OCCASION

This publication has been made available to the public on the occasion of the 50th anniversary of the United Nations Industrial Development Organisation.

DISCLAIMER

FAIR USE POLICY

CONTACT

Please contact publications@unido.org for further information concerning UNIDO publications.

For more information about UNIDO, please visit us at www.unido.org

UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION
Vienna International Centre, P.O. Box 300, 1400 Vienna, Austria
Tel: (+43-1) 26026-0 · www.unido.org · unido@unido.org

Annex D

20866

International Centre for Genetic Engineering and Biotechnology
United Nations Industrial Development Organization

Collaborative Research Programme

TERMINAL EVALUATION REPORT

UNIDO contract # 92/059

ICGEB ref. #: CRP/ ARG91-03

Project initiation: January 1992

Project termination: December 1994

International Centre for Genetic Engineering and Biotechnology
United Nations Industrial Development Organization

Collaborative Research Programme

TERMINAL EVALUATION REPORT

Part 1

Title of Project

REGULATION OF THE FIBRONECTIN GENE TRANSCRIPTION IN LIVER

Keywords: Transcriptional regulation, fibronectin

UNIDO contract # 92/059 **ICGEB ref. #: CRP/** ARG91-03

Project initiation: January 1992 **Project termination:** December 1994

Principal Investigator's name: Alberto R. Kornblihtt

Affiliate Centre mail address :
INGEBI-CONICET (SECyT)
Vuelta de Obligado 2490, 2°piso
(1428) Buenos Aires - ARGENTINA

Telephone no. 54-1-784-5516/783-2871 **Telex no.**
Fax no. 54-1-786-8578 **Email address** alberto@ingebi.uba.ar

Abstract:

ABSTRACT (as originally stated)

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.

Part 2

OBJECTIVES/METHODOLOGY

(proposed at the time of the submission of the research proposal)

1. **Characterization of the CREB and CAAT binding protein that interact with the FN promoter.** Many different CREBs and CAAT binding proteins have been described. In the case of the CREB/ATF it will be necessary to characterize by oligonucleotide competitions the differences with other CREB/ATFs that interact with the somatostatin CRE. Site contacts determine by methylation interference will be also performed to distinguish this CREB/ATF from others. The interaction of Jun/Fos or Jun/CREB heterodimers with the FN-CRE will be also investigated. A more precise identification of the member of the CREB/ATF family will be done by cross reactivity to antibodies directed to already characterized CREB/ATFs. In the case of the CAAT- binding protein we will perform competitions with the NF-1 oligonucleotide. Sequence identity analyses comparing target DNA sequences for CAAT-binding proteins such as NF-1, C/EBP-1, CP-1 and CP-2 will be carried out. Final characterization will result from the use of specific antibodies and partial purification.

The molecular contacts between the liver CREB/ATF and the CAAT binding protein that bind to the FN promoter will be confirmed by the covalent stabilization of the complex by glutaraldehyde cross-linking.

2. **Perturbation experiments to abolish the CREB/CAAT binding protein interaction.** The binding of the transcription factors to the DNA will be assessed by the techniques of DNaseI footprinting and gel mobility shift. We will test the effect of thermal denaturation and changes in the ionic strength and the pH on the protein/DNA and protein/protein affinities. A different kind of perturbation experiment will consist in separating physically the CRE and CAAT boxes at various distances by intercalating stretches of ds DNA of neutral information. In the FN promoter, the CRE and CAAT elements are separated by just 20 base pairs, i.e., two turns of helix. We plan to separate these elements by the insertion of plasmid sequences of 20, 28 and 44 base pairs, and assess the binding of CREB and CAAT binding protein to these constructs. One should expect to observe conservation of the binding of the CREB to the CRE, but abolition of the binding of the CAAT binding protein to the CAAT box. If, as we expect, increased spacing between CRE and CAAT decreases the affinity of the liver CAAT-binding protein for its target DNA, we will be able to determine the effects of spacing (i.e., simple separation), phasing (i.e., changes in the face of DNA sites on which the factors lie) and looping (i.e., approaching of distant target sites by DNA looping), by inserting segments of integral and non-integral numbers of helix turns between the CRE and CAAT sites.

3. **In vitro transcription of the FN gene promoter.** It will be crucial 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. For this, we will use liver nuclear extracts to transcribe in vitro fusion constructs between wild type and mutant segments of the FN promoter and a the TATA box of the Xenopus albumin promoter using the G-free cassette system (see Methodology). In this way we will analyze the regulatory effect of portions of the FN promoter region upon a neutral promoter. We expect to observe a decrease in the transcriptional activity when we abolish the interaction between the CREB and the CAAT binding protein, either by the physical separation of the two cis elements or by the competition with sequence-specific oligodeoxynucleotides.

4. **Transactivation domain of CREB.** Recent evidence indicates that the transactivation domain of the CREB (i.e., the domain involved in protein-protein interactions with other transcription factors leading to transcriptional activation) resides in a 14-amino acid segment, named alpha peptide, which is excluded from an abundant, widely distributed and inactive variant of the CREB, called ΔCREB, as a result of alternative mRNA splicing. We will test if the antibodies anti alpha peptide are able to affect our CREB/CAAT-binding protein interaction both in binding and in in vitro transcription studies. (continued)

OBJECTIVES/METHODOLOGY (CONTINUED)

5. **Variations with the cell type and status.** We will prepare nuclear extracts from a variety of organs and cells in culture (hepatomas, fibroblasts, brain, kidney, etc.) We will investigate the effect of the cell type, oncogenic transformation and aging on the transcriptional regulation of the FN promoter assessed by binding and in in vitro transcription experiments similar to those presented in 2. and 3. A similar approach will be applied using regenerating liver as the source of nuclear proteins. Changes in the expression of FN have been detected in rat regenerating liver (after partial hepatectomy, Prof. F.E. Baralle, unpublished results). We will assess in this way acute phase effects over the FN promoter activity.

6. **Purification of the transcription factors.** The CREB/ATF and the CAAT binding protein from liver that bind to the FN promoter will be partially purified using HPLC, heparin-Sepharose and affinity chromatography on oligonucleotide-Sepharose columns. The idea is to separate physically the two transcription factors and to perform reconstitution experiments in order to characterize the DNA/CREB, the DNA/CAAT binding protein and the DNA/CREB/CAAT binding protein complexes. These complexes will be analyzed by the "South-Western blot" technique.

7. **Correlation between the FN promoter activity and the alternative splicing of the FN primary transcript.** As said before, liver cells show a specific occupation of the FN promoter and a differential pattern of FN RNA splicing that never includes in the mature messengers neither the exon EDII nor the EDI. It will be interesting to know if there is a correlation between these two events. To test this possibility we will construct vectors carrying the human FN promoter or the spacing mutants described in 2., upstream of an alpha globin/ human FN gene hybrid. The FN portion will be an internal segment of the human FN gene that carries either the EDII exon, its neighboring introns and flanking exons, or a similar gene fragment corresponding to the EDI. With these constructs we will transfect the liver cell line Hep 3B and analyze the type of splicing achieved. Hep3B cells normally exclude the EDII exon from the mature FN mRNA, behaving like normal liver. So, we expect that the construct having the wild type FN promoter will lead to the transient expression of globin/FN mRNA hybrids all lacking the EDII segment. However, we want to test if the constructs in which the promoter has been modified by the separation of the CRE and CAAT boxes change the type of splicing achieved. The rational for this experiments id the following. It is known that alternative splicing is determined by two factors: the existence of certain informative sequences in the primary transcript and by the tissue-specific expression of splicing protein factors that act in trans. Apparently, the sequence determinant allows a particular folding of the primary transcript that is "seen" by the splicing machinery in such a way that results in a particular splicing product. If the primary transcript adopts a different folding, the spliceosome then processes it in a different way, resulting in another splicing variant. What seems to determine which kind of folding will be adopted by the same primary transcript in different cell types is the differential chances of the primary transcript to encounter and bind to RNA-binding proteins that prevent or restrict the spontaneous folding. We believe that transcriptional activity, measure in terms of speed and promoter force, may have deep influence in the steady state kinetics of the primary transcript, which in turn will influence the alternative splicing pathway.

METHODOLOGY

We will use standard techniques in molecular biology and recombinant DNA. The principal investigator has long experience and expertise in this field. Promoter analysis methodologies will be also used together with protein characterization and purification.

Subclonings: Several fragments of the human fibronectin promoter will be subcloned into the SmaI site of the plasmid vector pUC18. The fragments will represent the regions

-223/+45, -155/-55, -221/-156, -1500/-500 and the generated clones will be named pUC-220, pUCCAAT, pUCCRE and pUC-1500 respectively. These clones will be used in experiments of binding of transcription factors present in crude nuclear extracts or partially purified.

Spacing mutants: A series of mutants will be constructed from pUC-220. Essentially, the spacing between the CRE and CAAT sites will be increased by the insertion of polylinker sequences of different lengths: 20, 28 and 44 base pairs. These constructs will be used in binding assays to determine the occupancy affinities of the CRE and CAAT sites by their respective binding proteins.

Oligodeoxynucleotides: A series of double stranded oligodeoxynucleotides will be synthesized to be used both in competition of binding and transcription experiments and as radiolabelled probes for gel mobility shift assays. Most oligodeoxynucleotides will be designed with sticky 5' protruding ends to facilitate their labelling by filling-in with Klenow and their eventual subcloning in plasmid vectors:

FN-CRE 5'AATTCCCCCG**TGACGTCA**CCG 3'
3' GGGGGC**ACTGCAGT**GGCCTAG5'

FN-CAAT 5'GATCCCGGAGCCCGGG**CCAAT**CGGCGCA 3'
3' GGCCTCGGGCCC**GGTTA**GCCGCGTTCAG5'

FN-CRE-CAAT

5'
GATCCACAGTCCCCCT**GACGTCA**CCCGGAGCCCGGG**CCAAT**CGGCGCA3'
3'
GTGTCAGGGGG**ACTGCAGT**GGGCCTCGGGCCC**GGTTA**GCCGCGTTCGA5'

AP-1 5'ACCAGC**TGACTCA**GATGTCCT 3'
3' CG**ACTGAGT**CTACAGGATGGT5'

NF-I 5'TATTTTGGATTGAAGCC**AAT**ATGATAATGA3'
3'ATAAAACCTAACTTCG**GTTA**TACTATTACT5'

Nuclear extracts: Intact cell nuclei will be isolated from perfused rat liver according to Gorski et al, 1986. Nuclear proteins will be extracted from highly purified nuclei following the procedure of Dignam et al, 1983. Protein extracts will be concentrated by ammonium sulfate precipitation, dyalised, frozen in liquid nitrogen and stored at -70°C. When necessary, DNA-binding proteins will be partially purified by affinity chromatography on heparin-Sepharose or specific oligonucleotide-Sepharose columns or by ionic exchange chromatography on HPLC. All manipulations will be performed with buffers containing a cocktail of protease inhibitors: 2mM benzamidine, 0.5mM PMSF, 5μg/ml leupeptin and 5 μg/ml aprotinin.

DNase-I footprinting: Definition of the precise sequences of the FN promoter occupied by transcription factors will be assessed by DNase-I footprinting (Galas and Schmitz, 1978). Specificity of binding will be confirmed by absence of displacement in the presence of a molar excess of poly-dI-dC (unspecific competitor) and by successful competition with sequence-specific ds oligodeoxynucleotides.

Gel mobility shift: Complexes between DNA and transcription factors will be detected by the simple gel mobility shift assay (Garner and Revzin, 1988). This technique is more sensitive than the footprinting and also allows the determination of the DNA contact sites by methylation interference.

***In vitro* transcription**: The transcriptional activities of the wild type FN promoter as well as that of the spacing mutants will be assessed by the *in vitro* transcription system developed by Sawadogo and Roeder (1985). Different variants of the FN promoter, all of them lacking their own TATA, box will be fused to the TATA box of the albumin gene of *Xenopus lævis* leading the transcription of a 380 bp synthetic ds DNA segment lacking guanosines i its sequence (G-free cassette). In this way, if transcription is performed in vitro using nuclear extracts as the source of RNA polymerase II and transcription factors, and in the absence of GTP with one of the other three ribonucleotides radioactive, a single RNA transcript of 380 nucleotides will be observed in denaturing polyacrilamide gels. The intensity of the transcript band will be proportional to both the extract protein and template DNA concentrations and will reflect their efficacy.

Transfections: When possible, transcriptional activity of the wild type and different spacing mutants will be assessed by transfection of stable cell lines with FN promoter-CAT gene constructs. Transfections by the calcium phosphate method and chloramphenicol acetyl transferase assays will be performed according to Gorman et al (1985).

FN promoter-FN mini gene hybrids: The different variants of the FN promoter will be fused to the FN mini genes corresponding to either the EDII or EDI regions. These mini gene constructs have been successfully used by Vibe-Pedersen et al (1984) and Barone et al (1989) to reproduce the alternative splicing of the FN gene upon transfection of different cell lines. The new hybrid construct will be transfected into cell lines of hepatic and non hepatic origins to detect the splicing variants produced quantitatively and qualitatively.

RNA extraction: Total RNA will be extracted from confluent monolayers by the method of Favaloro et al (1980).

Detection of FN mRNA splicing variants: It will be done either by the S1 mapping assay used by Barone et al (1989) or by Northern blotting.

RESULTS

(compare against the set objectives)

I. Original objectives:

1. Characterization of the CREB and CCAAT 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.
6. Purification of the transcription factors.

Results obtained:

Binding of nuclear proteins to the cAMP response element (CRE) and the CCAAT box of the FN gene promoter, which are separated by only twenty base pairs, i.e. two turns of double helix. 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 CCAAT 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 44 bp 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, measured *in vitro* using the G-free cassette system, 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.

By the use of specific antibodies in western blots and "supershift" experiments we showed that the new CRE factor is an heterodimer between a 43 kDa and the "73 kDa" CRE-binding proteins and identify the latter as ATF-2 (also named CRE-BP1), a protein implicated in recruiting transcriptional activators to promoters, able to forms heterodimers with Jun and for which a sequence-deduced MW of 55 kDa had been reported before. This was confirmed in DNA-protein covalent cross linking studies. These results were published in the *J. Biol. Chem.* and in *FEBS Lett.*, and reviewed in the *FASEB J.* (see publications).

We have also begun to characterize the liver factors that bind to the CCAAT box in the FN gene promoter. Preliminary results indicate that both NF-1 and CP-1, but not CP-2 nor C/EBP are among the families of CCAAT proteins involved.

II. Original objective:

4. Transactivation domain of CREB.

Results obtained:

No experiments were performed in this line of research.

III. Original objective:

5. Variations with the cell type and status.

Results obtained:

The liver specific occupation of the CRE and CCAAT sites was commented above. Experiments performed by Dr. Andrés Muro in Dr. Baralle's laboratory at ICGEB-Trieste, as part of this CRP, showed no relevant differences in the binding to this sites using extracts from regenerating liver or liver from aging rats.

(continued)

RESULTS (CONTINUED)

IV. Original objective:

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

Results obtained:

Having characterized the tissue-specific architechture of the FN promoter occupancy and the importance of protein-protein interactions in the FN gene transcription, the task of correlating this with the alternative splicing of the primary transcript becomes daring and exciting. Unfortunately we did not have time to accomplish this objective, but it will be the main project for the renewal of our CRP.

As far as the FN mRNA alternative splicing is concerned, in a collaboration with the Canadian group of Dr. Benoit Chabot, we studied the molecular mechanisms of the ED1 (EDA or EIIA) exon inclusion:

The inclusion of the 270-nucleotide human fibronectin ED1 exon in HeLa cells requires the presence of a centrally located 81-nucleotide exon sequence. We have conducted a series of in vitro experiments aimed at understanding the structural and functional features associated with this splicing enhancer (SE). Using hybrid model pre-mRNA substrates, we showed that the SE element markedly stimulates the use of the 3' splice site of ED1. Deletion and replacement analysis identifies the stimulating sequences as a purine-rich stretch of 9 nucleotides (GAAGAAGAC). The SE element stimulates splicing to the ED1 3' splice site from various positions within the exon except when placed beyond 293 nucleotides downstream from that 3' splice site. The action of the enhancer is not limited to the ED1 acceptor site because the SE element stimulates human beta-globin splicing and also induces the use of a 3' splice site in a prokaryotic sequence in vitro. We have explored the mechanism of action of the fibronectin splicing enhancer and found that the SE element is required for efficient assembly of early splicing complexes, allowing a more efficient interaction of the U2 snRNP with branch site sequences. In competition experiments, an RNA containing mainly SE sequences specifically abolished the action of the SE element, suggesting that factors bind the enhancer element to mediate stimulation of splicing. Using RNA mobility shift assays we show that SR proteins interact specifically with the SE element. These results demonstrate that exon sequences lying in the SE element play a crucial role in specifying splice site recognition through interactions with factors binding to the 3' splice site. These results were published in *Genes and Dev.* (see publications).

V. Results unforseen in the original project

V.I A new and simple method that emerged from our footprinting studies of the FN gene promoter.

The G + A reaction of the Maxam and Gilbert procedure for DNA sequencing can be performed by omitting all steps but the last one (i. e., heating at 90°C the labeled DNA in formamide-containing gel loading buffer) of the original protocol (1), provided that formamide is not kept deionized.

In fact, instead of following the classical G + A reaction, which takes 2 to 3 hr. (incubations, ETOH-precipitations, evaporations, etc.), labeled DNA is directly boiled for 10 min in 2 volumes of "aged" formamide-loading buffer (80% not-deionized formamide, 1 X TBE, 0.1% bromophenol blue, stored at room temperature). Immediately before loading the gel, an aliquot is diluted with 3-4 volumes of normal deionized formamide-loading buffer and boiled again for 10 min. to guarantee complete denaturation.

Besides its application in footprinting studies, this rapid and reproducible method is also useful to confirm the identity of end-labeled DNA molecules of known sequence, for S1-nuclease mapping and for any approach needing single strand-DNA markers of small size differences.

(continued)

Furthermore, our results indicate that it is critical to use thoroughly deionized formamide in the gel loading buffer in DNase I footprinting reactions. Otherwise, a G + A degradation may occur during the final heat-denaturation, after the DNase I digestion step, giving rise to a pattern of bands which superimposes with the DNase I pattern and may mask protected regions, producing artifactual results. These results were published in *Trends in Genetics* (see publications).

V. II. Extending our experience in promoter analysis to an evolutionary study, not related to the FN gene expression.

The major satellite DNA of the subterranean rodent *Ctenomys*, named RPCS, contains several consensus sequences characteristic of the U3 region of retroviral long terminal repeats (LTRs), such as a polypurine tract, CCAAT boxes, binding sites for the CCAAT/enhancer-binding protein (C/EBP), a TATA box and putative polyadenylation signals. RPCS presents an enormous variation in abundance between species of the same genus: while *C. australis* or *C. talarum* have approximately 3 x 10^6 copies per genome, *C. opimus* has none. A sequence (RPCS-I) with identity to the SV40-enhancer core element, present in all the repeating units of the satellite is specifically protected in DNase I footprintings. Competitions of band-shift assays with different transcription factor binding sites indicate that binding to RPCS-I is specific and involves CCAAT proteins related to NF-I, but not to C/EBP. By the use of quantitative protein/DNA binding assays we determined that, despite of their conspicuous difference in RPCS copy number, *C. talarum* and *C. opimus* have equivalent amounts and identical quality of RPCS-binding proteins. These results are consistent with the observation, by *in situ* hybridization, that RPCS is clustered in heterochromatic regions, where it might have restricted accessibility to transcription factors *in vivo*. This is the first report of the binding of transcription factors to a satellite DNA of retroviral origin. It was published in *DNA Seq.* and *Nucleic Acids Res.*, and reviewed in *Rev. Chil. Hist. Nat.* (see publications)

Part 3

Work plan and time schedule (originally envisaged)

FIRST YEAR (1992)
OBJECTIVES:
1. Characterization of the CREB and CCAAT binding protein that interact with the FN promoter. (START)
2. Perturbation experiments to abolish the CREB/CAAT binding protein interaction. (START)
3. In vitro transcription of the FN gene promoter. (START)
4. Transactivation domain of CREB. (COMPLETED)

SECOND YEAR (1993)
OBJECTIVES:
1. Characterization of the CREB and CCAAT binding protein that interact with the FN promoter. (COMPLETED)
2. Perturbation experiments to abolish the CREB/CAAT binding protein interaction. (COMPLETED)
3. In vitro transcription of the FN gene promoter. (COMPLETED)
4. Transactivation domain of CREB. (COMPLETED)
5. Variations with the cell type and status.(START)
6. Purification of the transcription factors. (START)
7. Correlation between the FN promoter activity and the alternative splicing of the FN primary transcript. (START).

THIRD YEAR (1994)
OBJECTIVES:
5. Variations with the cell type and status.(COMPLETED)
6. Purification of the transcription factors. (COMPLETED)
7. Correlation between the FN promoter activity and the alternative splicing of the FN primary transcript. (COMPLETED).

Work plan and time schedule
(actual)

Project landmarks, duration of individual tasks (use bar charts); evaluation criteria (publications, patents, services, training)

FIRST YEAR (1992)
OBJECTIVES:
1. Characterization of the CREB and CCAAT binding protein that interact with the FN promoter. (PARTIALLY COMPLETED)
2. Perturbation experiments to abolish the CREB/CAAT binding protein interaction. (COMPLETED)
3. In vitro transcription of the FN gene promoter. (COMPLETED)
4. Transactivation domain of CREB. (NOT DONE)

SECOND YEAR (1993)
OBJECTIVES:
1. Characterization of the CREB and CCAAT binding protein that interact with the FN promoter. (COMPLETED FOR THE CRE-BINDING PROTEINS)
4. Transactivation domain of CREB. (NOT DONE)
5. Variations with the cell type and status.(COMPLETED)
6. Purification of the transcription factors. (PARTIALLY COMPLETED)
7. Correlation between the FN promoter activity and the alternative splicing of the FN primary transcript. (START).

THIRD YEAR (1994)
OBJECTIVES:
1. Characterization of the CREB and CCAAT binding protein that interact with the FN promoter. (START FOR THE CCAAT-BINDING PROTEINS, RESEARCH CONTINUES)
7. Correlation between the FN promoter activity and the alternative splicing of the FN primary transcript. (START, RESEARCH CONTINUES).

PUBLICATIONS:
See corresponding section.

TRAINING:
Under this CRP, Dr. Andrés Muro completed the experiments for his doctoral thesis, presented in 1992. He obtained the top qualification (10/10). At present, he works as a post doctoral fellow in Dr. Baralle´s laboratory at ICGEB-Trieste.
Four graduate students are now doing their PhD thesis in the laboratory: C. Gustavo Pesce, Claudio R. Alonso, Paula Cramer and Santiago Werbajh.

NETWORKING

Networking with the group of Prof. Francisco Baralle, at ICGEB-Trieste, was aimed to the following set of experiments:

-Study of the liver specific occupancy of the FN gene promoter and transcriptional activity in the aging rat and in regenerating liver.

- Study of the *in vivo* interaction of the CRE and CCAAT sites of the FN gene promoter, by transfecting different hepatic and non-hepatic cell lines with chloramphenicol acetyl transferase constructs carrying wild type and spacing mutants of the FN gene promoter.

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

Progress has been made mainly in the first two subjects. Preliminary results indicate that there are no major changes in the transcriptional activity of the FN gene promoter, assessed by *in vitro* transcription, when using nuclear extracts either from aged rats o regenerating liver, compared to normal rat liver. Transfections of hepatoma cell lines (Hep3B or HepG2) with CAT constructs carrying the CRE-CCAAT spacing mutants show an opposite effect to the one observed in *in vitro* transcription, i.e., increased spacing between the CRE and CCAAT elements seems to increase transcriptional activity. We are currently performing DNase I footprinting experiments in order to explore the molecular bases for the difference observed between the results obtained with *in vitro* transcription with liver nuclear extracts and by transfections of hepatoma cell lines.

The experiments to study the correlation between the FN promoter activity and the alternative splicing of the FN primary transcript are being done currently.

PUBLICATIONS

Numbers in bold indicate articles directly related to the original research proposal.

1. Muro, A.F., Bernath, V.A. & Kornblihtt, A.R. 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 (1992).

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

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

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

PUBLICATIONS (CONTINUED)

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

6. Lavigueur, A., La Branche, H. , Kornblihtt, A.R. & Chabot, B. A splicing enhancer in the human fibronectin alternate ED1 exon interacts with SR proteins and stimulates U2 snRNP binding. Genes & Dev. 7, 2405-2417 (1993).

7. Pesce, C.G., Rossi, M.S., Muro, A.F., Zorzópulos, J. & Kornblihtt, A.R. Binding of nuclear factors to a satellite DNA of retroviral origin with marked differences in copy number among species of the rodent *Ctenomys*. Nucleic Acids Res. 22, 656-661 (1994).

8. Kornblihtt, A.R. La inesperada complejidad de los genes eucariotas. Premio Nobel de Medicina o Fisiología 1993, Phillip Sharp y Richard Roberts. Medicina. Buenos Aires 563-565 (1994).

9. Rossi, M.S.; Pesce, C.G.; Kornblihtt, A.R. y Zorzópulos, J. Origin and evolution of a major satellite DNA from the South-American rodents of the genus *Ctenomys* . (Review article). Rev. Chil. Hist. Nat., in press.

10. Kornblihtt, A.R., Alonso, C.R., Cramer, P., Pesce, C.G., Srebrow, A., Werbajh, S.E. & Muro, A.F. The fibronectin gene as a model for transcription and splicing studies. Submitted for invitation to the FASEB J.

11. Pesce, C.G., Melen, G.J. & Kornblihtt, A.R. Identification of a hidden brake in the 28S ribosomal RNA of the rodent *Ctenomys*.. In preparation.

12. Alonso, C.R., Pesce, C.G. & Kornblihtt, A.R. Characterization of factors that bind to the CCAAT box of the fibronectin gene promoter. In preparation.

Part 4

STATEMENT OF EXPENDITURES

To be filled by ICGEB		To be filled by the Affiliated Centre	
Budgets as per original proposal		Summary of expenditures *	
1) Capital equipment	US$	1) Capital equipment	US$ 12,875
2) consumables	US$	2) consumables	US$ 41,386
3) training	US$	3) training	US$ -
4) literature	US$	4) literature	US$ 5,272
5) miscellaneous	US$	5) miscellaneous	US$ 10,459
TOTAL GRANT	**US$**.....	**TOTAL**	**US$** 69,992

Please itemize the following budget categories (if applicable)

Capital equipment

500 V Power supply
1500 V Power Supply
Dialysis System
Electric homogenizer
Electrophoretic transfer cell
Eppendorf microcentrifuge
Geiger radiation detector
Gel dryer
Perspex radiation protection screens
Sequencing electrophoresis cell
Stirrer-hot plate
Timers
Vacuum pump
Vortex Mixer

Training (provide names, duration of training, host laboratory)

Literature

SUBSCRIPTIONS:
Annu. Rev. Gen.
Cell
Genes & Dev.
Trends Cell Biol.
PNAS
EMBO J.
Nucleic Acids Res.

BOOKS:
Transcriptional Regulation (CSH)

PUBLICATION COSTS:
DNA Seq.
J. Biol. Chem.

* Please do not send invoices, receipts etc.; these should be kept by the Affiliated Centre for future reference and sent to ICGEB upon request.