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I.C.G.E.B. COLLOQUIUM ON: "EUCARYOTIC GENE REGULATION AND EXPRESSION"

FINAL REPORT TO CONTRACT: GE/GLO/89/001 - UNIDO CONTRACT No 90/79

This meeting funded by UNIDO was organized in Greece with the goal of bringing together senior scientists from Affiliated Centers as well as young scientists from the above countries to discuss results on Eucaryotic gene regulation and Expression. Encouragement of scientific collaboration was one of the goals of the meeting. Although no resolutions were adopted the filling on the high quality of the meeting its scientific interest and the benefit due to the exchange of Scientific information was shared by the participants. It was evident that such meeting are valuable and strengthen the Scientific potential of the affiliated centers through information and collaboration. Organizing similar meetings in different affiliated countries as well as keeping up with similar meeting or workshop held at the International Centers was a view shared by the participants.

It is important that UNIDO maintains support on the exchange of Scientific information and encourage collaborative links between different laboratories by such meetings that are in accordance to the philosophy of the establishment of the International Centers and their Networks in the affiliated countries.

I.C.G.E.B. Colloquium on "Eucaryotic gene Regulation and Expression"

Crete, May 22-24, 1990

List of Participants

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I.C.G.E.B Colloquium on:

"Eucaryotic Gene Regulation and Expression"

International Center for Genetic Engineering and Biotechnology, Trieste, Italy

Institute of Molecular Biology and Biotechnology, Heraklion, Crete, Greece

May 22 - 24

Crete, 1990

TALKS' ABSTRACTS

Studies on DNA replication origins of human cells

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The search for replication origins of mammalian cells as autonomously replicating sequences has been so far disappointing. In order to study the sequence requirements and properties of mammalian replication origins, it seems, therefore, advisable to do it in their natural context, namely in the chromosome.

A library of early replicating DNA sequences was constructed by purifying and cloning the DNA synthesized by human HL60 promyelocytic cells synchronized at the onset of S phase. These sequences should be representative of a subset of origins firing at the very beginning of the S-phase. A survey of the overall structural properties of these sequences did not show any distinctive features except for an enrichment in Cot0 DNA.

One of the fragments was used to retrieve adjacent sequences in a human genomic library, and the features of a 137 kb region encompassing the original early replicated fragment were studied in detail. Hybridization experiments with DNA replicated at different S-phase intervals using this region as probe would prove that it is indeed completely replicated within the first two minutes of S-phase. The region is heavily transcribed: at least three transcripts can be recognized in Northern Blot experiments, two of which possibly deriving from alternative splicing of the same messenger. All transcripts point toward the same direction.

A core portion of the region contains a number of outstanding features: five regions of homology to the origin of the human Papova viruses JCV and BKV, three possible thermodynamically stable stem-and-loop structures, a 600bp long CpG-rich region with the properties of an HTF island. Several transcription signals are observable in the CpG-rich region: three putative Sp1 binding sites located within a short range. In the stem-and-loop area: a 9bp sequence 70% homologous to the SV40 and human Ig (K chain) enhancer, and, at one extreme of the HTF island, a sequence which binds specifically a nuclear factor. The 17bp recognized sequence, TTCGTCACGTGATGCCA, which is palindromic (with one mismatch) in the twelve central nucleotides, turned out to be nearly identical to

the upstream element of the Major Late Promoter of Adenovirus 2. Interestingly enough, a very similar sequence has been described as a general cis-regulating element conserved throughout evolution: e.g. as the promoter of the mouse methallothionein I gene, of the rat γ-fibrinogen gene and of the human growth hormone gene. Surprisingly, a similar sequence can also be found in the LTRs fo HIV-1, in yeast centromeres and upstream of two homeobox genes of *Xenopus* and mouse. All these sequnces were proved to complete for the same human factor in gel retardation assays. Oligonucleotides mutated in the core portion of the sequence showed that the central CpG in the core portion of the sequence is essential for factor recognition, and methylation experiments with this dinucleotide proved that its specific methylation prevents the protein binding.

An array of probes of this genomic region hybridizing with DNA extracted at different intervals after entry in S-phase was used to follow the actual movement of the replication fork in the living cells. Preliminary results suggest that a replication origin is present upstream of the site of initiation of transcription and fires bidirectionally.

Initiation of DNA replication in Eucaryotic Cells

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One of the main functional differences between eukaryotic and procaryotic cells is that in the former DNA is functionally divided into discrete replication units called replicons. Replicons replicate according to specific time tables and reinitiation of replication never occurs before the next cell cycle. The most principal question concerning initiation of DNA replication is whether it begins at specific genetically determined replication origins, or at random. Both possibilities have been examined and confusing enough, both have been experimentally supported. Nevertheless, during the very recent 2-3 years the idea that DNA replication in eukaryotic cells begins at permanent genetically determined sites prevailed in the scientific literature over the idea of random initiation.

On the other hand, despite the systematic and persistent effort of many laboratories including ours, we still don't know what exactly these illusive replication origins are like to. In the few cases in which regions of initiation of DNA synthesis were mapped, no specific DNA sequences were found there, and in cases where mixture of different putative replication origins were isolated, they didn't show any specific features and had kinetic complexity similar to that of random DNA. Using a method developed in our laboratory to isolate putative mouse replication origins, we cloned and analyzed 30 of them and didn't found any common DNA sequences. On the other hand about 30% of them were able to bind specific protein factors present in actively dividing cells only. A conclusion is drawn that initiation of DNA synthesis is not absolutely sequence dependent but occurs at areas close to promoter regions which can bind specific protein factors and are often rendered single-stranded due to their active transcription.

Histone H1 and regulation of transcription of Eucaryotic Genes

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Nowadays it is known that histone H1 plays a role in the formation of chromatin structure both at the level of the nucleosome particle itself and at the level of the higher-order structures. Histone H1 is regarded as a part of a general repression of tissue-specific genes. In addition to serving a general repressor function for relatively involved in a finer control of individual gene transcriptional activity. In this lecture the recent data concerning the role of H1 in transcriptional control and the possible mechanisms through which such a role can be exerted with be discussed. An attempt will be made to evaluate the existing data in favour of one or another mechanism and also to point out some issues that would be difficult to reconcile at present with the existence of a given specific mechanism.

The enhancers and promoters of the *xenopus laevis* ribosomal spacer have a distinctive non-nucleosomal structure on active and inactive genes.

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The chromatin structure of the *X. laevis* ribosomal gene spacer and flanking gene regions have been studied by nuclease protection and indirect end-labelling. In erythrocyte chromatin the gene regions and the region upstream of the first spacer promoter were found to be nucleosomal and highly protected, while the spacer promoters and enhancer repeats were non-nucleosomal and sensitive to both DNAase I and micrococcal nuclease cleavage. The repeated spacer promoters were found to be hypersensitive to micrococcal nuclease, both at their 5´ boundaries and at their initiation sites (± 30bp). In tissue culture cells the gene regions and the whole of the spacer were non-nucleosomal, suggesting most ribosomal genes were actively transcribed. The spacer promoter and enhancer repeats however retained the distinct digestion kinetics seen in erythrocytes.

Among other things, we conclude that the spacer promoters and enhancers of both the inactive erythrocyte and the active tissue culture genes have the same or similar chromatin structures.

Structure and Expression of human hnRNP protein A1 gene

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Heterogeneous nuclear RNAs (hnRANs) are associated in the nucleus of mammalina cells with specific proteins to form ribonucleoprotein complexes called hnRNP particles. These indications of their possible involvement in the splicing process. We have concentrated our attention of hnRNP protein A1 that is one of the most abundant component of the complex. Protein A1 is structurally and antigenically related to other basic hnRNP proteins (type A and B). A1 cDNA cloning and sequencing revealed a peculiar two-domain structure with the RNA-binding comain (N-terminal 195aa) linked to a glycine-rich C-terminal domain (125aa) probably involved in protein-protein interactions.

20-30 A1 specific sequences are detectable in the human genome. Most of these sequences correspond to inactive speudogenes of the processed type. One active gene was selected and completely sequenced. It extends over 4.6 Kb and is split into 10 exons with one exon exactly separating the two structural domains. 700 bp upstream of the transcription start site were also sequenced and shown to contain a very strong promoter as judged by CAT assay. Further analysis of the A1 gene revealed the existence of an alternative splicing event resulting in a second A1 specific mRNA containing an extra exon of 156 nt in the 3'-end portion. The translation product of the second A1 mRNA is a protein of 38 kd (named $A1^B$) with a larger glycine-rich portion. The $A1^B$ protein corresponds to a minor protein of the hnRNP particles previously identified as B2 by some authors.

Alternative splicing of the A1 mRNA seems to be regulated in a tissue-specific fashion.

These and other results indicate that many hnRNP proteins could be produced by the differential splicing of a limited number of mRNAs. Such diversification mechanism rises interesting questions on the role of hnRNP complexes in RNA processing.

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The regulation of the potato proteinase Inhibitor II gene expression

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The proteinase inhibitor II gene family is constitutively expressed in tubers and in young floral buds of potato plants. Mechanical wounding triggers the accumulation of its mRNA in the aerial parts of the plant. This activation of the gene expresion is not restricted to the tissues in the immediate vicinity of the wound site but rather leads as well to its accumulation in the non-wounded, systemically induced, aerial organs.

The phytohormone abscisic acid (ABA) mediates the wound induction of the proteinase inhibitor II gene. Proteinase inhibitor II mRNA accumulation is detected after spraying potato with its involvement in the wound response, the endogenous ABA levels increase 2 to 3 fold upon wounding, both in the directly injured as well as in the systemically induced leaves. To provide further support for the involvement of ABA in the wound induction we have aken advantage of the ABA-deficient potato (droopy) and tomato (sit) mutants which show reduced levels of the hormone upon water stress. The proteinase inhibitor II gene is not activated by wounding in these mutant plants. However, ABA treatment leads to its accumulation thouthout the aerial organs in both droopy and sit mutants.

A reporter gene (GUS) was cloned into a proteinase inhibitor II cassette, consisting of a 1.3 kb promoter fragment and a 0.27 kb fragment containing the polyadenylation signals, and subsequently transferred to both tobacco and potato by Arobacterium-mediated techniques. This plants, thus following the expression pattern of the proteinase inhibitor II gene family. Hstochemical analyses of the transgenic plants show the proteinase inhibitor II promoter driven GUS activity being strongest in the mesophyll cells close to the vascular tissue.

Deletion analyses of the proteinase inhibitor II promoter were carried ot using transgenic tobacco plants. A 800 bp promoter drives wound-inducible activity of the fused CAT gene, which is several fold weaker than the activity shown in plants transformed with the 1.3 kb promoter. The CAT activity from a 550 bp promoter is below the detection limits, thus indicating the presence of elements in the 5´ upstream region required for gene expression. When the CaMV 35S enhancer is fused to these proteinase inhibitor II truncated promoters,

wound-inducible CAT activity is recovered with the 550 bp and in some cases with a 440 bp promoter. Plants transformed with a 210 bp promoter construction display only constitutive CAT activity. Moreover, a promoter fragment from -1300 to -195 is able to confer in both orientations wound-inducible activity to an otherwise inactive 35S truncated promoter-CAT gene construction, thus displaying features of an enhancer element. These results suggest that sequences downstream of -195 are not required for the wound induction of the proteinase inhibitor gene.

To gain an insight into the mechanisms of the proteinase inhibitor II wound response we have searched for protein factors acting on the cis-regulatory sequences responsible for the wound induction. For this purpose we have divided the proteinase inhibitor II promoter fragment in subciones suitable for analysis of DNA-protein interactions by the gel retardation assay. Protein-DNA complexes are formed with some of the fragments used. The binding activities remain constant upon wound induction and are also present in nuclear extracts from potato tubers. The interaction of a nuclear protein to an upstream element has been further characterized by DNAse I footprinting and DNA methylation interference experiments. The sequence requirements of the interaction have been determined from the binding properties of a series of mutated oligonucleotides. The protein binds to the sequence GAGGGTattttCGTAA where mutations at the nucleotides indicated with lower case letters do not disturb the binding specificity. Deletion analysis of the promoter points to a constitutive enhancer function for this upstream element.

Expression, processing and targeting of modified 2S albumin storage proteins.

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2S albumines are small, water soluble proteins present in large amounts in the seeds of dicot plants. The isolation and characterization of the *Arabidopsis thaliana* 2S albumin genes revealed four tightly linked genes. The specific probes and *in situ* hybridization showed different spatial and temporal transcription profiles. 2S albumin seed storage proteins undergo a complex series of post translational proteolytic cleavages. Expression of a complete *Arabidopsis* 2S albumin gene in transgenic tobacco shows tissue specific and developmentally regulated expression. Protein sequencing and immunocytochemical studies demonstrated that the *Arabidopsis* 2S albumin is correctly processed and targeted to the protein bodies. Modified versions of the *Arabidopsis* 2S albumin gene encoding more methionine or containing synthetic peptide encoding sequences were constructed and expressed in different transgenic plants, resulting in the accumulation of the modified protein in the transgenic seeds.

Regulation of the transcription of a wheat chlorophyl α/β binding protein gene

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The expression of the major chlorophyll a/b binding protein gene (Cab-1) of wheat is under multiple regulatory control: (i) it is light induced, light induction being mediated by the photoreceptor phytochrome; (ii) it is tissue specific (leaf specific), and (iii) it is modultaed by an endogeneous circadian clock. The aim of the experiments presented here was to identify the cis-regulatory sequences of various regulatory signals. To this end various deletion mutants and other gene cost·acts were prepared *in vitro*, transferred to tobacco plants via *Agrobacterium* trnasformation, and the expression of the transgenes was evaluated by S_1 protection experiments.

We identified two positive (enhancer-like) and one negative elements for light induced expression. We also located two positive regulatory elements for leaf specific expression, one of which overlaps with one of the light-inducible enhancer-like elements, and one root-specific silencer. For circadian cotnroi one, presumably negative regulatory element was identified. *In vitro* footprinting experiments revealed five distinct protein binding sites along the Cab-1 promoter the position of which is in good correlation with the cis-regulatory sequences identified above.

Cell type specific trans-acting factors are involved in alternative splicing of human fibronectin pre-mRNA

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ED-A and ED-B are facultative type II homologies of fibronectin, encoded by alternatively spliced exons, in man and in rat. A hybrid α-globin-fibronectin minigene containing the ED-A and ED-B regions from the human gene has been transfected in human cell lines derived from various tissue, in order to study the processing of the generated precursor RNA in the different cell environments. In most tested lines the pre-RNA is alternatively spliced and produces mature RNAs, with and without the ED-A or ED-B exon, in different ratios that closely resemble the corresponding endogenous firbronectin RNAs. In a hepatoma cell line, Hep 3B, only one class of RNA is produced, in which the AD-B exon is absent; the same pattern of splicing is observed in liver. The data show that all the information required to produce accurate and regulated alternative splicing of the ED-B exon is contained in the fragment used and cell specific factors are necessary for the pre-RNA to be differentially spliced in the various cell lines. In contrast, expression in Hep 3B of the minigene containing the ED-A segment failed to reproduce the liver specific splicing pattern. Therefore regulation of ED-A processing is likely to involve different mechanisms to those responsible for control of ED-B splicing. The physiological variations in differential expression of ED-A, ED-B and IIICS regions of rat fironectin pre RNA during embryonic development and aging in several tissues will be discussed.

Translational regulation of GCN4 mRNA and the control of protein synthetic rates in Yeast.

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In yeast, amino acid limitation initiates a signalling cascade that results in the reduction of overall protein synthetic rates and the concomitant activation of the translation of the GCN4 mRNA. The resulting increase in the amount of the GCN4 protein enhances the transcription of amino acid biosynthetic genes. The GCN1, GCN2, and GCN3 proteins are nodes in this signalling pathway that has as an end result the modification of the protein synthetic machinery. The step in protein synthesis that is affected is the formation of 43S preinitiation complexes. All available information suggests that the mechanism responsible for this translational activation resides on an increased ability of scanning 40S ribosomal subunits to reinitiate at the GCN4 coding AUG. This occurs only for those 40S subunits that have participated in the translation of the 5' most proximal ORF (ORF1) located in the 5' UTR of the GCN4 mRNA. We believe that recharging of those subunits with met-tRNA is the regulated step.

In order to verify directly the proposed mechanism, we have initiated an in vitro approach. We have established the conditions through which a yeast cell free extract can elicit translational regulation of the GCN4 mRNA that mimics the in vivo situation: ORF1 dependent translational activation and at the same time a reduction in the rate of 43S formation. Using this system we have shown that a more proximal signal for this translational activation is uncharged tRNA molecules.

The importance of uncharged tRNA molecules as signals in this system has been verified in vivo. We have shown that overexpression of a yeast gene encoding for $tRNA^{val}$ results in the translational activation of the GCN4 mRNA translation. This tRNA molecule neither can be charged in vivo nor does it affect the function of valyl-tRNA synthetase. Furthermore, its presence in high amounts relates directly a signal to the protein synthetic machinery, since it can bypass the requirements for the function of the GCN1, GCN2 and GCN3 gene products which are nornaly required for the signal transduction pathway. With the hope of

identifying the primary process modified through the action of uncharged tRNA molecules we are currently analysing the nature of isolated mutant strains that cannot respond to increased dosage of this tRNA.

One major node in the signaling cascade is the GCN2 protein which is involved in the modification of the protein synthetic machinery. GCN2 is a serine/threonine protein kinase. It is autophosphorylated in vitro and copurifies with the ribosomal fraction. Biochemical approaches to identify the substrate of this kinase have not been succesfull. Genetical approaches have revealed novel properties for this protein that result from mutations mapping at the ATP binding domain. These mutant proteins render cells resistant to amino acid analogs independently of the GCN4 protein. The involvement of the GCN2 protein kinase in additional or parallel functions is currently under investigation.

Finally, survival of of yeast cells under severe amino acid limitation conditions, depends as well as on the function of the GCN5 and the CDC4 gene products. gcn5 and cdc4 strains are unable to grow under such conditions although the translational regulation of the GCN4 mRNA and the resulting transcriptional activation of amino acid biosynthetic genes is not inpaired. Recent evidence indicates that these gene products could be involved in the recovery of cells from the side effects of the amino acid starvation shock.

Erythroid genes expression in anemic rat tissues

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The Belgrade b/b rat has an autosomal, recessive mutation which in homozygous state induces severe anemia. Anemia syndrome results from iron transport deficiency as a basic defect, with pleiotropic consequences. It is manifested phenotypically by red cell hypochromia, microcytosis, reticulocytosis, splenomegaly and low hemoglobin.

We analyzed, by dot blot hybridization, "b" mutation effect on transcription of some erythroid specific genes: β major globin, α globin and anion transporter protein. The amount of these mRNA is measured in total RNA of reticulocyte, bone marrow, spleen and brain (as a control), from normal (B/B), heterozygous (B/b) and anemic (b/b) rat.

Large amount of globin transcripts detected in anemic spleen and heterozygous spleen and reticulocytes, is a result of a) extensive erythropoesis in homozygote (increased percentage of erythroid cells in spleen), and b) "b" gene activated compensatory transcription of globin genes.

Oct-4: a germline-specific transcription factor mapping to the mouse *t* - complex

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Members of a family of murine octamer-binding proteins interact specifically with the octamer motif, a transcription regulatory element found in the promoter and enhancer regions of many genes[1,2].

Oct-4 is a maternally expressed octamer-binding protein encoded by the murine *Oct-4* gene[3]. It is present in unfertilized oocytes, but also in the inner cell mass and in primordial germ cells. The ectopic expression of Oct-4 in HeLa cells is sufficient for transcriptional activation from the octamer motif, indicating that Oct-4 is a transcritpion factor. Therefore, Oct-4 is the first trancription factor described specific for the early stages of mouse development. The spatial and temporal expression pattern was further determined using *in situ* hybridization. Before gastrulation Oct-4 expression was found in the embryonic ectoderm. Between day 8 and 8.5 p.c. expression decreased dramatically and was restricted at day 8.5 to the primordial germ cells which were identified at the base of the allantois by histological alkaline phosphatase staining. The primordial germ cells migrate from the base of the allantois to the genital ridges where they were easily detected both by their alkaline phosphatase activity and their Oct-4 expression. Therefore Oct-4 is a transcription factor specifically expressed in cells participating in the generation of the germline lineage. Linkage analysis using BxD recombinant inbred mouse strains demonstrates that Oct-4 maps to the distal regicn of the mouse *t* - complex in or near the major histocompatibility complex. Several, mouse mutants affecting blastocyst and embryonic ectoderm formation map to this region. Correlations between the Oct-4 expression pattern and the phenotype of certain *t* - haplotypes lethals are discussed.

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2) Schöler, H.R., Balling, R., Hatzopoulos, A.K., Suzuki, N. and Gruss, P. (1989) *EMBO J.* **8**, 2551-2557.
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Immunoglobulin gene expression

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Rearrangement of immunoglobulin (Ig) gene segments in lymphoid cell brings a V gene promoter into the activational distance of enhancer elements located in the J-C introns of both heavy and K light-chain genes. Recently, additional enhancer elements have been identified downstrieam of both K lightc-hain and heavy-chain constant region genes; the presence of additional enhancer elements in these loci invites a re-evaluation of the role of the intron enhancer in regulating Ig gene expression. The immunoglobulin enhancers are functional only in lymphoid cells and composed of several motifs. A detailed analysis of the Ig heavey-chain J-C intron enhancer demonstrates that the multiple motifs are functionally redundant. Lymphoid specificity of the heavy chain J-C intron enhancer can be attributed to at least two motifs which are preferentially active in lymphoid cells. Combinatorial interactions between certain motifs can create either lymphoid-specific elements or elements of more widespread activity, demonstrating the importance of such interactions in determining transcriptional cell-type specificity.

The Regulation of the Human Beta-Globin Domain

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The dominant control region of the human β-globin locus has been found to be necessary for the high level expression of the genes of the β-globin cluster. The action of this region has been studied by a variety of different approaches. Constructs containing the human α, β and γ-globin genes in different combinations, have been introduced into transgenic mice and the parameters affecting developmental regulation ahve been examined.

By injecting human α and β^s genes it has been possible to generate transgenic mice expressing sickle hemoglobin and showing symptoms of sickle cell anemia. The DCR has also been subjected to functional dissection and these functional studies will be discussed in conjunction with data on the binding of protein factors to the region.

Major Histocompatibility Class II gene regulation

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MHC Class II surface proteins are important determinants of the immune response through their participation in antigen presentation and Thymic education of T lymphocytes. MHC expression is restricted to the B lymphocyte lineage during mature stages of their differentiation and in addition is controlled by lymphokines in various cell types (macrophages, dendritic cells, fibroblast, etc).

In order to understand the molecular basis of MHC expression- a prerequisite to further study normal immune regulation and its aberrations i.e. in Autoimmunity - we have studied extensively the promoter region of Eα and Eβ mouse genes.

Multiple control elements have been identified that regulate constitutive expression in B lymphocytes and partly overlap with additional response elements that mediate transcriptional activation by IFNγ and TNFα.

Using mouse placental cultures we have observed Class II gene induction by IFNγ and 5′ Azacytidine, acting upon distinct cell populations. Interestingly, 5-Azacytidine when given *in vivo* to pregnant mice caused embryo malformations and abortions, an action suggested to involve immune reaction due to aberrant class II gene expression.

Response to lympho-cytokines is mediated by, *in vivo* titratable, *trans* acting factors. Furthermore cooperation between adjacent and possibly distal regulatory regions is shown by oligomerization of various segments that are unable to function when used in a single copy form.

We have shown binding of protein factors using nuclear extracts from various cell types to selected sites within the gene regulatory region. Screening of λgt11 libraries by target oligonucleotide binding, has led to the isolation of a group of cDNA clones that are currently under structural characterization and analysis for their effects on class II gene expression.

Poly/ADP-ribosylation in chromatin structure, differentiation and carcinogenesis

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There is compelling evidence showing that repair of damaged DNA depends on the synthesis of poly (ADP-ribose) molecules at specific sites on histones and other proteins in nuclei of injured cells. Additional evidence links this modification to DNA replication, sister chromatid exchange integration of foreign DNA into host chromosomes and chromain remodeling in development. All these nuclear processes involve the formation of DNA strand breaks. In turn, DNA strand breaks activate the otherwise inactive enzyme poly (ADP-ribose), polymerase, responsible for this modification. Our laboratory has studied the relationship between strand breaks and histone poly (ADP-ribosylation) and determined the molecular structure of poly (ADP-ribosylated) histone H2B [PNAS (1989) 86, 3499-3503]. We have identified histones as the major acceptors of poly (ADP-ribose) after DNA methylation-damage of cells in culture [EMBO J (9186) 7, 57-67] and found histone hyper (ADP-ribosylation) to take place after stimulation of human lymphoid cells in culture with a phorbol ester of PHA [Exp Cell Res (1990) 187, 77-84]. We have proposed that poly (ADP-ribose) assembles histones into octamers facilitating formation of new nucleosomes at the replication fork (JBC, 1990, in press]. Analysis of poly (ADP-ribose) molecules on 100cm-long DNA sequencing gels of polyacrylamide permits us to determine its size and concentration. Treatment of cells in culture with cell cycle blockers (hydroxyurea, ara-C, BrdU, Butyrate, colcemid) or nicotinamide followed by DNA methylation-damage reveal a synergism between selective pairs of drugs in lowering total protein poly (ADP-ribosylation) in response to DNA methylation-damage. These data permit us to uncouple the effect of replication from repair on poly (ADP-ribosylation). Studies on chromatin and nucleosome structure using zero length carbodiimide crosslinking reveal an opening up of nucleosomes into H2A-H2B dimers and $(H3\text{-}H4)_2$ tetramers to taking after histone mono (ADP-ribosylation) only in specific chromatin fractions associated with the nuclear matrix but not in bulk chromatin. Attachment of longer chains of poly (ADP-ribose) too histones has more dramatic effects on chromatin structure, causing the complete dissociation of histones from the DNA.

Expression of endogenous and transforming genes of tumour necrosis factor in human cells

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The human tumor necrosis factor alpha (TNF) is a lymphokine with significant antitumor potentials. It is produced as a membrane bound precursor protein of 26 kDa. We are interested in the posttranslational processing of TNF and the antitumor activity of the precursor forms. Several cell lines showing different tissue specificities protease pattern and growth character (e.g. HeLa, WISH, F4, etc.) were transformed with DNA constructions containing the natural or recombinant TNF gene. TNF producing clones were selected and the ration of preTNF to TNF was established. While some of the cell lines produce large amounts of TNF with only trace amounts of preTNF, plasma-membrane of others harbor relatively high percentage of the precursor. Site directed (point) mutations in the 3rd exon of TNF gene cause the accumulation of significant amounts of preTNF in the plasma membrane of HeLa cells.

The usefulness of TNF in tumor therapy is limited because serum proteins inactivate the circulating TNF so high doses are required to achieve cytotoxic effects. On the other hand, serious side effects accompany the administration of these high doses. Since vesicle bound preTNF is not inactivated by serum proteins the cytotoxic activity of the precursor form of TNF seems to be of significant pharmacological importance.

Structural aspects of protein-nucleic acid interactions

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ROP is a small dimeric protein that participates in the mechanism which controls the copy number of plasmid of the ColE1 family, via an interaction with two complementary RNAs. We report a model for the protein-RNA interaction based on the 3-dimensional structure of ROP and data obtained by site-directed mutagenesis.

UV Laser-induced crosslinking of proteins to DNA: a tool for studying the Eucaryotic gene regulation and expression

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Irradiation of nuclei and whole cells with UV laser was recently shown to crosslink proteins to DNA (Angelov et al., Nucl. Acids Res., 16 (1988) 4525-4538; Stefanovsky et al. Nucl. Acids Res. 23 (1989) 10069-10081). The advantages of the method over the other crosslinking techniques as well as some characteristics of the laser crosslinking are briefly discussed. The implications of the method for studying *in vivo* existing protein-DNA interactions are illustrated by following the binding of histones to the coding regions and the regulatory sequence motifs of individual genes.

The experimental strategy consists in: 1) irradiation of isolated nuclei by picosecond UV laser, 2) isolation of the crosslinked protein-DNA complexes by antibodies to the different histone species to select DNA fragments carrying a given histone and 4) identifying of selected DNA sequences in the immunoprecipitated DNA by hybridising to specific probes.

It was found that the highly transcribed ribosomal genes from *X. laevis* embryos were associated with core histones as were the silent genes from erythrocytes, whilst the content of histone H1 is reduced. Similar data were obtained for the enhancer-promoter regions of the *X. laevis* ribosomal spacer: sequences were found to contain normal amounts of core histones and twice less histone H1 as compared to bulk DNA.

Isolation of the chicken middle-molecular weight neurofilament (MF-M) gene and characterization of its promoter

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We have isolated and sequenced genomic DNA clones covering the coding region of the chicken middle-size neurofilament (NF-M) gene and 1 kb of its 5′ upstream region. The NF-M gene contains two introns which both are located within the highly conserved C-terminal region of the rod domain. The 5′ end of the corresponding mRNA was assigned to a G residue 40 nucleotides upstream of the translation start site and in appropriate distance from a potential TATA box. To functionally analyze the NF-M promoter, constructs carrying 112, 222, and 1026 nucleotides of the 5′ upstream region in front of a luciferase reporter gene were tested for their capability to direct luciferase expression after transient transfection into various cell lines. Significant luciferase activity was recorded both in rat phaeochromocytoma (PC12) cells and murine fibroblasts. In PC12 cells, in which neurite outgrowth is induced by nerve growth factor (NGF), expression was stimulated up to 13-fold within 3 days of NGF treatment. This closely resembles expression of the endogenous NF-M gene in response to this hormone.

Synapsin II: Neuron-Specific phosphoprotein, its cloning and expression

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Synapsin II is a neuron-specific phosphoprotein composed of two highly homologous polypeptides, Synapsin IIa and Synapsin IIb. Together with Synapsin I (Ia and Ib) it represents a family of phosphoproteins that coat synaptic vesicles and are believed to function in regulation of neurotransmitter release.

Rat brain cDNA libraries have been screened with antibodies and oligonucleotide probes and two groups of cloned, for IIa and IIb, have been obtained. Blotting experiments with RNA showed the existence of two polyA-RNA species in the brain and confirmed their tissue-specific, expression. Sequence analysis revealed the complete homologies of 5′ non-coding and most of the coding region among IIa and IIb mRNA. Taken together with Southern blot analysis, these results showed that Synapsin II is encoded by a single-copy gene whose primary transcript is differentially spliced into two mRNA for Synapsin IIa and Synapsin IIb.

The revealed pattern of Synapsin II gene expression thus represents another example of alternative splicing being the mode of amplifying gene expression plasticity in the brain.

POSTERS' ABSTRACTS

Initiation of DNA replication in the amplified dihydrofolate reductase domain in CHO Cells

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In an attempt to localize the origins of replication in the amplified dihydrofolate reductase (DHFR) domain in CHO cells we blocked the progress of replication forks emanating from origins at the beginning of the S phase by introduction of trioxsalen crosslinks at 1 to 5 kb intervals in the parental double stranded DNA. The small DNA fragments synthesized between crosslinks and centered around replication origins were then used as hybridization probes on digests of cosmids and plasmids from the DHFR domain. These studies suggested that DNA replication initiates at two sites in a 28 kb replication locus. The two initiation sites are separated by 22 kb and lie between the DHFR gene and a second transcription unit.

The downstream site contains unique (average) DNA while the upstream site contains repetitive sequence elements. A 3 kb fragment containing the 0.49 kb Pvu II restriction fragment coinciding with the upstream initiation locus has been sequenced. A notable feature of the initiation site is that it contains an Alu element and a long run of thymidines mapping just 5′ to the Alu element. In the region 3′ to the Alu element there are several clusters of A-rich tracts appearing at approximately 10bp intervals that flank a pair of inverted sequences.

Diagnostics of Cystic Fibrosis by DNA Amplification for detection of major CF mutation and by RFLPs analysis

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Fifty two subjects from sixteen Yugoslav CF families were typed for major CF mutation -3bp deletion ΔF_{508} and for RFLPs detected with tightly linked probes metH, metD, J3.11, KM19 and XV2c.

PCR was used for ΔF_{508} detection Genomic DNA samples from patients and their parents were amplified with oligonucleotide primers C16B and C16D, flanking ΔF_{508} deletion and products were analysed on polyacrylamide gels.

In ten of analysed families CF children were homozygous for ΔF_{508} mutation. In such families this rapid nonradioactive method can be applied for accurate prenatal diagnosis and carrier detection.

Four other patients were heterozygous for ΔF_{508}, and two had no ΔF_{508} chromosomes. In such cases RFLP typing provides powerful diagnostic system for both carrier detection and prenatal diagnosis.

A new putative protein kinase in yeast

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An oligonucleotide, corresponding to a conserved region in v-mos oncogene, was used as a hybridization probe. One or two discrete bands were identified on an yeast Saccharomyces cerevisiae genomic Southern blot using different restriction enzymes. Several positive clones were isolated from an yeast genomic library. One of them was further characterized. The sequence analysis revealed an open reading frame coding for more than 100 aminoacids. A comparison with the protein data bank showed partial homology to a number of eukaryotic proteins, a significant part of them (more than 30%) being protein kinases. This raises the possibility that our clone corresponds to a novel yeast protein kinase. A sequencing work in progress would elucidate this possibility, as well as the extent of homology to v-mos oncogene.

Mutational analysis of HTLV-I tax

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The human T-cell leukemia virus type I (HTLV-I) encodes a 40 kD protein known as tax, which activates the proviral long terminal repeat (LTR) and also several cellular genes (e.g. IL-2, IL-2α, c-fos), suggesting that tax plays a role in the development of adult T-cell leukemia. Transcriptional activation by tax occurs in trans, without direct binding of the protein to DNA. Similar trans-activators were found in other retroviruses like bovine leukemia virus (BLV) and HTLV-II.

These tax proteins contain a highly conserved cystein-rich N-terminal region. In this region, single amino acids of the HTLV-I tax were substituted using oligonucleotide-directed mutagenesis, leading to a dramatic decrease in trans-activation on the HTLV-I LTR. The same result was obtained with a deletion mutant, lacking the 69 C-terminal residues of the protein's 353 amino acids and with a hybrid protein containing amino acid residues 1-26 of the BLV tax and 60-353 of the HTLV-I tax. Evidence was found, that the point mutated proteins as well as the deletional protein were still able to bind to ceilular transcription factors mediating the activation of the LTR by native tax. However, the hybdrid protein lacked this ability. The present results suggest that:

(i) amino acid residues Asp-27, Cys-29, Ary-39, Cys-49 and the region between amino acid residues 285-353 are crucial for the function of tax as a trans-activator of the proviral LTR,

(ii) binding of tax to cellular transcription factors is not or not completely depentent on these amino acids and

(iii) the N-terminal region from residues 1 to 59 as a whole is crucial for both trans-activation of the LTR and binding of tax to cellular transcription factors.

Cloning, expression and mutagenesis of the cystein proteinase inhibitor Stefin B

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Stefin B is a cystein proteinase inhibitor, belonging to the stefin family of cystatins. It is present intracellularly in most of the tissues of the human and probably protects the cell against the uncontrolled proteolysis. We have synthesized the gene coding for the stefin B. Expression of the active inhibitor with the amino acid sequence of the natural inhibitor was done in E. coli using expression vector pKP1500. Protein was purified and its identity was confirmed both immunologically and by the determination of the N-terminal amino acid sequence. Variants of human stefin B were constructed by cassette mutagenesis. VAl^{47} as the constituent of highly conserved QVVAG sequence was substituted by hydrophobic amino-acids of increasing size-Ala, Ile and Phe. Recombinant proteins were expressed in E. coli and Ki values for the inhibition of papain were determined. Substitutions did not cause a major change in Ki value and we conclude that the interaction with the proteinases is not the reason for the conservation of the pentapeptide. Recently the crystal structure of the complex stefin B: papain prepared from our recombinant inhibitor was determined.

Storage protein expression in alfalfa embryos

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The electrophoretic profiles of proteins soluble in 0.2 M NaCl (7S) and in 1M NaCl (11S) extracted from seeds, (zygotic embryos) and somatic embryos of different alfalfa species species (M. falcata 47_1, M. sativa 2 and M. arabica).

The results obtained, reveal species' specificity of those proteins and confirm that somatic embryos accumulate lower quantity of storage proteins in comparison to zygotic embryos. The one-dimentional electrophoretic profiles (SDS-PAGE) of 7S and 11S of M. Falcata 47_1 were identical unlike the profiles in the other species investigated. There are no notable differences of electrophoretic profiles of 0.2 and 1 M NaCl soluble proteins at different stages of development of the somatic embryos, i.e. stage-specific proteins. The quantity of named proteins is lower in the globula stage and the higher-in cotyledons. The electrophoretic pattern in somatic embryos is more complex that in zygotic embryos. The presence of 1% PEG in culture medium induced synchronization of somatic embryo development and changes of electrophoretic profile of 0.2 M and 1 M NaCl-soluble proteins mainly between torpedo and cotyledon stages.

Nuclear Matrices from transcriptionaly active and inactive plant cells

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The topological organization and functioning of eucaryotic DNA gets a new dimension in the last 10 years with the intensive investigations of the nuclear matrix. It became evident that the nuclear matrix participates in such vital processes as replication and transcription of DNA, binding of regulatory proteins and hormones, etc.

In the same time there are almost no data about the nuclear matrix in plant cells. In connection with this we have undertaken a series of investigations. Here we show the morphology and the protein composition of nuclear matrices with and without DNA, from dry embryo (transcriptionaly inactive tissue) and from meristematic parts of the roots (transcriptionaly active tissue) from maize. These preliminary results are the first step to search for the elements that anchor chromosomal loops for localizing the matrix associated regions (MARs) in plant nuclei in transcriptionaly active and inactive tissues.

Characterization of a cDNA clone, probably encoding copper binding protein in *Nicotiana plumbiginifolia*

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A cDNA clone, hybridizing with the *Arabidopsis* gene for copper binding protein was isolated from cDNA library of *Nicitiana plumbiginifolia* in pUC18. The clone was sequenced by the dideoxy method of Sanger. The computer analysis of the sequence shows that block homologies between this clone and the *Arabidopsis* gene exist.

Structural changes in chromatin of H8P 70 Genes upon transcription

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Using "protein-image" hybridization technique combined with various cross-linking methods for formaldehyde-prefixed muclei we have analysed changes induced by activation in the chromatin structure of hsp 70 genes. From the cross-linking data it follows that chromatin of actively transcribed genes undergoes some structural rearrangements resulting in certain weakening of the contacts between DNA and the globular parts of histones so that the histones remain bound to DNA through their N-terminal regions. In addition, two specific regions with a reduced content of histones have been found: the 5′ promoter of hsp 70 gene and a region distanced by approximately 1kb from the 3′ end of the gene.

Human β1 Interferon Gene Expression in NIH 3T3 Cells in Culture

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The human structural β1 interferon gene with the 350 bp 5′ flanking region has been introduced into a simian virus (SV40) hybrid plasmid vector. The Interferon gene was inserted in each of the two possible orientations with respect to the SV40 polyA site: The Y-ZG in the right orientation and the GZp in the opposite one.

These vectors have been introduced into cultured mouse NIH 3T3 cells by DNA transfection induced by polyI-polyC, and superinduced by polyI-polyC in the presence of cycloheximide.

In our experimental model, human interferon gene is silent without induction, and expresses 32 U/ml of interferon in induced cells regardless of the presence of SV40 3′ untranslated region, and not superinducible. As the 3′ untranslated region of SV40 small t antigen cannot restaure superinducibility, we suppose that this feature of interferon gene is dependent on original interferon 3′ untranslated region.

Rat β globin locus: The Existence of Haplotypes Differing in the Number of Active Genes

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The number of adult α and β globin chains in rat was estimated to be 4 to 7. For more than 20 years it has been known that there are two haplotypes of adult hemoglobins and the polymorphism was attributed to the β chain locus.

We have determined the genomic organization of three haplotypes of β globin genes in order to resolve the question of their number. We have found two five gene haplotypes and one three gene haplotype. Sequences of 6.5 genes from two haplotypes convinced us that they are active adult β-globin genes. It seems that all 13 genes from three haplotypes are active. If this is the case a question can be asked about the mechanism ensuring balanced expression of α and β-globin genes, in both 3 and 5 β gene haplotypes.

Loss of E7 requirement in transformed BRK cells correlates with increased C-Myc expression

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We had previously shown that cells tranformed by inducible HPV-16 E7 and EJ-ras had an absolute requirement for continued E7 expression for maintenance of the tranformed phenotype. Using this system we attempted to isolate mutant Cell lines which had lost the requirement for E7 expression. Cells were placed in hormone free medium for 8-10 weeks, after which 6 slow g:)ing clones were obtained. These were expanded and shown to be hormone independent. On replacement of hormone E7 expression was resumed and the growth rate of the cells increased dramatically. All mutant cell lines retained the ability to grow in soft agar in the absence of E7 expression. Further analysis revealed that all clones isolated overexpressed cellular myc protein and in two instances this correlated with amplification of the c-myc locus. These results indicate that in the BRK cell system E7 has some function in common with c-myc.

Molecular cloning of venom gland cDNA from *Vipera Ammodytes*

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Phospholipases A_2 (PLA_2) are important constituents of the European viper *Vipera ammodytes)* venom. They show high degree of similarity in their amino acid sequences, but differ greatly in pharmacological properties. Besides ammodytocins A, B and C, which are highly potent presynaptic neurotoxins, also non-toxic PLA_2 homologues were found in the venom. The mechanism of their toxic activity in correlation to their enzymatic activity has not been completely solved yet. Therefore, a few amino acids were proposed, on the basis of computer modelling of the three-dimensional structure, that might contribute to the toxicity of the molecule. Expression of different natural PLA_2 and their mutants could help to elucidate the molecular mode of their toxic action. A venom glands cDNA library of *Vipera ammodytes* was prepared and screened with two different oligonucleotide probes. The first one was deduced from the unique Glu(4)-Ile(9) region of ammodytoxins, and the second one corresponding to the stretch of amino acid residues Tyr(24)-Gly(29), which is a part of the conserved region of PLA_2 responsible for Ca^{++} binding. Five different cDNAs coding for all three ammodytoxins and two non-toxic A_2 homologues, ammodytin L and i2, were found. In spite of significant differences in toxicity of ammodytocins A, B and C, the similarity between their nucleotide sequences exceeds 95%. In ammodytin L, one of the most interesting exchanges is the replacement of Asp(49) in the enzymatic active site by Ser residue. Ammodytin i2 is composed of 121 amino acids, one amino acid less than it is usual for other known PLA_2 homologues from the *Vipera ammodytes* venom. All cDNAs encoding above mentioned PLA_2 homologues have a highly conserved 16 amino acid residues long signal peptide.

Histones associated with transcribed ribosomal genes are not hyperacetylated

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Evidence was recently presented that highly transcribed ribosomal genes from *X. laevis* embryos were associated with histones (manuscript submitted). The acetylation state of histones bound to ribosomal DNA was studied in Guerin ascites tumor cells, grown in the presence of butyrate. Isolated nuclei were treated with formaldehyde to crosslink histones to DNA and the purified protein-DNA complexes were immunoprecipitated with an antibody that specifically recognizes -N-acetyl lysine. The DNA fragments carrying hyperacetylated histones were purified and analysed for the presence of ribosomal DNA sequences by dot hybridization to specific ^{32}P-labeled DNA probes. It was found that these DNA fragments were not enriched for ribosomal DNA sequences, i.e. the ribosomal genes, including transcribed ones, are not associated with hyperacetylated histones. Such a conclusion is supported by immunofluorescent microscopy, showing no staining of nucleoli. The absence of modified histone molecules on the ribosomal genes is discussed in the light of the role of acetylation in gene expression.

Anemic rat globin gene expression

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Anemic rat's globin gene expression was investigated by measuring the amount of globin mRNA transcribed in different erythropoetic tissues. RNA was isolated from spleen and bone marrow from anemic, normal and heterozygous animals and further analysed by dot-blot analysis.

According to these experiments, most of globin mRNA in normal rat is synthesized in bone marrow although some globin expression is present in adult spleen. In contrast, globin expression in spleen of anemic rat is ten times more efficient then in bone marrow. The most interesting result is obtained with heterozygous animals, which, although phenotypically normal and with functional bone marrow, they exhibit significant increase in globin expression in the apparently normal spleen.

Molecular Bases of Cytoplasmic male sterility of sunflower

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Cytoplasmic male sterility (cms) is a maternally inherited trait, resulting in the failure of the mature plant to produce functional pollen, while not affecting female fertility. Leclercq first described cms in sunflower as a result of interspecific crosses of H. Petiolaris and H. Annuus. The molecular mechanism which leads to cms in sunflower so far is not clarified. A cms specific polymorphism, associated with the atpA gene, for the HA_{89} isonuclear male sterile and male fertile lines has been reported (Siculella and Palmer 1988).

We have investigated the mitochondrial (mt) genomes of several valuable Bulgarian sunflower cms lines. The mtDNA was analyzed for cms specific RFLPs (restriction fragment length polymorphism) by utilizing probes corresponding to different clones of mt genes. We found a cms specific polymorphism, generated by several restriction enzymes, only in one locus encoding for atpA gene. A fragment covering this locus has been cloned. The bulk of the mt genomes of sterile and fertile lines appear to be similar as indicated by the absence of polymorphism using other mt probes.

Thus we can conclude that the atpA locus is somehow involved in the cms phenotype of all studied lines developed on the basis of H. Petiolaris cytoplasm.

Our clone, including atpA locus, could be utilized either for identification of sunflower lines carrying sterile cytoplasmic genomes, developed on the basis of H. Petiolaris or looking for similar polymorphism in all cms-candidate lines, developed on the basis of other sunflower species.

The biochemical mechanism of the cms phenomenon in sunflower remains to be elucidated.

Purification and Characterization of DNA Helicase-I from HeLa Cells

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We have initiated the characterization of the DNA helicases from HeLa cells, and we have observed at least 4 molecular species. One of these, DNA Helicase I has been purified to homogeneity. The helicase activity was measured by assaying the unwiding of radioactively labelled single stranded oligonucleotide (17 mer) annealed on M18 single stranded DNA. The helicase required a divalent cation for activity (Mg^{2+}>Mn^{2+}>Ca^{2+}). The helicase reaction was dependent on hydrolysis of ATP or dATP, CTP, GTP, UTP, dCTP, dGTP, dTTP, ADP, AMP and non-hydrolyzable ATP analogues such as ATPγS were not effective for the helicase activity. The best pH range for helicase activity was pH8.0 to pH9.0. The DNA unwinding activity was stimulated by monovalent cations KCl and NaCl up to 200mM concentration. The helicase reaction was inhibited by KPO4 (100mM), EDTA (5mM) and trypsin. The unwinding was also inhibited competitively by the coaddition of single stranded DNA which acts as cofactor of the ATPase activity. The purified fraction was free of DNA topoisomerase, DNA ligase and nuclease activities.

The direction of unwinding reaction is 3′ to 5′ with respect to the strand of DNA on which the enzyme is bound. The enzyme also catalyses the ATP dependent unwiding of a DNA:RNA hybrid consisting of a radioactively labelled single stranded oligonucleotide (18 mer) annealed on a longer RNA strand. The enzyme does not require a single stranded DNA tail on either strand at the border of duplex regions: i.e. the replication fork-like structure is not required to perform DNA unwinding. The purification of the other helicases is in progress.

Regulation of oxytocin and vasopressin mRNA in homozygous Brattleboro rats during dioestrus and lactation. Effects of dDAVP.

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Homozygous Brattleboro (DI) are deficient in neurohypophysial vasopressin (AVP) but are able to produce and secrete substantial amounts of oxytocin (OXY). As a result DI suffer from polyuria. These symptoms can be ameliorated to a large extent by administration of AVP or the AVP analogue 1-desamino-8-D-arginine-vasopressin.

In this study we demonstrate that the levels of AVP mRNA of DI and normal Wistar rats are regulated upon treatment with dDAVP and that in both strains of rat the supraoptic (SON) and paraventricular (PVN) nucleus of the hypothalamus work in concert to achieve the appropriate expression of the AVP gene. Our protocol shows that the levels of AVP mRNA in dioestrous DI rats can be down regulated and increased by stimuli involved in pregnancy, parturition and lactation.

In contrast to AVP mRNA levels, total OXY mRNA levels raised in control post-partum animals of both strains. However the increase was higher in DI than Wistar rats. The results indicate that upon dDAVP treatment the SON and PVN react in directly opposite way with regard to the regulation of OXY mRNA levels.

In this respect, it is of interest to note that although the genes for AVP and OXY show extensive sequence similarity, this is not the case for the 5′ regions, which suggests independent regulation.

We conclude that the OXY and AVP mRNA levels of DI and Wistar rats are modulated by dDAVP treatment and stimuli related to pregnancy, parturition and lactation.