmid libraries (pSV50 and pMOcosX) and a lambda phage library (EJ1). T3/T7 riboprobes and RFLP segregation analysis were used to walk and to find linkage relationships, respectively. To cover larger DNA regions we are using a YAC library constructed in the vector pYAC41. By protoplast transformation and phenotypical rescue we have cloned the gene *eth-1* (ethionine resistance), linked to CEN. Molecular cloning of *eth-1*-cDNAs, and partial nucleotide sequencing, demonstrated that *eth-1* encodes S-adenosyl-methionine synthetase. Physical distances between LGI linked loci, distant from the region of putative strong centromeric crossover suppression, are 3 to 5 times higher than expected from classical genetic data. We have proposed that in *N. crassa* recombinational activity is less in large chromosomes than in small ones.

Background: In addition to microtubules and microfilaments (McKerracher & Heath, 1987), fungi show a diversity of cytoplasmic filaments whose biochemical nature and function are unknown (Rosa *et al.*, 1990a). Recently, we have purified and characterized giant 8-10 nm diameter filament bundles present in the filamentous fungus *Neurospora crassa* (Rosa *et al.*, 1990a,b). The filaments are complex homopolymeric arrays of a 59-kDa polypeptide (P59Nc) that is encoded by the locus *cfp* on the right arm of LG VII, as was defined by restriction fragment length polymorphism mapping (Haedo *et al.*, 1992). P59Nc is immunologically unrelated to tubulin or actin and is aberrantly assembled in a particular type of *N. crassa* morphological mutant (Allen *et al.*, 1974; Rosa *et al.*, 1990a,c; Temporini & Rosa, 1993). We have recently reported that P59Nc is pyruvate decarboxylase (PDC), which is the key enzyme in the pathway of ethanol production by fungi and some plants and bacteria (Ullrich, 1982; Bringer-Meyer *et al.*, 1986). This finding represented the first demonstration of a complex supramolecular arrangement of this enzyme. The presence of abnormally sized and shaped bundles of PDC filaments in the mutants *snowflake* suggests that *sn* may encode a posttranslational modifier of the supramolecular properties of assembly of PDC (Rosa *et al.*, 1990c).

Gene expression of yeast and bacterial PDCs is dependent on the carbon source present in the culture medium (Brau & Sahmn 1986; Butler & McConnell 1988; Kellermann & Hollenberg 1988; Hohmann & Cederberg 1990). We have recently demonstrated that the cytoplasmic abundance of the 8-10-nm filaments is dependent on the metabolic growth conditions of the fungus (Alvarez *et al.*, 1993). This finding may shed light on the nature of other abundant and cryptic filament bundles in filamentous fungi and yeast (see Rosa *et al.*, 1990a), as well as bundles of filaments present in plants (Powell *et al.*, 1982).

Objectives: Present work in our laboratory concerns to the study of the physiological role of the *N. crassa* pyruvate decarboxylase filaments. The present ICGEB project focuses on the molecular mechanisms underlying gene regulation of the P59Nc gene and the study of the *snowflake* locus. Specific targets are: study of regulatory *cis acting* sequences at the 5' region of the P59Nc gene and molecular cloning of the *snowflake* gene by chromosome walking on the centromeric region of LGI.

Publications:

Haedo, S., Temporini, E., Alvarez, M.E., Maccioni, H.J.F. and Rosa, A.L. (1992) Molecular cloning of a gene (*fpp*) encoding the cytoplasmic filament protein P59Nc and its genetic relationship to the snowflake locus in *Neurospora crassa*. Genetics 131, 575-580.

Haedo, S. Mautino, M. and Rosa, A.L. (1992) Non Oak Ridge RFLP patterns in the *Neurospora crassa* multicent-2 strain. Gunfal Genet. Newslett. 39, 23.

Temporini, E. and Rosa, A.L. (1993) Pleiotropic and differential phenotype of two *sn* (snow flake) mutant alleles in *Neurospora crassa*: analyses in homokaryotic and heterokaryotic cells. Curr. Genet. 23, 129-133.

Alvarez, M.E., Rosa, A.L., Temporini, E., Wolstenholme, A., Panzetta, G., Patrito, L. and Maccioni, H.J.F. The 59-kDa polypeptide constituent of 8-10 nm cytoplasmic filaments in *Neurospora crassa* is a pyruvate decarboxylase. Gene 30, 253-258.

Mautino, M.R., Haedo, S.D. and Rosa, A.L. (1993) Physical mapping of meiotic crossover events in a 200 Kb cloned region of *Neurospora crassa* Linkage Group I. Genetics 134, 1077-1083.

Mautino, M.R. (1994) A computer program for circular restriction maps construction. CABIOS (accepted for publication).

ROLES OF POLYAMINES IN THE CONTROL OF PARASITE PROLIFERATION

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Keywords: polyamines, *Trypanosoma cruzi*, *Leishmania mexicana*

Abstract: During the present period we have investigated different aspects of the three topics included in our original project. The main achievements have been:

1) A DNA fragment of *Leishmania mexicana* ODC gene has been amplified by PCR using appropriate primers, and then cloned in the pBluescript SK(-) vector and sequenced. This fragment will be labelled by nick translation or random priming techniques and used as a probe for hybridization analysis of *Trypanosoma cruzi* and other parasites DNA and for screening of *T. cruzi*, *Leishmania mexicana* and *Crithidia fasciculata* genomic libraries.

2) *Leishmania mexicana* ODC has been extensively purified and some of its catalytic and structural properties have been investigated. Apoenzyme preparations completely free of cofactor were obtained and the process of enzymatic activity restoration by addition of pyridoxal 5' phosphate was studied.

3) We have investigated different characteristics and properties of polyamine transport systems in *T. cruzi*, *Leishmania mexicana* and *Crithidia fasciculata*. A regulatory mechanism of putrescine uptake in *Leishmaniamexicana* by feedback repression has been found and described.

Background: Many biochemical studies on human parasites have been carried out to understand the metabolism and life cycle of these organisms at the molecular level with the aim of finding appropriate chemotherapeutic strategies against parasitic diseases.

It is known that polyamines play important roles in cell proliferation and differentiation (Bachrach, U. and Heimer, Y.M. The Physiology of Polyamines. CRC Press, 1989; Pegg, A.E., Cancer Res. 48, 759, 1988). This conclusion led to the investigation of polyamine biosynthesis, degradation and transport systems in a variety of cells. Among all the enzymes catalyzing the different steps involved in the biosynthesis and interconversion of polyamines in eukaryotic cells, ornithine and S-adenosylmethionine decarboxylases, as well as the acetyltransferases of spermidine and spermine are the key enzymes which regulate the whole pathway (Bachrach, U. and Heimer, Y.M. The Physiology of Polyamines. CRC Press, 1989).

Ornithine decarboxylase (ODC), the most extensively studied enzyme of the polyamine pathway, can be regulated inside the cell at different levels of gene expression including post-translational modifications and protein degradation (Hayashi, S., Ornithine Decarboxylase: Biology, Enzymology and Molecular Genetics. Pergamon Press, 1989). These properties of ODC can explain why this enzyme is considered a putative good target to block cell proliferation by using specific inhibitors of polyamine biosynthesis (McCann, P.P. *et al.*, Inhibition of Polyamine Metabolism. Biological Significance and Basis for New Therapies. Ac. Press, 1987). This approach has been followed to induce polyamine depletion in cell cultures as well as in whole animals. In this way it has been possible to decrease the growth of tumors *in vivo* (Kingsnorth, A.N. *et al.*, Cancer Res. 43, 4031, 1983) and to block parasite proliferation in infected animals (Pegg, A.E. and McCann, P.P., ISI Atlas of Science: Biochemistry, pp. 11, 1988; Bitonti, A.J. *et al.*, Exp. Parasitol., 64, 237, 1987).

Difluoromethylornithine (DFMO) and other related compounds are potent inhibitors of ODC. It has been shown that African trypanosomes are extremely sensitive to these drugs, probably due to the unusual stability of the parasite ODC compared to the short life of the mammalian cell host enzyme (Coffino, P., in Ornithine Decarboxylase: Biology, Enzymology and Molecular Genetics, Hayashi, S., Ed., Pergamon Press, pp. 135, 1989).

We have shown that ODC of *Leishmania mexicana* is remarkably stable *in vivo* (Sánchez, C.P. *et al.*, Biochem. Biophys. Res. Commun., 161, 754, 1989) and that the parasite multiplication can be arrested by DFMO when synthetic growth medium was used, but not with rich media (González, N.S. *et al.*, Biochem. Biophys. Res. Commun., 180, 797, 1991). In the latter case, although putrescine biosynthesis is inhibited, *Leishmania* can overcome polyamine depletion by taking up these substances from the external environment.

Recent experiments have suggested that in *T. cruzi* polyamine biosynthesis might involve arginine decarboxylase (ADC) rather than ODC (Majumder, S. *et al.*, J. Parasitol., 78, 371, 1992). However, only traces of ADC have been detected in *Trypanosoma cruzi* (Algranati, I.D. *et al.*, in The Biology and Chemistry of Polyamines, ICSU-IRL Press, pp. 137, 1990), and polyamines might be preferentially obtained by the parasite from the external medium. For these reasons we are investigating the transport of polyamines and its regulation in *T. cruzi* and *Leishmania* as well as the structure of genes related to polyamine metabolism and their expression in different forms of both parasites.

Objectives: Our original research plan involved the following topics related to polyamine metabolism and the molecular mechanisms of their regulation in *Leishmania* and *Trypanosoma cruzi*:

1) Search for a putative ODC gene and studies on the regulation of its expression in *Trypanosoma cruzi*.

2) Polyamine metabolism regulation and gene expression amastigote and promastigote forms of *Leishmania*.

3) Transport systems of polyamines in *T. cruzi* and *Leishmania mexicana*.

Work Progress: The present technical report describes the advances achieved in the above mentioned topics during this period:

1) In order to look for the ODC in the *Trypanosoma cruzi* genome, we have prepared a probably appropriate probe from the DNA of another trypanosomatid; we have used *Leishmania mexicana* promastigotes for this purpose.

a. **Amplification of a Leishmania DNA fragment by PCR**: After comparison of the amino acid sequences corresponding to ODC from different organisms (mouse, yeast, *Trypanosoma brucei* and *Leishmania donovani*) we have selected several regions showing almost complete sequence identity and we have prepared six oligonucleotides corresponding to these polypeptide segments, either to the coding or to the complementary DNA strand.

We have added the nucleotide sequence CCGGAATTC which contains the Eco R restriction site at the 5' terminal end of the synthesised oligonucleotides in order to use this site for cloning of the amplified fragments.

Different combinations of sense and antisense pairs of these synthetic oligonucleotides were assayed as primers in Polymerase Chain Reactions (PCR) carried out under different conditions of stringency using *Leishmania mexicana* DNA as template.

We have chosen the optimal conditions which allowed the

amplification of a *Leishmania* DNA fragment with the correct size (approximately 170 base pairs) as expected from the oligonucleotides used as primers. In two other cases we got amplification of *Leishmania* DNA, but the fragments obtained did not match with the sizes expected from the primers used.

b. **Purification of the amplified DNA fragment of 170 bp**: We have pooled several preparations of the amplified DNA fragment and after confirming its putative nucleotide sequence by looking at unique sites corresponding to different restriction enzymes, the DNA was precipitated with ethanol in the presence of ammonium acetate. The amplified DNA fragment was extensively digested with an excess of the restriction enzyme Eco R_I and about 1 µg of the product obtained was submitted to gel electrophoresis using a minigel of 2% low melting point extra pure agarose (Sea plaque GTG agarose, FMC). The gel band containing the amplified DNA fragment was excised under UV light and the DNA then used as an insert to be ligated to the chosen vector.

c. **In-Gel Ligation of the *Leishmania* DNA fragment to the vector pBluescript SK(-) and transformation**: After heating the gel segment containing the amplified *Leishmania* DNA fragment for 10 min at 68°C and subsequent dilution, different aliquots of the insert DNA were ligated to the pBluescript SK(-) phagemid vector previously digested with the restriction enzyme Eco R_I.

The ligation products were used for transformation of competent *E. coli* XL-1 Blue cells and white colonies presumably containing the recombinant DNA (pBluescript vector with *Leishmania* DNA insert) were selected.

d. **Analysis of plasmid DNA from transformed colonies**: A number of selected colonies were grown in the presence of ampicillin (100 µg/ml) and minipreps of plasmid DNA were obtained from the bacterial lysates. All plasmid DNA preparation were digested with Eco R_I enzyme and after electrophoretic analysis on 2% agarose gels, the samples showing a DNA fragment of 170 bp were chosen as those containing the *Leishmania* DNA insert. We have confirmed this conclusion by analysis of DNA segments obtained with different restriction enzymes.

e. **Purification of recombinant DNA and sequencing of the *Leishmania* DNA insert**: A maxiprep of plasmid recombinant DNA was obtained from 500 ml of bacterial culture, purified by using Qiagen resin and then precipitated with isopropanol. The redissolved DNA was submitted to the sequencing protocol using Sequenase version 2.0 (T DNA polymerase) and the SK or KS primers. In this way we were able to sequence both strands of the *L. mexicana* DNA insert which showed almost complete identity with an homologous fragment from *Leishmania donovani* (Hanson, S. *et al.*, J. Biol. Chem., 267, 2350, 1992).

The *L. mexicana* DNA fragment obtained will be used as a radioactive probe in the search for the ODC gene and its expression in *T. cruzi* DNA and RNA preparations, as well as in the screening of *Leishmania mexicana* and other protozoa genomic libraries.

2) We have characterized and studied the enzymatic reactions related to polyamine biosynthesis in *Leishmania mexicana* promastigotes.

a. Ornithine decarboxylase, the first enzyme in polyamine pathway, has been partially purified and the corresponding apoenzyme prepared after complete removal of pyridoxal 5' phosphate by treatment with hydroxylamine. Apoenzyme preparations showed an absolute requirement for the cofactor to recover enzymatic activity. This restoration of ornithine decarboxylase activity seems to involve some time and/or temperature-dependent activation processes which are currently under investigation in our laboratory.

b. We are setting up conditions for the *in vitro* transformation from *Leishmania* promastigotes to amastigotes and *vice versa*, by using synthetic media and changing the temperature of cultures from 24-26°C to 32 or 37°C (Shapira, M. *et al.*, EMBO J. 7, 2895, 1988). At the same time we are investigating the effect of polyamine depletion of the differentiation process.

3) The characterization of polyamine transport systems has been carried out in *Leishmania mexicana* promastigotes and *Trypanosoma cruzi* epimastigotes.

a. **Differential properties of polyamine transport systems in *T. cruzi* and *Leishmania mexicana***: Our studies have indicated that the constitutive uptake of putrescine and spermidine in *T. cruzi* epimastigotes was markedly higher than in *Leishmania mexicana*. When parasite cultures were treated for a period of 2-3 generation times with DFMO, an irreversible inhibitor or ornithine decarboxylase, putrescine uptake was strongly stimulated in *Leishmania* by increasing considerably the Vmax of the process. In contract, putrescine transport in *T. cruzi* was not affected by the same treatment.

The measurements of polyamine uptake in the absence or presence of other polyamines added as putative competitors have indicated that at least two different transport systems might exist in trypanosomatids: one specific for putrescine and another one for both spermidine and spermine (González, N.S. *et al.*, An. Asoc. Quim. Argent., 81, 117, 1993).

b. **Regulation of polyamine uptake in *Leishmania mexicana* promastigotes**: We have found that putrescine uptake of *Leishmania mexicana* is tightly regulated by polyamine intracellular levels. This uptake can be markedly stimulated by polyamine depletion and strongly repressed by exposure of *Leishmania* cultures to exogenous putrescine or its derivative dimethylputrescine. In contrast, spermidine, spermine, diaminopropane and cadaverine are unable to decrease putrescine transport.

Our experiments have shown that putrescine uptake induction as well as its specific feedback repression by increased endogenous concentrations of this polyamine require protein synthesis since they were abolished after addition of cycloheximide for several hours. The results have indicated that the putrescine transporter is a stable protein which can be reversibly inactivated by a relatively unstable repressor.

Publications:

González N.S., Ceriani, C. and Algranti, I.D. (1992) Differential regulation of putrescine uptake in *Trypanosoma cruzi* and other trypanosomatids. Biochemical and Biophysical Research Communications, 188, 120-128.

Monte, M., Klein, S., Jasnis, M.A., Davel, L., Algranati, I.D. and de Lustig E.S. (1993) Inhibition of lymphocyte and tumor-induced angiogenesis by the administration of difluoromethylornithine. The Cancer Journal, 6, 147-150.

González N.S., Ceriani, C. and Algranti, I.D. (1993) Transport of polyamines in trypanosomatids. Anales de la Asociacion Quimica Argentina, 81, 117-125.

González N.S., and Algranti, I.D. Regulation of putrescine uptake in *Leishmania mexicana* promastigotes. To be submitted.

Networking: We are analyzing our recent results on the regulation of polyamine metabolism and transport systems in trypanosomatids and comparing them with those obtained in different mammalian cells by professor Grillo's research group at the University of Torino (Italy).

Prof. Grillo's characterization of polyamine transport mechanisms in lymphocytes and hepatocytes can serve as a model for the isolation of polyamine transporter proteins in parasites and eventually for cloning their corresponding genes.

At present we are exchanging information on the results obtained and the techniques used in both our laboratories. We plan to arrange with Prof. Grillo a short visit of a member of the Buenos Aires group to her laboratory with the purpose of performing some preliminary experiments of our joint project.

CHARACTERIZATION OF AN INTESTINAL POLYPEPTIDE FROM *TRIATOMA INFESTANS* THAT ACTIVATES *TRYPANOSOMA CRUZI* ADENYLYL CYCLASE AND DETERMINES THE DIFFERENTIATION OF EPIMASTIGOTE TO TRYPOMASTIGOTE FORMS

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ICGEB Reference No. CRP/ARG92-05

Keywords: *Trypanosoma cruzi*, Chagas' disease, *Triatoma infestans*, metacyclogenesis, adenylyl cyclase, hemoglobin

Abstract: A peptide from hindguts of the *Triatoma* hematophagous Chagas' Insect vector activates adenylyl cyclase

activity in *Trypanosoma cruzi* epimastigote membranes and stimulates the *in vitro* differentiation of epimastigotes to metacyclic trypomastigotes. Hindguts were obtained from insects fed two days before with chicken blood. Purification was performed by gel filtration and high performance liquid chromatography on C_4 and C_8 columns. SDS-PAGE of purified peptide showed a single band of about 10 KDa.

The following sequence for 20 residues of the amino terminus of this peptide was determined: H-N-Met-Leu-Thr-Ala-Glu-Asp-Lys-Lys-Leu-Ile-Gln-Gln-Ala-Trp-Glu-Lys-Ala-Ala-Ser-His. This sequence is identical to the amino terminus of chicken a^D-globin. After Western blotting, the peptide immunoreacted with a polyclonal antibody against chicken globin D.

Background: *Trypanosoma cruzi*, the etiological agent of the Chagas' disease, undergoes complex morphological changes through its life cycle in both, the insect vector and the vertebrate host. Metacyclogenesis is the differentiation process that occurs within the hindgut of the hematophagous *Triatoma* insect vector. This process converts *T. cruzi* epimastigotes which are proliferative but not infectious, to metacyclic trypomastigotes, the infectious and non-proliferative parasite forms. Metacyclogenesis can be induced in epimastigote liquid cultures under a variety of conditions such as growing epimastigotes with *Triatoma infestans* hindgut extracts or addition of exogenous cyclic AMP or its analogue to the cultures. Studies on the mechanism that causes *T. cruzi* differentiation revealed that metacyclic trypomastigotes have higher intracellular cyclic AMP levels than epimastigotes and that *T. cruzi* epimastigote membranes posses adenylyl cyclase associated to Gi$_a$ subunits. Gi$_a$ and Gi$_b$ polypeptides are also located in these membranes.

Objectives: General -To find and characterize a molecule that would be present in *Triatoma* hindgut with the capability of activating epimastigote adenylyl cyclase and thereby causing metacyclogenesis.

Particular -Peptide purification and characterization of its biological effects. -Peptide sequencing. - Synthesis of peptides and characterization of their biological effects. -Labelling of peptides and receptor characterization. -Preparation of antibodies. -*In vivo* and *in vitro* characterization. Receptor cloning.

Work Progress: Peptide purification: completed. Peptide characterization: completed. Peptide sequencing: completed.

Publications:

Fraidenraich, D., Peña, C., Isola, E.L., Lammel, E.M., Coso, O., Dia Añel, A., Baralle, F., Torres, H.N. and Flawiá, M.M. (1993) An a^D-globin fragment from *Triatoma* hindgut stimulates *Trypanosoma cruzi* adenylyl cyclase and promotes metacyclogenesis. Biol. Res. 26, 279-283.

Communications:

Fraidenraich, D., Diez, C., Burgos, M., Baralle, F., Isola, E., Flawiá, M.M. and Torres, H.N. A *Trypanosoma cruzi* epimastigote surface receptor form Hemoglobin Derived Peptides. XX Reunião Anual de Pesquisa Básica em Doença de Chagas, Caxambu-Brasil, Nov. 1993.

Torres, H.N., Burgos, M., Fraidenraich, D., Paveto, C., Baralle, F., Esteva, M., and Flawiá, M.M. Activación de la Adenilil ciclasa de *Trypanosoma cruzi* por un fragmento de hemoglobina aislado de intestino de *Triatoma*. Efecto a nivel de la metaciclogenesis. IV Congresso Argentino de Protozoología, Catamarca- Argentina, Nov. 1993.

Torres, H.N., Burgos, M., Fraidenraich, D., Paveto, C., Baralle, F., Esteva, M. and Flawiá, M.M. Receptor de superficie en Epimastigotes de *Trypanosoma cruzi* para peptides derivados de hemoglobina. XXIX Reunion Anual de la Sociedad Argentina de Investigacion Bioquimica, Cordoba- Argentina, Nov. 1993.

Networking: Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (CONICET) y Facultad de Ciencias Exactas y Naturales, Obligado 2490, 1428 Buenos Aires, Argentina; Instituto de Química y Fisicoquímica Biológicas (CONICET) and Facultad de Farmacia y Bioquímica, Departamento de Microbiología, Facultad de Medicina, Universidad de Buenos Aires and International Centre for Genetic Engineering and Biotechnology, Trieste (UNIDO).

BRAZIL

NUTRITIONAL IMPROVEMENT OF ROOT CROPS BY ENGINEERING

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ICGEB Reference No. CRP BRA88-02

Keywords: root and tuber specific promoters, nutritional improvement, transgenic plants, methionine rich protein gene, taro corm protein genes

Abstract: Previous report described background and objectives of this project as well as work in progress related to the objectives there presented. In this report we will focus on recent results on gene expression patterns occurring on corm development upon studies conducted with a corm globulin encoding cDNA clone designated here as TC1 and in previous report as PCE5; as well as on recent results on corm protein analysis. For details on the information here presented please see Castro *et al.*, 1992.

Background: In this laboratory two genes, BE2S1 and BE2S2, coding for methionine-rich albumins of Brazil nut 2S albumins have been cloned and their sequence determined. The fact that the Brazil nut 2S albumins have a high methionine content make them interesting candidates of correction of methionine-deficient crop plants via recombinant DNA technology.

Most root and tuber crops have low protein content (usually between 1% to 5%). Some roots like cassava and carrots have only 1% protein. Attempts to improve the protein content of some root crops by classical breeding have not been successful. Thus, we are now trying to improve the quantity and quality of storage roots and tuber via genetic engineering.

We have isolated genes that are highly expressed in taro corms. Our goal is to introduce these genes plus the gene that codes for the MRP of Brazil nut into various tropical roots and tubers.

Objectives: The final goal of this project is the expression of the methionine-rich albumin of Brazil nut seeds in tuber and roots, such as potato, cassava and sweet potato. For this purpose, the identification of DNA sequence(s) capable of driving gene expression in these tissues, and of DNA sequences capable of leading the protein to proper compartmentalization in the cell is essential.

The genetic transformation systems for cassava and sweet potato as well as for Brazilian potato cultivars will be established.

Work Progress:

1) Corm Protein Analysis.

We identified four prevalent protein groups that are regulated during corm development, referred as Globulins G1 and G2, and Albumins A1 and A2 (Castro *et al.* op. cit.). Sequencing studies of the globulin sub-groups indicated that G1 globulin proteins (14 Kd) are related to the curculin sweetener and taste-modifying protein. By contrast, the G2 globulins (24 Kd) are related to the sporamin storage protein, the Kunitz trypsin inhibitor, and the miraculin taste-modifying protein (Theerasilp *et al.*, 1989). We sequenced the N-terminal amino acids of the corm G1 (G1a, G1b, G1c and G1d) and G2 (G2a and G2b) corm globulin proteins. No amino acid homologies were observed between the N termini of proteins in the G1 and G2 globulin groups. By contrast, significant amino acid homologies were observed between the N termini of proteins within a give group.

The G1a and G1c N-terminal amino acid sequences are 88% identical to each other, where as the G1b and G1d sequences are 96% identical. The G2a and G2b N-terminal sequences are 68% identical to each other. By contrast, the G1b/G1d and G1a/G1c subgroups had only 25% identity in their N-terminal sequences. Together, these data indicate that the G1 and G2 globulins comprise small families of unrelated proteins and that the G1 protein family contains two subfamily groups that are more closely related to each other.

Furthermore SDS gel analysis of G1 globulins indicate that they constitute homodimers (28 Kd) that are processed into subunits of approximately 14.0 Kd. N termini of G1d and G1a are homologous to sequences of a genomic clone 100% homologous to a cDNA clone TC1 (Bezerra *et al.*, submitted to

Plant Molecular Biology).

2) Temporal and Cell-Specific Gene Expression during Corm Development.

We identified cDNA clones representing prevalent corm mRNAs to study the gene expression during corm development. One cDNA clone, TC1, encodes the curculin-related G1 globulins.

The TC1 globulin G1 mRNA accumulates during early corm development and is concentrated preferentially within corm apical regions, similar to that of the G1 globulin protein. The TC1 globulin G1d mRNA is not detectable within leaves and is present at a very reduced prevalence within roots.

In situ hybridization studies indicated that the TC1 globulin G1 mRNA is preferentially concentrated in corm storage parenchyma cells and is not detectable in most other corm cell types. The absence of a TC1 mRNA signal within roots emerging from stage C4 corms indicates that this mRNA probably has a reduced prevalence in most root cell types, rather than being highly concentrated in a few cells within the root.

3) Isolation and characterization of potential gene promoters.

Four genomic clones have been isolated from a genomic DNA library of taro corms. Sequencing of one of them evidenced 100% homology to the 5' region of a cDNA TC1. This clone has a promoter-like region presenting a TATA box like sequence and a potential ATG followed by a sequence of nucleotides coding for potential ATG followed by a sequence of nucleotides coding for hydrophobic amino acids, typical of a signal-peptide. A 3.0 kb fragment containing the promoter-like region has been ligated to the B-glucuronidase (GUS) gene in the vector pGV 1501 Gus.3. These tests are underway by biolistic method. This construction was utilized for transformation of tobacco by an Agrobacterium mediated system and 2 regenerated plants are being analyzed.

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IDENTIFICATION OF REGULATORY FACTORS INVOLVED IN INDUCING PLANT-DEFENSE RESPONSE DURING PLANT-PATHOGEN INTERACTIONS

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ICGEB Reference No. CRP BRA91-01

Keywords: biocontrol, genetics, molecular biology, plant resistance, plant-pathogen interactions, signal transduction

Abstract: The mechanisms of activation of genes involved in plant defense response are not yet understood. The study of the regulatory factors involved in the defense response will provide the necessary tools to initiate a detailed analysis of the precise mechanisms of plant-pathogen interaction. It will allow the development of a broad approach towards biocontroling, spread and infection of phytophatogenic organisms. The present project is concerned with the identification of the plant genes whose encoded products participate in the transduction pathway activated in response to microbial attack.

In this project we use the interaction between *Arabidopsis thaliana* and *Xanthomonas campestris* for the identification of regulatory factors involved in inducing the plant defense response. We have established the conditions to induce HR in Arabidopsis, using *X. campestris* mass spray inoculation. Of special interest was the finding that the HR is also induced in plants with seed pods. This finding allows us to carry out the screening of mutated plants at this stage of development.

The time course analysis of gene induction during the defense response have shown that transcripts can be observed as early as one hour after *X. Campestris* inoculation. Large amounts of RNA have been isolated at this time point to prepare a cDNA library to isolate genes which are inducted early during the plant pathogen interaction. The promoters of such genes will be used for identification of regulatory factors involved in inducing plant defense response. We propose to obtain Arabidopsis plants transformed with selectable markers and reporter genes under control of regulatory sequences from genes induced during the plant defense response.

Background: Our knowledge about the plant defense response is still elementary due to the lack of an appropriate model system featuring tractable molecular genetics and easily studied pathogens. Our results and those of several other groups show that *Arabidopsis thaliana* is a useful model for the analysis of plant/pathogen interactions of many sorts. It has already been shown that *A. thaliana* is a host for several pathogens as bacteria, viruses, fungi and nematodes. Among there, *Xanthomonas campestris* pv *campestris*, the causal agent of black rot of the cruciferous family, has been identified as a potential pathogen of *A. thaliana*.

Objectives: The objective of this proposal is to identify the early factors that govern transcription of genes involved in plant defense response.

The projects includes the following steps:

1) Isolation and molecular characterization of plant defense genes from tobacco and Arabidopsis plants. Analysis of the expression patterns of the isolated genes.

2) Fusion of the characterized promoter sequences to selectable and reporter genes and introduction of the chimeric constructs in the Arabidopsis genome by *Agrobacterium*-mediated transformation.

3) Analysis of the transgenic plants and mutagenesis of the F2 seeds obtained from the selected transformants.

4) Selection of plant mutants where the expected expression pattern conferred by the utilized promoter has been altered. Analysis of the expression characteristics of the reporter genes and determination of the effects that the mutation exerts in other plant defense compounds.

Simultaneously, direct mutagenesis of an Arabidopsis variety showing hypersensitive response to *X. campestris* will be performed and mutants with alterations in their response to pathogens will be selected and characterized.

Work Progress: We have established the conditions to induce the HR in *A. thaliana* using *X. campestris* mass spray inoculation. Certain features of an HR have been observed in this interaction: i) appearance of dry necrotic lesions is rapid (24hrs.); ii) the restriction of bacterial growth in an incompatible interaction in comparison to a compatible interaction. We have performed an extensive phenotypical, molecular and enzymatic investigation of this HR, including plant bacterial growth curves and comparison between compatible and incompatible interactions. The growth curves of *X. campestris* in the incompatible interaction results in an initial growth followed by rapid decrease. The compatible interactions induces rapid growth which is, however, sustained.

In general, the pathogenesis related (PR) proteins are rapidly induced during an incompatible reaction of plant to pathogenic microorganisms. Isometric-focusing analysis of total protein extract of control and infected plants showed the presence of at least two b-1,3-glucanase isoforms (one basic and one acidic isoform). Both were strongly induced two days after infection. The levels of peroxidase activity increases 5 fold after two days of infection. Isoelectric-focusing analysis of the protein extracts of untreated plants allowed to identify at least six distinct peroxidase isoforms. Infection with *X. campestris* strongly increases the levels of three isoforms and induced three new isoforms.

Two cDNA libraries were prepared, one with transcripts of non inoculated plants and another with mRNA of plants inoculated with *X. campestris* for 1 hour. The libraries were constructed in the lambda / ZAP vector system (Stratagene), generating 3.2×10^7 and 10^6 pfu/ml for non-inoculated and inoculated leaves respectively. The random analysis of 10 independent clones from each library showed insert sized varying from 0.3 kb to

more than 5 kb. The amplification of both libraries did not change the general pattern. The chosen strategy is the preparation of a subtracted library that will be analysed by differential screening using the transcripts of the leaves induced or not as probes. With this approach we expect to isolate genes which are induced early during plant-pathogen interaction. The promoters of such genes will be used in the follow up of the project for identification of regulatory factors involved in plant response.

We are applying two mutational strategies for the identification of the earlier factors involved in inducing plant-defense during interaction between *A. thaliana* and *X. campestris*: i) isolation of arabidopsis mutants in a hypersensitive response to the pathogen *X. campestris* and, ii) mutagenesis of plant transformed with chimeric constructs containing regulatory *cis*-acting sequence obtained from genes induced during plant defense response.

For the first strategy we have screened a total of 20700 showed EMS mutagenised col O seeds (col-O is an *A. Thaliana* ecotype which show the HR to *X. campestris*). After several successive screenings, 19 plants responded with an absence of HR (HR$^-$) after spraying and showed attenuated phenotypes when infiltrated. Three plants showed a hyper hyper sensitive reaction (HHR) and five showed symptoms much closer to a compatible interactions. Two HR$^-$, three HHR and five susceptible candidates were selected for segregation analysis and microscopic and molecular characterization.

For the second mutant strategy, chimeric constructs containing the selectable marker gene *npt* and marker gene *gus* under the control of the promoter of *X. plumbia genipolia* b-1,3-glucanase (*gnl*), were introduced in *A. thaliana* via *Agrobacterium* mediated transformation. The putative transformants obtained are currently under characterization by PCR to confirm that they are transgenic, using oligonucleotides specific to the *nptII* gene.

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Networking: Dr. Carmen Castresana, Centro de Investigaciones Biologicas (CSIC), Madrid, Spain.

BULGARIA

PROTEIN-DNA INTERACTIONS IN CELL NUCLEUS: A STUDY WITH UV LASER CROSSLINKING *IN VIVO*

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Keywords: protein-DNA crosslinking, UV laser, histone acetylation, ribosomal genes, vitellogenin gene

Abstract: Having shown that the active ribosomal genes in rat tumor cells are associated with hyperacetylated histones, we next studied the acetylation of histones bound to those of the ribosomal gene copies, that are organized in nucleosomes and thought to be inactive. Unlike the results obtained with the whole gene population, nucleosome-organized ribosomal DNA sequences were found associated with histones that were not hyperacetylated.

Another question we addressed was the presence of histones on the vitellogenin II gene in roosters upon induction with oestradiol. The actively transcribed gene was found to be associated with histones as was the permanently repressed gene. Finally, we analysed the relationship between lamina proteins and DNA and demonstrated the existence of lamin-DNA complexes upon irradiation of nuclei with UV laser, thus proving their *in vivo* existing contacts.

Background: The aim of the project is to study the link between the chromatin structure of some individual genes and their transcription. We are interested in the fate of histones upon transcription of RNA polymerase I and II genes. Whether histones remain bound to transcribed sequences and if so, what is their acetylation? Are there any RNA polymerase molecules in vicinity to histone molecules? A separate study aims to gain an information about the contacts of lamina proteins with DNA: there are some indications, not firmly proved yet, that lamins provide points for attachment of DNA to nuclear perifery.

For such a problem to be successfully studied, *conditio sine qua non* is to preserve *in vivo* existing protein-DNA interactions. A reliable way to meet such a requirement is "to freeze" these interactions in a very short time in order to avoid protein redistribution. This was carried out by crosslinking proteins to DNA by UV laser. This technique, first introduced for *in vivo* studies in our laboratory (NAR, 14, 4525, 1988; TIBS, 16, 323, 1991) allows crosslinking to occur in picosecond time intervals. Laser crosslinking in combination with immunochemical techniques for selection of covalent complexes between DNA fragments and the protein of interest is the experimental strategy used in this study. Briefly, it can by summarized as follows: 1) Irradiation of isolated nuclei with UV laser to crosslink chromosomal proteins to DNA; 2) Separation of the covalently linked protein-DNA complexes from non-crosslinked DNA and proteins by centrifugation in CsCl; 3) Selection of DNA fragments crosslinked to a given protein by means of specific antibodies (immuno-precipitation); 4) Purification of immunoprecipitated DNA; 5) Analysis of DNA thus obtained for the presence of coding, regulatory and other DNA sequences by hybridization to specific probes.

This strategy is particularly useful for studying interactions of DNA with proteins which, due to their high affinity to DNA, can easily form artifactual complexes in the course of various cell fractionation procedures. The fact that UV light is a zero-length crosslinking agent helps to justify *in vivo* existing intimate contacts between DNA and some proteins.

Objectives: As we mentioned in the first year's report, histones associated with the ribosomal genes are hyperacetylated. The question is whether all ribosomal gene copies are bound to acetylated histones or this modification is restricted to those of them that are active at the moment. To distinguish active from inactive ribosomal genes in a given cell population is very difficult task. There are evidence that nucleosome-organized ribosomal DNA is inactive, whilst the active sequences do not exhibit such an organization. The study we undertook was to analyze the state of acetylation of histones associated with the ribosomal genes in isolated oligonucleosomes.

After the demonstration that transcribed ribosomal genes are associated to core histones and H1, it was interesting to follow the fate of histones when an RNA pol II gene is transcribed at very high rate. One example is the permanently repressed gene for the yolk protein vitellogenin in roosters: the administration of oestradiol induces a very high expression of the gene.

The existence of *in vivo* contacts of lamina proteins with DNA is not definitely proved mainly because these proteins easily bind *in vitro* to any DNA. The use of laser crosslinking is the method of choice because the extremely short time of irradiation excludes protein redistribution to occur and, besides, the yield of crosslinked material is very high.

Work Progress:

Acetylation of histones associated with the ribosomal genes, that are organized in nucleosomes.

Guerin ascites tumor cells were grown in the presence of butyrate to inhibit histone deacetylation and used to isolate nuclei. They were digested with micrococcal nuclease and passed through a sucrose gradient. An oligomer fraction containing mono- and dinucleosomes only was collected and immunoprecipitated with anti-acetyl antibody. Precipitated DNA was purified, labelled and hybridized to coding sequences from the rat ribosomal DNA repeat, which has been immobilized on filters. The content of the ribosomal genes in the precipitated DNA was 3.5 times lower than that found in total rat DNA. This result markedly differed from that obtained upon immunoprecipitation of crosslinked histone-DNA complexes, generated by laser irradiation of nuclei, where a significant

enrichment for ribosomal sequences has been observed. As far as acetylation of histones has been correlated with transcription of protein coding genes and, on the other hand, in the light of the evidence that inactive ribosomal genes are organized in nucleosomes (Conconi *et al.*, Cell, 57, 753, 1989) our conclusion is that unlike active gene copies, those organized in nucleosomes (and claimed to be inactive) are associated with histones that are not hyperacetylated. Since our antibody does not react with mono- and diacetylated molecules; their presence in the inactive gene copies cannot be ruled out. Such a result is related to a question that concerns the organization of the work of the ribosomal genes. According to the data from studies of these genes with psoralen crosslinking, about 50% of them were found to exist in a nucleosomal conformation and, respectively, thought to be active. It is not clear whether the cell uses one and the same gene copies, the other being silent, or it plays by turning on or off all available gene copies. Or, in case of a need for an increased production of ribosomes, whether the answer is increased rate of transcription or switching on new gene copies? Acetylation of histones in active genes and the lack of such a modification in nucleosome organized (inactive) genes favours the first possibility.

Presence of histone on the vitellogenin II gene upon induction for transcription.

Young roosters were treated with oestradiol and the expression of the gene for vitellogenin was registered by following the appearance and the accumulation of the yolk protein in the blood. Livers from these animals were used to isolate nuclei which were irradiated with UV laser. The covalently linked histone-DNA complexes were purified and immunoprecipitated with antibodies to histones H1, H2A, H3 and H4. Precipitated DNA was purified, labelled and analysed for the presence of coding vitellogenin sequences by hybridization to specific probes. The amount of the coding sequences in DNA immunoprecipitated from induced liver cells was similar to that obtained with the non-induced cells, i.e. the vitellogenin II gene is associated with core histones and H1 as is the silent gene, which is never expressed.

Contacts of lamina proteins with DNA.

Ehrlich ascites tumor cells were irradiated with UV laser to crosslink chromosomal proteins to DNA. Nuclear lamina was isolated by digestion with DNAse and RNAse and treatment with 2M NaCl. Under these conditions as much as 2% of input DNA was found in the final preparation. The obtained material was dotted on filters and reacted with antibodies to lamins A and B. The positive reaction we observed was interpreted as an indication for a direct *in vivo* contact between lamina proteins and DNA.

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RFLP ANALYSIS AND BIOCHEMICAL STUDIES ON RESISTANCE OF RICE TO *CERCOSPORA ORYSAE*

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ICGEB Reference No. CRP/BUL92-01

Keywords: RFLP, rice, cercosporin, resistance, enzymes

Abstract: Resistance to *Cercospora orysae* is believed to be controlled by a single gene. RFLP analysis is performed to find out whether there is a high level of polymorphism between Red rice (resistant) and elite cultivars with different levels of resistance. There is preliminary step to mapping gene for resistance on Red rice genome. Crosses between Red rice and elite cultivars have been done. F1 and F2 progeny will be screened by RFLPs to find out the segregation of resistance with mapped probes.

The action of *Cercospora orysae* is assayed on the main enzymes in cell protective enzyme complex (peroxidase, superoxide, dimutase and catalase) and chlorophyll content on the leaves in several rice cultivars with different level of resistance. The quantitative differences of isozymes have been determined after the treatment of the leaves with the toxin, especially these in peroxidase system. The distruction of chlorophyll has been found to be connected with the resistance.

The glutelin electrophoretic pattern analysis have been used as an approach demonstrating the genome characteristics and to follow the way of introgression of the gene(s) for resistance to *Cercospora orysae* in Red rice and Labelle.

Background: The inheritance of resistance to *Cercospora orysae* has been studied in US. The only results obtained so far show that a single dominant factor for resistance does not explain all cases satisfactory. The *Cercospora orysae* is known to produce the red toxin cercosporin which plays an important role in symptom development of the disease.

We have investigated the toxicity of the pure cercosporin at plant and tissue level and we have measured the toxicity of cercosporin in terms of inhibition of callus growth. The results show the correlation between the reaction to the fungi *in vivo* and *in vitro* and also to the toxin.

Three commercial cultivars from US are used in there experiments: Leah and Lemont with intermediate resistance, Labelle - highly susceptible to *Cercospora orysae*. The annual weed form of rice, known as Lousiana Red rice, is resistant to all races of this pathogen and is also included in this study.

1) RFLP Analysis.

The gene mapping programme involves the use of RFLPs. Rice contains 12 haploid chromosomes for which a map of over 350 DNA markers has been prepared by Steve Tanksley's group at Cornell University. The clones mapped by the Cornell's group have been made available to us for mapping of genes which confer resistance to *Cercospora orysae*.

a. Mapping the DNA markers of the Red rice genome, Leah, Labelle and Lemont.

b. Crosses between Red rice (carrying the genes for resistance) and elite cultivar Labelle has been done to obtain F1 and F2 progenies and to use RFLP analysis to find out the linkage of the genetic markers with the gene(s) for resistance to *Cercospora orysae*.

For extraction of DNA, restriction, pick translation and labelling with P we used the methods by Roberto Bernatzky (Plant Molecular Biology Manuel C, 1988). The protocols for capillary transfer of DNA and hybridization from Gene Screen Plus (1987) have been applied.

2) Biochemical Analysis.

The aim of the biochemical investigation is to clarify the phenomenology of action of the toxin cercosporin on rice callus and plants from resistant and susceptible cultivars.

The main enzyme in cell protective complex have been investigated such as peroxidase, using the method of Kroehko J.B. and Bewly J.D. (Electrophoresis, 1988, 9, 751-763) and SOD, using the method of Beauchamp C.O. and Fridovich I. (Annal. of Biochem., 1971, 44, 276).

The extraction and electrophoresis of total soluble proteins have been carried out according the procedure of Sarkov R. and Roma S.W. (TAG, 1991, 85, 127-132).

Objectives: Crosses between resistant and susceptible rice cultivars and RFLPs will give more information about the nature of gene(s) action. The F2 population will explain how many genes are involved in resistance. Rice genome mapping will provide the information with which genetic marker the gene(s) for resistance to *Cercospora orysae* are linked.

The aim of biochemical investigations is to clarify the phenomenology of action of the toxin cercosporin on rice cultivars with different levels of resistance.

This year our investigation are concentrated on isozyme spectrum of the main enzymes in the cell protective enzyme complex into the leaves: pervidase, SOD, and catalase.

Effect of the cercosporin on photosynthesis, especially chlorophyll content was established.

Storage seed protein and amino acid composition are known to be different form one genus to the other, from species to the other and even from one cultivar to the other. The application of glutelin electrophoretic patterns is an useful approach to monitor gene(s) transfer in the obtained resistant material.

Work Progress: In search for RFLP's between rice cultivars resistant and susceptible to *Cercospora orysae* rice DNA was digested with HindIII restriction enzyme, blotted and hybridized with 31 genomic prose (P) labelled by nick translation.

Hybridizations with 22 different RG probes showed polymorphism between complete resistant Red rice and the other elite rice cultivars with different levels of resistance to *Cercospora oryzae*. Hybridizations with RG 190 and RG 381 show polymorphism between Red rice and cultivar Leah. There is no polymorphism between the cultivars Lemont and Labelle. Hybridization with GR 118 shows polymorphism between Red rice Labelle and Lemont. The probe RG 236 shows polymorphism between Labelle and the three other cultivars. Nine of RG probes after hybridization did not show any polymorphism between the resistant and susceptible cultivars. This results detect high RFLP between Red rice and three elite rice cultivars (7%). The obtained F1 plants will be screened and allowed to produce F2 families.

Electrophoretic spectra of peroxidase isozyme in the leaves were investigated. In resistant plants of Red rice we observed no changes in peroxidase activity 24 hours after treatment with the toxin. In susceptible plants from Labelle the intensity of the bents increases especially those molecular components with relatively small electrophoretic abilities. The results from the cultivars Leah and Lemont are the same. Peroxidase activity is strongly augmented in the leaves of all cultivars 72 hours after treatment when the tissue damage is more severe. The new peroxidase isoforms have not been established. Therefore, the peroxidase increase depends on the degree of tissue damage which is connected with the different accumulation of the toxin into the cells.

Catalase, whose function is to destroy the hydrogen peroxide (toxic to the cell) exhibits characteristic changes, depending on the time of toxin action and resistance of the plant. 24 hours after treatment with the toxin there are no changes in the intensity of fractions and their number of resistant Red rice. In susceptible plants from Labelle the catalase isozymes increase their activity, generally of a quantitative nature effecting the levels of all isozymes. The result is the same for the Lemont cultivar but the spectra of cultivar Leah shows decreases of the intensity of fractions with low mobility and increase the intensity of a fraction with the lowest mobility. 72 hours after treatment with cercosporin the catalase activity declines in all cultivars. This concerns mainly the fractions with low mobility. Probably the hydrogen peroxide which is responsible for cell damage accumulates during the longer period of the toxin action in both susceptible and resistant plants. A similar situation is established with superoxide dismutase.

The preliminary experiments on the influence of the toxin on the peroxidase spectra of calli from Labelle cultivar have been done. The treatment with cercosporin on leaves influences the chlorophyll synthesis. The larger area of damage on plant tissue is connected with more sensitive decrease of chlorophyll contents which is connected with the resistance of investigated cultivars.

On the basis of the electrophoretic analyses of the rice glutelins the following conclusions could be made. There is a basic similarity in the glutenin profiles of the experimental cultivars. The differences between Red rice and elite rice cultivars are qualitative. The presence of two glutelin bands with M 45 KD and one band with M 29 KD are established only in Red rice.

(Due to limited space the figures and tables are not included in this Activity Report)

CHILE

STUDIES OF THE STRESS RESPONSE IN BIOMINING MICROORGANISMS. POSSIBLE IMPLICATIONS IN THE IMPROVEMENT OF THE BIOLEACHING PROCESS

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ICGEB Reference No. CRP/CHI90-01

Keywords: bioleaching, stress response, heat shock, starvation, *Thiobacillus ferrooxidans*

Abstract: The biomining or bioleaching of minerals is a process by which metals such as copper, gold and uranium are solubilized from the ores containing them by the action of acidophilic microorganisms. During this process, bacteria are subjected to different kinds of environmental stress such as temperature or pH changes and lack of essential nutrients that may affect the activity of the bacteria, therefore, affecting the efficiency and rate of metal extraction. Knowing the stress responses of extreme acidophiles at the molecular level is not only interesting from the fundamental point of view, but will help also to monitor the *in situ* state of these microorganisms, making possible to improve their activity in industrial processes. In response to these stressing conditions, bacteria are known to change the genetic expression of several cellular polypeptides, inducing stress proteins and others as a defence or remediating mechanism to overcome the adverse condition. Experimentally, we have studied the global gene expression changes by using two-dimensional polyacrylamide gel electrophoresis and have identified some of the polypeptides induced by microsequencing of their N-terminal ends. We have previously reported the existence of a heat shock and pH-stress response in *Thiobacillus ferrooxidans* (ICGEB Activity Report 1992).

During this period, we have analyzed in detail the proteins synthesized by *T. ferrooxidans* when subjected to heat shock, the lack of nutrients such as phosphate and the global gene expression changes when *T. ferrooxidans* is switched from growth in ferrous iron to elementary sulfur.

Background: Bacterial leaching is a process of metal extraction in which different microorganisms are involved. This biodegradation of minerals is of great industrial importance, since it may contribute about 10% to yearly world copper production, and it may also be important for the recovery of gold and uranium. The main advantages of the biohydrometallurgical process are: 1) it can be applied to low grade ores; 2) low cost; and, 3) is a low contaminating process compared with the standard extraction procedures.

However, it is a process which is rather slow. The speed of bioleaching can be affected by several factors which influence the microorganism activity, such as stressing conditions, lack of nutrients, presence of toxic metals. Knowing the response mechanisms that the bacteria employ to adapt to these environmental changes could help to improve the rate of yields of the biomining process.

Objectives:

1) To know the components specifically induced in response to temperature and nutrient changes.

2) To prepare either antibodies or nucleic acid probes *to detect* the components and the levels in which they are induced.

3) To assess the relative physiological condition of the bacteria in a given bioleaching operation (for example, if it is preferentially oxidizing sulfur or if starved for a given nutrient) by using the probes prepared. Decisions could then be made, if possible, to change the conditions to improve the local bacterial activity (e.g. add phosphate, etc.)

Work Progress:

Phosphate-starvation response.

Cultivation of *T. ferrooxidans* in the absence of added phosphate not only reduced their growth rate and capacity to oxidize ferrous iron but induced a remarkable filamentation of the cells. Two dimensional gel electrophoresis revealed at least 25 proteins whose levels of synthesis were increased upon phosphate limitation, as well as some polypeptides that were exclusively synthesized under the starving condition. One of the proteins induced by the lack of phosphate was an acid phosphatase with a pH optimum of about 3.8, and a M.W. of 26 kDa, which was apparently located in the periplasm.

Several of the proteins induced by the lack of phosphate could be traced to the surface of the bacteria by total cell labelling in the presence of ^{125}I and analysis of the outer membrane from the microorganisms. One of these proteins was the *T. ferrooxidans* outer membrane protein (omp40), whose level of expression was apparently regulated by the external pH and the concentration of phosphorus. The amino terminal sequence of omp40 showed 38.5% identity to the outer membrane channel-forming protein NosA from *Pseudomonas stutzeri*, whose expression is also regulated environmentally.

The proteins induced by *T. ferrooxidans* upon phosphate starvation are probably part of a phosphate scavenging system utilized by the microorganisms in their natural environment, in which soluble forms of phosphorous could be limiting.

Changes in global expression of *T. ferrooxidans* when grown in sulfur.

There is a great interest in studying the structure, expression and regulation of the genes involved in the oxidation of ferrous iron and sulfur, since they make *T. ferrooxidans* and other bioleaching bacteria industrially important. Several proteins involved in ferrous ion oxidation by *T. ferrooxidans* are known, including rusticyanin, cytochromes a and c and other proteins. On the other hand, the proteins involved in sulfur oxidation have not been studied in detail and their cellular localization and exact roles are still unknown. It is also important to know whether *T. ferrooxidans* is using elemental sulfur, ferrous iron or both in bioleaching operations, since due to the chemical and biological leaching of sulfides, elemental sulfur is generated which tends to coat mineral particles, interfering with the process.

To identify some of the gene products involved in these oxidations, we have initiated the study of the changes in global gene expression in *T. ferrooxidans*, when the bacteria is grown on sulfur or iron. A few polypeptides were exclusively synthesized when the cells were grown in ferrous iron, and others were apparently synthesized only when *T. ferrooxidans* was grown in sulfur. By microsequencing of their N-terminal ends, and by specific Heme group staining, we have identified some of these proteins as cytochromes. In addition, we are preparing antibodies against some of the bacterial surface proteins that change their expression according to the environmental conditions. These antibodies will allow us to monitor the *in situ* physiological state of the bioleaching microorganisms.

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Networking: Hector Torres, Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI), Buenos Aires, Argentina.

SACCHARIFICATION OF STRAW: USE OF ENZYMES FROM NATIVE FUNGI

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Keywords: straw, lignocellulose, saccharification, cellulolytic fungi, cellulase, xylanases.

Abstract: Lignocellulose wastes represent the most important renewable source on earth for the production of fuels and chemicals. Enzymatic biodegradation of these materials is still too expensive for industrial use, but important progress is being made in the laboratory with the isolation of microbes that produce the required enzymes and by their characterization.

The purpose of this project is to analyze the cellulolytic and xylanolytic potential of a locally isolated strain of the soft-rot fungus *Penicillium purpurogenum*, which grows well on wheat straw. Optimal conditions for the production of the enzymes will be determined, the enzymes will be isolated and characterized and the use of enzyme mixtures in the degradation of straw will be studied.

Work performed so far has shown that the fungus can produce under appropriate conditions high levels of the different cellulases, beta glucosidase and xylanases. The beta glucosidase has been purified to homogeneity and its main catalytic properties have been characterized. Several xylanase isoenzymes are produced; they can be separated by chromatography; two of them have been obtained in a pure form and their properties have been studied. Several clones of Saccharomyces cerevisiae which secrete cellulase-free xylanase have been obtained by transformation with fungal DNA. Work is in progress in the characterization of auxiliary xylanolytic enzymes such as acetyl xylan esterase, beta-xylosidase and arabinofuranosidase which have also been found in the culture supernatant.

Background: Over 70% of all carbon in nature is in the form of lignocellulose. Therefore, this material represents by far the most abundant source of renewable resources on earth for the production of fuels and chemicals.

Lignocellulose biodegradation is a complex process, and has not as yet been implemented at the industrial level due to high costs. The resistance of lignin to decay and the crystallinity and insolubility of the cellulose components are major limitations.

Several approaches are being followed to optimize lignocellulose biodegradation. One is the identification, isolation and characterization of the enzymes involved, with the purpose of learning more about the process and optimize it.

This project is oriented to the study of the enzymes produced by a native strain of the fungus *Penicillium purpurogenum*, which grows well on wheat straw. Penicillia are fungi with good cellulolytic and xylanolytic potential but have been well studied, and straw is an inexpensive and abundant raw material, with relatively low lignin content and cellulose of lower crystallinity than other lignocellulosics.

Objectives:

1) Determination of culture conditions for the optimal production of cellulases and xylanases by *Penicillium purpurogenum* using straw as carbon source.

2) Identification, purification and characterization of the cellulases and xylanases obtained from these cultures.

3) Use of mixtures of the above isolated enzymes to find optimal conditions for the degradation of straw glycans.

4) Preparation of a gene bank of Penicillium for future cloning of the most promising enzymes.

Work Progress:

1) Culture conditions for the optimal production of cellulases and xylanases by *Penicillium purpurogenum*.

P. purpurogenum was grown in liquid media using Mandel's salt mixture (Process Biochem. 13, 6, 1978), cellulose, xylan or wheat straw as carbon sources and several nitrogen sources. Optimum temperature for growth was found to be 28 °C.

Best cellulolytic activities were found with cellulose as carbon source and corn steep liquor as nitrogen source: endoglucanase 29.2 U/ml; activity against filter paper 0.75 U/ml; beta glucosidase 1.0 U/ml. All data are for 10 day cultures.

Best xylanase activity was found using wheat straw as carbon source and Mandel's nitrogen source: 11.31 U/ml. Beta xylosidase

was produced optimally using xylan as carbon source (1.15 U/ml).

2) Identification, isolation and characterization of cellulases and xylanases from *Penicillium purpurogenum*.

Work performed has been oriented mainly to the study of beta glucosidase and xylanases.

Beta-glucosidase: the enzyme has been purified to homogeneity as shown by SDS-gel electrophoresis. It is a monomeric glycoprotein of molecular weight 90,000. Two isoenzymes have been isolated by chromato- and isoelectrofocusing, with pI's of 4.2 (major form) and 6.0. The major form was further characterized. Enzyme activity is optimal at pH 3.5 and 60 C. The enzyme is stable in the pH range 2.5-9.5 for 24 hrs at 4 C. The enzyme hydrolyzes a wide range of substrates including aryl glucosides, cellubiose and amygdalin.

Xylanases: By gel filtration chromatography, at least 3 forms of xylanase have been separated from the culture supernatant. Two of these xylanases (called A and B) have been purified to homogeneity. Xylanase A has an optimal temperature of 60 C, an optimal pH of 7.0, a molecular weight of 33,000 and a pI of 8.5. Xylanase B has an optimum pH of 5.0 and an optimal temperature of 50 C; its pI is 5.9 and the molecular weight is 23,000.

3) Cloning of xylanase genes.

Saccharomyces cerevisiae was transformed with fungal DNA to obtain strains capable of secreting xylanases. Several clones have been isolated which secrete xylanases to the medium. One of them has been studied further. Both xylanases A and B, as determined with electrophoresis and immunoblots are found in the culture supernatant, suggesting that these genes are organized in a cluster. The xylanase activity produced by the clone is cellulose-free.

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Networking: Mrs Jeannette Steiner, Department of Biochemistry and Molecular Biology, Faculty of Chemical and Pharmaceutical Sciences, Universidad de Chile, Santiago de Chile.

PROTEIN PHOSPHORYLATION AS A REGULATORY MECHANISM FOR CELLULAR PROLIFERATION

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ICGEB Reference No. CRP/CHI91-02

Keywords: protein kinases, casein kinase I, casein kinase II, cDNA cloning, site-directed mutagenesis, *Xenopus laevis*, *Baculovirus*

Abstract & Work Progress: The project has significantly advanced towards its general and specific objectives of gaining new knowledge about the structure and function of the casein kinases (CKI and CKII), abundant protein kinases present in the nucleus of all eukaryotic cells.

cDNAs coding for the two subunits of casein kinase II from *Xenopus laevis* oocytes have been cloned and sequenced and both recombinant proteins have been expressed in bacteria. The recombinant a and b subunits have been purified and shown to interact *in vitro* to generate a fully active holoenzyme. Using synthetic peptides it has been shown that the holoenzyme is preferentially inhibited by peptides with the Y-1 over the sequence E-Y, showing that the enzyme recognizes tyrosine residues in a similar position to serines and threonines in substrates. The isolated a subunit, however, does not differentiate between these peptides. It was also shown that CKII can be inhibited by tolylpolyglutamate derivatives that are present *in vivo*.

The study of the effect of metal ions on CKII activity has demonstrated that Mn^{++} and Co^{++} can replace Mg^{++} and that in the presence of Mn^{++} the enzyme uses GTP more efficiently than ATP as a phosphate donor, while the Mg^{++} the reverse is true. Site directed mutagenesis has changed the serines 2 and 3 of the b subunit for glycines, eliminating the autophosphorylation site of this subunit. The mutant b subunit that cannot be phosphorylated is not affected significantly in its capacity to increase the catalytic activity of the a subunit.

Five other mutations of the b subunit have been produced and sequenced and their effects on the function of this subunit is being tested. Mutation of the a subunit replacing lysines 75 and 76 for glutamic acids have been produced.

The mutant, CKII a E75,76E is active and has similar capacity as the wild type subunit to interact with b and a similar app. km with casein. However, CKII a E75,76E is much less sensitive to inhibition by polyanions such as heparin, copoly glutyr (4:1), poly U and 2,3 bisphosphoglycerate.

The a and b subunits of CKII from the *Zebra* fish have also been cloned at the cDNA level and at the genomic level.

The cDNA for the *Xenopus* and human CKI has been cloned and partially sequenced.

Background: Protein phosphorylation and dephosphorylation is the most important post-translational modification of proteins in the regulation of their biological activity.

More than 200 different protein kinases have been discovered and have been shown to participate in many fundamental steps of signal transduction.

Protein kinases play key roles in the regulation of cell proliferation and many oncogenic proteins have been shown to have protein kinase activity.

Casein kinases I and II are abundant in the nucleus of all eukaryotic cells and are know to phosphorylate key proteins involved in genetic replication and expression such as DNA polymerase a, RNA polymerase I and II, DNA ligase, jun, fos, myc, max, SRF, Rb, p53, T antigen of SV-40 and many other proteins. The regulation and function of these enzymes are still unknown and require further biochemical and molecular biology research.

Objectives: The general objective of the project is gain understanding on the role of some nuclear protein kinases in the control of cellular proliferation.

Specifically, using the combined techniques of protein chemistry and molecular biology, four specific studies were proposed:

1) The structures and expression of the *Xenopus laevis* genes coding for casein kinase I and casein kinase II.

2) The specificity of substrate recognition through the use of synthetic peptides and model substrates and pseudo-substrate inhibitors.

3) The structural and functional relationships of some important domains of these enzyme through site-directed mutagenesis.

4) The relation of these protein kinases to cellular transformation by specific oncogenic products.

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Networking: Collaboration with Dr. Sandor Pongor from the Trieste Laboratory of the ICGEB has been extremely important in the design and synthesis of peptide substrates and inhibitors and also in the installation of a core facility for nucleic acid and peptide synthesis and microsequencing in the Chilean Laboratory.

Dr Raul Aguirre, who received an ICGEB fellowship to the Pongor Laboratory, is now the technical director of this facility.

Active collaboration has also been established with the laboratory of Dr. Mari Armelin from the University of Sao Paulo, Brazil. Two members of the Allende's laboratory have spent a total of 4 months in Dr. Armelin's laboratory in studies leading to the transfection of CKII subunits in animal cells. Dr. Armelin has also visited the Chilean laboratory on two occasions.

The Allende's laboratory has provided post-doctoral training to an ICGEB fellow, Dr. Jose Luis Daniotti, from Argentina, who has worked in the isolation of the CKII clones from *Zebra* fish.

PHASEOLUS VULGARIS: IN VITRO REGENERATION AND OTHER BIOTECHNOLOGIES FOR DISEASE CONTROL AND RESISTANCE

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Keywords: *Phaseolus vulgaris*, bean, *in vitro* culture, disease resistance, biological control, transformation

Abstract: Beans are the food basis for most of the people living in Latin America and Africa. Its production is limited by the world scarcity of new varieties with high potential yield, which includes resistance against phytopathogens. *Phaseolus vulgaris* and most of the grain legumes, are species which are considered difficult to regenerate *in vitro*. Therefore, an efficient and reproducible regeneration protocol is essential for studies which involve genetic modification to decrease susceptibility against bean pathogens. On the other hand, it is important to choose the best method for plant transformation and also to find alternatives to control bean phytopathogens based in their biological control. The present report includes results on the goals for the first year of project, related to the use of biotechnologies that could improve bean resistance to phytopathogens. The experimental approach was divided into two main topics: *in vitro* culture and regeneration of beans for the obtention of protocols that could be used in transformed bean, and the search of microorganisms to be used for bean transformation (*Agrobacterium*) and for biological control of bean phytopathogens (chitinolytic microorganisms).

Background:

1) *In vitro* culture of beans: *In vitro* culture and regeneration of beans, are still underdeveloped as compared with other plants, because of their difficulty to be regenerated *in vitro*. Bean *in vitro* regeneration was studied including four trials: a) culture of embryonic axis explants from pre germinated seeds, in low to normal BAP (Benzylaminopurine) and IBA (Indolbutiric acid) concentrations (using cv. Tortola INIA) and in low culture dates; b) embryo culture in low, normal and high BAP concentrations (using cvs. Tortola INIA, Coscorron and Pinto 114); c) embryonic axis explants (cotyledonary nodes) in high BAP concentrations (using cv. Tortola INIA); and d) apical meristem culture (shoot apices) under different ethylene concentrations (using cv. Pinto 114).

2) Isolation of fungal local strains which infect beans: *Fusarium solani* and *Sclerotium rolfsii* are among the fungal pathogens that infect bean plants in Chile. Plants with symptoms of the diseases caused by these two pathogens were used as the source for their obtention. Their identification was performed after growth in different culture media (potato dextrose agar, malt agar and Czapek agar) to determine macro and microscopic morphologies. Then ability to infect bean plants and produce the same symptoms (in green house) was tested inoculating them in already grown plants. Re-isolation of the fungal strains was performed from these infected plants. They were compared to the initial isolated strains.

3) Isolation of local strains of *Agrobacterium tumefaciens*: *Agrobacterium* has been considered the natural genetic engineer because of its ability to transfer part of its genome contained in a plasmid, to the infected plant tissue. The isolation of local strains of *Agrobacterium* was performed taking material from plants that showed typical symptoms of infection of *Agrobacterium tumefaciens* (Almond and Pear trees) and analyzing their development in minimal media, resistance to carbenicillin and ability to induce tumors in carrot slices. Its further characterization included the identification of the produced opines.

4) Bean infection by *Agrobacterium tumefaciens*, the use of *A. tumefaciens*, for the introduction of resistance genes into beans has been considered as a good alternative for transformation. Beans (cv. Pinto 114, Coscorron, Tortola INIA and Negro Argel) were germinated in the presence of *A. tumefaciens*, or germinated beans (4 days old) were inoculated with the bacteria in previously wounded hypocotyles, closed to cotyledons.

5) Isolation of chitinolytic microorganisms: chitin degrading microorganisms are useful for the control of pathogens that contain chitin in their wall. Soil samples were screened for the presence of both fungi and bacteria that could grow in solid media containing chitin as the sole carbon source. Those presenting this ability were further studied for their ability to excrete chitinase, growing the fungi in submerged cultures with chitin as the sole carbon source. Exochitinase activity was measured as the release of the N-acetylglucosamine from choloidal chitin. Endoquitinase activity, as well as chitinase isoforms, was estimated in native PAGE in which enzyme activity could be observed in an auxiliary (revealing) gel containing glycol chitin. The control of *Sclerotium rolfsii* was tested either using directly a culture filtrate to study germination of sclerotia and protection of bean seeds from infection; or pellets (containing fungal mycelium, alginate and maize flour) inoculated in the soil along with sclerotia to study protection of bean plants from infection by *S. rolfsii*. Controls were performed in the absence of culture filtrates or pellets. The original protocols and details of the experimental procedures used are available upon request.

Objectives: The objectives for the first year were: a) The evaluation of *in vitro* cultures techniques described in the literature for *Phaseolus* and protocols developed by Dr. Herrera Estrella's group, to develop an efficient *in vitro* regeneration system for local bean genotypes; b) the isolation of fungal local strains which infect beans, for further studies on local bean genotypes; c) the isolation of chitinase producing microorganisms, to be tested as biological controllers of bean pathogens; d) the isolation and characterization of local strains of *Agrobacterium tumefaciens*; e) the evaluation of the infection capacity of local strains of *Agrobacterium tumefaciens* in bean tissues.

Work Progress:

1) *In vitro* culture of beans: the results of Trial I indicated that the highest BAP concentration tested (3mg/L), free of auxin, gave the best shoot regeneration from cotiledonary node explants and shoot meristem explants, from the 7th day in culture on, with no significantly differences between them (2.6 and 2.7 shoots/explant, respectively). Regarding culture date, though the number of shoots formed was higher when culturing cotiledonary node explants in May as compared to December, this effect interacted with the growth regulator's effect and could not be analyzed independently. However, for shoot meristem explants, significant differences were observed between culture dates, being May also the best time to culture. In December cultures, more callus and roots were developed from the explants. Epicotylar explants showed no response to *in vitro* culture.

Trials II and III demonstrated that high BAP concentrations (8 or 16 mg/L) were able to induce much higher number of shoots from cotiledonary nodes or shoot meristems, specially when these embryo parts were not separated as independent explants from the complete embryo. Nevertheless, the dissection of one cotyledon from the embryo, did not produce statistical differences in shoot formation compared to complete embryo culture.

Significant differences in shoot regeneration were observed among genotypes. Cultivar Tortola INIA produced the highest number of shoots (>100; 20-3 shoots counted through the flask, which after dissection showed that each one of them was formed

of 3 - 8 smaller shoots) when complete embryos were cultured under 16mg/L of BAP.

When embryo parts were cultured under 8 and 16 mg/L, no statistical differences in shoot number were found (= or > 2.5; 5.26 and 5.30 counted through the flask, respectively), indicating that 8 mg/L could be an appropriate concentration for the culture of complete embryos.

The hystological study showed that root regeneration from cotyledonary nodes was initiated from the performed axillary buds, which under high cytokinin concentrations develop new meristematic tissue giving rise to new apices and shoots. These, in turn, originate new shoots following the same process, forming a massive rosette of shoots at each axil.

Trial IV was able to demonstrate that high ethylene concentrations (1.73 - 3.13 ppm) accumulated in the culture flask produced an inhibition of shoot growth, but did not significantly affect shoot number. Results also showed that cultivar Pinto 114 generates significantly more ethylene when cultured *in vitro*. This could in part explain the difficulty of this species to regenerate *in vitro*, as this cultivar was the least responsive in shoot formation in Trial II.

It can be concluded that *in vitro* culture of complete embryos or with only one cotyledon in *Phaseolus vulgaris*, subjected to high cytokinin concentrations (8-16 mg/L of BAP), produces massive regeneration of shoots from the apical meristem or from the cotyledonary node. Significant differences in shoot regeneration response are found among different *Phaseolus* phenotypes. The presence of auxin in the culture medium inhibits shoots formation in all cultivars under study. The culture of embryonic axis explants produces significant less shoots than the culture of complete embryos, even when supplemented with high cytokinin concentrations. Time or date of culture, even for embryos, seems to be an important factor in shoot regeneration; more detailed physiological studies are necessary to explain this effect. The origin of the regenerated shoots when culturing embryos or embryonic axis explants corresponds to the apical embryo apex and to preformed axillary buds at the cotyledonary node. When subjected to high cytokinin concentrations these regions develop new meristematic tissue at the base of the present bud (6 - 8 days in culture) and then from the new formed shoots (10 - 12 days in culture), thus producing a cascade-like regeneration process. Bean is a species which generates high ethylene concentrations when being cultured *in vitro* as compared to other species of very easy *in vitro* regeneration. This characteristic could be contributing to its well-know regeneration difficulty.

2) Isolation of fungal local strains which infect beans: bean plants with symptoms corresponding either to *Fusarium solani* or *Sclerotium rolfsii* infections were selected for the isolation of local strains of these bean pathogens. The isolated fungi were characterized, and their morphologies corresponded to *Fusarium solani* and *Sclerotium rolfsii*, as expected. Bean plants inoculated with these fungi, developed the same symptoms present in those selected at the field level and the new isolates corresponded to the identified fungal strains. These fungi were used for further studies of biological control, and for preliminary studies of resistance of local bean genotypes to infection by them.

3) Isolation of local strains of *Agrobacterium tumefaciens*: an *A. tumefaciens* strain was isolated from crown galls of almond trees. Samples taken from pear trees did not give positive results for the presence of *Agrobacterium*. The strain isolated from almond trees could grow in minimal culture media, was resistant to carbenicillin and induced tumors in carrot slices. The ability of this strain to metabolize opines such as nopaline and octopine was also determined.

4) Bean infection by *Agrobacterium tumefaciens*: beans from cv. Pinto 114, Coscorron, Tortola INIA and Negro Argel, germinated in the presence of the *A. tumefaciens* strain that induced tumors in carrot slices, did not induce tumors in any of the bean varieties tested. In addition, pre-germinated beans from the same varieties, inoculated with the same strain of *A. tumefaciens* did not develop tumors. Studies are in progress to improve protocols to obtain transformation of beans with this bacteria.

5) Isolation of chitinolytic microorganisms: the hypothesis for the isolation of microorganisms which could antagonize growth and/or condition of pathogenic fungi was chitinase producing microorganisms should be able to degrade chitin present in phytopathogen's cell wall and by doing so antagonize growth of the pathogen. The ability of fungi to grow on chitin as sole carbon source was used as a selection criteria for the isolation of chitanase producing organisms. 30 fungi were isolated from soil sample. Their ability to antagonize bean phytopathogens was assayed *in vitro*. One of the isolates, *Penicillium purpurogenum*, could inhibit growth of *Alternaria alternata*, *Fusarium solani* and *Sclerotium rolfsii*. The other isolate corresponded to *Trichoderma harzianum* V that could inhibit growth of *S. rolfsii*. Preliminary results show that culture filtrates from *P. purpurogenum* contain both endo- and exochitinolyties activities. This filtrate inhibits 90 - 95% germination of sclerotia and protects ca. 75% of bean seeds from fungal infection. On the other hand, pellets containing *T. harzianum* protect ca. 50% of bean plants from infections with *S. rolfsii*.

6) **Other studies**: during the first year we have begun the study of the response at the molecular level of different local bean genotypes (cv. Pinto 114, Coscorron, Tortola and Negro Argel) against infection by *S. rolfsii*. Kinetic experiments have been run to determine Phenylalanine ammonia lyase (PAL) and chitinase activation. Preliminary results show that phenylpropanoid pathway is poorly activated in all the bean varieties tested, and that both endo and exochitinase activities present basal activities.

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Larenas, C. and Montealegre, J. (1994) Use of *Trichoderma harzianum* in the control of *Sclerotium rolfsii* in beans. (Uso de *Trichoderma harzianum* en el control de *Sclerotium rolfsii* en frejoles). VII Congreso Latinoamericano de Fitopatologia (VII Asociacion Latinoamericano de Fitopatologia). Resumenes pag. 15 (Res. 025).

Pozo, P. and Perez, L.M. (1994) Chitinases are induced in *Phaseolus vulgaris* as a response to infection by *Sclerotium rolfsii*. (Las quitinasas se inducen en *Phaseolus vulgaris* en respuesta a la infeccion por *Sclerotium rolfsii*). VII Congreso Latinoamericano de Fitopatologia (VII Asociacion Latinoamericano de Fitopatologia). Resumenes pag. 111 (Res. 017).

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Vecchiola, A., Zaldivar, M. and Perez, L.M. (1994) *Penicillium* spp.: antagonists of *Sclerotium rolfsii* isolated from *Phaseolus vulgaris*. (*Penicillium* spp.: antagonistas de *Sclerotium rolfsii* aislado desde *Phaseolus vulgaris*). VII Congreso Latinoamericano de Fitopatologia (ALF. Asociacion Latinoamericano de Fitopatologia). Resumenes pag. 112 (Res. 0175).

Networking: The 3rd Latinamerican and 3rd Chilean Congress on Biotechnology took place in Santiago in November 1993. A researcher from Dr. Herrera Estrella's group planned to come for the meeting, to take the opportunity di discuss and exchange regeneration protocols for beans. Specifically, results from both laboratories were compared related to culture media composition, date of culture and bean varieties used. It was concluded that micropropagation from apical meristems had no problem at all, and that induction of an organogenic or embryogenic process has not been accomplished routinely and successfully by any of the laboratories. Exchange of further modified protocols will be done in the future, in order to find the best one to be used for possible transformed explants.

PRODUCTION AND USE OF SAPONINS WITH ADJUVANT ACTIVITY FROM THE BARK OF *QUILLAJA* SAPONARIA MOLINA

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ICGEB Reference No. CRP CHI92-02

Keywords: vaccines, adjuvants, saponins, *Quillaja* saponaria

Abstract: Effective vaccine production required the use of appropriate antigens and adjuvants to optimize protective humoral and cell-mediated immuno responses. One very effective type of adjuvants are saponins, which are mixtures of triterpene glycosides present in many plant extracts. Commercially, saponins for vaccine production are obtained from the bark of the tree *Quillaja* saponaria Molina. This tree is native to Chile and its bark contains about 10% saponins, with proven adjuvant capacity. However, due to severe local reactions at the site of injection, its use is limited to animal vaccines (e.g. foot and mouth disease).

The project objective is to develop a scalable process for the production of a purified fraction of *Q. saponaria* saponins with enhanced adjuvant activity. This fraction is effective at smaller doses than crude saponins (1mg vs 5 mg), minimizing adverse reactions at the site of injection. Besides, this purified fraction has the capacity to form Immuno Stimulating Complexes (ISCOMS), which are novel submicroscopic particles used to present virus envelope glycoproteins. At present, only the firm Superfos from Denmark manufactures this purified fraction (QUIL-A), derived from the bark of Chilean Quillaja.

The process is based on the size reduction, aqueous percolation, diafiltration- to remove low molecular weight contaminants (e.g. sugars), ion-exchange- to adsorb the adjuvant rich fraction, which is negatively charged-, the lyophilization of the final product. Process studies will be conducted at lab-scale, and then scaled up to pilot plant level, to produce 50 g of product per batch. Product quality with animal vaccine animal preparations will be assessed in collaboration with the Department of Immunology of the Catholic University as well as the Butantan Institute in Sao Paulo, Brazil (ICGEB Affiliated Centre).

The project duration is three years with a total budget of US$ 55,000: US$ 40,000 provided by ICGEB and US$ 15,000 by the School of Engineering of the Catholic University.

Background: Saponins derived from the bark of the Chilean endemic tree *Quillaja* saponaria Mol. have been used for decades as adjuvants in animal vaccines. However, because they are surface active, many saponins are hemolytic to red blood cells and induce severe local reactions when injected parenterally. A partial solution to this problem was provided by Danish researcher Dr. K. Dalsgaard, through purification of Quillay saponins in order to isolate the fraction with highest adjuvant activity. The product composition is still complex, consisting of a mixture of very closely related triterpene saponins with high adjuvant activity. However, if is effective in less quantities than crude saponins (1/5 of normal doses), and therefore show less local reactions at the site of injection. This discovery provided the basis for the industrial production of a purified fraction (Quil-A), by Superfos (Denmark), which is used for the preparation of vaccines for large animals, i.e. cattle, pig, and sheep. Besides, Quil-A has the capacity to form Immuno Stimulating Complexes (ISCOMS), which are novel submicroscopic particles formed by the interaction of Quil-A, lipids (particularly cholesterol) and virus envelope proteins. In recent years they have received increased attention, particularly due to their potential to produce more stable and highly immunogenic vaccines. Since the amount of Quil-A required is minimum, practically no adverse effects have been reported. More recently, an alternative purification technique for Quillay bark adjuvants based on silica and reverse phase chromatography has been reported by Cambridge Biotech Corp., USA. It is claimed that the method produces a better purification of the different components with adjuvant activity, but the process is still in a developmental stage.

In Chile, Quillay bark from 20-25 year old trees has been exported for many decades for its use as an emulsifier, particularly by the photographic industry. Since the tree runs the risk of being over exploited, its use is now regulated by the Ministry of Agriculture. Present export levels, primarily to USA and Germany are about 600-800 tons/y at an average price of US$ 1,000/ton. Only a minor quantity of bark is processed to obtain a crude extract of saponins. Based on our estimates of the market for a purified saponin fraction, only about 5 tons bark/year are required, thereby reducing any environmental impact due to an over exploitation. Moreover, the extraction process considers primarily the use of water, thus minimizing treatment of process effluents.

Objectives:

1) To develop a scalable process for the production of a purified fraction of saponins derived from the Chilean tree *Quillaja* saponaria Mol.

2) To test these purified fraction as adjuvants in animal vaccines and for the preparation of ISCOMS.

3) To develop analysis procedures for saponin based on HPTLC and HPLC.

4) To test *Q. saponaria* trees from different locations in Chile, and evaluate its saponin composition.

Work Progress: During this first research period we have performed the following tasks:

1) Optimization of a lab-scale procedure for the extraction and purification of *Quillaja saponaria* Mol. saponins.

2) Determination of process parameters to scale up the process.

3) Product quality comparable to existing commercial product (Quil-A, Superfos, Denmark).

4) Verification that the final product forms Immuno Stimulating Complexes (ISCOMS).

5) Product samples were sent to the Butantan Institute in Sao Paulo, Brazil.

6) Acquisition of equipment to scale up the process during the second research period.

7) Acquisition and operation of HPTLC analysis system for quillaja saponins.

Process Development: What follows is a brief description of the main unit operations of the process under development.

Extraction: all experiments are performed with air dried bark (about 10% MC). Defibration in a hammer-mill is necessary to improve extraction yields. The process is carried out recirculating water at room temperature through the bark. Approximately 7.5 lts of water are added per kg of dry bark. Solubilization of the saponins is very fast (0.5-1 h). Two extractions with fresh water result in a yield of 14.0% extractives, based on air-dried bark. The pH of the pool is 5.5.

Diafiltration to remove low molecular weight compounds (e.g. sugars): diafiltration using water is performed using a 10,000 Daltons membrane that allows for the retention of the saponins, since they form miscelles. Filtration rates are 12 lts/m²-h, at 15 psig inlet pressure. About 70% of the solids pass

through the membrane.

Ion exchange: Ion exchange with DE-52 resin (Whatman) is used to adsorb the saponins. The capacity of the column in the range of 40 mg/ml. An important problem refers to the irreversible adsorption of coloured compounds. This has been reduced by using the internal part of the bark which is whiter.

Diafiltration to remove salts: the final step is to remove the salts used in the ion exchange using diafiltration with 10,000 Dalton membrane.

Product properties: The average yield of product is 1% (w/w). Colour: white-brownish. Its composition compares well with the commercial product Quil-A from Superfos. ISCOM formation was demonstrated by Dr. K. Dalsgaard, from the State Veterinary Institute for Virus Research, Denmark.

(Due to limited space the figure is not included in this Activity Report)

Publications:

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Networking: Instituto Butantan, Sao Paulo, Brazil

STUDY OF THE WATER FERN AZOLLA FOR MUNICIPAL WASTERWATER TREATMENT AND ITS UTILIZATION AS BIOFERTILIZER

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ICGEB Reference No. CRP CHI92-03

Keywords: azolla, biofertilizer, wastewater treatment

Abstract: Azolla fern, an aquatic plant having the ability to fix atmospheric nitrogen is being used in warm and tropical countries as a source of low-cost biofertilizer. In this project the study growing of azolla and its use in municipal wastewater and as biofertilizer in the north of Chile is proposed. Given the scarcity of water and the poor soil quality for agricultural, a double benefit is expected to be obtained as a result of azolla utilization: improved water quality and soil fertility.

Background: Azolla, a quick-growing fern, occurs in ponds, ditches and rice field of tropical to temperate region. A lot of authors have reported its use for agricultural purposes, its capacity to fix N from the atmosphere is due to symbiosis with a heterocystous N-fixing blue-green alga (*Anabaena azollae*) found within the cavities of upper and dorsal lobes of axolla fronds. Latry, Peter (1991) has indicated that the dominant symbiont in association with *Azolla spp.* is more closely related to *Nostoc spp.* than to free-living *Anabaena spp.* Tarrapico (1991), has also suggested that bactria (*Arthrobacter*) are the third partner of the symbiosis, because these bacteria were always present as a permenent member of the prokaryotic colony at different stages of leaf development of *Azolla spp.*

In Chile, only the specie *Azolla filiculoides* Lam (Reed, 1965) has been descripted and information about distribution, environmental requirement and nutrient requirement is documented (Vidal and Longeri, 1988, 1990a). The same authors have evaluated in experimental ponds the monthly growth and N fixation of 52 ecotypes from 6 azolla species., to come both from Chile and foreign collections. They found significant differences in growth and amount of N fixed; the best specie was *Azolla rubra*, native of New Zealand (Vidal and Longeri, 1990b).

Recently, considerable attention has been paid to minimize dependence on inorganic nitrogen fertilizer, hence there is a need to explore the possibilites of using and expanding natural sources of plant nutrition. The use of biological nitrogen fixation through azolla is an alternative. Other uses of azolla as a decontaminant in sewage treatment and as animal food.

Recently, various aquatic plants (*Eichhornia, Spirodela, Ipomoea, Lemna, Nasturtium*) have been proposed to remove N and P. The average ratio of N to P in plants is 10:1. Thus, a favorable uptake of N and P by aquatic plants will be observed in a culture solution containing N and P at a ratio of about 10:1. Nutrient concentration, however, varies widely with waste water treatment plants. In most cases of waste water treatment by aquatic plants, P nutrient will remain after N removal. We try azolla as a decontaminant of waste water because it grows by fixing atmospheric N and P removal can be expected even after the N has been consumed.

Azolla cultured in waste water can be harvested for use as green manure, further reducing waste water treatment cost. This could be an additional benefit of azolla use in sewage treatment.

Objectives:

1) To evaluate the potential of azolla as a decontaminant in sewage treatment and measure its biomass production.

2) To determinate the effect of azolla green manure on N uptake and yield of rice and other crops. To compare the recoveries of N from azolla and inorganic fertilizer sources, using ^{15}N labelled materials.

3) To determine the growth of some azolla ecotypes in paddy rice with different fertilization (P,K,Mg).

Work Progress:

1) Growth of azolla ecotypes in wetland rice with P-K-Mg fertilization. This study was started in 5 farmer's rice field at Parral, San Carlos and Chillan (Chile). The growth and N fixation of 5 azolla ecotypes under different fertilization treatment will be measured. Two harvest in the season will be carried out and dry weight, N%, N fixed and foliar concentration (P,K,Ca,Mg,Na) will be determined.

Experimental plots were laid out in a split plot design with 4 replications. The whole-plots were azolla ecotypes and the subplot correspond to fertilization treatment. The treatments and sybols used are:

Ecotypes

UCA 162 = *Azolla rubra* (new Zealand)
UCA 110 = *Azolla mexicana* (California, USA)
UCA 109 = *Azolla mexicana* (Brazil)
UCA 106 = *Azolla filiculoides* (Brazil)

Fertilization treatment

Control : without fertilization
P : 60 kg P2O5/ha as triple superphosphate
P+K : 60 kg P2O5/ha and 80 kg K2O/ha, as triple superphosphate and potassium sulfate.
P+K+Mg : 60 kg P2O5/ha as triple superphosphate, 80 kg K2O/ha and 40 kg MgO/ha as magnessium-potassium double sulfate.

2) Nitrogen uptake and recovery from azolla and urea in wetland rice by isotope techniques. The described study is to investigate the availability of azolla-N to rice plants when incorporated before sowing or when grow simultaneously with rice. The availability of azolla-N will be compared to that of urea, the N fertilizer used in Chilean rice fields.

A field and greenhouse experiment were conducted using a randomized complete block design. In the field experiment the treatments with 4 replications were:

I. Control = Without N
II. U80 = 80 kg N (Urea)/ha
III. U40+A40 = 40 kg N (Urea)/ha + 40 kg N (azolla)/ha incorporated
IV. U40+As = 40 kg N (Urea)/ha + Azolla growing simultaneously with rice
V. U40 = 40 Kg N (Urea)/ha

Plot size was 9 m2. For 15N studies, one 0.8x0.8m microplot was installed nearby each main plot. Each microplot was surrounded by 0.2m high border made of wood, inserted to a depth of 0.3m into the soil. A no-fertilizer control treatment was also included as microplot.

The greenhouse experiment had the same treatments and were used pot with 8 kg soil with 4 replicates and the treatments were as follows:

I. Control = Without N
II. U80 = 80 kg N (Urea labelled *) /pot
III. U40+A40 = 40 kg N (Urea labelled *)/pot + 225 mg N (azolla)/pot
IV. U40+As = 225 mg N (Urea labelled)/pot + Azolla growing simultaneously with rice
V. U40 = 225 mg N (Urea labelled)/pot

(*) Urea labelled with 3% a.e. ^{15}N.

Yield, dry wt, N% and ^{15}N atom excess will be measured in rice.

3) Azolla as a decontaminant in sewage treatment. As the previous experiment the azolla growth in sewage coming from Antofagasta city (Chile) is been measured. We are wocking with trays (200 cm2) containing 1 lt of medium and we are using 3 water sources. i) Control (solution Hoagland 40%). ii) Treated municipal waste water from stabilization ponds and iii) Untreated municipal waste water. The following ecotypes are being used:

UCA17 *A. filiculoides*. Chilan, Chile
UCA 106 *A. filiculoides*. Brazil. CNPAF 6.
UCA 109 *A. mexicana*. Brazil. CNPAF 9
UCA 110 *A. mexicana*. USA. California
UCA 162 *A. rubra*. New Zealand. U.L.

Apparatus for culturing azolla in continously flowing water and with major capacity trays will be cuilt in the next months. Waste water will be pumped at different flow rate and effluent will be sampled to measure the removal rate.

Networking: Luis Longeri. Ivan Vidal. Universida de Concepcion. Facultad de Agronomia. Chillan. Chile.

Luis Caceres. Rene Contreras. Erika Gluttner. Universidad de Antofagasta. Instituto del Desierto. Antofagasta. Chile.

Jorge Etchevers. Roberto Quintero. Colegio de Postgraduados. Centro de Edafologia. Montecillo. Mexico.

CHINA

STUDIES ON STRUCTURAL MECHANISM OF PROLONGED-ACTING AND HIGHLY POTENT HUMAN INSULIN

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Keywords: insulin, prolonged action, highly potency, X-ray structural analysis

Abstract: In the clinical medicine, insulin is used as an injected therapeutic agent for the treatment of diabetes, therefore is very critical for the relative therapeutics. The present insulin preparations have, however, had some significant disadvantages and the supply of insulin in the market of China is very short. Obviously it is significant to explore some new therapeutic agents with certain attractive properties (e.g. prolonged action, high stability and superpotency, low immuniogenicity etc.) for improving the clinical use. For these scientific goals it is central to understand the structural mechanism of such new molecular behaviors, namely the relationship between structure and new properties.

The main task of our research project is, by using X-ray crystal structural analysis, to determine the three-dimensional structures of a series of insulin mutant prepared by protein engineering and partly by semisynthesis and possessed some desirable properties related to prolonged-biological action, high potency or highly chemical stability. On basis of these, the structure mechanism of these new features will be elucidate. Furthermore, the rational molecular design and molecular modelling for the more effective prolonged-acting and highly stable or potent insulin derivatives will be investigated. The results will provide new idea and information to the insulin engineering which may benefit the medical practice related to diabetes. The investigation will also give the chance to gain new insight into the structure-function relationship of insulin, especially the new features of insulin as an allosteric protein.

Background: Insulin is a well known protein hormone which mainly controls the glucose content in blood to keep it at physiological levels. In the clinical medicine insulin is a specific drug for the therapy of diabetes mellitus, thereby is very critical for the relative therapeutics. In China and also in other countries, the medical requirement of insulin is tremendous and non-substitutive. But the present insulin preparation have some significant disadvantages and the supply of insulin in the market of China is very short. Therefore, it is a challenge for scientists to explore some new therapeutic agents with certain attractive properties (e.g. prolonged action, high stability and potency, low immunogenicity etc.) for the treatment of diabetes. Understanding the structural mechanism of such new molecular behavior is central in these scientific goals. In recent years some new insulin derivatives, which possess prolonged action, high stability or potency, or low immuniogenicity, were prepared by means of protein engineering and biochemical synthesis in some laboratories. Nevertheless few studies only related to the structural mechanism of these properties was reported so far. Our laboratory in Beijing have been working on X-ray structure for quite a long time and recently we obtained a series of crystals of insulin mutants and derivatives which provide the sound basis of the research project.

Objectives:

1) Determining X-ray crystal structures of 5~7 insulin mutants and derivatives which are prepared by protein engineering or semi-synthesis and possessed certain attractive properties.

2) On basis of the comparisons between the determined three-dimensional structures of insulin mutants and that of the native insulin, elucidating the structural mechanism of the prolonged action and other engineered properties of mutants and providing new idea and information to further insulin engineering.

3) Gaining new insight into the structure-function relationship of insulin, especially the new allosteric properties of insulin.

Work Progress: The main work progress can be summarized as the following:

1) Prolonged-acting insulin. X-ray structures of two prolonged-acting insulin, including ArgB31-human insulin (ABHI) and LysB31-human insulin (LBHI), have been determined at 2 Å and 2.1 Å resolution respectively. The refined structure of ABHI shown similar features to that of LBHI. The conformation of B-chain C-terminal residues is more stable than that in the native molecule. The striking structural feature is composed of an additional ion pair formed between arg-B31 or Lys-B31 of molecule 1 and Glu-B21 of molecule 2 in a dimer and thereby three ionic bonds between the neighbour molecules appeared on the surface of the hexamers. This structural characteristic should make the dissociation rate of ABHI and LBHI hexamer slow down when it was injected into the body and the property of protraction should be produced by a "depot effect". This ought to be the main structural basis of the prolonged effect. The results observed here may provide a principal which could be used in the further search for new preparation.

2) High stable insulin. X-ray crystal structures of three insulin mutants with high stability, including GlyA21 HI, SerA21 HI and AspA21 HI, have been determined all at 1.8 Å resolutions. The main structural information from these three mutant in relation to the high stability can be summarized as: The enhancements of the chemical stability of the mutants are just caused by the elimination of the chemically unstable -CO-NH2 group of A21-Asn through substitutions by Gly, Ser and Asp.

3) High potent insulin. In collaboration with NOVO Institute of Denmark, two partners with high-low potency respectively from the mutation at the same residue position have been prepared, including A21-Ala HI (with potency of 140% of insulin) and A21-Glu HI (with potency of 8% only), as well as B25-Tyr (160%) and B25-Asp (0.1%). Therefore, we found two comparable partners which possess unusual high and low activities respectively that are very useful for understanding the structural basis of high potency. Besides, a derivative from a substitution of residue Lys for B3-Asn of native insulin was prepared and displays also a high potency of about 170%. This is quite interesting because no report before shows that residues at B-chain N-terminal segment could relate to the increase of insulin potency. So far three of them, including A21-Ala, B3-Lys and B25-Asp, have been crystallized and the preliminary X-ray analysis for them have also been completed.

4) New structural types of insulin allosteric intermediate observed in some mutants. Some recent finding from X-ray structural analysis and spectroscopic analysis revealed that the insulin hexamer displays many of the classical elements of an allosteric protein. In our X-ray structure analysis of the mutants A21-Ser HI and A21Ser-B21 Arg-B30Thr-NH2 HI, two new types of allosteric structure were observed which were considered as new forms of insulin allosteric intermediate and designated as 2Zn-T3R3 and Zn-free-T3R3 structures.

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A new structural type of zinc insulin observed in a mutant of (A21, Ser)-human insulin. Peptide-Biology and Chemistry. PP. 241-244. ESCOM. 1993.

Networking: China National Center for Biotechnology Development. Laboratory of Protein Engineering. Institute of Biophysics. CAS.

CUBA

TRANSFORMATION OF SWEET POTATO (*IPOMOEA BATATA L.*) FOR INCREASING ITS NUTRITIONAL VALUE AS HUMAN FOOD AND ANIMAL FEED

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UNIDO Contract No. 91/050

ICGEB Reference No. CRP CUB90-02

Keywords: sweet potato, sporamin, plant transformation, amino acid content

Abstract: In 1993, the work aimed at obtaining genetically transformed variants of sweet potato sporamin, was continued. Synthetic DNA fragment insertion was used to enrich the sweet potato nutritional value. Moreover, obtaining at least one culture transformation method has acquired great importance to facilitate the accomplishment of the final goal. In the second year of this project the results obtained were satisfactory and in accordance with the expectations lined in this years objectives.

Reporter genes were utilized to set up transformation methods, with the use of *A. tumefaciens*, for two sweet potato commercial varieties, besides, with the employment of previously obtained clones containing the synthetic DNA fragment, encoding for limiting amino acids in sporamin, different cloning strategies for such fragment in different sporamin gene sites, were designed and performed. The constructions obtained were tested by molecular methods.

Background: Sporamin is the major storage protein in tuberous roots with 60-80% of total soluble protein, which has an apparent molecular weight of 25,000 daltons (1). Sporamine is considered a kind of storage protein, and during the sprouting of the next generation it is specifically degraded (1,2), but it may have protease inhibitor activity, considering also the homology between amino acid sequence of sporamin and Kunitz type trypsin inhibitors (3,4). Sporamin is encoded by a multigene family and it is possible to distinguish two subfamily codings for sporamin A and B. Both types of proteins are immunologically very similar (3). Isoelectric points for sporamin A and B are 5.1 and 5.2 respectively (5). The Northern blot test demonstrates the tissue specific expression of these genes detecting sporamin-mRNA in tubers but not in other organs (5). The improvement of the storage protein nutritional value is an important target in plant genetic engineering (6,7,8). One important aspect in this kind of work is the choice of insertion site in the storage protein gene, because a drastic change in the special structure could provoke low protein yields or protein degradation (9). Different versions of the modified sporamin were analyzed from nucleotide secondary sequence structures and compared with the natural protein. The most important version of synthetic fragment insertion was determined. The modifications of the sporamin gene consist in the deletion of 84 b FokI - MluI fragment by substitution of 60 b SalI (SI blunted) - NruI fragment by SI blunted 300 bp synthetic fragment, encoded for essential amino acids. The synthetic fragment was prepared according to the established methodology (10).

Objectives: For the second year, the two main objectives proposed were:

1) Construction of required plasmids expressing modified sporamin in *E. coli*.

2) Establishment of transformation methodology by *A. tumefaciens*. Obtainment of km resistant plants.

Work Progress: Following the strategies designed from previously obtained clones carrying high content essential amino acids in the synthetic fragment, as well as sporamin structural gene carrier-clones, different transformed variants were obtained. Such fragment was totally, or partially inserted in the variants, in different positions which as to structural predictions won't affect the main protein features. All this work was performed with the use of restriction enzymes and prefixed cloning sites. The three variants chosen were tested through restriction and also sequenced. Their expression in *E. coli*, though did not reach high levels, was proven by means of Western-blot, utilizing sporamin specific antibodies. The difference in size, as regards with native sporamine, supported our expectations in relation with the transformation made. Both sequencing and expression results confirmed the effectiveness of the variants used.

According to our tissue regeneration works on sweet potato varieties existing in our germoplasm collection, a new method for the variety Jewel was set up, using MS basal medium, and combination of BAP and NAA hormones. For the variety CEMSA 78-354, which occurs over 50% of sweet potato cultivated lands in Cuba, it was necessary to apply hormone combinations such as 2,4D and Zeatin, because it is very recalcitrant variety both against organogenesis and embriogenesis. In both varieties, the optimum time for explant cocultivations with *A. tumefaciens* was 20 hours in darkness at 28 Celsius degrees. The gene used as reporter in the transformation of both varieties was NPTII; therefore, the selection of apparently transformed clones was made through Km resistance at different levels in the culture, higher than the natural resistance shown in previous experiments. Following the selection of Km resistant clones, the confirmation of their transgenic character was carried out using PCR in order to amplify the fragment corresponding to the NPTII gene and Southern-blot, to detect the same incorporated genetic information. The results of both procedures proved the effectiveness of the transformation methods used.

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Publications:

Cloning of three different constructions containing modified sporamin in *E. coli*. Abstract Book of the 3rd International Conference on Plant Biotechnology. Santa Clara, Cuba, June 1993.

Regeneration of plants through organogenesis and somatic embriogenesis in two sweet potato varieties. Abstract Book of the 3rd International Conference on Plant Biotechnology. Santa Clara, Cuba, June 1995.

MECHANISM OF CELL TRANSFORMATION BY HPV-16

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ICGEB Reference No. CRP CUB91/04

Keywords: IFN (interferon), ISRE (interferon stimulated response element), HPV (human papilloma virus), ISG (interferon stimulated genes), ISGF (interferon stimulated gene factor), IRF-1 (interferon regulatory factor 1), LCR (long control region), RSV (Rous Sarcoma Virus), LTR (Long Terminal Repeat)

Abstract: Concerning the probable connection between HPV biology and the IFN system, we have previously reported a partial phenotype reversion of HeLa cells by long-term treatment with alpha IFNs in association to the down-regulation of HPV-18 mRNA (J. IFN Res. 13, 369-375, 1993). The purpose of the project is to identify more interaction targets between gene products from Human Papillomavirus (HPVs) with host genes involved in cell-growth control as a potential mechanism of malignant transformation displayed by these DNA viruses. Also we are investigating how does IFN alpha could repress the high risk HPV expression using different *in vitro* cell models. In short-term studies, we observed that IFN alpha 2b reduced the HPV-18 mRNA on HeLa cells in a time-dependent way but this inhibitory effect was differential for both viral transcripts and it was maximal after 48 hours of incubation. 3.4 Kb mRNA was down-regulated about 50% compared to 1.6 Kb mRNA which was reduced about 80%. Simultaneously, the 2-5 Oligo A synthetase gene was clearly induced as cellular response marker to IFN action. The experiments using protein synthesis inhibitors suggested that the ongoing protein synthesis is required for the HPV-18 mRNA inhibition produced by IFN after 48 hours of treatment. Experiments better controlled for determining whether IFN reduces HPV-18 mRNA either by interaction with the LCR or by stimulation of transcripts degradation are currently underway. Otherwise, HPV-16 on CaSki cells remained to be resistant to IFN treatment. However, the 2-5 A synthetase gene was induced after 8 hours and mRNA levels were further maintained until 72 hours thus indicating that lacking of IFN response over the HPV-16 transcription was not due to neither the absence of type I IFN receptors nor a deficient IFN signal transduction pathway. We have also observed a reduction of HPV-16 mRNA by *in vivo* administration of IFN alpha 2b in biopsies taken from invasive carcinoma. We have also performed transfections of Hep-2 cells (a laryngeal adenocarcinoma cell line very sensitive to the antiviral activity of IFN) and RHEK-1 cells (human keratynocytes) with constructs we cloned containing HPV-16 E6 or E7 along with neo gene in tandem driven by RSV promoter-enhancer. Concerning the transfection with E6, we isolated two clones from Hep-2 and RHEK-1 cells which are resistant to 800 ug/ml of G418. On E7 transfection three different clones were obtained from RHEK-1 and one clone from Hep-2 cells using the same antibiotic dose. The biological characterization from respective clones in underway.

Simultaneously, on transient transfection experiments using RHEK-1 cells we have observed that E6 and E7 interferes with the inducibility of beta IFN promoter by classic inducers like Sendai virus when both constructs were co-transfected. These results were similarly seen when we used a construct containing the binding site of IRF-1 taken from the guanylate-binding protein promoter, fused to CAT and also driven by HIV truncated LTR. Similarly E7 appears to block the induction of IFN of one ISRE belonging to 5-15 gene (an ISG) fused to CAT. Results obtained from transient transfections will be correlate with those ones obtained on stable transfectant.

Background: Human papillomavirus (HPVs) are useful in control mechanism studies of gene expression, because its transcription programme appears to be closely linked to specific events for the control of growth and differentiation of squamous epithelium. Particularly, HPV-16 and -18, are usually integrated into host chromosomes in a specific way that ensures that E6 ad E7 early genes are selectively retained and transcribed. IFNs are a family of polypeptide response modifiers which exhibit antiviral, antiproliferative and immunomodulating functions. Binding of IFNs to their receptors triggers the assembly of a cytoplasmic protein complex and its translocation to the nucleus, where the activated complex can promote transcription by binding to ISREs. Only one of the three well-characterized ISRE-binding factors, ISGF-3, seems to be directly responsible for the transcriptional activation of ISGs. A lack of response toward IFN has been attributed to an impaired induction of ISGs in the case of IFN-resistant variants of Daudi cells and in connection with hepatitis B virus and adenovirus infection. In these cases, it has been possible to show that the adenovirus E1A and the hepatitis virus terminal proteins, respectively, mediate the aberration of ISG induction.

Similarly, cells expressing EBNA2 for Epstein-Barr virus also exhibit a resistant phenotype to the antiproliferative effect of IFN which was mediated by an aberration of ISG induction without inactivation of ISGF3. Endogenous beta IFN and the 2-5 oligo A synthetase (an ISG) seems to play a role in the regulation of cell growth and IFN has been related to cell differentiation process and arresting in G0/G1 phase during cell cycle.

Objectives: The main objectives of the project could be summarized as follow:

1) Identification of cellular targets for HPV-16 E6 and E7 mainly among IFN system.

2) Identification of those viral gene products related to the resistance toward IFN treatment and the mechanisms involved on such phenomenon.

3) To know how does IFN could repress the high-risk HPV expression?

Work Progress: The current status of the project includes the following outcomes:

Alpha IFN elicits down-regulation of the HPV-18 mRNA: Alpha IFN efficiently inhibited the HPV-18 mRNA levels on HeLa cells after 48 hours of incubation. The inhibition rate observed for 1.6 Kb mRNA was about 80% compared to that obtained for 3.4 Kb which was only about 50% when 40,200 and 1000 IU/ml were tested. The effect was time-dependent and after 72 hours was not further maintained.

HPV-18 mRNA inhibition by alpha IFN depends on the ongoing protein synthesis: Results clearly indicated that the HPV-18 mRNA inhibition observed after 48 hours of IFN treatment was completely abrogated when the protein synthesis inhibitor was included in the incubation. The results also suggest that any factor induced by IFN during the first five hours is likely involved on such regulatory effect.

Studies of IFN effect at the HPV-18 LCR level and over the stability of viral transcripts: We are now performing transient transfection experiments of RHEK-1 cells with constructs containing HPV-18 LCR fused to CAT gene and other containing Lac Z driven by Cytomegalovirus promoter enhancer in order to normalize the transfection efficiency. Experiments of HPV-18 mRNA stability will be normalize using a p53 cDNA probe because this gene is not modified by IFN at the transcriptional level but rather post-translational.

IFN alpha down-regulates the HPV-16 mRNA levels *in vivo*: Preliminary results indicated that intramuscular and topic IFN administration during three weeks reduced the HPV-16 mRNA levels from biopsies taken from three invasive cervix carcinoma patients. Simultaneously, up-regulation of the 2-5 A synthetase and p68 mRNA levels by IFN treatment was also evident in patients who did not receive IFN administration the effect was not observed neither on the HPV expression nor on the ISG induction.

HPV-16 E6 and E7 interfere the inducibility of beta IFN promoter: On transient transfection in RHEK-1 cells both viral proteins in different extent reduced the induction of beta IFN promoter by Sendai virus. In order to look whether IRF-1 which is involved on the activation of this promoter by its inducers, was affected by viral gene products, we co-transfected a construct containing the IRF-1 binding site upstream to the HIV truncated LTR and further fused to CAT along with others expressing E6 or E7 under RSV enhancer. We again observed similar results suggesting that this transcriptional factor could be a potential target for HPV-16 major transforming proteins.

HPV-16 E7 interferes with the induction of ISREs: On transient transfections on RHEK-1 we have also observed that the construction described above containing E7 is capable to block the induction by IFN of one ISRE fused to CAT in a dose dependent manner.

Networking: Some of the genetic constructions we used in our experiences were obtained from the Virology Department at the ICGEB. It includes those ones related to HPV-16 LCR fused to CAT gene and others which express E6 and E7 from HPV-16 (pJ4-E6 and pJ4-E7 respectively). HPV-18 LCR-CAT constructs were kindly provided by Mathias DÜrst (Germany). Some experiments related to the resistance of HPV toward IFN treatment were performed by Dr. Omar Lopez from our group in collaboration with Alex Von Gabain's group at the Bacteriology Department in the Karolinska Institute (Stockholm, Sweden).

GENERATION OF THERAPEUTIC ANTIBODIES AGAINST ENDOTHELIAL ADHESION MOLECULES

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UNIDO Contract No. 93/093

ICGEB Reference No. CRP CUB92-03

Keywords: acute inflammation, endothelial cell adhesion molecules, therapeutic monoclonal antibodies

Abstract: The project deals with the generation of mouse monoclonal antibodies against endothelial cell adhesion molecules, and their modification by genetic engineering techniques, the latter to increase their efficacy as therapeutic molecules. The monoclonal and recombinant antibodies will be assayed for their usefulness in blocking the adhesion of monocytes and polymorphonuclear leukocytes to endothelium in inflammatory sites.

Background: Adhesion of leukocytes to the endothelium is one of the first steps of the inflammatory response. Experimental data indicate that inhibition of leukocyte adhesion to endothelial cells (EC) could be a valuable therapeutic procedure for a variety of pathologies including respiratory diseases, septic shock and myocardial ischemia. Leukocyte adhesion is mediated by specific molecules expressed by the EC after activation with inflammatory cytokines, and can be inhibited by molecules interacting with either ligands or receptors expressed by the leukocytes and the EC. Monoclonal antibodies have been experimentally shown to be useful for such purposes. Antibody fragments, produced either biochemically or by recombinant DNA technology in bacteria, could also be potentially employed for such goals.

Objectives:

1) To generate a panel of mouse monoclonal antibodies capable of blocking *in vitro* the adhesion of human PMN cells to endotoxin-activated human endothelial cells.

2) To characterize the molecules recognized by the monoclonal antibodies.

3) To modify such monoclonal antibodies by genetic engineering techniques, either by 'chimerization' techniques, or through the development of single-chain antibody Fv fragments in bacteria, in order to increase their efficacy and safety characteristics.

Work Progress: The following results have been obtained:

1) The human melanoma line MEL-2/14, expressing endothelial cell adhesion molecules after activation with Tumor Necrosis Factor, was acquired.

2) The production of primary cultures of umbilical cord human endothelial cells was standardized, as well as the procedures for their activation with LPS.

3) An *in vitro* assay to measure the adhesion of PMN cells to activated endothelium was developed.

4) Using mice immunized with melanoma cells, we have generated eight hybridomas producing antibodies that block the adhesion of human PMN cells to LPS-activated endothelium.

Networking: Dr. Elisabetta Dejana, Vascular Biology Laboratory, Mario Negri Institute for Pharmacological Research, Milan, Italy

GREECE

STUDY OF MEMBRANE MOLECULES OF *LEISHMANIA*

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UNIDO Contract No. 92/265

ICGEB Reference No. CRP GRE92-04

Keywords: *Leishmania*, gp63, *Leishmania* transferrin receptor

Abstract: Our long term interests have been the identification and study of *Leishmania* membrane antigens that, based on both functional and immunological criteria, would prove to be good candidates for the development of a vaccine against leishmaniasis and/or potential chemotherapeutic targets. Our work is focused on the dynamics of two *Leishmania* membrane molecules, gp63 and the transferrin receptor, good candidates for protective immunity against leishmaniasis. Moreover, since drug resistance is considered to be an obstacle to the successful treatment and cure of leishmaniasis (TDR news no. 37 Nov. 1991), the expression of gp63 in drug-resistant parasites is also investigated.

In 1993 the following activities were pursued:

1) Synthesis of gp63 of SRYD-containing peptides, modelled from the putative cell adhesion domain of gp63 binding to macrophage receptors (Soteriadou *et al.* 1992, J. Biol. Chem. 267:13980) and investigation of their potential in mediating protection against *Leishmania* infection.

2) Study of the expression of gp63 in drug-resistant parasites and correlation with *Leishmania* virulence.

3) Production of monoclonal antibodies against *Leishmania* transferrin receptor and investigation of the role of iron on *Leishmania* growth.

Background: The development of controls tools against leishmaniasis, vaccines and new drugs, appears to be an urgent need. Despite the range of clinical disease, recovery from all forms of leishmaniasis appears to be dependent on specific T lymphocyte responses. Thus, the identification of defined parasite proteins and peptides that include or elicit beneficial T cells responses may contribute to vaccine development. One widely studied parasite antigen, gp63, is reported to be a potent stimulator of human T cell responses. More recently, T cell epitopes within gp63 have been identified and shown to induce partial protection against *L. major* infection (Russell and Alexander 1988, J. Immunol. 140; Jardim *et al.* 1990, J. Exp. Med. 172; Yang *et al.* 1993, Immunology 78). To this end we aim to investigate the effectiveness of synthetic peptides, modelled from gp63 cell adhesion (SRYD - containing peptides) in inducing protective immunity *in vivo*, (Soteriadou *et al.* 1992, J. Biol. Chem.). Moreover, gp63 is considered to be a key molecule in critical steps in *Leishmania* infection and the investigation of its expression in drug-resistant promastigotes as well as the identification of factors that regulate its expression may be proved of leishmanicidal importance. In this respect we study the expression and regulation of gp63 in sinefungine-resistant *Leishmania* promastigotes. In our previous work we have identified, purified and characterize the *Leishmania* TR transferrin receptor (Voyatzaki and Soteriadou, 1990, J. Biol. Chem. 265: 22380; Voyatzaki and Soteriadou, 1992, J. Biol. Chem. 267:9112). Clearly, much more information is required before firm conclusions can be made on the importance of TR to the parasite. Nevertheless, the available experimental results and theoretical considerations make the prospect of a vaccine based on this molecule not unrealistic. Our goal is to exploit the potential of *Leishmania* TR for chemotherapy of leishmaniasis.

Objectives: The main objectives of the project are the following:

1) Test the effectiveness of gp63 SRYD-containing peptides in inducing protective immunity. Synthesize peptide analogues and identify, using anti-gp63 SRYD antibodies, peptides with increased antigenicity. Test the effectiveness of the latter peptides in inducing protective immunity *in vivo*.

2) Study the regulation of gp63 in SI-resistant parasites where an overproduction of gp63 has been identified. Determine whether the control of gp63 gene expression in SI-resistant parasites occurs at the level of mRNA translation and/or if it is

transcriptionally regulated. Correlate the overexpression of gp63 in SF- resistant parasites with leishmanial virulence *in vivo*. We will also study gp63 expression in other SF-resistant *Leishmania* species.

3) Investigate the role of iron on the growth of *Leishmania* promastigotes and in experimental infection of mice. Produce mAbs to *Leishmania* TR and screen for mAbs that block its function. Study transferrin-mediated uptake and release of iron by *Leishmania* promastigotes at the molecular and ultrastructural level. Investigate the role of iron in experimental infection of mice.

Work Progress:

1) Test the effectiveness of gp63 SRYD-containing peptides in inducting protective immunity.

In the first series of experiments three different SRYD-containing produced in large quantities in soluble form by our collaborator Prof. C. Sakarellos, were used for immunizing mice. The two peptides, corresponding to residues 150-157 (IASRYDQL) and 150-164 (IASRYDQLVTRVVTH) contained the SRYD-adhesion site of gp63. The third peptide, a chimeric one (IASRYDQLQTHVKLFNRINK) corresponded to residues 150-157 of gp63 and residues 88-891 (QTHVKLFNRINK) of the sequence of the tetanus toxoid protein. This sequence is considered as a universal Th-cell epitope (Etlinger and Knorr 1991, Vacc. 9). Lymph node cells from immunized mice tested from proliferative responses *in vitro* against graded concentrations of various peptide preparations showed a very moderate response. Experiments conducted *in vivo* for induction of protective immunity to experimental challenge infection gave discouraging results. A series of experiments using other SRYD-containing peptides is now in progress.

2) Expression of gp63 in Sinefungin resistant parasites and correlation of the overexpression of gp63 in resistant parasites with *Leishmania* virulence.

We have surveyed gp63 expression in sinefungin (SF) resistant and wildtype *Leishmania* promastigotes. We demonstrated an overexpression of gp63 protein in SF-resistant *Leishmania* promastigotes versus wildtype respectively. Northern blot analysis showed that the increase in the amount of gp63 protein in SF-resistant parasites was concominant with an increase in gp63 mRNA. Treatment of SF-resistant and wildtype *L. donovani* promastigotes with cycloheximide resulted in an increase of the steady state levels of gp63 mRNA in the SF-resistant parasites to approximately 5 fold of the wildtype. After treating parasites with actinomycin D, estimated gp63 mRNA t1/2 in the wildtype was 40 min and increased up to 83 min in SF-resistant promastigotes. Therefore, the overexpression of gp63 may be mediated at least in part by a post-transcriptional stabilization of gp63 transcript by a protein factor. Down regulation of the latter factor may account for the observed increase in gp63 expression in SF-resistant promastigotes. Attempts to correlate gp63 expression with promastigote virulence suggested that the observed increase in gp63 expression did not result in a significant change in the virulence of SF-resistant versus wildtype *L. donovani* promastigotes (Soteriadou *et al.* Eur. J. Biochem. Accepted for publication).

3) Biochemical, immunological and functional studies on *Leishmania* transferrin receptor (TR).

a. Investigation of the role of iron on the *in vitro* growth of *Leishmania* promastigotes. The presence of TR on *Leishmania* promastigotes suggests iron requirements in the life cycle of the parasite provided by transferrin. We thus sought to examine whether an iron deprivation strategy may be used against *Leishmania*. To this end, the effect of iron chelators (DFO and hydroxypyridones L1 and CP94) on the *in vitro* growth was monitored. All three chelators used reduced the rate of promastigote replication in a dose dependent fashion whereas referrated DFO, L1 or CP94 did not. 150μM, 75μM and 50μM of DFO, L1 and CP94 respectively inhibited by 50% parasite growth clearly indicating the hydroxypyridones L1 and CP94 are more efficient than DFO. The growth of parasites, cultured in medium containing 125μM L1, was completely or partially restored when FeNTA in the culture, respectively. Binding assays using ^{125}I-transferrin performed on promastigotes cultured in the absence or presence of DFO. Showed that iron depletion from the culture medium results in an increase of almost 40% of TR expression compared to promastigotes cultured in the absence of DFO. In conclusion, we suggest that iron may play a crucial role in *Leishmania* infection. Submitted for publication.

b. Production of mAbs to *Leishmania* TR. Briefly spleen cells of BALB/c mice immunized with isolated *Leishmania* TR were fused with S194 mouse myeloma cells. Four stable hybridomas, producing mAbs to purified TR were produced by the direct cloning method. The characterization of these antibodies is in progress. The above antibodies will be useful tools for functional and molecular studies.

Publications:

Soteriadou, K.P., Tzinia, A.K., Mamlaki, A., Phelouzat, M. Lawrence, F. and Roberto-Giero, M. Expression of the major surface glycoprotein of *Leishmania*, gp63, in wildtype and sinefungin resistant promastigotes. (Eur. J. Biochem. In press).

Papavassiliou, P., Voyiatzaki, C. Boelaert, J. and Soteriadou, K.P. Effect of iron depletion on the *in vitro* growth of *Leishmania*, promastigotes. (Submitted for publication).

Networking: The proposed project involves the synthesis of synthetic peptides. Concerning this part of the work a fruitful collaboration, that has resulted in a publication (Soteriadou *et al.*, J. Biol. Chem. 267: 13980), has already been initiated with the group of Prof., C. Sakarellos (Department of Chemistry, University of Ioannina, Greece). The study of the TR-mediated endocytosis of transferrin and the uptake of Fe in *Leishmania* at the ultrastructural level necessities electron microscopy technology and expertise. To this purpose a collaboration with the laboratory of Prof. Pierre Courtoy (International Institute of Cellular and Molecular Pathology, Cell Biology Unit, Brussels), who has a long lasting expertise in the study of endocytosis, has recently been initiated. In the context, Dr. Pericles Papavassiliou, visited during 1993 Prof. P. Courtoy's laboratory. The work on drug-resistant parasites is carried out in collaboration with Dr. Malka Roberto-Giero, Institut de Chimie des Substances Naturelles CNRS Gif-Sur-Yvette France.

HUNGARY

CHARACTERIZATION OF DNA BINDING PROTEINS INVOLVED IN THE REGULATED EXPRESSION OF A WHEAT CHLOROPHYLL A/B BINDING PROTEIN GENE

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Keywords: trans-acting factors, wheat *Cab* gene, regulated gene expression

Abstract: The expression of the wheat Cab-1 gene encoding the major chlorophyll a/b binding protein is light regulated and tissue specific, it is mediated by and further modulated by a circadian clock. The regulation is exerted at the transcription level and by 5' deletion mutant analysis we defined several cis-acting regulatory sequences involved in this complex expression pattern. The analysis of *in vitro* constructed chimaeric genes in transgenic plants allowed us to localize the cis-regulatory sequences for leaf-specific expression, for light-induced expression and for circadian clock regulated expression.

1) We have recently defined the exact position and nucleotide sequence of the cis-acting elements by *in vitro* and footprinting;

2) We characterized by gel retardation and mutation analysis those DNA-binding proteins that specifically bind to the Cab-1 promoter regions thus defines;

3) We are presently engaged in isolating genes encoding the above described DNA-binding proteins (transcription factors);

4) In the near future we intend to determine the expression pattern of the genes encoding these various trans-acting proteins and to characterize them *in vivo* under various physiological conditions (light vs dark, leaf vs root etc.).

These studies will help to understand the molecular mechanism of nuclear gene transcription in higher plants and will provide information about the terminal steps of various signal transduction

chains affecting the expression of the Cab-1 gene. Judicious overexpression or inhibition of the genes encoding the trans-acting proteins involved can lead to the formation of novel regulatory circuits for gene expression in higher plants.

Background: In our research proposal we set the aim of gaining information about the light induced signal transduction chains leading to the transcriptional activation of the Cab-1 gene. One possible way to start such studies is to identify cis-regulatory sequences which are necessary for regulated expression and subsequently to identify those transcription factors which specifically bind to the cis regulatory sequences of the target promoter. Transcriptional activators and repressors have been extensively studied in other eukaryotic systems. However in higher plants this line of research has just been started in the past few years. Regulatory proteins interacting specifically with the promoters of plant viral or nuclear genes have been described. For example, both rbcS and Cab promoters of dicot plants have been shown to bind several different proteins (GT-1, GBF-1, AT-1) in a sequence-specific manner. The precise role of these factors in the regulation of these promoters is not fully understood, although it has been shown that mutation of the binding sites for GT-1 and GBF-1 transcription factors results in loss of expression.

We showed that the wheat Cab-1 promoter, similarly to the tobacco CabE promoter, contains several well-defined cis-regulatory sequence elements, which predicts that various DNA-binding proteins will interact specifically with these sequences *in vivo*. In addition, our data obtained so far by analyzing transgenic plants clearly show that the important cis-regulatory sequences of monocot (wheat) and dicot (tobacco) Cab promoters do not share significant nucleotide sequence homology, yet the wheat Cab-1 gene is faithfully expressed in transgenic tobacco plants, indicating a common regulatory mechanism in diverse plants.

We intent to isolate some of the transcription factors involved in the regulation of the wheat Cab-1 gene. We assume that the information obtained will help to determine the structure and function of transcription regulation in higher plants.

Objectives: We proposed to:

1) define the exact position and nucleotide sequence of the cis-acting elements of the wheat Cab-1 promoter;

2) isolate and characterize genes encoding DNA-binding proteins that specifically bind to the above mentioned Cab-1 promoter regions;

3) determine the expression pattern of the genes encoding these various DNA-binding (trans-acting) proteins;

4) characterize these trans-acting regulatory proteins *in vitro* under various physiological conditions (light vs. dark, leaf vs. root etc.)

Work Progress:

1) Cis- and trans-acting regulatory elements of the wheat Cab-1 promoter.

In our last interim report we described in detail our progress made in isolating cis- and trans-acting regulatory elements for the circadian clock responsive expression of the Cab-1 gene. During the last year we finished the characterization of cis-acting elements and submitted manuscripts (Szell *et al.*, Dallmann *et al.*) discussing these data. Progress has also been made in the identification and characterization of trans-acting factors interacting with the cis-regulatory elements (primarily with the sequence motifs Cab-E and Cab-D). We isolated and sequences several cDNA clones encoding putative DNA-binding proteins. Our major effort at present is aimed at identifying the biological function of these regulatory proteins *in vivo* using transgenic plants and microinjection.

2) The interaction between the circadian clock and the photoreceptor phytochrome

We found that the circadian clock itself is regulated by phytochrome, the major red far-red absorbing plant photoreceptor. Furthermore, we provided evidence that the circadian clock exhibits an extreme light sensitivity, therefore it should be regulated by the very low fluence (VLF) response of phytochrome (Nagy *et al.*, 1993, PNAS 90, 6290-6294).

To further characterize the interaction between the circadian clock and phytochrome, we isolated and studied the expression patterns of two tobacco genes encoding type A phytochrome (Adam *et al.* 1993, Plant Physiol. 101, 1407-1408). We showed that the expression of the *phy*A1 and *phy*A2 genes is not regulated by the circadian clock (Adam *et al.*, submitted to Plant Journal).

Finally, we obtained evidence that in addition to light, the circadian clock is also regulated by a developmental programme. We showed that this light independent regulation can be readily demonstrated in young dark-grown seedlings. We also proved that distinct cis-regulatory promoter elements are involved in mediating the light-dependent and the light-independent, circadian clock modulated expression of the *Cab-1* gene (Kolar *et al.*, submitted to PNAS). The analysis of the latter regulatory element is currently being undertaken.

In a separate series of experiments we studied the expression of nuclear genes in the *albostrians* mutant of barley. We demonstrated that *Cab* mRNA was transcribed at a low level in the mutant cells containing ribosome-deficient, white plastids; however, *Cab* transcription still retained its light-inducible, circadian clock modulated character. We showed that the chloroplast seems to be the origin of a signal chain involved in the regulation of the expression of *Cab* and other nuclear genes (hence the low level of expression in the mutant); however, functional plastid protein biosynthesis and photosynthesis are not prerequisites for the circadian regulation of *Cab* transcription (Hess *et al.*, Mol. Gen. Genet., in press).

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CELL CYCLE CONTROL AND EMBRYOGENESIS IN SOMATIC CELLS OF HIGHER PLANTS

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Keywords: alfalfa, Medicago, cell synchronization, p34 kinase, cyclin, cDNA, Northern analysis, p13 affinity binding, auxin shock

Abstract: The previously established embryogenic culture system and production of cDNA library from auxin-activated cells allowed us to identify several hormone responsive cDNA clones. The MsPRP cDNA corresponds to a proline rich protein that belongs to a group of cell-wall proteins with new amino acid repeat motif. The mRNA (650 bp) was rapidly accumulated after auxin shock. This plant gene failed to express in somatic embryos or plant organs. We also identified an other auxin-activated gene, encoding for a protein with Ca binding domain (MsCa). The transcripts from this gene are detectable in early globular embryos. As continuation of studies on alfalfa cell cycle genes, we have cloned new variants of cdc2 genes, furthermore, we provided evidences for existence of cyclin gene showed expression during G2/M phase. By production of promoter-gene fusion and transgenic plants, we have proved that a short (284 bp) sequence region from the alfalfa H3 histone gene was sufficient to provide S phase specific expression. These studies also indicated the possible involvement of the 3' end region of histone H3 genes in mRNA stability.

Background: The basic features of the embryogenic tissue culture system were described in our previous progress report. In the field of studies on somatic embryogenesis, limited publication activity could be recognized in the literature during the last year. In contrast, a very competitive situation has been generated in

identification of cell cycle control genes in higher plants. The present project could contribute to the start of this new field in plant molecular biology. The cloning of several cell cycle control genes from alfalfa opened the potentials to generate further molecular tools to analyze this very basic cellular function. Use of the antibodies against p34 -kinase and cyclin variants is an essential prerequisite to proceed with this project. We can see the first attempts to express cell division genes in transgenic plant in order to gain information about the functional significance of the key regulatory elements.

Objectives:

1) Identification of new auxin responsive genes (MsPrp, MsCa);

2) Cloning and sequencing of *cdc2* and cyclin gene variants from alfalfa cDNA library;

3) Detailed expression analyses by Northern blot hybridization;

4) Production of genomic library from DNA of *Medicago sativa* var. RA;

5) Synthesis of specific peptide and immunization in rabbits;

6) Production of transgenic plants with promoter element from histone H3 gene.

Work Progress: The preliminary data about expression of new genes as MsPRP and MsCa are published in our previous review article (Dudits *et al.*, 1992). The sequence data and results of Northern hybridization with cyclin genes are shown in publication by Hirt *et al.*, (1992). Description of *cdc2* gene variants was first presented during the European Cell Cycle Conference (La Rochelle, France). Analysis of 284 bp long promoter from histone H3 gene showed that short region of 3' end of the gene was sufficient for S-phase specific expression (Kapros *et al.*, 1993).

Publications:

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IDENTIFICATION OF MITOCHONDRIAL GENES FOR MALE STERILITY IN TOBACCO

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Keywords: cytoplasmic male sterility, mitochondrial DNA recombination, *Nicotiana*.

Abstract: The aim of the project is the identification and efficient transfer of mitochondrial genes responsible for cytoplasmic male sterility (CMS) in higher plants. Our approach could facilitate interspecific transfer of CMS without secondary effects which frequently lead to a deterioration of the cultivar, and elucidate a presently poorly explainable form of nucleus-organelle interaction. Our experimental object is tobacco, which is both a crop and a model species, and in which CMS is important in breeding but poorly analysed at the molecular level. We demonstrated that in alloplasmic male sterile tobacco plants the appearance of reduced growth characteristics were exclusively due to the presence of alien mitochondria. Repeated protoplast fusion-mediated mitochondrial DNA (mtDNA) recombinations were shown to result in the formation of stable recombined genomes. This finding, if generally applicable, should strongly increase the value of somatic cybrids for breeders. Mitochondrial DNA recombination proved to be a tool for separation of agriculturally useful and harmful forms of nucleo-cytoplasmic incompatibility (male sterility and reduced growth). Stable CMS plants were produced in which a very few, characteristic changes in the mtDNA fragment patterns can be located as putative sources of the cytoplasmic male sterility.

Background: Cytoplasmic male sterility (CMS) in higher plants is an essential tool in agriculture for large scale hybrid seed production as crop plants are generally bisexual and self-fertile. CMS is the result of a disturbance in the delicately concerted intracellular nucleus-mitochondrion cooperation, via a very poorly understood mechanism. Transfer of a cytoplasm causing CMS necessitates a lengthy back-crossing programme in the absence of biparental organelle inheritance in most crop species. CMS transfer might be further hampered from a breeder's view by a deteriorating effect on manifestations of agriculturally important traits caused by the presence of alien cytoplasmic genomes. The nature of mitochondrial gene action leading to the CMS phenotype is essentially a puzzle. Rearrangements in the mitochondrial genome supposed to be connected with CMS have been characterized especially in corn and petunia. In these cases, however, the casual relation of such changes to CMS remains to be confirmed in the absence of a transformation system for mitochondria in higher plants. The great number of mitochondria in a plant cell makes cell selection the key element in the production of a stable, uniformly modified mitochondrial genome population. In plants which are unable to obtain energy from fermentation, however, mitochondrial mutants carrying selectable markers are not available. Tobacco, a crop which is also a widely used model species of both cell and molecular genetic research, is especially suitable for an analysis of CMS and finding a solution to the above mentioned problems. In tobacco detailed analysis of mitochondrial genes or rearrangements responsible for CMS has not been performed yet. Although the connection of CMS with mtDNA has clearly been demonstrated, CMS is important in tobacco breeding in numerous developing and developed countries. In tobacco the notable scientific importance of CMS is the appearance of definite deviations in the normal flower mophogenesis indicating the existence of a set of mitochondrion coded transcription factors which controls cross-points of the organogenesis pathways.

Objectives: Analysis of the expression of characteristic CMS phenotypes in the nuclear background of different *Nicotiana* species after protoplast fusion mediated cytoplasm transfers. Investigation of stability and uniformity of the CMS phenotypes and the particular restriction fragment length polimorphisms (RFLP) of their mtDNA in sexual and somatic progenies. Selection of stable CMS lines with the least deviation from the fertile parent detected in RFLP and hybridization patterns of their mtDNA. Location of CMS specific mtDNA fragments by comparative analysis of RFLP and hybridization patterns in the particular mtDNA recombinant CMS lines. Modification of the standard DNA isolation and analysis methods to achieve high mtDNA RFLP pattern resolution in a large number of samples form small amounts of plant material.

Work Progress: We have performed experiments aimed at partial transfer of the mitochondrial genome (chondriome) in isonuclear-isoplastidic tobacco (*Nicotiana tabacum*) combinations, where cytoplasmic male sterility (CMS) marked the presence of alien chondriome in the donor (from *N. undulata*). Efficient cytoplasm transfer has been achieved by selection for a plastid marker (antibiotic resistance), an approach resulting in 98-100% cotransfer of CMS. In the first fusion experiment an alloplasmic tobacco line bearing stigmoid petaloid stamens, produced by back crosses, was used for protoplast fusion with wild type tobacco. An F1 (first fusion derived) CMS plant, possessing tobacco chloroplasts and a recombinant chondriome

intermediate between the fertile (*tabacum*) and CMS (*undulata*) types, was used in further fusion experiments. For a characterization of the chondriome, a comparison was made between the fusion-derived lines and the fusion parents using mitochondrial DNA (mtDNA) fragment patterns generated by five restriction endonucleases. After a subsequent fusion transfer (back-fusion to the fertile type), the fusion-derived F(2) plants demonstrated further mtDNA recombinations resulting in chondriomes much more similar to the fertile type (the mtDNA patterns contained less *undulata* specific and more *tabacum*-specific fragments). The chondriomes of individual fusion-derived plants were also different from each other. Most of the plants (95%) remained male sterile but showed different forms of stamen malformations (stigmoid-petaloid, petaloid-stigmoid, petaloid, arrow-headed).

A plant bearing the most frequent flower type (with petaloid-stigmoid stamens) was used in an additional back-fusion experiment. Surprisingly, the 20 randomly chosen F(3) plants, and their sexual F progeny, investigated showed no signs of further mtDNA recombination and their flower morphology was also identical to that of the donor F(2) plant. Very similar results were obtained in a taxonomically distant combination (tobacco and potato, from different subfamilies). After two rounds of mtDNA recombination (mediated by two successive cytoplasm transfers by protoplast fusion) a stable recombinant chondriome was formed, which remained unchanged after an additional back-fusion. Protoplast-derived regenerates (protoclones) of the F(1) plants from both combinations, the interspecific *Nicotiana* and *Nicotiana-Solanum*, were also investigated for the stability of their chondriomes. Extensive mtDNA recombinations detected in these lines further demonstrated the unstable nature of chondriomes of the first-fusion-derived plants. Experiments are in progress to complete molecular analyses of the chondriome recombinants.

Many of the alloplasmic plants produced by either back-crosses (as the above *Nicotiana tabacum [undulata]* line) or protoplast fusion (as *N. tabacum [plumbaginifolia]* lines in our laboratory) showed a reduced growth capacity in addition to their male sterility. For an elucidation whether the mitochondria have an exclusive role in these phenotypes or not, we used isonuclear alloplasmic plants in which the alien chloroplast were exchanged to those of the wild type by pollen-mediated plastid transfer. This sexual cybridization method is the only reliable way to produce new chloroplast-mitochondrion combinations without any change (recombination) in any of the organelles. We compared growth characteristics of the wild (fertile) type with those of the line containing wild type chloroplasts and alien mitochondria, and the line containing both alien chloroplasts and mitochondria. The results clearly revealed that both the CMS and slow-growth phenotypes were linked to the presence of alien mitochondria exclusively.

In the light of these data it was a notable observation that the F(2) and F(3) plants from the above described fusion experiments, possessing stable recombinant chondriomes, showed normal growth characteristics in a comparison with the original alloplasmic line and the F(1) plants. This result strongly indicated that the mtDNA recombination at the first and second fusion steps resulted in an adaptation of the chondriome directed by the nucleus-mitochondrion incompatibility. Stabilisation of a properly recombined chondriome, therefore, should mean the formation of a particular arrangement of parental mitochondrial genes able for normal interaction with the given nuclear genes. The stable recombinant chondriome, consequently, should be able for normal energy supply which enables the plant to grow normally. Presumably at the cell culture stage there is a selection advantage of cells which have better respiration ability, that is, properly recombined mitochondria. All the alien mitochondrial genes which are indifferent from the view point of nucleus-mitochondrion co-operation, however, probably are avoiding any counter selection at the cell culture stage, and can be stable maintained in the accommodated chondriome. These genes seem to involve the developmentally late expressed CMS genes, therefore most of the normal growing plants maintained a full male sterility. Experiments are in progress to complete data supporting the recombination mediated adaptation of mitochondrial genomes.

Publications:

Medgyesy, P., Thanh, N.D., Horváth, G.V., Rasochová, L. and Rusu, A. (1992) Nucleus-mitochondrion incompatibility drives the DNA recombination-mediated adaptation of the mitochondrial genome. NATO ASI Seminar on "Plant Morphogenesis-Molecular Approaches", Heraklion, Greece (lecture abstract).

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Networking: Dr. A. Atanassov, Institute of Genetic Engineering, Agricultural Academy, 2232 Kostinbrod 2, Bulgaria; Dr. N.D. Thanh, Institute of Biology, National Scientific Research Centre, Nghia do, Tu Liem, Hanoi, Vietnam.

STRUCTURAL AND ANTIGENIC PROPERTIES OF THE RABIES VIRUS GLYCOPROTEIN

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Keywords: DNA, cloning, sequencing, glycoprotein, rabies virus

Abstract: The primary structure of the glycoprotein gene of the rabies virus, strain Vnukovo-32, was determined.

The virus was propagated on primary hamster kidney cells or monkey kidney cell line 4647. After infection supernatants were clarified by ultrafiltration and viral particles concentrated by ultracentrifugation. Pellets were resuspended in 4 M Guanidinium thiocyanate and the viral RNA was isolated by phenol:chloroform extraction and ethanol precipitation.

The cDNA was made from RNA using 5'- and 3'- specific primers and PCR. PCR product was cloned into bacterial plasmid pUC19.

Both strands of cDNA was sequenced by the dideoxy-nucleotide termination method and DNA sequences were analysed by computer using GCG package version 7.1.

Background: Rabies virus, a member of the family Rhabdoviridae, contains a negative-stranded RNA genome encoding five structural genes. Glycoprotein G forms the 10 nm long peplomers on the external surface of the virus membrane and is responsible for the induction and binding of virus-neutralizing antibodies, for conferring immunity against lethal infection with rabies virus, and determination of virulence. To define the antigenic structure of rabies virus G protein, the nucleotide sequence of the virus-specific cDNA of several laboratory strains was determined. Recently, first structural studies on street strains were published.

In each of the strain the precursor of the rabies glycoprotein is 524 amino acids (aa) in length. The first 19 aa represent the hydrophobic signal peptide, the cleavage of which results in the production of the mature form of the glycoprotein. Next 439 aa from the N-terminus constitute the ectodomain, the following 22 hydrophobic aa -trans-membrane segment and C-terminal 44 aa represent the cytoplasmic domain.

Using neutralizing monoclonal antibodies directed against glycoprotein G several antigenic sites were localized on G protein, most of them recognized conformational epitopes. There was a great diversity in the antigenic structure of the glycoprotein among different strains of rabies virus.

The rabies virus, strain Vnukovo was propagated on primary hamster kidney cells or monkey cell line 4647. After infection supernatants were clarified by ultrafiltration and viral particles concentrated by ultracentrifugation. Pellets were resuspended in 4 M Guanidinium thiocyanate and the viral RNA was isolated by phenol/chloroform extraction and ethanol precipitation.

The cDNA was made from RNA using 5' end-specific primer 5' GGATCCAGGAAAGATGGTTCCT CAGGCTCTCCTGTTTG 3', overlapping the translation initiation (ATG) codon, and reverse transcriptase (Amersham).

The DNA was amplified using PCR (GeneAmp, Perkin Elmer Cetus) 5' and 3' specific primer 5' GCTGCAGC

AAGGGGAGGTGATCTTCAGACTTGGAT CGT - 3', containing stop-codon. PCR product was electrophoresed on 1% agarose gel and the band of expected size of double-stranded DNA was excised from the gel and cleaved with BamHI and PstI. The cleaved product was ligated with a similarly treated pUC19 vector. Clones were obtained after transformation *E. coli* DH5 bacterial cells and ampicillin selection on plates with X-gal.

Plasmid DNAs containing insert were identified by agarose gel electrophoresis after cleavage with BamHI and PstI. Both strands of cDNA were sequenced by the dideoxy-nucleotide termination method [17] with the Sequenase kit (USB, USA). DNA sequences were analysed by computer using GCG package version 7.1.

Objectives: To understand the genetic basis for the biological differences, and the antigenic diversity between the laboratory strains, we have determined the primary structure of the glycoprotein gene of an attenuated virus strain Vnukovo-32. The deduced amino acid sequence of the glycoprotein has been compared with attenuated strains HEP-Flury, SAD B19, ERA, as well as with pathogenic strains PV and CVS.

Work Progress: Our prime objective was to sequence the glycoprotein gene of the attenuated rabies virus strain Vnukovo-32.

The deduced sequence of polypeptide is identical in size and organization of most of previously characterized rabies glycoproteins. Polipeptide of 524 aa consists of a signal peptide of 19 aa, an ectodomain of 439 aa, a transmembrane domain of 22 aa and a cytoplasmic domain of 44 aa.

Sequence comparisons between different laboratory rabies virus strains indicated strong conservation along the glycoprotein G. When the nucleotide sequences of six glycoprotein G genes were compared, Vnukovo-32 strain had the greatest homology with ERA (99.4%) and with SAD B19 (99.1%).

Networking: Dr. Sandor Pongor, International Centre for Genetic Engineering and Biotechnology, Trieste - Italy. Computer analysis of the predicted structure of rabies glycoprotein.

Dr. V.I. Grabko, Laboratory of Genetic Engineering, Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino - Russia. Cloning and sequencing of glycoprotein cDNA.

Drs. V.V. Khozinski and M.A. Selimov, Institute of Poliomyelitis and Viral Encephalidides, Academy of Medical Sciences, Moscow - Russia. Purification of rabies virus, strain Vnukovo-32.

(Due to limited space the figure is not included in this Activity Report)

REGULATION OF METHIONINE SYNTHESIS IN YEAST AND POLYMET CODE INSERTED HYBRID

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Keywords: yeast, methionine, distribution polymet code DNA

Abstract: Two glucose non sensitive *C. guilliermondii* and *R. glutinis* and four glucose sensitive *Saccharomyces* strains were investigated for the effect of different glucose concentrations and aeration levels on methionine synthesis. We determined protein and methionine distribution among main protein fractions and protein subfractions from the physiologically active water soluble part.

CB 89 an authentic mutant *S. cerevisiae* strain was used as parent strain for the construction of a polymet DNA-hybrid. The synthetic DNA sequence was inserted into the polylinker region of the pVT100 U vector with 2 μ plasmid replicon. The vector-polymet insert construction was used for the transformation of CB 89 yeast, hybrids were selected and methionine contents were determined. The results showed that the polymet DNA code was active, two hybrids had higher methionine than the CB 89 strain and all had increased methionine content in comparison to the plasmid transformant.

Background. Methionine contents of yeast vary between broad ranges (0.2-1.0% on dry weight). In methionine biosynthesis carbon skeleton and sulphur incorporation are enzymatically regulated. In yeasts with higher methionine content the sensitivity against methionine, S-methylmethionine and methionine homologues is reduced. Our own investigation showed that high-methionine yeasts are influenced by aeration intensity in respect to methionine content.

In glucose sensitive yeasts metabolism switches to fermentative pathway at higher glucose concentration that involves not only the repression of several enzymes - mainly of the oxidative branch of catabolism - but also the formation carbon skeleton for further amino acid biosynthesis.

Methionine resistant yeast mutants have a higher methionine content than the wild type strains, overproduction is accumulated as free methionine in the amino acid pool (Lim *et al.*, 1990). Zn^{++} highly stimulates the intracellular production of L-methionine in mutant yeast strain by increase in the activity of homocysteine transmetylation (Tani *et al.*, 1988). Halasz *et al.* (1989) reported that methionine rich yeast mutants have increased sulfate and methyldonor requirements. Amino acid enrichment is highly efficient when poly - AA DNA is inserted into the gene.

Yeasts were grown on Petri-dishes and in batch cultures at 0.1 and 1.0% glucose concentrations and two aeration intensities 100 lh and 500 lh. In case of *S. pastorianus* and *S. cerevisiae* the effect of yeast extract was also investigated to confirm SO_4 uptake regulation.

Methionine content was determined from the whole yeast biomass and the main protein fractions (according to Osborne) after acid hydrolysis.

Protein content of whole biomass and separated fractions was evaluated by nitrogen determination.

Protein SDS-PAGE prints were prepared to characterize the subdivision of main protein fractions.

Yield values were calculated on the basis of utilized glucose and yeast biomass produced.

Methionine enrichment of yeast protein by insertion of polymet DNA vector into glucose sensitive strains:

1) A synthetic DNA sequence encoding a methionine polypeptide (oligos HPP5-Met Glu W and HPP5-Met Glu C) was inserted into the polylinker region of the pVT100 U vector with 2μ plasmid replicon.

The oligo DNA sequences were determined according to Hinnebush and Liebman, 1991 (The molecular and cellular biology of the yeast Saccharomyces. Protein synthesis in Saccharomyces: 627-735. Codon usage, pg. 632-635).

HPP5-Met3Glu2W:
5' CTAGAATGATGGAAATGGAATGATAG 3'
HPP5-Met3Glu2C:
5' GATCCTATCATTCCATTTCCATCATT 3'
To form dsDNA insert:
XbaI
5' CTAGAATGATGGAAATGGAATGATAG 3'
3' TTACTACCTTTACCTTACTATCCTAG 5'
Bam HI

2) After transformation of *E. coli* HB101 cells, the efficiency of the ligation and transformation was checked by digesting the minipreps with different restriction enzymes. The restriction digest resulted in a 26bp fragment, which corresponds to the size of the inserted oligoDNA.

3) The vector-polymet insert was used for the transformation of CB 89 yeast cells grown on minimal media (YNB) containing the selection markers (ade, leu, trp and/or his, leu, trp). The resulting 10 colonies/plate were inoculated onto uracilless minimal media. The methionine content was determined by the microbiology test using Leuconostoc mesenteroides.

Objectives: Aim of our research was to investigate the possible role of glucose effect on the methionine synthesis of *C. guilliermondii*, *R. glutinis* and four *Saccharomyces* strains (*S. pastorianus* CBS 1503, *S. cerevisiae* CBS 1395 and two *S. cerevisiae* auxotrophic mutants CB 69 and CB 89) to find out the distribution of methionine among the protein fractions and if this is influenced by change in the whole methionine content.

Separation of protein subfractions and detection of special bands which correlate with total methionine content.

Influence of glucose concentration, aeration level and yeast extract (permease regulation).

Construction of a polymet DNA which encodes 3 MET and 2 GLU. GLU was involved to decrease hydrophobicity of the peptide and rigidity of the structure. Insertion of polymet DNA

into the vector and use for transformation of CB89 auxotrophic mutant. Propagation of the transformants and determination of methionine content.

Work Progress: We determined methionine distribution between the mean protein fractions for following yeast strains: *Rhodotorula glutinis*, *Candida guilliermondii*, brewer's yeast, *Saccharomyces pastorianus*, *Saccharomyces cerevisiae* and two auxotrophic mutants *S. cerevisiae* and two auxotrophic mutants *S. cerevisiae* CB 67, *S. cerevisiae* CB 89.

Greatest part of methionine content was in all strains in the water and salt soluble protein fractions (Osborne 1 and 2) with differences in the ratio.

Only brewer's yeast shows permease regulated SO_4 uptake. All *Saccharomyces* strains were glucose sensitive in respect of yield. Protein content of yeast biomass was positively influenced by the increase of sugar concentration in case of *C. guilliermondii* 812 and CB 89 (only at high aeration rate). Increased aeration rate resulted a decrease in protein content of *R. glutinis* 803, had seriously negative effect on CB 67, CB 89, *S. cerevisiae* and did not cause any changes in *S. pastorianus*.

Methionine content of yeast was glucose dependent in *S. cerevisiae*, CB 67. In both cases increase of glucose concentration resulted in the decrease of methionine content at high aeration rate. *Candida guilliermondii* 812 showed increase of methionine content at higher glucose concentration. *S. pastorianus*, brewer's yeast were not glucose dependent in case of batch fermentation.

Changes in whole methionine content are parallel to changes in protein fractions 1 and 2 in case of brewer's yeast, *C. guilliermondii*, *R. glutinis*, *S. pastorianus* but were not correlated in case of *S. cerevisiae*, CB 67 and CB 89.

Subfractionation of water soluable protein fractions by SDS-PAGE resulted different protein prints for the investigated yeasts and specific bonds seemed to correlate with changes in methionine content.

For the construction of a yeast containing the polymet encoding DNA the following synthetic DNA was synthesized: oligos HPP5-Met Glu W and HPP5-Met Glu C which had been inserted into the polylinker region of the pVT100 U vector with 2μ plasmid replicon.

After transformation of *E. coli* HB101 cells the efficiency of the ligation and transformation was checked by digesting the minipreps. The restriction digest resulted in a 26 bp fragment which corresponds to the size of the inserted oligo DNA.

Auxotrophic *S. cerevisiae* CB 89 was transformed with vector-polymet insert and with the vector only as well. For selection hybrids were grown on uracilless minimal medium and separate colonies were propagated on plates for methionine content analysis.

Results show that insertion of the plasmid resulted a decrease in total methionine in comparison to CB 89. Polymet encoding DNA hybrids were all increased in methionine in comparison to the plasmid-hybrid and 2 of them showed elevated met levels in comparison to CB 89, the original strain.

Publications:

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MEXICO

PAPILLOMAVIRUS, CELLULAR ONCOGENES AND HUMAN CANCER

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Keywords: HPV, cancer

Abstract: Cellular and viral oncogenes are involved in human cancer. Our laboratory has been working on the mechanism associated to human cancer, in particular to uterine-cervix carcinoma of extremely high incidence in Mexican and Latin American women, and leukemia, frequent in children and young adults. In relation to leukemia, we have developed methodology to detect by PCR the ber-abl and PbX1-E2A rearrangements in acute lymphoblastic leukemia and chronic granulocytic leukemia, we think that this is important in diagnosis and prognosis. With respect to uterine-cervix carcinoma, we have found (using Southern blot) that oncogenic HPV types are present in relatively low percentage of Mexican tumors; thus, it was interesting to determine if increasing the sensibility of the detection method (by PCR for example) increase the percentage of HPV positive tumors, or if a new type of HPV, or cellular oncogenes, are involved in these carcinomas. The study of 125 invasive carcinomas by PCR indicated that about 80% of the tumors are positive for PVP-16 (50%), HPV-28 (approx. 20%), or HPV 30S (10%). The identification of an early marker for premalignant lesions (antibodies against oncoprotein E7 in patient's serum) is an important step towards the control of this carcinoma and we have continued research involving the detection of antibodies against viral oncoproteins E6, E7 and cellular oncoprotein Ha-ras. Cellular oncogenes could be responsible for certain neoplasias; we have found frequent c-myc alterations in cancerous lesions and now we investigated c-myc alterations in precancerous lesions, the presence of HPV-16 sequences integrated within c-myc gene, and the level of myc proteins in cervical lesions to determine if a correlation could be established between the observed c-myc alterations and gene overexpression. Our laboratory analyzed the mechanism by which E2 gene product of HPV repress the expression of early viral genes. We compared (in cotransfection experiments) the effect of the E2 transregulator on the activity of genital or cutaneous promoters (J. Gen. Virol. 73, 1395, 1992). Finally, we continued with the analysis of the specific binding of epithelial enriched nuclear factors to sequences present in the control region of HPV and cytokeratin genes.

In conclusion, we continued with research on the involvement of cellular and viral oncogenes in neoplasias of high incidence in Mexico. We hope that these studies will improve methods for diagnosis, prognosis and therapy of human cancer.

Background: In Mexico and Latinamerica, the incidence of uterine-cervix carcinoma represents about 30% of all malignant tumors in women, constituting one of the main causes of death. This is obviously a major health and economic problem for a large number of people. The development of improved methods for diagnosis and therapy of uterine-cervix carcinoma is extremely important. During the process of neoplastic transformation the epithelium of the uterine cervix can give rise to a variety of clinically and histologically distinct entities. Uterine cervix carcinoma has been found to be associated with one or more types of human papillomavirus (HPV). It is of great importance to understand the participation of the immune system, the type of HPV involved and of cellular oncogenes in the Mexican population if a vaccine against HPV is to be developed. In developing countries there are few Virologists and Molecular Biologists; through this project we can train several researchers in these disciplines and in modern techniques employed in genetic engineering and immunology. We are also trying to divulge this knowledge among medical doctors and clinicians both in Mexico City and in other cities of the country.

During the development of this carcinoma, both cellular oncogenes and anti-oncogenes are involved. Using Southern blot we found that only about 50% of the uterine-cervix carcinomas contained high risk HPVs. Last year we employed the PCR technique and determined that approximately 80% of the tumors had high risk HPV sequences. In addition, using Western blot we are detecting antibodies against ras (mainly in premalignant lesions) and against E7 (principally in malignant tumors). By *in situ* hybridization for mRNA, and immunohistochemistry we are detecting the expression of viral and cellular oncogenes.

In relation to the mechanism by which the E2 gene product of HPV repress the expression of E6 and E7 oncogenes we used cotransfection experiments and CAT assays. For the analysis of the specific DNA protein binding we frequently use gel shift assays and footprints.

Objectives:

1) Assimilate new technology; in particular, we recently developed the PCR technology, *in situ* hybridization (for both DNA or RNA) and immunohistochemistry.

2) Formation of human resources; we are training graduate

and undergraduate students in Molecular Oncology and Virology.

3) Determination of the molecular basis of uterine cervix carcinoma: in particular the involvement of viral and cellular oncogenes and of antioncogenes.

4) Transfer of technology to other institutions.

Work Progress: We had previously determined, using Southern blot, that oncogenic HPV types are associated to a low percentage (about 50%) of Mexican tumors. There are at least 3 possible explanations for this observation: 1) Low sensitivity of the methods utilized (Southern blot); 2) A new viral type is present in Mexican samples; 3) Cellular oncogenes or anti-oncogenes could be involved in the development of HPV-negative cervical tumors. With regard to the first possibility, we analyzed 125 uterine-carcinoma samples, using PCR and Southern blot. It can be seen that for all viral types, PCR was more sensitive (48.8%, 18.4% and 11.2%) than Southern blot (32.0%, 15.2% and 6.4% for HPV 16, 18 and 30s, respectively). Thus, by using PCR we were able to detect high risk HPV DNA in about 80% of invasive tumors. In relation to the second possibility, about a year ago we sent 20 HPV negative samples to Dr. Gerard Orth (Institute Pasteur, Paris) since he agreed in a collaboration to detect new viral types in our samples. Unfortunately, we have been very unlucky and it has not been possible so far to contact Dr. Orth. With respect to the third option, we have continued the study on oncogene and anti-oncogene alterations in uterine cervix carcinomas and cellular lines derived from them. For example, a study using 6 invasive tumors containing HPV DNA and 6 invasive tumors without viral DNA, indicated that in both cases c-myc is frequently altered. This study is in agreement with previous observations from our group (Ocadiz *et al.*, 1987). In addition, we have determined that c-myc alterations are less frequent in precancerous lesions (22%) than in cervical carcinomas (95%). In a few cases we have also observed that the Rb gene is altered in uterine cervix cancer.

In parallel with these studies we have continued with experiments related to c-myc expression. We employed Northern blot, Western blot, *in situ* hybridization for mRNA or immunohistochemistry. In some malignant or premalignant tumors we observed m-myc overexpression.

By using PCR, we have continued a molecular epidemiology study on the presence and typification of HPV in large populations (approx. 500 samples) in collaboration with the National Institute of Public Health, Cuernavaca, Morelos. We had to use the b-globin gene amplification as an internal control since many samples (cervical scrapes) contained a PCR inhibitor. This study is in progress and we still do not know if the HPV positive (or negative) samples are from clinically normal persons, high risk populations, CIN or Invasive Ca patients.

Finally, we made some progress on the collaboration with Dr. Lutz Gissman (Heidelberg, Germany) related to the presence of antibodies against HPV-16 proteins (E4, E7) and p21ras in sera from patients with cervical cancer. A higher prevalence of sera with antibodies to p21ras was observed in patients with premalignant lesions than in healthy individuals. Antibodies against HPV-16 oncoprotein E7 were detected in a higher proportion of cervical cancer patients compared to patients with CIN or normal individuals.

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Networking: As indicated in the proposal, our group has been assimilating new technology in molecular biology and genetic engineering from different laboratories (P. Chambon, Strasbourg; M. Dahmus, U.C. Davis; M. Yaniv, Pasteur Institute; T. Benjamin, Harvard; L. Gissman, Heidelberg). We recently developed the technology for *in situ* hybridisation (for both RNA and DNA), immunohistochemistry and PCR; we are now in the process of transferring it to different Institutions: a) in Mexico (National Institute of Public Health; National Cancer Institute; National Medical Center; etc.); b) in other countries (INOR, Cuba; National Cancer Institute, Rio de Janeiro, Brazil etc.).

We have trained students from hospitals, clinics and Universities in Mexico. In addition, our group trained Madely Ramirez from INOR, Cuba and Monica Pereira from the National Cancer Institute, Rio de Janeiro.

We have continued the collaboration with Pasteur Institute (N. Yaniv), Loyola University (L. Gissman), Princeton University (R. Weinman), Harvard (T. Benjamin), NIH (J. Di Paolo).

(Due to limited space the figures and tables are not included in this Activity Report)

STUDY OF PROTEASE AND ALPHA AMYLASE IN TEPARY BEANS (*Phaeolus acutifolius*) SEEDS

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ICGEB Reference No. CRP/MEX91-02

Keywords: enzyme inhibitors, plants defence mechanisms

Abstract: The study of two different proteinaceous enzyme inhibitors (protease and alpha amylase inhibitors), is on progress. They both have been isolated, purified and are being partially characterized.

In the case of the protease inhibitor, this shows a double function (usually known as double head inhibitors), recognizing not only trypsin-like enzymes, but also chymotrypsin-type enzymes. This property could be important, considering their possible role in the plant defense array of the plant.

Another unusual property consist of the formation of olygoforms, which could constitute a type of biological control of the enzymatic activity.

As for the amylase inhibitor, this protein has also been isolated and partially purified from the flour of tepary beans seeds, by ammonium sulphate fractionation, different types of chromatographic procedures, including affinity chromatography (as described in the work progress section).

The inhibitor belongs to the albumin type of protein and it is very likely by its behavior during the chromatography on concanavalin, a type of glycoprotein.

From the amylases tested so far, it only recognized that the beetle *Tribolium castaneum*

Objectives: The general objectives of this project is to find new natural proteinaceous compounds, with inhibitor activity against amylases and proteases from different sources, which could participate in the defense mechanisms of plants.

The specific objectives consists in isolating, purifying and characterizing the proteinase and the amylase inhibitors present in the seeds of Tepary beans.

Background: The development of new technologies for the introduction of foreign genes into a specific plant tissue, has increased the interest for the identification in plants of new genes which could confer resistance to the most important insect infestations. For this reason the study of proteins which participate in the defense mechanism of the plant, have become the target of different studies.

Among the different proteins related to resistance to insects and to some plant infections, are the enzyme inhibitors.

Naturally occurring proteinaceous enzyme inhibitors are found in a wide variety of plants and animal tissues. In the present project we are interested in the protease and in the amylase

inhibitors present in the seeds of tepary bean.

Some characteristics of the tepary bean (*Phaseolus acutifolius*):

Tepary bean is an annual legume adapted to the increasingly large arid and semi arid regions of the world, particularly in North America, where it has been cultivated for many centuries. Tepary beans have the characteristic of being relatively more resistance to insects and microbial predation that common bean (*P. vulgaris*) Thomas *et al* (1983); Coyne and Shuster, (1973).

Interest in these close relatives of the common beans (*Phaseolus vulgaris*) centers on both, their value as an arid land crop and as a source of stress resistant germ plasma. Tepary-common bean hybrids have been produced (Pratt, 1983) and it appears that the stable introduction of Tepary traits into common beans by hybridization is feasible (Thomas and Waines, 1980).

Some anti physiological factors such as its content of digestive enzymes and lectins have been studied (Elvira G. de Mejia, *et al.*, 1990).

Protease inhibitor: Proteins that form complexes with proteases inhibiting their proteolytic activity are widespread in nature. In addition to their roles in regulating proteolytic activities, they are also important in protecting fluids or tissues for degradation by unwanted proteolytic activities, and as storage proteins in the seeds (Neurath) (Neurath, H. 1984 Science 224:350-57).

Purification and characterization of the physical, chemical and kinetics properties of the inhibitor present in plants of the Phaseolus generous, have not been done in a comprehensive form, beside the fact that they are one of the sources of protein for the world population, specially in Latin America (Wu and Whitaker, 1990).

Alpha-amylase inhibitors: The alpha-amylase inhibitors were first reported in buckwheat by Chrzaszez and Janicki (1933). Since this first report, many other plants like maize (Blanco Labra and Iturbe-chinas, 1981), acorns (Stankovic and Markovic, 1960-61), sorghum (Kutty Pattabiraman, 1986), millet (Shivaraj and Pattabiraman, 1980), rye (Granum, 197; Manjunath *et al.*, 1983), Bajra (Chandrasekher and Pattabiraman, 1985), peanuts (Irshad and Sharma, 1981) and in many other cultivars, have also been reported.

In beans, alpha-amylase inhibitors were first reported by Bowman in 1945, and investigated again in 1968 and 1971 by Hernández *et al.*, and Joffe *et al.*, 1973. Detailed studies on bean a-amylase inhibitors have continued to increase in recent years.

Bean inhibitors are active in different extents depending on their source, against mammalian, human saliva, plant, bacterial or insect alpha-amylases. Among insect alpha-amylases affected by bean inhibitors are those from *Zabrotes subfasciatus*.

One of the characteristics of the alpha-amylase inhibitors of beans is the presence of sugars in the peptide structure. Molecular weights of alpha-amylase inhibitors range from 38,000 Da for the white kidney bean to 60,000 Da for inhibitor 1-1 from black bean. The isoelectric points vary from 4.35 to 5.0.

Work Progress: Partial progress has been obtained so far for the isolation and characterization of both inhibitors. Both have already been obtained as a partially pure protein (alpha-amylase inhibitor), and highly pure (protease inhibitor).

Proteinase Inhibitor: A protein proteinase inhibitor form flour extracts from seeds of Tepary beans (*Phaseolus acutifolius*), was isolated and purified to homogeneity by fractional precipitation with ammonium suphate, gel filtration chromatography and reverse-phase high performance liquid chromatography.

The purity of the protein was determined by re chromatography on HPLC and by gel electrophoresis. The molecular weight was determined by gel filtration chromatography for th, native protein, and by PAGE for the denatured sample. The results indicate the existence of oligomeric forms, being the trimmer the active form, with a molecular weight of 21,000 Da. The inhibitor belongs to the serin protease type and recognizes both trypsin and chymotrypsin proteases, contains no carbohydrate residues and shows a high resistance profile to high temperature.

Alpha amylase Inhibitor: An alpha-amylase inhibitor from Tepary bean (*Phaseolus acutifolius*) has been partially purified using ammonium sulfate fractionation, Sephadex G-75 gel filtration, DEAE-sepharose CL-6B, anion exchange chromatography and concanavalin-A affinity chromatography. Most of the inhibitory activity was detected in the albumin fraction of the Tepary bean proteins, which precipitated between 35 and 65% saturation with ammonium sulfate.

The inhibitor was further purified by preparative SDS-PAGE. In these analyses, the inhibitor showed an apparent molecular weight of 31,000 Da, and an isoelectric point of 4.5 assessed by isoelectric focusing.

The inhibitor is thought to the a glycoprotein by its ability to bind to the concanavalin-A in an affinity chromatography column. It was active against *Tribolium castaneum* alpha-amylases, and inactive against bacterial, mold and pancreatic procine alpha-amylases.

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Networking: We have been collaborating with Dr. Sandor Pongor from ICGEB at Trieste.

MOLECULAR STUDY OF THE PATHOGENICITY AND VIRULENCE GENES OF THE BASIDIOMYCETE *USTILAGO MAYDIS*

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ICGEB Reference No. CRP MEX91-04

Keywords: Ustilago, virulence, pathogeneicity, transformation

Abstract: The long term goal of our work is to elucidate the structure, regulation and role of genes involved in the pathogenic growth of *Ustilago maydis*. To this aim we have isolated several mutants of the mero diploid strain of *U. maydis* FB1 pub2-5 (a1, b1/b2) affected in the pathogenic process, which should facilitate the isolation of genes in it. We have also established two transformation systems for *U. maydis* both based on the resistance

to hygromycin B (Wang *et al.*, 1988; Tsukuda *et al.*, 1988).

In order to carry on the molecular genetic analysis of these genes and others, alternative more efficient transformation and selection systems will be developed. Among the possible dominant selectable markers two have already been defined, scrupper resistance conferred by the *cup1* gene of *Saccharomyces* and benomyl resistance conferred by a beta-turbulin gene of *Neurospora crassa*. Therefore, it will be possible to construct a genomic bank that could be used in complementation tests with the mutants, to clone wild type genes and to demonstrate their involvement in pathogenicity.

Background: *Ustilago maydis* is a good candidate to study plant-microbe interactions because it has well established genetics, almost all molecular biology techniques including transformation are available and it can be easily manipulated under laboratory conditions. However, little is known about the pathogenicity determinants in *Ustilago*, perhaps the only genes involved in pathogenicity that have been studied are the mating type factors (a and b). Using this information it has been established that mero-diploid strains for the b genes are pathogenic. The use of such strains for mutagenesis allows the detection of pathogenicity determinants even if they are recessive characters because except for the b locus they are haploid. It is important to mention that the mero-diploid strains have been obtained by transformation with a b gene different to that of the haploid strain (such as FB1 pub2-5) and that selection of these strains was done using hygromycin B resistance as a selectable marker.

The project consists on the development of new transformation procedures for the smut fungus *ustilago maydis* as well as the seek for alternative selection markers which could be used instead of hygromycin B. This information put together will allow us to (in the near future) introduce genetic material in strains which are already hygromycin resistant either naturally or due to previous genetic manipulations such as the strain FB1 pub2-5.

Several non-pathogenic strains of *Ustilago maydis* have been obtained by chemical mutagenesis of the mero-diploid strain FB1 pub2-5. In which the mutation does not affect the mating type.

Objectives:

1) Establishment of alternative selection systems which do not relay on hygromycin resistance.

2) Development of more efficient transformation system(s).

3) Construction of a genomic bank in a suitable vector using the alternative selection marker.

4) Complementation of non-pathogenic mutants.

Work Progress: First attempts were made to transform *Ustilago maydis* using biolistics and two plasmids previously used with success in the classical transformation procedure developed for this fungus (pHL-1 and pCM54), both conferring hygromycin resistance. Yeast like cells of the mero-diploid strain (FB1 pub2-5) were grown to logarithmic phase and aliquots taken to make a lawn of either $1 \cdot 10^7$ or $1 \cdot 10^8$ cell/50 mm Petri dish containing complete medium. Cells were bombarded using M10 Tungsten particles covered with either of the Hyg^R plasmids or in control experiments with pUC19. The helium pressure used in these experiments was 1200 psi. No transformants were recovered.

Further attempts to transform *U. maydis* using biolistics were made changing the selection strategy. In this case a double layer of complete medium was used. The lower layer contained 700 ug/ml hygromycin and the upper layer (applied just before the lawn of cells) contained no hygromycin. In these experiments bombardment was carried out just after the cells were spread on the Petri dish. The rest of the conditions were maintained as described above. In these experiments only a few transformants were obtained with plasmid pCM54. Further experiments are needed to determine the appropriate conditions of transformation and selection of transformants using biolistics in Ustilago. Recent experiments allowed us to establish a high efficiency transformation system using electroporation with which we can obtain up to 10^3 transformants per mg of DNA.

Screening with different antibiotics and/or possible growth inhibitors against *Ustilago maydis* was carried out using the mero-diploid strain (FB1 pub2-5). Among the most successful substances tested are: benomyl, copper sulfate and carboxin. Typical results obtained when using a variety of concentrations of the inhibitor against the yeast like growth phase of this strain indicate that the optimal concentrations to use are as follows: for copper sulfate 20 mM and for benomyl 2 mg/ml; for carboxin we have only tested the concentrations previously described (Keon *et al.*, 1991) and obtained very similar results to those reported in their publications. The transformation system established gives similar results using any of the selection systems proposed above.

The positive results obtained using carboxin as growth inhibitor lead us to construct a genomic bank in the vector pCBX122. For this purpose we made use of the unique BamHI site present in this vector to clone inserts ranging from 5-10 kb obtained by partial digestion of total DNA of *U. maydis* with the enzyme Sau3A1. Chromosomal DNA was obtained by the method of Raeder and Broda (1985) and partially digested with the endonuclease Sau3A1. Three different concentrations of enzyme were chosen for a large scale digestion (0.062 U/mg, 0.031 U/mg and 0.015 U/mg). The products of the partial digestion were fractioned in a Sucrose gradient and checked by agarose gel electrophoresis, and the selected fragments ligated into BamHI cut and dephosphorylated vector. Insertion of DNA fragments in this unique BamHI site does not affect the expression of the carboxin resistance gene.

REGULATION OF TRYPTOPHAN DECARBOXYLASE FROM *CATHARANTHUS ROSEUS* TRANSFORMED ROOTS

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Keywords: ajmalicine, alkaloids, catharanthine, *catharanthus roseus*, tryptophan decarboxylase, hairy roots.

Abstract: Two year old, transformed root cultures of *Catharanthus roseus* accumulate ajmalicine and catharanthine (0.57 and 0.355 mg/g DW, which resulted in a volumetric yield of 7 and 3 mg/L, respectively). Changes in the concentration of the medium components, as well as the addition of hydrolytic enzymes and biotic elicitors, were used as strategies to increase these alkaloid yields. Regarding the components of the medium, the results obtained, when sucrose was raised from 3 to 4.5% are noteworthy. The nitrogen source induced differential responses in the individual alkaloids. No net change in the alkaloid content was observed either with changes in the concentration of vitamins or macro- and micronutrients. Of all the biotic elicitors that were tested in the root culture, only *Aspergillus* treatment and the use of macerozyme increased the accumulation of ajmalicine selectively. Regarding tryptophan decarboxylase activity, it was evaluated through a culture cycle (36 d) along with variations of the tryptamine pool, as well as the accumulation of alkaloids. Ajmalicine and catharanthine contents in the tissue increased coordinately with an increase in TDC specific activity after 18 days of growth. No dramatic shifts were observed for the total alkaloid and tryptamine profiles.

Tryptophan decarboxylase (TDC) was purified from transformed root cultures of *C. roseus* following a classic scheme, which comprises the use of ammonium sulfate precipitation and ionic exchange chromatography and non-denaturing electrophoresis. The pure preparation had a molecular mass of 110 KDa, while the TDC subunit mass was 55 KDa as shown by SDS-PAGE. Antibodies were raised in New Zealand rabbits using the pure protein. The serum has a high antibody titer and specifically recognized TDC.

Background:

1) Optimization Of The Medium. The culture medium employed for transformed roots is Gamborg's B-5 at half strength, supplemented with 3% sucrose. For transformed cultures, the medium was free of growth regulators. For its optimization, the concentration of the major salts (potassium nitrate, ammonium sulfate, calcium chloride and sodium monobasic phosphate) were modified from total absence to 10-fold their usual concentration. In the case of the minor salts, such as cupric sulfate, zinc sulfate, cobalt chloride and manganese sulfate, the range was from 0 to 20-fold their usual concentrations. The same range was used for myo-inositol, pyridoxine, nicotinic acid and

thiamine. The roots were maintained in these mediums for 14 and 28 days, after which they were harvested and growth and alkaloid contents were evaluated.

2) Elicitation. In the case of fungal elicitors, four species were chosen: *Trichoderma viride*, *T. roseii*, *Rhodotorula marina* and *Aspergillus spp.* The fungi were grown in potato dextrose medium for 7 days and micelia were harvested by filtration. They were then homogenized and autoclaved for 20 min. Total soluble sugars were determined by the phenol-sulfuric acid method. Three concentrations were tested in 20-day old transformed root cultures and harvested after 72 hours of treament. Growth, total and individual alkaloid contents were evaluated.

For the elicitation studies using enzymes, pectinase, macerozyme, cellulose and quitinase were chosen. In the case of pectinase (Sigma), three concentrations were tested along a culture cycle. For macerozyme and cellulase, five concentrations were used in 20-days old cultures. Roots were harvested after 48 hours of induction. Growth, total and individual alkaloid contents were evaluated.

3) Tryptophan Decarboxylase: Determination Of Enzyme Activity And Purification. TDC activity was measured as reported by Eilert *et al.* (1987). For the purification of TDC, 50g of freeze-dried roots were homogenized in 500 ml of extraction buffer. The brei was filtered and centrifuged. The supernatant was fractionated using $(NH_4)_2SO_4$ 40-60%. The precipitate was dissolved in running buffer and loaded to a permeation column (Sephadex G-200). Fractions with TDC activity were pooled, concentrated by ultrafiltration to a minimum volume and loaded to an ion exchange column (DEAE Sephadex). TDC positive fractions were then loaded to another permeation column and the desalted preparation was applied to a native preparative gel. The gel was stained and the TDC band was cut and electroeluted. The purified enzyme was aliquoted and kept under liquid nitrogen.

4) TDC Antibodies. Two New Zealand rabbits were used to produce the TDC antibodies. The first immunization consisted of 250 µg of pure TDC mixed with complete Freund's adjuvant. A booster injection was applied 15 days later, using the same amount of TDC plus incomplete Freund's adjuvant. To test the specificity of the antibodies, Western blots were performed using positive and negative controls with immune and pre-immune sera.

Objectives:

1) Evaluation of the activity of TDC among the culture cycle of transformed roots of *C. roseus*.

2) Purification and characterization of TDC from transformed roots.

3) Development of specific antibodies versus TDC from these roots.

4) Production of TDC cDNA clone from these roots.

5) Development of the optimal concentration, both for growth and for the production of indole alkaloids, of each one of the components of the culture medium.

6) Determination of the effect of fungal and enzymatic elicitors on the synthesis of alkaloids and on the activity of TDC.

Work Progress: Medium composition, biotic elicitors and hydrolytic enzymes were evaluated with respect of biomass yield and productivity. The ajmalicine content reached 0.832 mg/m DW when the sucrose concentration was increased from 2 to 4.5%, while the amount of catharanthine stayed the same. An increase in the concentration of nitrate, in the absence of ammonium, caused an increase of 30% in the accumulation of ajmalicine content. There was no net change in the alkaloid content of the system following a treatment with vitamins and micronutrients.

In general, the biotic elicitors were effective, although the extent to which elicitation occurred differed from elicitor to elicitor. The addition of 0.005% of macerozyme increased the accumulation of ajmalicine 1.8 times in the tissues, while the release increased 34 times with 0.5% macerozyme. After 72 hours of macerozyme treatment, catharanthine increased 67% in the tissue. The fungal treatment with *Aspergillus spp.* (06 mg/glucose/ml) increased the ajmalicine content by 90% and the yield increased 65%.

The time course of TDC activity along the culture cycle that there is a peak of maximal activity between days 21 and 24 (63.7 nmoles min^{-1} mg protein^{-1}). The rest of the cycle, this activity remained between 20 and 30 nmoles min^{-1} mg protein^{-1}. The total alkaloids accumulation occurred in two different periods, one at the beginning of the lag phase and the second one during the stationary phase.

Tryptamine content in the transformed roots remained stable during the first 12 days of the culture cycle and just after day 24, at the end of the peak of maximal TDC activity, there was a moderate increase in the pool.

Both catharanthine and ajmalicine showed similar accumulation behaviors, i.e. after a decrease in their contents during the first days of the cycle, they showed a steady increase from day 15 onwards.

Only one peak of TDC activity was detected after solution through the ion exchange column, which might indicate that in transformed roots of *C. roseus* there is only one isoenzyme of TDC. The pure preparation showed an enrichment of 80-fold and showed only one band in native polyacrylamide gels strained with Coomassie. When SDS-PAGE was performed, three bands could be detected; nevertheless, the band around 55 KDa (which is the reported molecular mass for the TDC subunit) was the majoritarian one. The molecular mass calculated from the permeation chromatography for the native enzyme is 110 KDa.

The pure protein was used as antigen for the immunization of rabbits. Fifteen days after applying the booster injection, the rabbits were bled and the immune sera collected. To determine the specificity of the obtained antibodies, Western blots were run with positive and negative controls. The immune serum recognized specifically the 55 KDa band, which is the molecular mass corresponding to the TDC subunit.

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Thesis:

Moreno-Valenzuela O., Effect of biotic and abiotic elicitors on the production of indole alkaloids in transformed roots of *Catharanthus roseus*, M.Sc. thesis 1993.

Islas-Flores, I., Production of policlonal antibodies against tryptophan decarboxylase from transformed roots of *Catharanthus roseus*, M.Sc. thesis (in revision).

MOLECULAR CLONING AND CHARACTERIZATION OF GENES EXPRESSED DURING MANGO FRUIT RIPENING

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Keywords: genes, mango, fruit ripening, cDNA cloning, softening

Abstract: Ripening and postharvest physiology have constituted intense areas of study for decades, obvious interest centering on agricultural practice both in the field and in storage and transport. Fruit ripening and postharvest behaviour are of particular interest since they are amenable to experimental and agricultural interventions that influence yield, quality, timeliness of harvest and storage duration. For years, ethylene has been considered the quintessential ripening hormone and is still viewed as such although the interaction with other hormones has been proposed. To date, apart from extensive horticultural breeding research, emphasis has been on a mix of descriptive and analytical work, together with metabolic and enzymological investigations. Only of late, has it become possible to evaluate old questions concerning fruit maturation, ripening and senescence in molecular terms, particularly with respect to differential gene expression. Our work seeks to approach ripening and postharvest problems from the point of view of differential

gene expression. Genes expressing only during ripening will be isolated and analysed at the molecular level. Our model system is the mango fruit which is particularly susceptible to overripening. The information derived from such studies will provide the basis for biotechnology of fruits that will influence agricultural, storage and marketing practices alike. This project represents the first step in that direction.

Background: Fruit ripening is a complex process regulated during development. It is usually accompanied by dramatic increases in the respiratory rate of the tissue, and in ethylene production (Biale, 1960). These events are only two external manifestations of a series of internal biochemical reactions which reflect a pronounced metabolic activity. All processes during ripening are probably associated with alterations in a number of different enzyme activities and at least in some cases it has been demonstrated to be that the case (Brady, 1987). In this sense there is good evidence that the appearance of particular enzyme activities is determined by control of specific gene expression.

Fruits are normally active in RNA and protein synthesis, as has been shown by some laboratories in tomato (Rattanapanone *et al* 1977), apple (Lay-Yee *et al*, 1990) and in mango by our laboratory (López-Gómez and Gómez-Lim, 1992b). There are also specific changes in the mRNA populations during the ripening of all these fruits.

One of the main indicators of the quality of fruits is texture. This refers to the degree of softening that the fruit has reached during ripening. There is enough evidence that during tomato softening extensive degradation of the middle lamela occurs (Crookes *et al*, 1983). This structure serves as cement between several cells and therefore its dissolution is thought to cause a major change in firmness (Themmen *et al*, 1982). As the main component of this structure is pectin (Tucker and Grierson, 1987), the enzyme that has received most of the attention is pectinase or polygalacturonase (PG).

It has been suggested that PG is the enzyme responsible for fruit softening (Crookes *et al*, 1983, Themmen *et al*., 1982). Furthermore, the tomato mutants *rin*, *nor*, and *Nr* which do not soften significantly produce very little PG (Della Penna *et al*, 1987). Tomato PG has been cloned and sequenced by several groups (Della Penna *et al*, 1986, Frierson *et al*, 1986). Recently, work with transgenic plants has led to the role of PG in softening being questioned (Smith *et al*, 1988, Giovannoni *et al*, 1989). Fruits of plants engineered to express antisense PG mRNA produce only 10% of the normal level of PG during ripening (Smith *et al* 1988). Despite this reduction no difference in compressibility with control fruits was observed (Smith *et al*, 1988). When a line of tomato mutant *rin* was transformed with achimeric PG under the direction of an thylene-induced promoter, there was no significant effect on fruit compressibility in relation to control fruits when PG was induced (Giovannoni *et al*, 1989). The induction of PG in *rin* did not complement the mutation although the rin mutant may be pleiotropic. These results have suggested that PG is not the primary determinant of fruit softening.

Other cell wall-related enzymes have received some attention in the past. Tomato pectin methyl esterase (PME) has been cloned and sequenced and its expression studied during ripening (Ray *et al*, 1988). Avocado cellulase has also been cloned and sequenced (Christoffersen *et al*, 1984). In these two cases the enzyme activities are not as closely correlated with softening as the activity of PG, although cellulase does increase during avocado fruit ripening (Christoffersen *et al*, 1984). However the exact role these enzymes fulfil during fruit ripening is still controversial. Genetic transformation of tomato with the PME and cellulase in the antisense orientation have produced fruits with levels dramatically reduced of PME and cellulase activity but there was no difference in compressibility with control fruits, just as with the experiments with antisense PG (Tieman *et al*, 1992; Fray and Frierson, 1993).

Ethylene is the most important accelerator of fruit ripening (McGlasson, 1985) and therefore the enzymes related to its synthesis have been in the focus of interest of many laboratories. Several genes from various plants coding for ACC synthase have been cloned which has allowed the analysis of ACC synthase at the molecular level (Sato *et al*, 1989, Van der Straeten *et al*, 1990, Kende, 1993). This enzyme is likely to be the rate limiting step in the pathway. Furthermore the gene coding for ACC oxidase also called the ethylene forming enzyme (EFE) has also been isolated and sequenced from different plants (Hamilton *et al*, 1990, Kende, 1993). The transformation of tomato with the latter gene in antisense resulted in a reduction of up to 97% in ethylene production by the fruit (Hamilton *et al*, 1990). This delayed the softening significantly. The application of exogenous ethylene, on the other hand, restored the normal softening pattern. It is likely that the transformation of tomato with antisense ACC will have a similar result. These experiments have confirmed that ethylene production by the fruit is a major cause of overripening.

Mango fruit has been the focus of study by some laboratories in the past. There is a solid biochemical basis for this fruit. A number of enzymes activities have been reported to increase during fruit ripening (reviewed by Mattoo *et al*, 1975). Among them are PG, cellulase, invertase, sucrose phosphate synthase, phosphofructokinase and a-amylase. In addition, it has been isolated an inhibitor of some enzymes from mango (Mattoo and Modi, 1969) something never done in any other fruit. However, there is virtually no studies on the molecular biology of mango fruit ripening. We have shown in our laboratory that during ripening there are changes in gene expression, and that they can be detected in proteins sythesized both *in vivo* and *in vitro* (López-Gómez and Gómez-Lim, 1992b). The present project was proposed as continuation of this work with the isolation of ripening specific genes.

Objectives: Overall aim: isolation and analysis of genes involved in mango fruit ripening and softening.

Specific objectives:

1) construction of a cDNA library from ripe mango fruit
2) isolation of ripening-specific genes
3) study of the expression of ripening genes.

Work Progress: The first step in the project involved obtaining suitable plant material to prepare the library form. We have been obtaining the fruits from a research station in Veracruz. The station keeps a large germplasm of mango plants, besides doing research in various aspects in mango horticulture. The materials are well preserved and looked after. We have only been taking fruits from the same tree which is 14 years old. In order to asses properly the progress of ripening we decided to measure ethylene production since this is an accurate parameter to evaluate ripening (Tucker and Grierson, 1987). Nowaday ethylene determination is a simple process by gas chromatography.

We took fruits producing ethylene at maximum rate and used them to prepare poly A+ RNA. The RNA looked intact on gel electrophoresis. We extracted RNA from ripe and unripe fruit and *in vitro* translated it. The translation products clearly showed differences in the mRNA populations between the unripe and ripe fruits. Total proteins were also extracted from both types of fruit and the results were consistent with the aforementioned results. These results are in total agreement with those obtained using other fruits (Rattanapanone *et al*., 1977; Christoffersen *et al*., 1982; Callahan *et al*., 1989; Lay-yee *et al*., 1990; Gomez-Lim and Lopez Gomez, 1992b).

The library was prepared using common proveedures (Gubler and Hoffman, 1983) and the vector used lambda ZAP II, is a versatile phage vector (Short *et al*., 1988) which can be converted into a plasmid form. This allows easy handling. A section of the library was digested with Eco RI and analyzed on gel electrophoresis. The library contained inserts ranging in size from 500 to > 4000 bp. This range is very convenient for our purposes.

A preliminary screening was carried out using two heterologous probes: Apple ACC synthase and Avocado cellulase. These genes have been shown to be involved in fruit ripening (Christoffersen *et al*., 1984; Yang and Hoffman 1984; Sato and Theologis, 1989; Dong *et al*., 1991) and that is why we decided to use them. Several positives have been isolated from the primary screening. The screening with cellulase yielded similar results. Further rounds of screening are in progress with both probes.

In the original proposal, the work plan included the construction of the library during the first year at the preliminary screening. In this sense, not only have we completed the construction of the library, but also we have started the screening and obtained preliminary positives. For that reason we believe that our achievements this past year were beyond our expectation. We also have published two papers on the preliminary stages of our work. We have not published any of the results obtained this past year because the work is clearly not complete. We would

like to continue the work which will basically consist in the second year in the screening of our bank to look for as yet unidentified ripening genes as well as other known genes. We believe that we have done a successful start of our work and would like to continue it. Clearly the work needs completion. What we proposed for the second year was the isolation of ripening-specific genes and the analysis of their expression. In addition, the genes isolated will be sequenced to confirm the identification. Our future plans are as follows:

1) To sequence the clones obtained with the ACC synthase and cellulase probes to confirm the identification.

2) To continue with the screening using two approaches: differential screening and screening with other probes specific for ripening such as ACC oxidase and PG. By doing this we will be able to isolate ripening genes not previously characterized and genes with a well defined role in ripening.

3) To sequence the clones obtained. A computer search will be subsequently performed to try and identify the product they code for.

4) To perform expression studies (Northern blots) with the clones to study the time course of appearance. The organ specificity will also be looked at by Northern blots using RNA from other organs i.e. leaves.

5) To study the effect that stimuli like wounding have on the expression of the clones isolated. We will be analysing mRNA levels in Northern blots.

TUNISIA

OVEREXPRESSION AND SECRETION OF GLUCOSE ISOMERASE OF *S. VIOLACEONIGER* AND *S. OLIVOCHROMOGENES*

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Abstract: Using the promote probe vector pUT832 (Reynes *et al.*, 1989), we have succeeded in isolating some new strong promoters. We have rapidly focused our interest on the strongest one called P1. This promoter expresses phleomycine resistance more efficiently than the strong promoter of mel operon. To overexpress xylA genes the P1 promoter has been cloned behind the xylA gene of *S. violaceoniger* and *S. olivochromogenes* strains. This led to a strong and constitutive expression of these genes. To avoid instability of plasmid and glucose-isomerase activity, the P1-xylA gene of *S. violaceoniger* has been integrated into the chromosome of the latter, using the integrative vector pTS55 (Smokniva *et al.*, 1989). The resultant CBS1 strain has four to five fold higher glucose-isomerase activity in the absence o xylose compared to that of strain SV1 fully induced by xylose. In addition, glucose-isomerase specific activity of CBS1 strain, increases in the secondary growth phase, in contrast to wild type and SV1 mutant strains. The P1 promoter is carried by a fragment within 1250 pb. Determination of the sequence of this fragment has been done. Analysis of this sequence enables us to characterize potentials Open Reading Frames (ORF) truncated in their 3' ends. More analysis of this sequence with subcloning and determination of start transcription point are in progress.

On the other hand, and in order to try to secrete xylose isomerase activity, we have obtained the oligonucleotides which will be used to perform signal peptides. Cloning and verification of the sequence of these oligonucleotides are in progress.

Background: D-Xylose isomerase (XI) also known as D-glucose isomerase (GI) is a key enzyme used for production of sweet high fructose syrups from starch hydrolysates of corn, or other cereals. Because of the commercial importance of this activity, several D-xylose isolmerases have been studied, their genes cloned and sequenced. In the SWISS-PROT data base (Release 26, August 1993), there are 21 microbial xylose isomerase (xylA) genes, catalogued.

In most cases, the expression of the xylA gene is induced by xylose or derivatives, e.g. *B. subtilis*, *E. coli*, *S. typhimurium* and *Thermus aquaticus* HB8. Utilization of xylose for induction considerably increased the enzyme cost at the industrial level. This economic cost has prompted the construction of strains having constitutive xylose isomerase activity. The xylA gene of *S. violaceoniger* is also induced by xylose (Marcel *et al.*, 1987). *In vivo* this gene is involved, with others, in xylose utilization as the carbon source. The region carrying the xylose catabolism genes has been cloned (Marcel *et al.*, 1987) and the xylA gene characterized and sequenced (Drocourt *et al.*, 1988). This entire region was also independently sequenced (Bejar and Tiraby, unpublished results). The analysis of this sequence enabled characterization of the xylB gene which starts 195 bp in the opposite direction of xylA gene. A third Open Reading Frame (ORF-X) behind xylB and in the same orientation has been also identified (Tiraby *et al.*, 1989) and coincides with a regulatory region, sharing strong homology (data not shown) with the xylose operon repressor of *B. subtilis* (Kreuzer *et al.*, 1989). XI of *S. olivochromogenes* is also used in industrial scale. The latter shows a more interesting catalytic property than that of *S. violaceoniger* despite the amino-acid sequence homologies between the two proteins (Tiraby *et al.*, 1989). XI of both strains is cytoplasmic and used as immobilized mycelium at the industrial scale. Nevertheless, others are used as immobilized purified enzymes since they were naturally secreted. Besides studying many natural secreted proteins in the Streptomyces had permitted to have more information about signalpeptide which mediated secretion of these proteins. A typical Streptomyces signalpeptide has 30 amino-acids with a terminal portion carrying positively charged residues, an hydrophilic part with 8 to 20 residues and an uncharged C-terminal portion containing the cleavage site. In addition the number and the position of charged residues have been studied using the tendamistat secretion system (Sike and Engels, 1990).

Objectives: Both XI of *S. violaceoniger* and *S. olivochromogenes* are cytoplasmic and inducible by xylose. The use of xylose as inductive substrate on a large scale leads to an important increase in the enzyme production cost of enzymes. Our principal aim is to construct new strains having a strong, constructive and stable glucose isomerase activity. We are also planing, to try, to secrete this activity by coupling xylA gene with a known or synthetic signalpeptide which will be performed after compilation of some known signalpeptides. To overexpress both xylA genes, they will be cloned downstream of a new strong streptomyces promoter called P1. In order to have a strain having a stable GI activity, we will try to integrate the P1-xylA gene into the chromosome, "haphazardly", or by using the pTS55, a special integrative Streptomyces vector (Smokniva *et al.*, 1990). The promoter P1 is very interesting since it is stronger than the "reputed strong" promoter of the mel operon. This has urged us to determine the nucleotide sequence and the transcription initiation site to a better characterization of this new promoter.

This project is an extension of large subject development in the CBS which consist in the elaboration of a process for the enzymatic transformation of starch contained in wheat grits, into glucose or high fructose syrups to reduce Tunisia's sugar imports.

Besides and in general, this enables us to have a strong Streptomyces promoter and a functional signalpeptide. Therefore it would be of great importance for secreting heterologues proteins by Streptomyces and for other present or future themes developed in the CBS laboratories.

Work Progress:

1) Selection of new strong Streptomyces Promoter. In order to isolate new promoters, we have used the promoter probe vector pUT832 based on the phyleomycine resistance and SV10 as host strain. After cloning fragments with 300 to 1500 bp from partially Sau3A digested chromosomic *S. violaceoniger* DNA, we have succeeded in isolating some plasmid conferring a high level of phyleomycine resistance. This plasmid has an insert of 1250 bp carrying a promoter called P1. This promoter confers a phleomycine resistance (CMI=60mg/ml) higher that the reputed strong promoter of mel operon (CMI=40mg/ml) cloned in the same vector.

2) Construction of new strains having a strong, constitutive and stable glucose-isomerase activity.

Insertion of the xylA genes downstream of the P1 promoter: In order to test the ability of P1 promoter to express both xylA genes, they were cloned, from pre-existent plasmid, downstream of the P1 promoter in the pCBS1 plasmid. We have succeeded in obtaining two plasmids, pCBS6 for *S. violaceoniger* and pCBS8 for *S. olivochromogenes* which complement the

xylA-mutation of SV10 strain (Marcel *et al* 1987).

In addition, SV10/pCBS6 and SV10/pCBS8 strains have strong and constitutive glucose isomerase activities in the presence of a selective pressure (thiostrepton).

Integrate the P1 xylA of *S. violaceoniger* into the chromosome using the pTS55 special integrative vector: The fragment of plasmid pCBS6 harbouring P1-xylA gene was purified and ligated with linearized pTS55 vector. The ligation mixture was used to transform SV10 protoplasts, and thioR, xylose positive clones were searched. We isolated some clones that have both stable xylose positive and thiostreptionR phenotypes even after long culture without any selective pressure. One of these clones called CBS1 was used for further studies.

Stability of CBS1 strain: CBS1 strain was cultivated for more than 100 generations in liquid media lacking thiostrepton. Plating this culture on solid media both with or without thiostrepton, showed that 100% of clones were thiostrepton resistant. This situation contracts with SV10/pCBS6 strain in which the majority of clones lost both their plasmids and thiostrepton resistance after the same period of culture in the absence of selective pressure. The stability of the CBS1 strain proves that P1-xylA gene carried by pTS55 vector was integrated irreversibly into chromosome. This integration was also verified by Southern blot hybridization.

Glucose isomerase activity of CBS1 strain: The yield of intracellular GI activity restrained in mycelium of CBS1, SV1 and SV+ (Marcel *et al*., 1987) were measured after 48 hrs of culture. We have found that GI Isomerase activity of CBS1 strain is constitutive and approximately 4.5 and 7.5-fold higher than SV1 and SV+ respectively. Studying of production kinetics of GI pf CBS1 and SV1/pUT206 shows that the activity of the first strain increases regularly up to 92 hours. This contrasts with the situation encountered with SV1 and also SV+ (Tiraby *et al*., 1989). This difference could be linked to the nature of the promoter, P1 for CBS1 strain and natural promoter for SV1 and SV+ strains. This could indicate that P1 promoter is particular because it is expressed during both the primary and secondary metabolite growth phase. The possibility that there is more than one transcriptional activity in the fragment harbouring the 'P1 can not be ruled out'. Determination of the sequence of this fragment and the start transcription site should give information about this new Streptomyces promoter.

Integration of P1-gene xylA by recombinant: Construction of CBS2 strain. To avoid utilization of pTS55 which is patented, we have tried to integrate the P1-xylA gene without using this vector. The SV10/pCBS8 strain was cultivated for several generations without any selective pressure.

After plating this culture in solid media, the majority of clones had lost thiostrepton resistance. Nevertheless we succeeded in isolating very few clones which remained thiostrepton and xylose plus. One of these clones (CBS2 strain) has a xylose plus and thiostrepton stable phenotypes nearly the same as that of CBS1, in the absence of selective pressure. We believe that pCBS8 has been integrated into the chromosome. Nevertheless, after more that 100 generations of culture without selective pressure, we are able to detect a minor proportion of free plasmid in addition to the integrated one.

Determination of the sequence of the fragment carrying P1 promoter: The fragment carrying 'P1 promoter' was subcloned into M13 derivative vector. DNA sequencing was done by the dideoxy chain-termination method. Analysis of this sequence enables us to characterize potentials Open Reading Frames (ORF) truncated in their 3' extremities and situated on the same side of phleoR gene resistance in pCBS1 construction. There may be a transcriptional fusion between one of these ORF and phleoR gene in pCBS1 context or xylA genes in pCBS6 or pCBS8 context. Analysis of 5' region of the ORF did not allow us to characterize evident promoters. It is noticed that there is no consensus sequence for the Streptomyces promoter. Nevertheless, more analysis of this sequence with subcloning and a determination of the transcription starting point (in progress) will permit us to identify the(se) promoter(s).

Synthesis of signalpeptide: To synthesize the both signalpeptides (see project), 2 oligonucleotides have been performed (by Genset, France) for each of them. Each couple of oligo have 14 bases of homology. The strategy used is to hybridize the two oligo before filling the recessed 3' termini by large DNA polymerase fragment. The resulting reaction product will be cloned into M13 derivative vector and the clones having the correct sequence of signalpeptides will be selected (in progress). Besides we have succeeded in creating an NcoI restriction site into ATG (initiator) of *S. violaceoniger* xylA gene. This will permit us to couple signalpeptide to xylA gene.

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Bejar, S., Belghith, K., Gargouri, R. and Ellouz, R. Construction of new strains of Streptomyces violaceoniger, having strong, constitutive and stable glucose-isomerase activity. Submitted to publication in Applied Microbiology and Biotechnology.

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PRODUCTION OF TRANSGENIC POTATO PLANTS RESISTANT TO POTATO VIRUS Y

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Keywords: transformation, *Agrobacterium tumefaciens*, virus resistance, coat protein

Abstract: Potato virus Y (PVY) is one of the most important virus infesting potato in Tunisia and all over the world. The major aim of this project is to overcome PVY infection in potato plants by producing transgenic plants expressing the viral coat protein or an antisens ribozyme-like RNA sequence. Therefore, a cDNA corresponding to the coat protein gene of a Tunisian isolate of PVYn was synthesized and amplified in *E. coli*. Its insertion into a vector between the CaMV 35S promoter and the 3'nos polyadenylation signal is being performed. This latter vector contains also a reporter gene (GUS or bar) for selection of transgenic plants.

The second part of the project aims to express an antisens-RNA ribozyme in transgenic plants. Since the position of the GUC triplet (recognized by ribozyme for RNA cleavage) in the 5' non translated sequence of two different isolates of PVY is different from one to another, it was necessary to sequence this part of genomic RNA of Tunisian isolate. Therefore, the cDNA corresponding to the 5' non translated 200 nucleotides of PVY was synthesized and cloned into *E. coli*. The sequence of this cDNA will be determined soon for synthesis of the corresponding ribozyme.

The transformation will be mediated by *A. tumefaciens* system.

Background: PVY is the type member of the potyvirus group. Considerable efforts are being made all over the world to overcome propagation of this virus. Coat protein gene-expressing plants have been obtained from other viruses (reviewed in Beachy *et al*., 1990, Ann. Rev. Phytopath., 28, 458). Transgenic potato plants (var. Russet Burbank) expressing the coat protein gene of PVY strain o (PVYo) have been obtained (Lawson *et al*., 1989, Bio/Technol., 8, 127). The expression by transgenic plants of antisense RNA corresponding to viral sequences affords either no protection or poor protection of the transgenic plants against infection with the related virus (Powell *et al*., 1989, PNAS, 86, 6949). The introduction of an antisense ribozyme-like RNA into the plant genome is a new approach (Haseloff and Gerlach, 1988, Nature, 334, 585; 1989, Gene, 82, 43; Maddox, 1989, Nature, 342, 600). Haseloff and Gerlach (1988) have

adopted the consensus ribozyme structure to develop a sequence-specific endoribonuclease which can be tailored to cleave exogenous RNA molecules containing the GUC sequence. Lamb and Hay, 1990 (J. Gen. Virol., 71, 2257) have produced two ribozyme molecules that specifically cleave potato leafroll virus (PLRV) RNA *in vitro*.

Objectives: Two strategies of production of transgenic potato plants are envisaged in this research project. These transgenic plants have to overcome PVY infection or at least to be tolerant to virus attack. The first part of the project aims to express viral coat protein by plants. This implies the synthesis of the corresponding cDNA and its cloning in *E. coli*. The cDNA has then to be introduced into *Agrobacterium tumefaciens* for subsequent transformation of potato leaf discs.

The second part involves expression of antisens RNA ribozyme in plants. Before the synthesis of the ribozyme, it is necessary to sequence the target RNA molecule since a GUC triplet is needed for clivage. The part of the RNA to be clived in this case is the 5' untranslated region. Therefore, the nucleotide sequence of this 5' terminal region of PVY genomic RNA has to be determined for selection, synthesis and cloning of antisens RNA ribozyme. After introduction of the cDNA into A. tumefaciens, the potato leaf discs can be transformed.

Work Progress: The synthesis of PVY coat protein cDNA was performed by reverse transcription for the first strand using an oligo(dT) as primer since the PVY RNA possesses an oligo(A) at its 3' terminus. The second strand of cDNA was synthesized using the Klenow fragment of DNA polymerase and a synthetic oligonucleotide primer (45 nucleotides) which sequence corresponds to that of 5' part of coat protein gene of French isolate. Since all PVY proteins are generated by clivage of a polyprotein, this oligonucleotide contains an ATG initiation codon in frame with the coat protein ORF in addition to a consensus sequence recognized by ribosomes into plants.

The second oligonucleotide primer, possesses also at its 5' part of BamHI restriction site for subsequent cloning. The sythesized double stranded cDNA (1200 bp) treated by BamHI was inserted into pT7T3/a18 vector linearized with BamHI, and amplified in *E. coli*. The recombinant clones were screened for the size of their plasmid and then by different restriction enzyme digestions. We have tried twice to sequence this cDNA but we did not succeeded. However, the sequence determination has to be performed for this cDNA. It will be done as soon as possible.

Since the objective of these constructions is the expression of the cDNA by plant genome. The expression of the cDNA in plant cell is mediated by plant regulatory sequences. The cDNA is now being inserted in a second vector between the CaMV 35S RNA promoter and the (3'nos) polyadenylation signal of nopaline synthase gene. This latter vector contains also a reporter gene (either GUS or bar) that will be used for selection of transformed plants.

The second part of the project deals with synthesis and cloning of antisens RNA ribozyme.

The target sequence chosen among PVY RNA is the 5' untranslated region, in order to inhibit polyprotein translation.

Sequence comparison between two isolates of PVYn (French and Hungarian) shows that the location of GUC triplet required for ribozyme action is different from one isolate to another. It is thus necessary to sequence this RNA region in the Tunisian isolate of PVYn. Therefore the cDNA corresponding to the 5' terminal 200 nucleotides of PVYn was synthesized using two synthetic oligonucleotide primers kindly sythesized by the ICGEB.

The sequence of these primers corresponds to that of French isolate. Each primer contains a restriction enzyme recognition site (XbaI for primer 1 and BamHI for primer 2) for subsequent insertion in an expression vector. Once synthesized, the double-stranded cDNA was treated by BamHI and XbaI. The cDNA is then inserted into the pT7T3a18 vector treated by BamHI and XbaI. After transformation of *E. coli*, the recombinant clones were screened by the size of the plasmid they contain and then by different restriction enzyme digestions. As soon as the nucleotide sequence of these plasmid is determined, the corresponding ribozyme can be chosen.

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CLONING AND MODIFICATIONS OF CELLULASES GENES

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Keywords: cellulase, enzyme, purification, gene cloning

Abstract: In order to improve the cellulolytic capacity of our producing strains, we decided to clone the cellulase genes. Two different by complementary strategies have been developed during the first year of our project. One based on the purification of cellulolytic enzymes, partial sequencing of peptide fragments and synthesis of the corresponding oligonucleotides. These oligos can serve as well as primers in PCR experiments to amplify the cellulase genes fragments or as probes to clone the genes in a genomic or cDNA bank. The second strategy is based on the direct expression of a genomic bank in a cellulase negative host such as *E. coli*. We have applied both strategies on two cellulolytic microorganisms: the fungus *Penicillium occitanis* and the filamentous bacteria a Streptomyces sp. The first way led us to clone PCR fragments corresponding to endoglucanase and exoglucanase of the fungus; the second led us to clone two b glucosidaes from both microorganisms. In addition, we have cloned two Streptomyces genes that derepress the cryptic b glucosidase operon of *E. coli*.

Background: The breakdown of cellulose is carried by three enzymatic activities acting synergistically: endoglucanse, exoglucanase and b glucosidase. Depending on their molecular structure and their action on specific substrates, both bacterial and fungal endogluacanses and exoglucanases have been grouped in families. These extracellular enzymes, called cellulases, are synthesized in response to an inducer: the cellulose or its derivates. Usually, fungi and bacteria have more than one gene encoding each of these activities. Many genes have been cloned and sequenced by various laboratories. The comparison between fungal exo and endogluacanses revealed a highly conserved region of about thirty amino acids (the cellulose binding domain, CBD) separated from the active site (not conserved) by a hinge region, highly rich in Threonic, Serine and Proline. Few is known on b glucosidase and on the molecular regulation of cellulases.

Our previous works, before starting this project, dealed with the optimization of fermentation conditions of cellulolytic fungi (Penicillium and Trichoderma), hydrolytic efficiency of cellulases on local substrates (esparto grass pulp paper) and finally the isolation of cellulolytic Actinomycetes strains (see publications).

Our actual aims are: 1. to get more fundamental knowledge about cellulases produced by our strains by cloning genes and studying the biochemical properties of enzymes; 2. to do genetic modification of our strains in order to improve their capacities in cellulase production.

Objectives: Our main objectives during the first period were:

- Genomic banks construction of Streptomyces and Penicillium strains
- Functional screening of the genomic banks in *E. coli* and isolation of cellulases genes
- Purification of Penicillium cellulases and kinetic studies
- Amino acid Nterminus sequencing of some parts of these purified enzymes
- Oligonucleotides synthesis and PCR amplification of internal fragments of cellulases.

Our objectives for the next year will be:

- Complete sequence of the b glucosidase genes already clones, we will start by the Penicillium one. Isolation of the complete version of this gene (non truncated at the 5' end).
- Sequencing of the cellulase specific PCR fragments
- Genomic and cDNA bank construction and/or physical screening by oligonucleotides or PCR fragments
- Cloning of the small endoglucanase gene
- Northern analysis using the b glucosidase probes
- Elucidation of the mysterious functions encoded by pLA1 and pLA7 plasmids
- Transfer of the cloned genes into yeast, other fungi and streptomyces

Work Progress. Purification and characterization of *Penicillium occitanis* cellulases. *Penicillium occitanis* produces a complete cellulase system including two b glucosidases, two

cellobiohydrolases and endoglucanases when grown on cellulosic substrates. Cellulolytic enzymes have been isolated to homogeneity by gel filtration, ion exchange chromatography, chromatofocusing and preparative native PAGE.

We have characterized by chromatofocusing and native PAGE two distinct b-glucosidases activities. However, they have similar M.W. determined by SDS PAGE, similar amino acid composition and kinetic characteristics. Two different cellobiohydrolases activities (CBH I and CBH II) were also characterized. The differed in their M.W., amino acid composition structure and glycosylation. The kinetic characteristics of these two cellobiohydrolases are also distinct. Cellobiose inhibited competitively the CBH I activity and has no effect at 100 mM on the CBH II activity. The endoglucanase 28 KDa purified by this technique showed more interesting properties for some industrial applications than cellobiohydrolases. It was more active against crystalline and amorphous cellulose in large range of pH and temperature. Both cellobiose and glucose have no effect on endoglucanase activity. Cellobiose was the major end product of cellulose hydrolysis by the two cellobiohydrolases whereas cellotriose, cellobiose and glucose were the end products of cellulose hydrolysis by endoglucanase.

CBH I and CBH II acted synergystically to effect extensive hydrolysis of cellulose but endoglucanase acted synergistically only with CBH II.

These enzymes purified to homogeneity were used for the determination of some peptide sequences with a view to preparing the corresponding oligonucleotides. These oligonucleotides would be a useful material to select cloned genes in both genomic and cDNA banks. After proteolysis of CBH I by the V8 protease and CBH II by the achymotrypsine we have purified and sequenced two peptides from CBH I and five peptides from CBH II. Two oligonucleotides corresponding to the CBH I peptide have been synthesized and used for PCR and Southern experiments.

Genomic banks construction and isolation of two b-glucosidase and two regulatory genes: Two genomic banks have been constructed in pIC19, one form the fungus *P. ochrosporum* and one from the non identified local strain, LV125 (a Streptomyces sp). The functional screening of these banks in *E. coli* DH5alpha cells has been applied using chromogenic and fluorogenic substrates (XGlu: 5-Bromo-4-Chloro-3-Indolyl-b1-4-DGlucose and MUG: Methyl Umbellypheryl-b1-4- D Glucose) for the b glucosidase activity, Congo red staining of CMC plates for the endoglucanase and MUC (Methyl Umbellypheryl-b1-4 Cellobiose) for the exoglucanase activity. Four clones expressing the b glucosidase activity in *E. coli* have been isolated. One from the *P. ochrosporum* bank, named pAL1 and three from the bacterial bank, named pLA1.4.7. These inserts differ from each other by length (respectively about 7.2,1 Kb), restriction map and hybridization pattern with the LV125 genomic DNA.

We have sequenced the beginning of the pAL1 insert and found an open reading frame, in frame with the LacZ gene of the vector explaining the expression of an such eukaryotic gene in *E. coli*. Over the four hundred bases sequenced, a high content of Threonine (about 30%) characterizes the deduced protein that did not show any similarity to the known b glucosidases or other sequences of the Swiss-Proteins bank. Moreover, the Southern analysis with the pAL1 probe revealed the existence of two genes in *P. ochrosporum* genomes.

In fact, *E. coli* contains a cryptic bgl operon which becomes decryptified after insertion of an IS in the promoter region of the gene. We have obtained from Dr. Steinmetz two very useful strains, both bear an integrated prophage containing the b galactosidase reporter gene behind the PR promoter (PR: promoter of the bgl operon controlled by antitermination) but in one of these strains, MV152, the bgl locus is deleted and in the second, MV200, the bgl operon is decryptified by an IS insertion. The transfer of pAL1, pLA1, pLA4 and pLA7 in MV 152 allowed us to conclude that pAL1 (from Penicillium bank) and pLA4 (from Streptomyces bank) encode really the b glucosidase activity since they give to MV152 the capacity to hydrolyse Aryl b Glucosides such the XGlu and MUG. On the other hand, the other plasmids (pLA1 and pLA7) did not confer this ability to the MV 152 strain. They should probably act, in DH5alpha, by disturbing the regulation of the cryptic bgl operon of *E. coli*, enabling it to be transcribed. Although, they did not enhance the b galactosidase expression from the PR promoter in neither MV 200 nor MV 152. So the deregulatory function shared by these two plasmids remains to be elucidated.

Publications:

Hadj-Taieb, N., Ellouz-Chaabouni, S., Kamoun, A. and Ellouz, R. (1992) Hydrolytic efficiency of *Penicillium occitanis* cellulase: Kinetic aspects. Appl. Microbiol. Biotechnol. 37: 197-210.

Ellouz-Chaabouni, S., Hadj-Taieb, N., Mosrati, R. and Ellouz, R. (1993) *Penicillium occitanis* cellulase: a further useful system. Enzyme Microbiol. Biotechnol. (accepted)

Kamoun, A., Belguith, H., Hadj-Taieb, N. and Gargouri, A.F. (1993) Screening and isolation of cellulolytic actinomycetes from Tunisian soils. Research in Microbiology (Institut Pasteur France) (accepted).

Networking: We have collaborated during this first period with various laboratories in Tunisia and France:

1) INRST-Tunisia (Institut National de la Recherche Scientifique et Technique): Laboratory of Prof. Rachid Ghrir, who has determined the amino acid composition of our purified proteins. He gave us also very useful HPLC columns.

2) Faculte de Medecine de Sfax-Tunisia: Laboratory of Dr. Hamadi Ayadi, where we have produced the rabbit antibodies raised against the purified exo and endoglucanases. We have also performed our PCR experiments in this laboratory since we have not yet the Thermocycler apparatus.

3) CEA Saclay-France: Laboratories of Prof. Andre Menez and Andre Sentenac, where one of us, Nejib Marzouki, has spent forty days in these laboratories in order to perform the sequencing of peptide fragments and oligonucleotides synthesis.

4) "Batiment d'Enzymology" of the CNRS at Gif-sur Yvette-France: Dr. Antoine Le Caer, who has also performed for us some sequencing analysis of peptide fragments.

5) Centre de Genetique Moleculaire of the CNRS at Gif-sur Yvette-France: Laboratory of Prof. Piotr Slominski, where we have synthesised some oligonucleotides.

6) INRA de Versailles-Grignon-France: Dr. Michel Steinmetz, who gave us two very useful *E. coli* strains, one deleted and on decryptified at the bgl locus.

VENEZUELA

INTRODUCTION OF GENETIC RESISTANCE TO TOMATO YELLOW MOSAIC VIRUS IN THE CULTIVATED TOMATO (*Lycopersicon Esculentum*)

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Keywords: TYMV, geminivirus, Lycopersicon, somatic hybrids

Abstract: Several virosis, but specially TYMV, are attacking Venezuelan tomatoes plantations with millions of dollars loses during the last year. In spite of the number of tomato cultivators the yield of commercial tomato suffers a loss from different diseases including plant viruses. Especially virus diseases are wide-spread in the tropical and subtropical areas so far as it is concerned with prolonged period vegetation and significant quantity of insect-pests which can transfer virus of great importance. Yield reduction attributed to TYMV infection are usually greatest in Venezuela and other tropical countries where the conditions are ideal for geminivirus spread. Some wild species of *Solanaceae* family show high variability to virus resistance and can be used as a good material for improvement of commercial tomato germplasm by methods of cell engineering (somatic hybridization). Also it is possible to construct gene vectors from this viruses and to obtain viral protection in transgenic tomato plants expressing the TYMV coat protein or its antisense RNA.

Background: Genetic resistance is the more practical way to deny virus infection in cultivated plants, it is easy to apply, cheap and needs no induction. On the other hand it is environmentally safe. *L. esculentum* is a member of relatively small genus, *Lycopersicon* within the large family *Solanaceae*. The

commercial tomato, a self-pollinated plant, has very limited genetic variability, as it was shown by isoenzyme variation among cultivars, due to this fact it is necessary to use some wild relatives with natural resistance to virus in order to improve genetically this very important crop. It is possible regeneration of tomato plants from explants and protoplasts, which led to the development of efficient transformation procedure and somatic hybridization. A number of laboratories have successfully produced interspecific hybrids between tomato and wild species of *Solanum cae*. Then the possibility to transfer in tomato resistance to TYMV is concerned with finding of such natural resistance in some wild species. However, suitable resistance genes frequently are inseparably linked to undesirable traits. By classical breeding techniques few years are required for the incorporation into tomato plants virus resistance, with somatic hybridization less time is required, because it is possible the control and the characterization at molecular level of the hybrids at cellular stage. However, other strategies have been followed using genetic engineering and plant transformation techniques like:

1) Expressing viral proteins in transgenic plants;
2) Expressing antisence viral RNA in transgenic plants;
3) Expressing satellite RNA in transgenic plants.

Advances in plant and viral molecular biology have identified "geminivirus" genes involved in replication of viral DNA, spread of virus in the plant and insect transmission. Gene replacement experiments suggest that useful plant gene expression vectors can be constructed on the basis of the tomato yellow mosaic virus (TYMV) and can be transformed into tomato cultivars. TYMV belongs to the geminivirus group and is transmitted by the white fly *Bemisia tabaci*, twin isometric (geminate) virions particles with 18-20 mm in length are the principal characteristics of the viruses-single stranded circular DNA. Geminivirus DNA is usually present in 2 ssDNA molecules, A and B, in TGMV DNA-2588n,DNA-2508n. Both molecules show little homology except for strongly conserved 200 base sequence, which is believe to participate in DNA replication. In this common sequence there are 18 bases which are strongly conserved among all the white fly transmitted viruses.

Objectives: Several virosis, but specially TYMV, are attacking Venezuelan tomatoes plantations with millions of dollars loses during the last year. In this project we attempt to introduce genetic resistance to TYMV by two different paths. We look for wild species of *Solanaceae* family to find natural resistance to the virus, in order to use cell genetic and somatic hybridization to transfer this resistance to cultivated tomatoes and we will use genetic engineering techniques to construct vectors capable to introduce resistance.

Work Progress: In this work we studied genetic resistance/susceptibility to TYMV infection in different members of the *Lycopersicon* genus. When we began to work with TYMV collected from different areas of Venezuela only one virus per sample was found. Symptomatology and type of disease was the same after insect transmission and mechanical inoculation. In open field the same symptoms of TYMV disease were also observed. This is important because there are few geminivirus diseases described in tomato which have more or less similar symptoms than TYMV and are transmitted by *B. tabaci* like: Tomato Golden Mosaic Virus(TGMV); Tomato Yellow Leaf Curl Virus (TYLCV); Chino del Tomate Virus, Potato Yellow Mosaic Geminivirus (PYMG). Nevertheless, all geminivirus subgroup B. Disease of TYMV was first reported in Venezuela during 1963 as a virus transmitted by the *B. tabaci*. We tested plant material to TYMV infection in green house and open field near infected tomato plantation. We found that *L. cheesmanii*, *L. esculentum*, *L. chmielewskii*, *L. parviflorum*, *L. pennelli* LA 1926, *L. peruvianum* LA111, *L. peruvianum* v. *humifusum*, *L. pimpinellifolium* LA2187 were very susceptible to the virus. The species *L. hirsutum*, *L. peruvianum* v. *glandulosum*, *L peruvianum* v. *dentatum* are less susceptible and have a tolerance to TYMV. After testing accession LA1963 and LA 1969 of *L. chilense* we found some plants to be highly resistant. In our knowledge this is the first time genetic resistance to TYMV in *Lycopersicon* genus have been described. We are now attempting to better characterize the resistance found in *L. chilense*, testing other geminiviruses for resistance/susceptibility, in the other hand, this species will be used as a germplasm source for introgressive hybridization, in order to transfer this resistance to cultivated tomato.

Publications:

Nicolai M. Piven, Rafaela C. de Uzcategui, Diogenes Infante. Testing Tomato Yellow Mosaic Virus Resistance/Susceptibility in Different Species of *Lycopersicon* Genus. Submitted for publication.

Networking: We select the tomatoes varieties and perform the field test in collaboration with Fondo Nacional de Investigaciones Agropecuarias (Fonaiap), Barquisimeto, Lara and Fundacion de Servicio al Agricultor (Fusagri), Cagua, Aragua.

Training Programme: Long-term

Fellowships

In line with the original mandate of the Centre, namely to strengthen the research capabilities of its members through training, the short and long-term training programmes continue to be among the most important activities of ICGEB.

General statistics concerning the long-term fellowship programme for the year 1993 can be summarized as follows:

- applications received: 207 (144 post-docs and 63 pre-docs)
- fellowships offered by ICGEB: 38 (33 post-docs and 5 pre-docs)
- fellowships accepted: 31 (26 post-docs and 5 pre-docs)

As in the past, the post-doctoral fellows have been assigned to research teams located in either of the two Components, in selected Italian Institutes and in a few Affiliated Centres (the latter being in connection with a Collaborative Research Project financed by ICGEB).

The pre-doctoral fellowship programme, developed within the framework of the Ph.D. course in molecular genetics jointly organized by ICGEB and the

Figure 1: ICGEB Fellowships Awarded in 1993

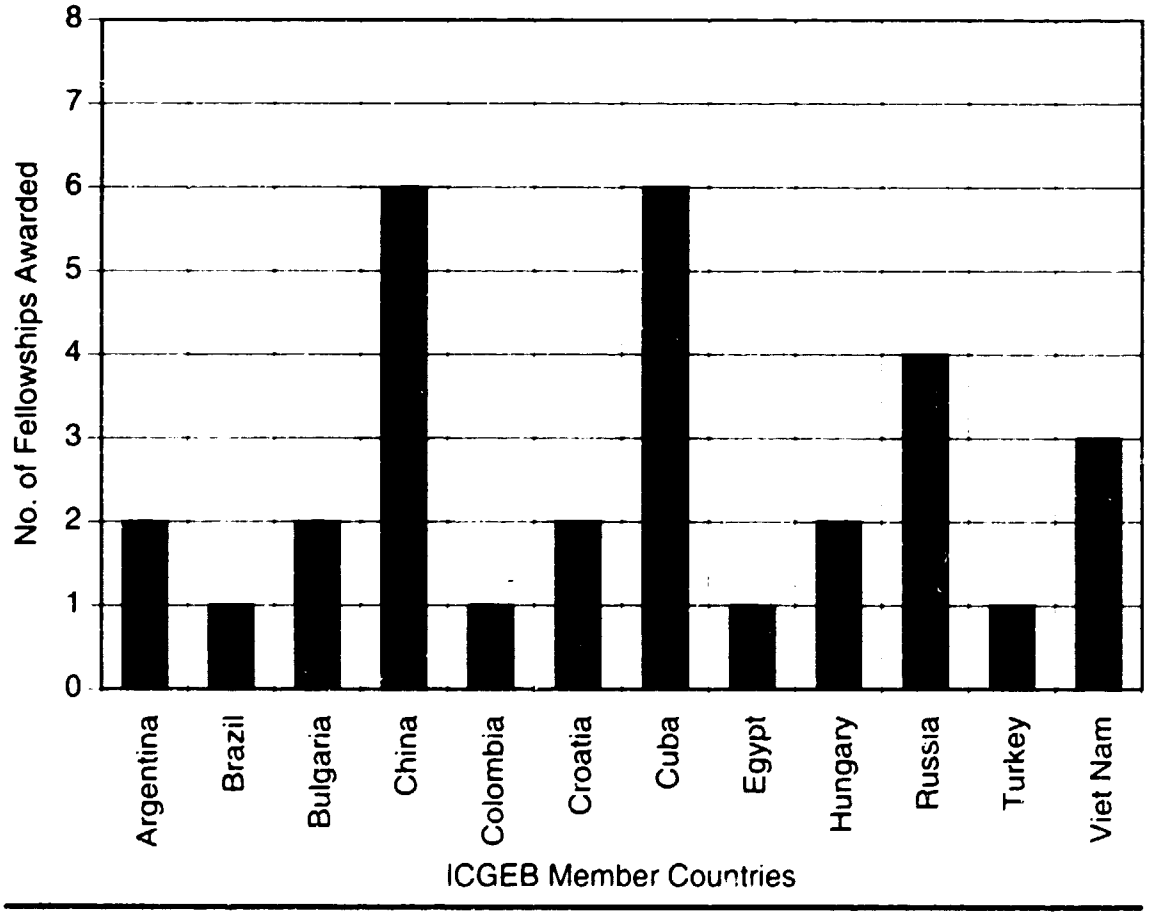


Table 1: Geographical Distribution of Fellowships Awarded in 1993 (31)

COUNTRY	FELLOW	HOST LABORATORY
Argentina	Monica FULCHIERI	University of Florence, Italy
	Facundo BATISTA*	ICGEB, Trieste, Italy
Brazil	Jefferson Costa da CUNHA	IBME/CONICET, Argentina
Bulgaria	Ivka AFRIKANOVA*	ICGEB, Trieste, Italy
	Anna DRAGOEVA	Biological Research Centre, Szeged, Hungary
China	Wenxia QIE	ICGEB, Trieste, Italy
	Ping SONG	ICGEB, Trieste, Italy
	Tao ZU	IRIS, Siena, Italy
	Jinqiu CHEN*	ICGEB, Trieste, Italy
	Yalin JIANG	ICGEB, Trieste, Italy
	Huiwen MA	ICGEB, New Delhi, India
Colombia	Olga CAMACHO VANEGAS	University of Rome "Tor Vergata", Italy
Croatia	Dean LAZAREVIC	CIB, Trieste, Italy
	Miranda MLADINIC*	ICGEB/SISSA, Trieste, Italy
Cuba	Manuel PENICHET PRADO	IGBE/CNR, Pavia, Italy
	Gabino GARRIDO	Mario Negri Institute, Milan, Italy
	Juan ROCA	ICGEB, New Delhi
	Ariel ARENCIBIA RODRIGUEZ	University of Pavia, Italy
	Adrián SUAREZ	IGBE/CNR, Pavia, Italy
	Lincidio PEREZ SANCHEZ	San Raffaele Hospital, Milan, Italy
Egypt	Mostafa EL-SHEEKH	ICGEB, New Delhi, India
Hungary	Péter FÁBIÁN	ICGEB, Trieste, Italy
	Zsolt HATSAGI	ICGEB, Trieste, Italy
Russia	Elena GAZINA	IGBE/CNR, Pavia, Italy
	Olga MINENKOVA	University of Rome "Tor Vergata", Italy
	Kirill DEGTIARENKO	ICGEB, Trieste, Italy
	Leonard KHIROUG*	ICGEB/SISSA, Trieste, Italy
Turkey	Mehmet OZDENER	ICGEB, New Delhi, India
Viet Nam	Quang Binh DO	University of Pavia, Italy
	Thi Lan Oanh LE	ICGEB, New Delhi, India
	Vo Thi THU	ICGEB, New Delhi, India

*Predoctoral Fellows

International School of Advanced Studies (ISAS) of Trieste, is now reaching its steady state. The first Doctorates in Philosophy were awarded during 1993 and more students will complete the course in 1994. A similar programme for establishing a Ph.D. course at the New Delhi Component is envisaged.

The 1993 fellowships were awarded to scientists originating from 12 Member Countries; Figure 1 gives the collective data relating to this component of the programme, whereas Table 1 provides a comprehensive list of placements. Figure 2 gives a geographical and numerical overview of the fellowship programme in its five years of operation.

Due to the pluriannual nature of most of the fellowships awarded, the real dimension of the output of ICGEB for the long-term training programme becomes clearer when considering the number of trainee/years funded by the

Figure 2: Progress of the Fellowship Programme 1989-1993

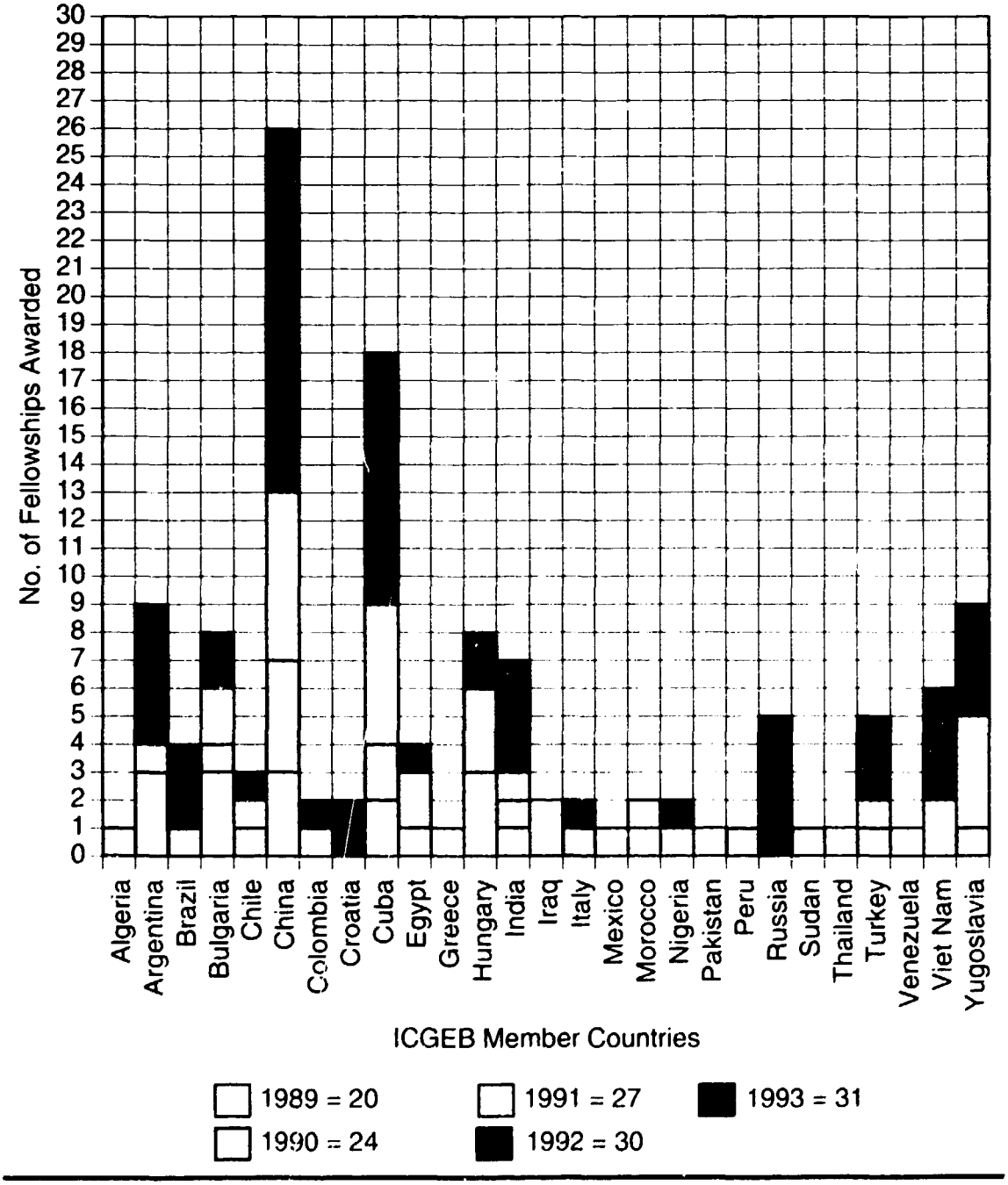
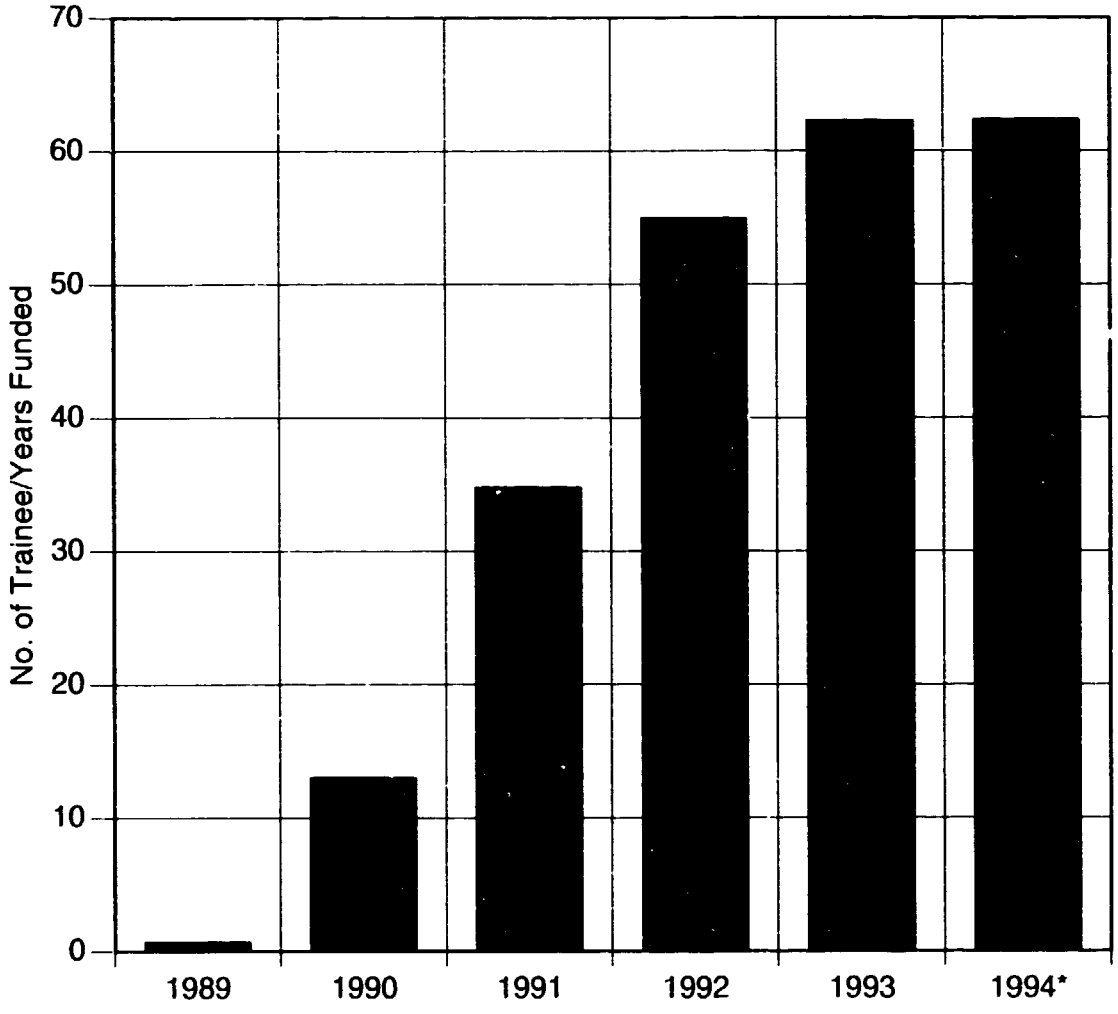


Figure 3: Trainee/Years Funded



* showing funds committed for 1994

Centre, as shown in Figure 3. In 1993, the overall programme reached a total of 62 trainee/years funded and the commitments for 1994 have already reached this figure. With the present funding, this has become the steady level of the fellowship programme.

As recommended by the delegates of the Second Forum of Scientists (30 September - 1 October, 1992) and approved by the Preparatory Committee, a scheme for the awarding of short-term (maximum 3 months) fellowships has been initiated by ICGEB. Numerous applications were received during 1993 and 2 such fellowships have already been awarded; the fellows are scheduled to start during 1994.

Contacts between ICGEB and scientists who formerly held an ICGEB fellowship remain concrete and operational. In fact, many of the applications received from Affiliated Centres for funding in the framework of the Collaborative Research Programme have originated from research groups where former ICGEB fellows have returned to their home institutes. The link existing between the fellowship and the Collaborative Research Programmes thus becomes concrete, demonstrating the validity of the ICGEB approach.

Training Programme: Short-term

Meetings and Courses

This other important component of the ICGEB training programme continued its gradual and constant expansion during 1993, as can be seen in Figure 5, which shows the number of participants in the various courses organized by the Centre since its inception.

The high quality of the courses and meetings focussed on specialized research topics or techniques attracts an increasing number of scientists from Member Countries as well as the interest of other international organizations and funding agencies. Contributions for the short term training activities were received by ICGEB during 1993 from the United Nations Environment Programme (UNEP), the European Molecular Biology Organization (EMBO) and the Rockefeller Foundation.

The geographical distribution of the participants who have attended the various events since the inception of ICGEB is given in Figure 6. Table 2 shows a comprehensive list of courses held in 1993, whereas those scheduled to take place in 1994 are shown in Table 3. The list of teachers who contributed to the courses in 1993 is shown in Table 4.

The 1993 programme has experienced an important increase in the number of training courses organized in Affiliated Centres through the funding of ICGEB. This important aspect has a dual relevance since, on the one hand, it consolidates the relationship between ICGEB and the network of Affiliated Centres and, on the other hand, it promotes the realization of courses which can have an important regional impact.

Figure 5: Short-term Training Programme 1988-1993

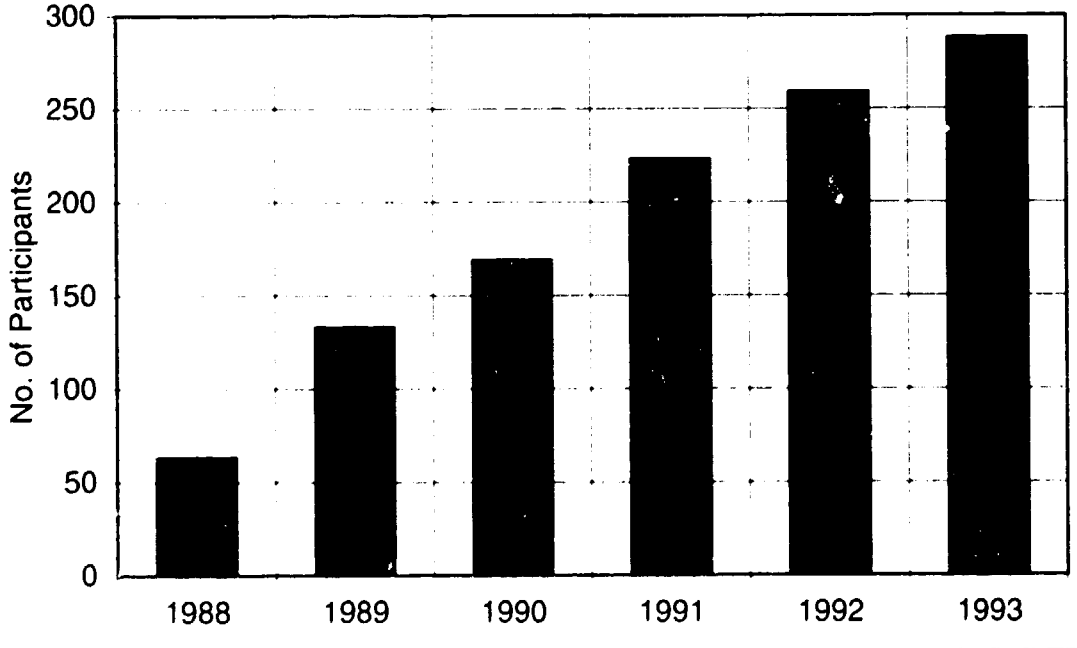


Figure 6: Geographical Distribution Short-term Training Programme 1988-1993

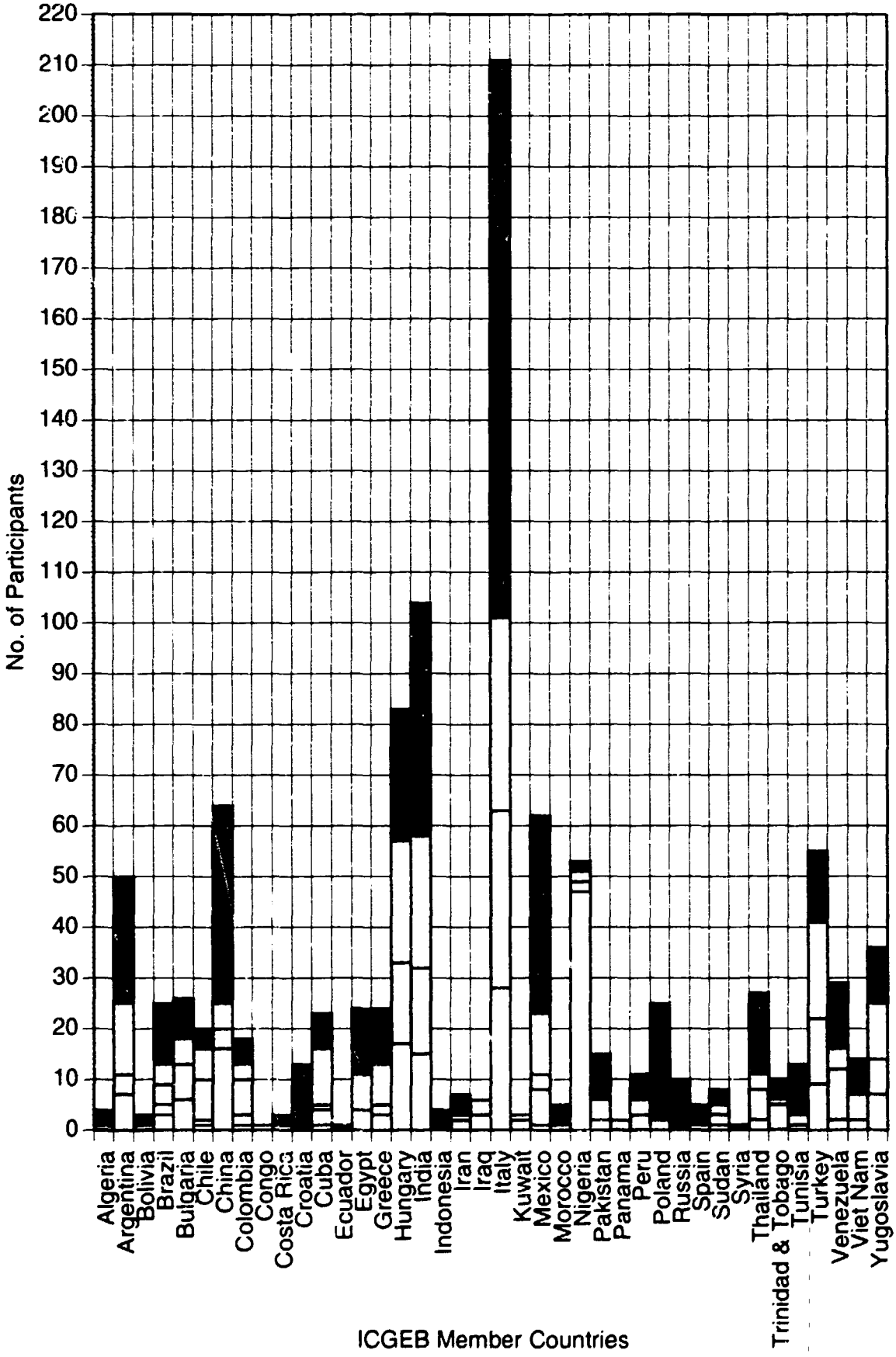


Table 2: Meetings and Courses 1993

TITLE	DATES and LOCATION	ORGANIZER(S)
Conference: PROTEIN KINASES, CELL PROLIFERATION AND ONCOGENESIS	6-8 January Santiago, Chile	Jorge Allende
Conference: BIOTECHNOLOGY FOR ENVIRONMENT AND AGRICULTURE	6-9 January Karachi, Pakistan	Nuzhat Ahmed
Practical Course: GENOME ANALYSIS OF PLANTS, PESTS AND PATHOGENS	8-27 February New Delhi, India	John Bennett
Theoretical and Practical Course: BACTERIAL GENETICS	1-12 March Trieste, Italy	Carlo Bruschi Thomas Silhavy
Practical Course: SITE-DIRECTED MUTAGENESIS	2-19 March New Delhi, India	Vijay Kumar
Theoretical Course: RNA STRUCTURE AND FUNCTION	29 March - 1 April Trieste, Italy	Glauco Tocchini-Valentini
Conference: INTRACELLULAR CHANNELS, ORGANELLES AND CELL FUNCTION	21-23 April Trieste, Italy	Enrico Cherubini
Theoretical and Practical Course: YEAST MOLECULAR GENETICS	26 April - 7 May Trieste, Italy	Carlo Bruschi Glauco Tocchini-Valentini
Practical Course: FUNGICIDE RESISTANCE RESEARCH: BIOTECHNOLOGICAL APPLICATIONS	17-23 May Nanjing, China	Jiewei Wei Mingguo Zhou
Practical Course: PREPARATION, ANALYSIS AND APPLICATIONS OF SYNTHETIC OLIGONUCLEOTIDES	5-17 July Mexico City, Mexico	Gabriel Guarneros Francisco de la Vega
Theoretical Course: ENVIRONMENTAL APPLICATIONS OF BIOTECHNOLOGY: SCIENTIFIC RISK ASSESSMENT METHODOLOGIES	28 June -2 July Trieste, Italy	Gilbert Howe
Course: MOLECULAR BIOLOGY AND DIAGNOSIS OF HUMAN CHLAMYDIAL INFECTIONS	6-10 July Trieste, Italy	Giulio Ratti
Practical Course: COMPUTER METHODS IN MOLECULAR BIOLOGY	14-23 July Trieste, Italy	Sándor Pongor
Practical Course: BACTERIAL EXPRESSION OF ANTIBODY FRAGMENTS	5-18 September Havana, Cuba	Jorge Gavilondo Marta Ayala
Practical Course: HUMAN GENOME DIVERSITY	13-17 September Trieste, Italy	Luca Cavalli-Sforza Francisco Baralle
Practical Course: PLANT BIOTECHNOLOGY: TISSUE CULTURE AND BEYOND	9-21 October Cairo, Egypt	Hamdy Abdel-Aziz Moursy
Practical Course: APPLICATIONS OF THE NEW BIOTECHNOLOGIES TO AGRICULTURE	1-21 November Buenos Aires, Argentina	Alejandro Mentaberry Hector Torres

Table 3: Meetings and Courses 1994

TITLE	DATES and LOCATION	ORGANIZER(S)
Symposium: MOLECULAR MECHANISMS OF GENE EXPRESSION	5-7 January Santiago, Chile	Jorge Allende
Theoretical and Practical Course: BACTERIAL GENETICS	14-25 March Trieste, Italy	Carlo Bruschi
Theoretical Course: RNA STRUCTURE AND FUNCTION	28-31 March Trieste, Italy	Glauco Tocchini-Valentini
Workshop: BIOTECHNOLOGY AND FOOD	10-23 April Awka, Nigeria	Nduka Okafor
Workshop: OPEN QUESTIONS IN MOLECULAR EVOLUTION	18-23 April Guanacaste, Costa Rica	Giorgio Bernardi Gabriel Macaya
Theoretical and Practical Course: YEAST MOLECULAR GENETICS	18-29 April Trieste, Italy	Carlo Bruschi
Theoretical Course: BIOTECHNOLOGY AND AGRICULTURAL IMPROVEMENT IN DEVELOPING COUNTRIES	21 May-3 June Marrakech, Morocco	Zaid Abdelouahhab Oihabi Abdellah
ICGEB Inaugural Conference: EMERGING BIOTECHNOLOGIES AND INDUSTRIAL OPPORTUNITIES	June, tentative Trieste, Italy	Francisco Baralle George Tzotzos
Practical Course: BIOINFORMATICS: COMPUTER METHODS IN MOLECULAR BIOLOGY	13-22 July Trieste, Italy	Sándor Pongor
Theoretical Course: MEDICAL GENETICS IN DEVELOPING COUNTRIES	21-26 August Beijing, China	Giovanni Romeo Qin Xinhua
Theoretical Course: ENVIRONMENTAL APPLICATIONS OF BIOTECHNOLOGY: SCIENTIFIC RISK ASSESSMENT METHODOLOGIES	19-23 September Trieste, Italy	Gilbert Howe
Practical Course: INSECTICIDAL ENDOTOXINS	25 October-12 November New Delhi, India	Raj Bhatnagar
Practical Course: PLANT TRANSFORMATION	22 November-10 December New Delhi, India	Swapan Datta
International Symposium: PLANT MOLECULAR BIOLOGY AND BIOTECHNOLOGY	14-17 December New Delhi, India	Krishna Tewari

Tabel 4: List of Teachers Participating in ICGEB Courses - 1993

ABELSON John, California Institute of Technology, Pasadena, USA
ANDRUS Alexander, Applied Biosystems Division of Perkin Elmer, Foster City, USA
AYALA Marta, CIGB, Havana, Cuba
BAIROCH Amos, CMU, Geneva, SWITZERLAND
BENSON Dennis, National Library of Medicine, Bethesda, USA
BISHOP Martin, Molecular Genetics Department of Medicine, Cambridge, UK
BOWCOCK Anne, University of Texas, Dallas, USA
BOZZONI Irene, Universita' degli Studi "La Sapienza", Rome, ITALY
BREMER Erhard, Max Planck Institute, Marburg/Lahn, GERMANY
CAVALLI-SFORZA L. Luca, Stanford University, Stanford, USA
CEVENINI Roberto, Ospedale S. Orsola, Bologna, ITALY
CHAKRABORTY Ranajit, University of Texas, Houston, USA
CHOUDHURY Vijay, University of Delhi, New Delhi, INDIA
COMANDUCCI Maurizio, IRIS, Siena, ITALY
COVACCI Antonio, IRIS, Siena, ITALY
DAHLBERG James, University of Wisconsin, Madison, USA
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ICGEBnet

Sándor Pongor, Valeria Bevilacqua and Zsolt Hátsagi

ICGEBnet is a central biocomputing resource located in Trieste that currently provides login facilities to over 600 users world-wide via INTERNET and X.25 connections. ICGEBnet provides a computer environment that allows molecular biologists to analyse nucleotide and protein sequences. ICGEBnet provides access to a large variety of databases (biosafety, genetics, biodiversity, etc.) and to various tools of electronic communication (bulletin boards, electronic mail and wide area information services). Access to ICGEBnet is available free of charge to all ICGEB Member Country scientists; however, preference is given to those scientists whose research is directly related to the research goals of ICGEB.

Molecular Biology Services

ICGEB hosts copies of the most important biological sequence data banks, including GenBank, EMBL, PIR, Swiss-Prot and Prosite. In addition, ICGEB provides access to virtually all molecular biology databases available world-wide, via the INTERNET. Analysis software includes three major programme packages for biological sequence analysis (GCG, Staden and IG) and a large variety of specialized software (see list below).

Information Services, Biosafety

ICGEBnet provides on-line access to a large number of databases pertinent to genetics, molecular biology and biotechnology. Special emphasis is given to biosafety and the release into the environment of genetically modified organisms. ICGEBnet collects documents and guidelines on biological and chemical laboratory safety, lists of experts, biosafety committees and so forth. Using the gopher wide area information server, access is provided to specialized data collections on biodiversity, microbiology, cell culture collections, etc. The aim of the biosafety information service, the first such service worldwide, is to assist national authorities, academic institutions and industries in ICGEB Member Countries in the safe evaluation of the environmental effects of genetically modified organisms and to help in the creation of national regulations.

In addition, ICGEBnet provides on-line access to all computer programme descriptions as well as a complete user manual. The programmes of ICGEB courses, fellowships and application forms are also available on-line.

Electronic Communication and Other Services

ICGEBnet provides electronic mail services (mm, elm), access to bulletin boards (nn), and wide area information servers. While being increasingly popular in industrialized countries, these communication tools are only rarely available in the developing world. Presently ICGEBnet appears to be one of the main INTERNET gateways for biologists in ICGEB Member Countries. ICGEBnet also provides access to a variety of UNIX programmes and facilities.

Research and Training Activities

Research at ICGEBnet concentrates on computer methods for detecting distant protein homologies and the maintenance of SBASE, a comprehensive and annotated collection of protein domain sequences. UNIX biocomputing utility programmes (menu interfaces, database update, sequence retrieval, etc.) are developed in-house and are available through the anonymous ftp facility (ftp.icgeb.trieste.it).

ICGEBnet also serves as a training facility with 2-3 yearly computer courses. The practical course "Computer Methods in Molecular Biology" is held in July each year and provides an introduction to bioinformatics and biological sequence analysis.

User Support

User consultation is available via telephone and electronic mail in addition to on-line and hard copy documentation of the major programmes. User training is provided through the computer courses organized at ICGEB.

International Collaboration

ICGEBnet is a member EMBnet, the Biological Information Network funded by the European Economic Community. The databases are maintained in collaboration with EMBL, the European Molecular Biology Laboratory (Heidelberg, Germany), NCBI, the US National Center for Biotechnology Information (Bethesda, Washington DC), USDA, the US Department for Agriculture and other national and international agencies. The Biosafety Archives are maintained in collaboration with the Agricultural Biotechnology Center, Gödöllö, Hungary. ICGEBnet is also providing know-how for Member Countries to set up biocomputing resources based on the UNIX utilities, menus and manuals developed at ICGEB.

Recent System Additions

- Version 2.0 of the SBASE protein domain library was released.
- The BIOSAFETY archive of laboratory and environmental safety documents was made available through the gopher wide area information server. We also installed an E-mail document server which sends documents of the Biosafety Archive in response to electronic mail requests (docserver@icgeb.trieste.it).
- Databases/software developed at ICGEB are now available through direct file transfer from our anonymous ftp server (ftp.icgeb.trieste.it).
- A protein domain homology E-mail server was installed (sbase@icgeb.trieste.it). This server carries out automated searches for functional domains in query sequences and returns the results by electronic mail. The procedure is based on the SBASE protein domain library, developed at ICGEB in collaboration with the ABC Institute of Biochemistry and the Protein Research, Gödöllö, Hungary, where we installed a domain homology server based on a different principle (domain@hubi.abc.hu).

Computer Environment

ICGEBnet is based on a cluster of UNIX workstations. The central unit of ICGEBnet is "genes", a SUN4/390 computer that is accessible through X.25 and INTERNET lines. "Genes" runs under the UNIX operating system version SunOS 4.1.1. Currently, "genes" has 32 MB memory, 4.5 Gbyte disk space and a CD-ROM drive. "Genes" is providing the on-line services for remote users as well as for the local users of the Trieste Component who access the system through PC's connected to an Ethernet based local area network.

On-line Accessible Software

1. GCG. Staden. IG: Comprehensive programme packages for representing and manipulating nucleic acid and protein sequence data:
2. FASTA . TFASTA and BLAST for sequence similarity searching:
3. CLUSTAL for multiple sequence alignment:
4. PHYLIP for the construction of phylogenies:
5. GM for the automated analysis of eukaryotic sequences:
6. PROSEARCH and PLSEARCH for the detection of protein motifs in amino acid sequences:
7. SRS for sequence retrieval:
8. MM and ELM for electronic mail and NN for accessing the electronic bulletin boards:
9. Gopher info-server for information on ICGEBnet and for accessing over 150 free databases world -wide: and.
10. General purpose UNIX programs including TEX for text formatting and GAWK for text pattern scanning/processing.

On-line Accessible Database Library

1. EMBL: EMBL nucleic acid sequence data bank (updated daily):
2. GenBank: NIH nucleic acid sequence data bank (updated daily):
3. PIR: Protein Identification Resource protein database:
4. Swiss-Prot EMBL/University of Geneva protein database:
5. SEQDB: PRF Osaka Peptide/Protein Sequence Database:
6. SBASE: ICGEB Trieste Protein Domain Library:
7. Enzyme: Restriction Enzyme Data Bank:
8. HIV-NA/AA: HIV nucleic acid and protein database:
9. KeyBank and KeyTool: IG sequence pattern databases:
10. Prosite: EMBL/University of Geneva protein pattern database:
11. Vectorbank: Cloning Vector databank:
12. Bibliography: Molecular biology computer applications:
13. PLSEARCH: Protein superfamily sequence motif database:
14. OMIM: V. McKusick's Database of Human Genetic Disorders:
15. Brookhaven: 3-D structure of proteins (and other macromolecules):
16. LIMB: A comprehensive list of molecular biology databases:
17. TFD: Transcription factor database: and.
18. CLDB: Interlab Cell line database.

ICGEBnet Biosafety Archives*

In addition. the ICGEBnet gopher server provides remote access to virtually all molecular biology databases currently available.

1. Laboratory Chemical and Biological Safety (general):
   - Material Safety Data Sheets
   - US Institutional Biosafety Committees
   - Collection of documents. institutional regulations
2. Environmental Biosafety:
   - Genetically Modified Organisms
   - US Regulations
   - US GMO Permit Applications Forms
   - US Field Test Permit Approvals
   - ICGEB Documents
   - European Documents
   - Useful Infos and Contacts (Legislation. Experts)
3. Biodiversity Issues:
   - UNCED documents
   - Biodiversity gopher servers

*available through the gopher server and. also. partly through E-mail (doeserver@ icgeb.trieste.it).

Biosafety

ICGEB continued to make considerable resources available in order to strengthen institutional capability in Member Countries in the area of biosafety.

The annual courses, sponsored by the United Nations Environment Programme, covering theory and methodologies in biological risk assessment, continued successfully. Two such courses took place in 1993 in Trieste and Cairo, respectively. The latter, which was specifically intended to cover Africa, was co-organized by the Agricultural Biotechnology for Sustainable Productivity (ABSP) project and the Agricultural Genetic Engineering Research Institute of Egypt.

In addition, ICGEB has been requested to provide assistance to Member Countries to develop biotechnology regulations and/or to set up regulatory oversight mechanisms. One such request from the Russian Federation (National Committee for Elaboration of Legislation for Work with Genetically Modified Organisms) soliciting assistance in the development of national legislation, was met by expediting an expert meeting to take place in Moscow. During the meeting, international experts engaged by ICGEB, Russian scientists and senior political figures – including Vice-Ministers for Agriculture, Ecology, Science and Technology – considered possible regulatory options and implementation mechanisms. It is anticipated that draft biotechnology regulations for the Russian Federation will be prepared in the course of 1994.

ICGEB continued to collaborate closely with UNIDO on the establishment of a Biosafety Information Network and Advisory Service (BINAS). This service is expected to contribute greatly to the fulfilment of some of the recommendations of Agenda 21 of the Earth Summit and will be launched in the first half of 1994. In addition, arrangements are being made to establish the exact nature of ICGEB's technical support to UNIDO in meeting its mandate as Task Manager for the UN system wide follow-up and reporting on Chapter 16 of Agenda 21 (Environmentally Sound Management of Biotechnology).

Annex 1: ICGEB National Scientific Focal Points (*ICGEB Affiliated Centres)

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