Instituto Juan March
de Estudios e Investigaciones

44 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Selective Gene Activation by Cell Type Specific Transcription Factors

Organized by

M. Karin, R. Di Lauro, P. Santisteban and J. L. Castrillo

H. Arnold
M. Beato
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J. L. Castrillo
A. Celada
P. Charnay
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Instituto Juan March (Madrid)

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Instituto Juan March (Madrid)

INDEX

PAGE

P. Santisteban and J.L. Castrillo: Introduction............ 7

FIRST SESSION
Chairperson: Moshe Yaniv.................................... 11

Robert G. Roeder: The role of ubiquitous and cell specific coactivators in transcriptional activation....... 13

Michael Karin: GHF-1 and the control of GH gene expression and pituitary development...................... 14

Roberto Di Lauro: Homeo-,forkhead-, and paired-domains in thyroid specific transcription.......................... 15

Pilar Santisteban: Hormonal regulation of thyroid-specific transcription factors............................ 16

SECOND SESSION
Chairperson: Miguel Beato................................... 19

José Luis Castrillo: Transcriptional regulation of placental-specific genes................................ 21

Antonio Celada: Phosphorylation as a clue for activation of the tissue specific transcription factor PU.1.......... 22

Valeria Poli: C/EBPβ-deficient mice present specific defects in the myeloid and lymphoid compartments.......... 24

Short presentations:
Dionisio Martín Zanca: Analysis of the promoter region of the mouse NGF receptor gene,*TRKA*.................... 26

Beatrix Holewa: Transcriptional hierarchy in *Xenopus* embryogenesis: HNF4 a maternal factor involved in the developmental activation of the gene encoding the tissue specific transcription factor LFB1 (HNF1)...... 27

Instituto Juan March (Madrid)

PAGE

THIRD SESSION
Chairperson: Robert G.Roeder.................................... 29

Moshe Yaniv: Hepatocyte nuclear factor 1 (HNF1) inactivation results in phenylketonuria and renal fanconi syndrome.. 31

Ken S. Zaret: Mechanism of gene activation at the beginning of liver development............................ 32

Erwin F. Wagner: Functional analysis of transcription factor AP-1 in mice.. 34

Ueli Schibler: Leucine zipper proteins in circadian gene expression.. 35

FOURTH SESSION
Chairperson: Michael Karin................................... 37

Eric N. Olson: Transcriptional control of muscle gene expression.. 39

Hans-Henning Arnold: The regulatory network of transcription factors controlling skeletal muscle development in vertebrates................................. 41

Günter Schütz: Molecular genetic analysis of cAMP and glucocorticoid signaling in development and physiological and pathological processes.................. 42

Short presentations:

Jeffery D. Molkentin: Characterization of the DNA binding and transcriptional activation domains of MEF2C... 44

Gillian Z. Hastings: Inhibition of Pax 5 activity by expression of its DNA binding domain..................... 45

Instituto Juan March (Madrid)

PAGE

FIFTH SESSION
Chairperson: Mariann Bienz.................................. 47

Frank G. Grosveld: Transcriptional regulation of the β globin gene cluster.................................... 49

Beverly M. Emerson: Developmental regulation of β-globin gene transcription in reconstituted chromatin and synthetic nuclei.................................... 50

Miguel Beato: Participation of chromatin structure in regulated transcription.................................... 52

Rudolf Grosschedl: Assembly and function of multicomponent enhancer complexes.......................... 54

SIXTH SESSION
Chairperson: Roberto Di Lauro............................ 57

Mariann Bienz: Target sequences for growth factor-like signals in Drosophila................................... 59

Juan Modolell: Transcriptional control of *ac/sc* expression in proneural clusters.......................... 60

Patrick Charnay: Functional analysis of *Krox-20* during CNS and PNS development................................... 62

POSTERS.. 63

Natalia Arenzana: The promoter region of the human *C4BPB* gene... 65

Juan J. Arredondo: Analysis of the Paramyosin and mini-paramyosin expression in *D.melanogaster*.................. 66

Fabrizio d'Adda di Fagagna: Molecular interactions between transcription factor USF and the long terminal repeats of HIV-1....................................... 67

Mireille Delhase: Identification of cis-active promoter elements responsible for autoregulation of the human Pit-1/GHF-1 promoter.................................. 68

Instituto Juan March (Madrid)

PAGE

Cristina López-Rodríguez: Transcription factors involved in the leukocyte-restricted and the regulated expression of LFA-1 and p150,95 integrins.................. 70

Lourdes Ortíz: Hormonal regulation of thyropeoxidase promoter gene transcription.............................. 71

Ma. Isabel Rodrigo: Regulation of E-cadherin promoter in mouse epidermal keratinocytes.......................... 72

Ma. Teresa Sáenz: Untitled abstract........................ 73

Cathy Tournier: TSH stimulates MAP kinase in human thyroid and rat astrocytes............................... 74

Miguel A. Vega: AML1 (PEBP2/CBF) transcription factors regulate the expression of the CD36 gene in myeloid cell lines... 75

Nicola Zambrano: Initiator-mediated, neuron-specific expression of the rat FE65 gene.......................... 76

LIST OF INVITED SPEAKERS................................... 77

LIST OF PARTICIPANTS.. 83

Instituto Juan March (Madrid)

INTRODUCTION

P. Santisteban and J. L. Castrillo

Instituto Juan March (Madrid)

Selective gene activation by cell type specific transcription factors

Introduction

Pilar Santisteban and José Luis Castrillo

A major challenge in biology and molecular genetics is to decipher the mechanism that control the utilization of genetic information in a cell-type specific manner. Most tissue-specific transcription factors are expressed in more than one cell type. However, these transcription factors activate different target genes in different cells. How does this occur has been the aim of this workshop. During three days specialist in this field have presented new data on a number of tissue-specific transcription factors, transcriptional enhancers and tissue-specific co-activators. One of the important point have been the study of how this transcription-factors and co-activators interact with the basic transcriptional machinery, mainly presenting data about the existence of positive and negative co-factors and their interaction with the TATA-binding subunit. An important point common to the mechanism of action of either transcription-factor or co-activator is that in both cases are activated after phosphorylation/dephosphorylation processes, involving the study of different kinases.

Another important studies is the fact that many tissue-specific transcription factors are expressed in more that one tissue controlling the expression of different genes in each tissue. The general model to understand this phenomena is that in every tissue there is also co-activators or repressors that in collaboration with general transcription factors regulate more specifically, the expression of a determined gene.

How in a determinate cell type a gene is transcribed in response to a external signal has been another of the objective presented. Data have been reported about

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the signal cascade that occurs from the membrane to the nucleus. The question debated, but still not answered is : How does occurs the specificity in response to a different signals when downstream of the membrane the cascade signal is the same?

Many data have been reported about the role of tissue-specific transcription factors in embryos development and cell differentiation and proliferation. By the technology of transgenic and knockout mice have been presented data about roles, until this moment unknown, of constitutive and tissue-specific transcription factors.

How the nucleoprotein structures interacts with enhancers in the context of chromatin and what is the role of the chromatin structure in tissue-specific transcription have been another interesting question treated during the symposium. The structure of different factors and their interaction with the DNA or another nucleoproteins has been very well presented from several specialists.

We can conclude that during this three days all of us have been listening new experiments from laboratories working in different systems but with very related questions (and answers) on the role of tissue-specific transcriptions factors in the regulation of gene expression.

Finally we would like to thank all the speakers and the Instituto Juan March de Estudios e Investigación, specially its director Andrés González, to give us the opportunity to organize this workshop.

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FIRST SESSION

Chairperson: Moshe Yaniv

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THE ROLE OF UBIQUITOUS AND CELL SPECIFIC COACTIVATORS IN TRANSCRIPTIONAL ACTIVATION.

Robert G. Roeder*, Cheng-Ming Chiang, Jeffrey DeJong, Hui Ge, Marcus Kretzschmar, and Yan Luo. Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, New York, NY 10021.

The TATA-binding subunit (TBP) of natural TFIID and a number of other general initiation factors suffice for basal transcription by RNA polymerase II from most core promoters. In contrast, the in vitro function of gene-specific DNA-binding activators requires, in addition: (i) one or more of the other TFIID subunits (TAFs), (ii) initiation factors (e.g., TFIIA) that may facilitate but are not essential for TBP-mediated basal transcription, (iii) one or more separable positive (PC1, PC2, PC3, PC4) and negative (NC1, PC3) components purified from the general cofactor USA, that may act individually or in concert and (iv) in some cases, tissue-specific coactivators (e.g., the lymphoid specific OCA-B) that show independent interactions with specific activators. Ongoing efforts have resulted in the further purification, cloning and characterization (structure and/or function) of various cofactors and selected results relevant to activation mechanisms will be discussed, as exemplified by the following. First, consistent with previous studies implicating TFIID as an activator target, studies of human TFIID have revealed direct interactions of the derived $TAF_{II}55$, through different domains, both with $TAF_{II}250$ (which may anchor $TAF_{II}55$ to TFIID) and with the DNA binding domains of certain activators (which may provide a novel mechanism for activator functions). Second, studies of PC4 have indicated that it can enhance function of a number of distinct activators up to 90 fold, apparently by virtue of its ability (as an adaptor) to interact directly both with activation domains and with TBP-TFIIA complexes. The ability of TFIIA to prevent interactions of negative cofactors with TBP and to interact directly with $TAF_{II}136$ suggests these components as possible downstream targets for PC4. PC4 is also subject to regulation by phosphorylation, which inhibits its coactivator function. Third, studies of OCA-B, previously shown to be responsible for the lymphoid-specific expression of immunoglobulin promoters through direct interactions with promoter bound Oct-1 or Oct-2, have provided new information on its mechanism of action; this includes promoter recruitment through the Oct POU domain and synergistic function with Oct activation domains, in addition to a requirement for another (USA-derived) general co-activator(s). Along with previous studies these observations indicate that transcriptional activation may involve stepwise or concerted interactions of multiple activators/activation domains with different components of the basal transcriptional machinery, either directly or through intermediates (coactivators), and that coactivators may be general or activator specific as well as ubiquitous or cell-specific.

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Y. Luo and R.G. Roeder. Cloning, functional characterization, and mechanism of action of the B-cell-specific transcriptional coactivator OCA-B. Mol.Cell.Biol., in press, 1995.

Instituto Juan March (Madrid)

HOMEO-, FORKHEAD-, AND PAIRED-DOMAINS IN THYROID SPECIFIC TRANSCRIPTION.

M.I. Arnone, G. Cobellis, M. De Felice, K. Sato, S. Zannini, and R. Di Lauro, Stazione Zoologica "Anton Dohrn", Villa Comunale, 80121 Naples, Italy.

The thyroglobulin and thyroperoxidase genes are exclusively expressed in the thyroid and insights on their transcriptional regulation could help in elucidating the mechanism of the differentiation of the thyroid cell type.
At least three factors are necessary for transcription of the thyroglobulin and thyroperoxidase promoters: Thyroid Transcription Factor-1 (TTF-1), Thyroid Transcription Factor-2 (TTF-2) and Pax-8.
TTF-1 is an homeodomain-containing protein, it is able to bind to both the thyroglobulin and thyroperoxidase promoters and the presence in these promoters of at least two binding sites for TTF-1 is required for thyroid-specific transcription. Transactivation studies performed in non-thyroid cells demonstrated that TTF-1 is able to activate transcription from both thyroglobulin and thyroperoxidase promoters.
The Pax-8 gene, a member of the murine family of paired box containing genes (Pax genes), is expressed in adult thyroid and in cultured thyroid cell lines. We have shown that the Pax-8 protein binds, through its paired domain, to the promoters of both thyroglobulin and thyroperoxidase genes at a single site. Interestingly, in both promoters, the binding site of Pax-8 shows a complete overlap with one of the TTF-1 binding sites. Pax-8 activates transcription from both thyroperoxidase and thyroglobulin promoters, indicating that it may be involved in the establishment, control or maintenance of the thyroid differentiated phenotype.
The cDNA for the Thyroid Transcription Factor-2 (TTF-2) has recently been cloned. The clone has been identified as TTF-2 on the basis of the following criteria: it is able to bind to the sequences on the thyroglobulin and thyroperoxidase promoters that had been previously described as binding site for a thyroid-specific factor named TTF-2; it is tightly regulated at the transcriptional level by insulin as it has been described for the TTF-2 binding activity. In situ hybridization experiments have shown that TTF-2 is expressed very early during mouse development suggesting that it could play a role in the commitment of the thyroid cell precursors of the embryo. Moreover its expression is restricted to the thyroid and to the anterior pituitary both during development and in adult tissues.
At variance from TTF-1 and Pax-8 that are differently affected upon transformation with a variety of oncogenes, TTF-2 is very sensitive to the action of oncogenes, being absent in all transformed thyroid cell lines tested.
The full length cloning of the cDNA will allow to investigate its role in the thyroid specific expression of the thyroglobulin and thyroperoxidase promoters and hence in the establishment of the differentiated thyroid phenotype.

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GHF-1 AND THE CONTROL OF GH GENE EXPRESSION AND PITUITARY DEVELOPMENT. Michael Karin, Ph.D. University of California, San Diego, Department of Pharmacology, School of Medicine, La Jolla, CA 92093-0636, and Department of Reproductive Medicine, School of Medicine, La Jolla, CA 92093-0674.

GHF-1 (aka Pit-1) is a transcriptional activator that belongs to the POU sub-group of homeoproteins. GHF-1 is specifically expressed in cells that belong to the somato/mammotrophic lineage of the anterior pituitary where it activates expression of the growth hormone (GH) and prolactin (PRL) genes. *GHF-1* gene transcription is first detected in somatotropic progenitors within 24 hrs of the individualization of Rathke's pouch in the mouse. Due to translational delay in appearance of GHF-1 protein, transcription of the target gene is detected only 2-3 days later. The Snell dwarf mouse strain carries a point mutation within the *GHF-1* gene affecting a conserved residue in the homeodomain and resulting in a protein with severely reduced DNA binding activity. These dwarf mice suffer from anterior pituitary hypoplasia due to the absence of somatotropic cells. A role for *GHF-1* in proliferation of somatotropic cells was demonstrated by using antisense RNA. To study the control of *GHF-1* transcription during development, the control region of the gene was fused to SV40 T antigen and introduced into the fertilized mouse eggs. Transgenic mice that expressed this gene developed pituitary tumors that express low levels of GHF-1 but no GH. Further characterization of the tumor cells indicates that they might correspond to immortalized somatotropic progenitors. Interestingly, the *GHF-1* promoter in these cells is activated by a different constellation of transcription factors than in mature GH- or PRL-expressing cell lines. In addition to GHF-1, the *GHF-1* gene encodes a second protein, GHF2, with different regulatory properties. Recently we succeeded in inducing the differentiation of the *GHF1*-Tag immortalized cells by culturing them on extracellular matrix in the presence of FGF. This system will be useful for dissecting the molecular events in GH and PRL gene activation as GHF-1 is not the sole activator of these genes.

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HORMONAL REGULATION OF THYROID-SPECIFIC TRANSCRIPTION FACTORS.

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The identification of the *cis*-regulatory elements and the transcription factors mediating the hormonal regulation of gene expression has become one of the attractive field in the last few years. This, together with the fact that certain genes are transcribed only in a determined cell type (1) make the thyroid cells an excellent model for both studies. Thyroid cells are specialized cells that express differentiated functions to synthesize and secrete thyroid hormones. Thyroperoxidase (TPO) is the primary enzyme involved in thyroid hormone biosynthesis by iodinating and coupling the tyrosine residues in Thyroglobulin (Tg) (2). The expression of TPO and Tg genes is restricted to thyroid cells since only in this cell type are present three thyroid-specific transcription factors TTF-1, TTF-2 and Pax-8. The three specific transcription factors bind to both promoters (3-6).

In this work, it has been studied the molecular mechanisms involved in basal transcription and hormonal regulation of Tg and TPO genes. We have demonstrated that the pituitary hormone thyrotropin (TSH) through cAMP and insulin/IGF-I, stimulate 4 to 7 fold Tg and TPO mRNA levels (2). This hormonal regulation takes place at the transcriptional level. Transient transfection experiments demonstrate that the minimal Tg and TPO promoters that confers thyroid-specific expression also confer responsiveness to TSH and IGF-I. We have found in both promoters the *cis* DNA sequences and the trans-acting proteins involved in the hormonal transcriptional regulation. Two hormones responsive elements have been defined in both promoters that correspond to the binding sites for the thyroid-specific transcription factors TTF-1 and TTF-2. The mechanisms involved in the action of both of them are very different. Electrophoretic mobility shift assays (EMSA) demonstrate that TTF-2/DNA complex is absent in nuclear extracts from cells depleted of hormones and is induced by TSH/cAMP and by insulin/IGF-I. This effect requires on going protein synthesis. Heterologous promoter constructs containing four, eight or 12 tandem repeats of an oligonucleotide that includes the TTF-2 binding site increase their activity in response to the hormones (7-8). However, that a high copy number of the TTF-2 binding site is needed to work efficiently as a hormone response element suggests a role for other transcription factors either TTF-1, Pax-8 and/or ubiquitous factors present in both promoters. This hypothesis is now under study in our laboratory trying to demonstrate a role for such transcription factor binding sites in the TPO/Tg promoters context and if these sites affect the hormonal response of the TTF-2 binding site.

How TTF-1 works is much more complex, involving phosphorylation (9-10) as the main mechanism of regulating its activity. We have observed that the levels of TTF-1 phosphorylation are lower in cells growth in the presence of hormones. This means that TTF-1 is less phosphorylated in the situation where the protein is more active. We have also

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investigated the role of protein kinase A and C in TTF-1 activation/inactivation process. All these mechanisms will lead to an equilibrium between inactive-phosphorylated and active-dephosphorylated TTF-1. Studying Harvey-**ras** transformed thyroid cells where TTF-1 is present but inactive we have confirmed the importance of protein phosphorylation in this transcription factor activation.

The role of TSH and IGF-I in thyroid cells is not only regulating Tg and TPO gene transcription, but also regulating thyroid cells proliferation (2). Since the thyroid transcription factors TTF-1 and Pax-8 are homeo- and paired-box containing genes respectively that are responsible for thyroid development and thyroid cell differentiation (10-11), we have investigated if such factors play a role in thyroid cell proliferation. The antisense oligonucleotide strategy was used to clarify this point. Treatment of quiescent thyroid cells with TTF-1 or Pax-8 antisense oligonucleotides caused a significant reduction in TSH and IGF-I-stimulated cell proliferation, measured as DNA synthesis and cell number. The same result was obtained with forskolin indicating that the TTF-1 or Pax-8 role mediating TSH growth effect occurs throughout the cAMP pathway. The effect was higher with TTF-1, since the blockage of this factor caused a 65% decrease in cell proliferation compared to the control. Pax-8 blocking only leads to a 30% decrease. The blocking of both thyroid transcription factors together did not result in an additive effect. These data provide direct evidence that both homeo and paired box gene expression is essential for thyroid cell proliferation, with each one possibly playing a different regulatory role.

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SECOND SESSION

Chairperson: Miguel Beato

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TRANSCRIPTIONAL REGULATION OF PLACENTAL-SPECIFIC GENES

V.Vila, O.Jiménez, A.Güell, M.de la Hoya, J.Echave, J.Cosano and J.L.Castrillo*

Centro de Biología Molecular "Severo Ochoa". Univ. Autónoma de Madrid. Cantoblanco. Madrid-28049.

The human growth hormone gene family includes three members of physiological and clinical importance: Growth hormone (GH), Prolactin (PRL) and Placental lactogen (PL), also called chorionic somatomammotropin. The individual members of the human growth hormone gene family have been isolated and their complete nucleotide sequences determined (1). It has been shown that the five closely related genes coding for GH and PL are clustered within a 66 Kb region located on chromosome 17 (17q22-24): The Human GH Locus (2). The gene order is: HGH-N, HPL-1, HPL-4, HGH-V and HPL-3. This clustering and the degree of sequence homology of these genes indicate a recent evolutionary origin, probably by gene amplification (1,3).

While the GH-N and PRL genes are expressed in two related cell types of the anterior pituitary, the somatotrophs and lactotrophs, the PL genes are expressed in the completely unrelated placental syncytiotrophoblast. What is even more unique for the GH family is that the two more divergent genes, GH-N and PRL, are both expressed in the anterior pituitary, while the sites of expression of the closely related genes, GH-N and PLs are not similar at all. This is particularly intriguing considering that the GH-N and PLs genes are 95% homologous in the first 650 bp of their promoters.

We are interested in the study of: (1)*cis* elements responsible for the tissue-specific expression of the GH-N gene in the anterior pituitary, and PLs genes in placenta. And (2) the recognition of these elements by *trans* -acting DNA-binding proteins specifically presents in pituitary and placenta tissues.

The human GH Locus presents a unique model to investigate the molecular mechanisms by which a cluster of recently evolved and thus closely related genes has become specialized to express related hormones in two distinct tissues.

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Phosphorylation as a clue for activation of the tissue specific transcription factor PU.1

Antonio Celada

Department of Physiology (Immunology), School of Biology, University of Barcelona

PU.1 is a tissue-specific transcription factor expressed in macrophages, granulocytes and B lymphocytes (1). PU.1 binds to a purine-rich requence that contains a central core with the sequence 5′-GGAA-3′. The DNA binding domain, which is located near the carboxy terminus is homologous to the *ets* family of DNA binding proteins.

PU.1 migrated with an apparent molecular weight of 43.5 kD, with minor amounts of 44.5 and 38 kD species. Analysis of the phosphoamino acid content of the three species indicated that they were all phosphorylated, exclusively on serine residues. PU.1 protein synthesized in bacteria could be phosphorylated *in vitro* on serines by the protein serine/threonine, casein kinase II (CKII). Predicted CKII target sites in PU.1 were mutated and the altered protein was expressed in mammalian cells. Mutation at the sites, none of which lies in the DNA binding domain of the protein, had no effect on the ability of the protein to bind to DNA (2).

PU.1 recruits the bind of a second B cell-restricted nuclear factor, NF-EM5 to a DNA site in the immunoglobulin κ 3′enhancer. Binding of NF-EM5 to the DNA requires a protein-protein interaction with PU.1 and specific DNA contacts. The protein-protein interaction is mediated through a 43 aminoacid region with PEST sequence homology. Phosphorylation of serine 148 (Ser^{148}) is required for protein-protein interaction. Expression of the wild type PU.1 increased nearly six fold the expression of a reporter construct containing the PU.1 and NF-EM5 binding sites, whereas the Ser^{148} mutant forms only weakly activate transcription (3).

Macrophages transfected with a vector containing PU.1 showed an enhancement of M-CSF dependent proliferation (4). However, a reduced macrophage proliferation was found when antisense PU.1 or the binding site of PU.1 were transfected. The deletion of the PEST sequence of the PU.1 protein does not affect the raise of proliferation. However, serines at positions 41 and 45 are critical for macrophage proliferation.

We conclude that depending of the genes or the tissues, different areas of the PU.1 protein or different phosphorylation sites are required to induce transcription activation.

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C/EBPß-DEFICIENT MICE PRESENT SPECIFIC DEFECTS IN THE MYELOID AND LYMPHOID COMPARTMENTS.

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Transcriptional activators and repressors of the C/EBP family constitute a large group of nuclear proteins that play important roles as regulators of cell proliferation and differentiation, and as mediators of intracellular signalling from extracellular stimuli. All C/EBP proteins show a high degree of homology in the carboxy-terminal basic and leucine zipper domains, responsible for DNA binding and dimerization. Homology does not extend to the amino-terminal portion of the protein, which confers specific *trans*-activating properties. Each polypeptide can form both homo- and hetero-dimers with the other family members.

C/EBPß (previously referred to as IL-6DBP for IL-6 Dependent DNA Binding Protein, or as NF-IL-6 for Nuclear Factor IL-6) was originally identified as an important component of the Interleukin 6 (IL-6) signalling pathway: its *trans*-activating potential is enhanced by IL-6 in transfected hepatoma cells, where it acts as an inducer of acute phase response genes (Poli et al., 1990), and its binding to the IL-6 gene promoter is thought to be required for transcriptional induction by IL-1/IL-6 (Akira et al., 1990). Subsequent studies demonstrated that C/EBPß can also be activated by IL-1 and TNFα, and pointed to several other potential functions of this factor, including transcriptional regulation of cytokine genes other than IL-6 (IL-1ß, TNFα and G-CSF), and regulation of the differentiation of B cell and myeloid cell lineages. C/EBPß mRNA was shown to generate two proteins with opposite functions, a longer form acting as *trans*-activator and a shorter form named LIP (Liver Inhibitory Protein) (Descombes and Schibler, 1991), which does not have a *trans*-activating domain and is believed to act as a repressor of all C/EBP factors activity.

The differential and modulated expression of the various C/EBP genes and the complexity of the C/EBP network does not allow the clear distinction between unique and redundant functions. In an effort towards the clarification of this issue, we generated mice in which the C/EBPß gene was inactivated by gene targeting.

The analysis of the acute phase response in the C/EBPß-/- mice revealed defects in the late phases of induction (Poli et al., manuscript in preparation), showing that the major role of this factor in the control of acute phase genes transcriptional activation is to maintain the induction.

Strikingly, with age the C/EBPß-/- mice develop a pathology similar to mice over-expressing IL-6 and nearly identical to multicentric Castleman's disease in human patients, with marked splenomegaly, peripheral lymphoadenopathy and enhanced hemopoiesis (Screpanti et al., 1995). Interestingly, C/EBPß-/- mice show an age-related increase in IL-6 levels, suggesting that this factor is not required for IL-6 transcription. Humoral, innate and cellular immunity are profoundly distorted. Nitric Oxide (NO)

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as well as IL-12 production by macrophages are defective, and the mutant mice show an increased susceptibility to *Candida albicans* infection, most probably due to an altered T helper function, and characterized by development of a non protective Th2 response instead of the protective Th1 response showed by the control mice. These data show that C/EBPβ is crucial for the correct functional regulation and homeostatic control of hemopoietic and lymphoid compartments, and reveal a previously unsuspected role in the regulation of the T helper response.

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ANALYSIS OF THE PROMOTER REGION OF THE MOUSE NGF RECEPTOR GENE, *TRKA*

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The *trk* gene family encodes high affinity tyrosine kinase receptors for the nerve growth factor family of neurotrophins. The different neurotrophins are responsible for the survival, differentiation and maintenance of specific populations of neurons in the developping and adult nervous system. *trk*A gene expression is restricted to a subset of neural crest-derived sensory neurons of the trigeminal, superior, jugular and dorsal root ganglia (DRG), *trk*A is also expressed in sympathetic neurons. In the CNS it is expressed only in cholinergic neurons of the basal forebrain. Thus, *trk*A expressing neurons closely match those that are dependent on NGF for survival and differentiation *in vivo*. Recent "knock-out" experiments have shown that *trk*A is essential for the survival of those neurons during embryonic development. *trk*A gene expression marks specific groups of neurons for survival in response to NGF, this factor rescues them from death during development and induces their differentiation and mantains their functional phenotypein the adult. *trk*A expression may be part of a developmental program or may actively contribute to define it. In either case, understanding the mechanisms that control *trk*A expression will help define, at the molecular level, some of the events leading to the adquisition of a specific neuronal phenotype.

Our goal is to disect the *trk*A promoter to identify the regulatory sequences acting in *cis*, as well as the transcription factors acting in *trans* which are responsible for the exquisite pattern of *trk*A expression *in vivo*. Detailed analysis of the 5' region of the mouse *trk*A gene, including nucleotide sequencing of 4.5 Kbp around the first exon, followed by Southern blot analysis of genomic DNA, has allowed us to identify a CpG island around the *trk*A first coding exon. By primer extension and S1 nuclease protection analysis, using RNA from mouse embryo DRGs (E12), we have mapped the *trk*A transcription start site 70 nucleotides upstream of the ATG translation initiation codon.

A series of transcriptional fusions beetwen *trk*A and reporter genes have been generated and used in transient transfection assays on cells that express *trk*A (PC-12, N2A, SY5Y) as well as in others which do not express it (NIH3T3). These experiments have allowed us to define a 700 bp fragment 5' to the ATG of *trk*A which shows promoter activity in cells of neuronal type and not in fibroblasts. Primer extension analysis using RNA from PC12 transfected with *trk*A-*lac*Z fusions indicates faithful initiation of transcription in those cells. Thus, a relatively short fragement of the 5' region of trkA contains the promoter and some regulatory sequences capable of directing its transcription to cells of neuronal type. Detailed analysis of this region is in progress, in our laboratory.

Finally, nucleotide sequence analysis of the 5' region of mouse *trk*A revealed the close presence of another gene, which is transcribed in the opposite direction, and whose trascription start site, as described in rat, is only 1,600 bp from that of *trk*A. This gene encodes a receptor tyrosine kinase very similar to the insulin receptor, called IRR (insulin receptor-related receptor) and whose ligand is unknown. IRR expression pattern during embryonic development is essentially identical to that of *trk*A. This fact, together with the proximity beetwen *trk*A an IRR, suggest that both genes could share a bidirectional promoter or, alternatively, that they may use different promoters with some common regulatory sequences. We are using dual reporter constructs to analyze both possibilities.

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Transcriptional hierarchy in *Xenopus* embryogenesis: HNF4 a maternal factor involved in the developmental activation of the gene encoding the tissue specific transcription factor LFB1 (HNF1)

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The tissue specific transcription factor LFB1 (HNF1) is expressed in vertebrate liver, kidney, stomach and gut. During *Xenopus* development LFB1 gets transcriptionally activated shortly after midblastula transition, when zygotic transcription starts suggesting a role in early development. In tailbud stage *Xenopus* embryos LFB1 is expressed in pronephros, liver and gut paralleling the expression in the adult.

By microinjection into fertilized *Xenopus* eggs a 387 bp LFB1 promoter fragment is sufficient to be activated in the middle part of developing larvae reflecting the expression pattern of the endogenous LFB1 gene. Mutational analysis of this *Xenopus* LFB1 promoter fragment showed that the LFB1 and HNF4 binding sites are essential for proper embryonic regulation of the gene. By injecting synthetic HNF4 mRNA into fertilzed *Xenopus* eggs, we induced ectopic activation of the endogenous LFB1 gene, revealing a transcriptional hierarchy between HNF4 and LFB1 during embryogenesis. To analyze whether HNF4, a member of the steroid receptor superfamily is present at the correct time and place to be the endogenous LFB1 inducer in early development, we cloned the *Xenopus* HNF4 homolog. We detected low level of HNF4 mRNA from early gastrula onwards and a dramatic increase in the amount of the mRNA during the transition between late neurula and tailbud stage when pronephros differentiation occurs. In hatched larvae HNF4 mRNA could be localized in pronephros, liver and gut paralleling the expression of LFB1. Since on the RNA level HNF4 expression seems not to precede LFB1 activation, we analyzed embryonic HNF4 protein accumulation. HNF4 protein could be detected as a maternal component from the egg stage onward throughout early development. Thus, HNF4 qualifies as the natural embryonic inducer of LFB1. Since the HNF4 protein is localized within an animal to vegetal gradient in early cleavage stage embryos we assume, that this gradient contributes to a regional expression of specific genes including LFB1 and plays a significant role in the differentiation of different cell fates during early development.

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THIRD SESSION

Chairperson: Robert G. Roeder

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HEPATOCYTE NUCLEAR FACTOR 1 (HNF1) INACTIVATION RESULTS IN PHENYLKETONURIA AND RENAL FANCONI SYNDROME.

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HNF1 (Hepatocyte Nuclear Factor 1) is a liver enriched variant homeoprotein that binds and transactivates the promoters of a large set of hepatic genes like albumin, α-1 antitrypsin, β-fibrinogen etc (1,2). To study its function we inactivated the gene by replacing the first exon, with a β-galactosidase cassette. Two independent embryonic stem cell clones with this targeted mutation colonized the germ line of several mice. Heterozygotes were normal and the detection of the β-galactosidase activity revealed expression in liver, renal proximal tubules, pancreas, stomach and intestine. Inter-crosses between heterozygotes produced homozygous pups with roughly the expected ratio showing that HNF1 deficiency is not embryonic lethal. However postnatal development was strongly perturbed. Already by the first week of life homozygotes showed a marked decreased growth rate. Homozygotes life-span averaged less than 30 days and more than 75% died between days 20 and 40, after a progressive wasting syndrome. Homozygotes showed a drastic liver enlargement associated with hyperphenylalaninemia, hypercholesterolemia and higher hydroxyproline plasma levels. Surprisingly, hepatic albumin mRNA accumulated at the same levels in wild type, heterozygotes and homozygotes. However nuclear run on assays revealed that the transcription rate of albumin was reduced several fold in the homozygotes, indicating that a post-transcriptional stabilisation of the messenger could compensate for the reduced transcription rate. The decreased or even normal rate of many HNF1 target genes may be explained by the upregulation of vHNF1 (HNF1β), a closely related gene that shares similar expression pattern and identical DNA binding specificity. The only hepatic transcriptional defect so far identified involves the phenylalanine hydroxylase gene which was the cause of the hyperphenylalaninemia observed in the homozygotes. In addition to liver dysfunction, we observed a severe defect in the reabsorption process in the kidney proximal tubules of the homozygous animals (Fanconi syndrome). This results in strong glucose and amino acids wasting that in turn causes a marked polyuria. Homozygotes loose up to 1.3 gr of urinary glucose per day and produce an amount of urine that in some cases is 85% of their body weight per day. This urinary glucose loss, which is not completely compensated by the actual polyphagic behaviour of the animals, could explain the growth defect, the wasting syndrome and the mortality of the homozygotes (3).

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Mechanism of Gene Activation at the Beginning of Liver Development

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Transcriptional enhancers activate genes in a cell-specific manner by binding particular combinations of tissue-enriched and ubiquitous regulatory proteins. Many studies have shown that protein-protein interactions are necessary for enhancer activity, yet little is known about the higher-order structures created by the multitude of factors that typically bind enhancer elements. We are interested in how nucleoprotein structures form at enhancers in the context of chromatin, in the importance of such higher-order structures for transcription, and in how the structures are assembled in a developmental context.

As a model system, we are investigating an enhancer that exists 10 kb upstream of the serum albumin gene. The albumin enhancer confers liver-specific activation of a reporter gene in transgenic mice and in transfected hepatic cell lines, similar to the high specificity of expression of the endogenous albumin gene in hepatocytes. Studies of the albumin enhancer in mouse tissues revealed the surprising finding that the enhancer is organized into a short array of three precisely positioned nucleosomes, or nucleosome-like particles, only in the liver, where the enhancer is active. The particles were designated N1, N2, and N3, and span a host of essential transcription factor binding sites. Furthermore, in vivo footprinting studies showed that the factor binding sites are occupied in the liver and map to the positions of the N1-N3 particles, not the linker sequences between the particles. In non-liver tissues where no factors bind the enhancer, nucleosomes are not translationally positioned over the sequences. Detailed nuclease mapping studies in liver suggest that N2 is a conventional nucleosome with factors bound to its surface and 3' edge, whereas N1 either contains factors bound to a disrupted nucleosome or it consists solely of an aggregate of factors bound to DNA. We suggest that similar higher-order structures could exist at other tissue-specific enhancers.

Understanding the steps involved in assembling the N1-N3 particles in liver development will help us reconstitute the particles in vitro, with the ultimate goal of testing the relationship between higher-order structure and function of the enhancer. To this end, we have used in vivo footprinting to determine the time at which different enhancer-binding factors first occupy their sites in the N1 region in hepatic ontogeny. The results were unanticipated. HNF3 is the first known liver transcription factor to be expressed in the lineage, and it weakly binds one of its two sites on the albumin enhancer in gut endoderm, which is the precursor to the liver. In vitro binding studies show that this site, designated eG, binds HNF3 with about 1.5 fold greater affinity than the other HNF3 site, eH, 20 bp away. HNF3 is homologous to the *Drosophila fork head* protein, which controls gut development in the fly; both proteins contain the "winged helix" DNA binding domain and function as monomers. In the hepatic primordia at 9.5 days gestation in the mouse, both the eG and eH HNF3 sites are occupied as well as sites that bind an uncharacterized factor designated eF, which is present in adult liver, and a new factor, eY, which appears specific to the embryo liver. After the liver is formed, at 12.5 days gestation, HNF3 no longer binds the eH site and the eF site is no longer occupied, and NF1 now binds at eH. In the adult liver, the eY factor is gone whereas the eF site and the HNF3 site at eH are re-occupied, along with HNF3 at eG and NF1 at eH. In sum, in liver development factors bind the albumin enhancer, some are released, new factors bind, and then the released factors re-bind. We believe that this "sequential remodeling" strategy

Instituto Juan March (Madrid)

may be necessary to create the specific nucleoprotein architecture that defines the active N1 particle of the enhancer. We anticipate that sequential remodeling may occur at other complex regulatory sequences.

Using the knowledge of the developmental order of factor binding, we are now trying to reconstitute the albumin N1 particle in vitro. Because factors normally bind the gene in a chromatin context, we are using different nucleosomal templates that span the N1 sequence. Mononucleosome core particles have been reconstituted in vitro using core histone proteins purified from liver. Various mapping techniques show that the enhancer DNA has some intrinsic ability to position nucleosomes translationally. However, positioning of a nucleosome at N1 is not dominant in polynucleosome arrays in the absence of HNF3-type binding factors. Thus we conclude that positioning of N1 in liver is probably due to a combination of instrinsic sequence preference of the DNA and specific binding proteins. Purified HNF3 DNA binding domain is capable of binding nucleosome cores with an affinity about 10-fold lower than that for free DNA, which is still much higher than that seen for other nucleosome-binding proteins. Upon HNF3 binding, changes in nuclease sensitivity can be detected between the HNF3 sites on the nucleosome, suggesting structural perturbation. We are currently investigating how the binding of HNF3 to a nucleosome may potentiate the binding of other factors that appear later in development.

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Functional analysis of transcription factor AP-1 in mice.

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Embryonic stem (ES) cells and transgenic mice are being used to investigate the function of AP-1 (*fos* and *jun*) through ectopic expression during development or following gene inactivation in mice. Overexpression of c-*fos* reproducibly leads to the development of osteogenic tumors[1] whereas c-*jun* transgenic mice exhibit no apparent phenotype. In contrast, the inactivation of c-*jun* leads to embryonic lethality, implying an important function of c-*jun* during mouse development[2]. Various strategies to define the molecular defects including rescue experiments with transgenes will be described.

Mice lacking c-*fos* are viable but develop the bone disease osteopetrosis which is characterized by deficiencies in bone remodelling and hematopoiesis[3,4]. Analysis of different bone cell types indicated that while osteoblasts are apparently unaffected osteoclasts are not formed in the absence of c-*fos*.[5] The role of c-Fos in osteoclast differentiation and its possible involvement as a key regulator of osteoclast/ macrophage lineage determination will be discussed.

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LEUCINE ZIPPER PROTEINS IN CIRCADIAN GENE EXPRESSION

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DBP (1), TEF (2), and HLF42 and HLF36 (3,4,5) are four highly related basic region-leucine zipper (bZip) proteins with identical *in vitro* DNA-binding specificity. The consensus recognition sequence for these so-called PAR (proline-acidic amino acid-rich) bZip proteins has been identified as RTTA(T/C)GTAAY (5). This sequence is also avidly bound by members of the C/EBP transcription factor family, whose basic regions share considerable sequence similarity with the DNA-binding domains of PAR proteins. However, C/EBP proteins are much more promiscuous with regard to their DNA cognate sites and occupy many sequences that are not recognized by PAR proteins. A single V to A amino acid substitution within the basic region of C/EBP approaches its DNA sequence specificity to the one observed with PAR proteins (5).

All four PAR family members accumulate in rat liver nuclei according to a circadian rhythm with daily amplitudes of 160 for DBP (6,7), about 50 for TEF (7) and HLF 42, and about 3 for HLF36 (8). DBP and HLF may be involved in the regulation of cholesterol homeostasis by controlling circadian expression of cholesterol 7α hydroxylase, the rate limiting enzyme for the synthesis of bile acids (7,9).

The DBP activation domains have been delineated by a set of nested deletions, and by transplantation of various DBP peptide segments onto the DNA binding domain of the yeast transcriptional activator GAL4. In the context of the DBP protein, two adjacent DBP peptide regions, one enriched in the amino acids E and D, and the other in SP motifs, are required for efficient trans-activation of all examined promoters. Stimulation of transcription from the albumin promoter requires an additional N-terminal DBP region, rich in basic and acidic amino acids. Thus, DBP appears to contain both general and promoter-specific activation domains. Interestingly, in the context of GAL4-DBP fusion proteins, only an about 30 amino acid peptide encompassing the acidic activation domain is neccessary for efficient target gene activation. This minimal activation domain shares significant sequence identity between the four members of the PAR family. By using a GST-pulldown approach we found a chromatin-associated serine protein kinase that binds to this conserved peptide segment. This serine protein kinase exhibits high phosphorylation activity for the C-terminal domain of the largest RNA polymerase II subunit (CTD). Mutations in DBP that reduce the affinity for this CTD kinase activity also interfere with transcription activation. The possible significance of these findings for transcription activation will be discussed.

In spite of their identical *in vitro* DNA binding specificity, and their highly conserved activation domain, not all PAR proteins are functionally redundant. TEF, and HLF36, while capable of activating transcription efficiently from the albumin promoter, are unable of enhancing transcription from the cholesterol 7α promoter. Conversely, DBP and HLF42 are more potent stimulators of the the cholesterol 7α promoter than of the albumin promoter. Unexpectedly, subtle differences in the bZip regions, rather than divergent N-terminal peptide sequences appear to account for the different promoter preferences od DBP and TEF (7). In contrast, in the case of the two HLF proteins, 49 N-terminal amino acids that are present in HLF42 but absent from HLF36 appear to be responsible for their different target promoter specificity (8).

Recently, we established two mouse lines with different DBP mutant alleles. Homozygous DBP-/- mutant animals of both transgenic lines are

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viable and fertile, but show subtle differences in circadian metabolism and behavior. In one mutant allele, the entire coding region of DBP was replaced with a bacterial lacZ gene driven by the DBP promoter. This allowed the relatively easy identification of individual cells (putatively) expressing DBP. In 9 to 14 day embryos, x-gal staining is observed in most if not all sensory ganglia, and in the adult brain, it is present in parts of the hippocampus and in subregions of the cortex that are responsible for the processing of sensory information. It is thus conceivable, that DBP is a transcriptional regulator of genes involved in sensory processing.

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FOURTH SESSION

Chairperson: Michael Karin

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Transcriptional Control of Muscle Gene Expression.

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Skeletal muscle in vertebrate embryos is derived from mesodermal progenitors in the somites which become committed to the myogenic lineage and subsequently express muscle-specific genes. The muscle-specific bHLH protein myogenin acts as a genetic switch to activate skeletal muscle-specific gene expression during mouse embryogenesis. Transcriptional activation of the *myogenin* gene is dependent on the MEF2 family of MADS box proteins, which bind to the *myogenin* promoter. Four *MEF2* genes, designated MEF2A-D, have been cloned in mice and their products bind as homo- or heterodimers to an A+T-rich DNA sequence in the control regions of numerous muscle-specific genes. During mouse embryogenesis, *MEF2* genes are expressed in myogenic progenitors of the three muscle lineages, suggesting that MEF2 factors may control aspects of the myogenic program common to these lineages. To further define the role of MEF2 within the genetic pathway leading to myogenesis, we have cloned a homolog of MEF2, referred to as D-MEF2, from *Drosophila*. D-MEF2 expression is first detected within uncommitted mesoderm prior to formation of the somatic, visceral, and cardiac muscle lineages. During late gastrulation and germ band extension, the expression pattern of D-MEF2 is similar to that of *twist*, which regulates mesoderm formation, and precedes expression of the myogenic bHLH gene *nautilus*. Expression of D-MEF2 is dependent on the mesodermal determinants *twist* and *snail* but independent of the homeobox-containing gene *tinman*, which is required for visceral muscle and heart development. Embryos homozygous for a *D-mef2* null allele fail to express muscle structural genes in somatic, cardiac, or visceral muscle, but they contain precursors of these lineages that are normally positioned and specified. Together, these studies reveal an essential role for the MEF2 family in the establishment of the three muscle cell types, skeletal, cardiac, and smooth muscle, and they suggest that the genetic pathways that specify these cell lineages are highly conserved from *Drosophila* to mammals.

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The regulatory network of transcription factors controlling skeletal muscle development in vertebrates. H. Arnold and T. Braun, Department of Cell and Molecular Biology, University of Braunschweig, Spielmannstr. 7, 38106 Braunschweig, F.R.G.

The roles of myogenic regulatory genes during mouse development have been studied in mouse knock-out models. Animals carrying a MyoD null mutation are viable and have no obvious defects in skeletal myogenesis. Similarly, mice lacking the Myf-5 gene also develop normal skeletal musculature, however, this mutation results in perinatal death owing to a severe defect in rib formation. Double mutants lacking both the Myf-5 and MyoD genes fail to form any skeletal muscle and myogenic precursor cells. Taken together, these observations suggested that Myf-5 and MyoD are independently capable of specifying muscle development due to overlapping or partially redundant functions. Indeed, in Myf-5 mutant mice skeletal muscle development is considerably delayed but resumes when MyoD begins to be expressed.

To study the mechanism by which MyoD can rescue the muscle defect in Myf-5 knock-out mice, we targeted one Myf-5 allele in Es cells with the HSV TK gene which allows selective ablation of Myf-5 expressing cells with nucleotide analogues, such as FIAU. Despite complete elimination of Myf-5 positive cells from embryoid bodies, MyoD expressing cells appear and readily differentiate into myotubes. We also targeted the second Myf-5 allele with the LacZ gene to obtain Myf-5 null cells which can be traced by β-Gal staining. Upon ES cell differentiation one finds activation of the Myf-5 locus and MyoD expression in different cells but both genes are never active within the same cell. These results indicate that Myf-5 and MyoD are expressed in distinct mesenchymal stem cells and each gene determines a different skeletal muscle cell lineage This view is consistent with the observation that MyoD - and Myf-5 positive cells are found in separate and distinct muscle regions in mouse embryos.

In contrast to MyoD, myogenin and Myf-6 functions do not overlap with Myf-5. A factual double mutant lacking Myf-6 and Myf-5 develops essentially normal muscle, although deep back muscles appear reduced in size and few genes encoding isoforms for contractile proteins are slightly down-regulated. Thus, myogenesis can be maintained in the absence of two of the four myogenic regulatory genes when functional complementation by the two remaining genes is ensured.

The rib phenotype in Myf-5 minus mice is not a cell-autonomous defect as Myf-5 is not expressed in sclerotomal cells. FGF4 and FGF6 emenating from the early myotome are possible signals which may affect sclerotomal development. Both growth factors are not expressed in Myf-5 knock-out mutants. Somite cultures respond to FGF4 and FGF6 together with TGFβ with enhanced chondrogenesis. These observations suggest that development or maintenance of part of the sclerotome may be dependent on FGF4 and / or FGF6.

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MOLECULAR GENETIC ANALYSIS OF cAMP AND GLUCOCORTICOID SIGNALING IN DEVELOPMENT AND PHYSIOLOGICAL AND PATHOLOGICAL PROCESSES

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To understand the role of glucocorticoid and cAMP signaling during development, we have disrupted the CREB and the glucocorticoid receptor gene by homologous recombination in mouse embryonic stem cells. Most of the mice with a disrupted glucocorticoid receptor gene die within the first hours after birth due to severe atelectasis of the lungs, possibly resulting from impaired surfactant and amilioride-sensitive Na+-channel synthesis. Perinatal induction of gluconeogenic enzymes in liver is impaired. Regulation of glucocorticoid synthesis via the hypothalamic-pituitary-axis is perturbed leading to increased corticosterone and ACTH levels. Interestingly, there is a significant increase in these hormones in mice heterozygous for the mutation. Activation of the hypothalamic-pituitary-axis results in extensive hypertrophy and hyperplasia of the cortical zones of the adrenal gland and induction of genes involved in steroid biosynthesis. The adrenal medulla is disorganized and severely reduced in size; no cells capable of epinephrine synthesis can be detected. It is not yet clear whether this deficiency results from impairment of proliferation and/or survival and/or misrouted migration of sympathoadrenal precursors. Thymocytes were found to be resistant to glucocorticoid induced apoptosis. Mice deficient in the CREB protein appeared healthy and exhibited no impairment of growth and development. We find that CREB and two other members of the family of CRE-binding proteins, CREM and ATF1, are capable of mediating cAMP dependent transcriptional responses. CREM expression is upregulated in CREB deficient mice as well as a novel CREB mRNA isoform. Both processes may be responsible for compensation of CREB function during development. Future analysis will be directed to understanding the role of these signaling pathways in development and physiological and pathological processes, such as memory formation, the acute phase response, circadian rhythmicity and drug addiction.

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Characterization of the DNA binding and transcriptional activation domains of MEF2C

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MEF2 was originally identified as a muscle-specific DNA binding factor that is induced as myoblasts differentiate into myotubes. MEF2 has recently been cloned and is encoded by four genes, termed mef2a,b,c,d, in both humans and mouse. MEF2A, MEF2B, and MEF2D transcripts are ubiquitously expressed while MEF2C expression is restricted to skeletal muscle, brain and spleen. These proteins contain a MADS domain, which is a 56 amino acid motif that mediates DNA binding and dimerization as well as a highly conserved domain referred to as the MEF2 domain that is of unknown function. In this study, we characterized the regions of MEF2C that were responsible for transcriptional activation, DNA binding, and subunit dimerization. We identify the individual amino acids that are involved directing both DNA site specificity as well as subunit dimerization. Mutagenesis of the highly conserved MEF2 domain immediately adjacent to the MADS domain, demonstrated that it plays an important role in DNA binding affinity, but not in dimerization. The MEF2 domain contains a consensus casein kinase II motif that is important for high affinity DNA binding and transactivation potential. We show that site-specific mutation of amino acids in the MADS-box that are critical for DNA binding, result in proteins that function as dominant negative mutants, which are capable of dimerizing with endogenous MEF2 activities. Dominant negative MEF2 proteins are shown to down-regulate expression of both the myogenin promoter and a artificial MEF2-dependent reporter gene in C2C12 myotubes. It has previously been shown that a MEF2 site in the myogenin promoter is critical for conferring temporal expression of the myogenin gene during skeletal muscle development. Hence, MEF2 is part of a coordinated regulatory circuit with the myogenic bHLH proteins to augment the establishment of the differentiated phenotype of skeletal muscle.

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Inhibition Of Pax 5 Activity By Expression Of Its DNA Binding Domain.

The B-cell specific transcription factor pax 5 encodes the B cell specific activator protein (BSAP) and is expressed at all stages of B cell development except in terminally differentiated plasma cells (Adams et al., 1992). BSAP has also been shown to regulate the expression of the B cell specific antigen CD19 (Kosmic et al., 1992). This suggests that the expression of BSAP plays a role in B-cell commitment and development.

To investigate this role we have attempted to express BSAP activity in non-B cells using CAT reporter constructs containing 1, 2, or 4 BSAP binding sites. However, the reporter gene was not expressed. This suggests that while BSAP is necessary for transactivation of a target gene, it is not sufficient (ie. a second B-cell specific factor must be required). Therefore we produced a construct in which the BSAP DNA binding domain is linked to the VP16 transactivation domain (Dalton and Treisman 1992). Co-transfection of this construct with the CAT reporter constructs resulted in the expression of CAT activity. The level of expression is dependant on both the number of BSAP binding sites and the amount of the expression vector used. This effect could be inhibited by transfecting cells with a further plasmid expressing either native BSAP or a truncated pax 5 gene product demonstrating that the BSAP DNA binding domain alone can act as a competitive inhibitor of BSAP binding (Hastings and Adams, 1995).

Work is in progress to produce stable lymphocyte cell lines in which BSAP activity has been either expressed or inhibited. These cell lines will then be used to identify the genes regulated by BSAP and to investigate the role of BSAP in B cell commitment and differentiation.

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FIFTH SESSION

Chairperson: Mariann Bienz

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Frank Grosveld, Erasmus University Rotterdam, The Netherlands.

Transcriptional regulation of the ß globin gene cluster.

The globin gene cluster is regulated by several different cis acting mechanisms. The entire locus is regulated by the Locus Control Region (LCR) and each of the genes contains regulatory regions that are important for its stage specific expression. The transcription factors involved in this mechanism will be discussed. The genes are further regulated via a competitive mechanism between the genes leading to the suppression of the adult genes early during development. We have postulated that the interaction of the LCR and the genes is mediated via a stochastic looping mechanism. A number of experiments to distinguish such looping from tracking mechanisms will be presented. Lastly we will discuss the dynamics of this looping mechanism in vivo by presenting a novel method of measuring transcription in nuclei in vivo.

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Developmental Regulation of ß-Globin Gene Transcription in Reconstituted Chromatin and Synthetic Nuclei.

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Overview of Laboratory Interests:

We are interested in defining the mechanisms involved in tissue-specific gene expression using the chick and human ß-globin gene families and the mouse T-cell receptor α-chain gene (TCR α) as model systems. Our approach is to use in vitro transcription and chromatin/nuclear assembly systems with cloned ß-globin and TCR α genes to recapitulate major aspects of transcriptional regulation that are known to occur in vivo. In this way, we can decipher how transcriptionally active chromatin structures are formed by individual or multiple proteins in a tissue- and developmental stage-specific manner and how nuclear processes such as DNA replication and the cell cycle affect regulated gene expression. We are particularly focussing on the mechanism by which long-range communication between DNA elements within a complex chromosomal locus is established. This includes interactions between promoters and distal enhancers as well as tissue-specific Locus Control Regions (LCRs) which regulate the transcriptional activity of large genomic domains.

Current Research:

We have analyzed the transcriptional properties of the ß-globin chromosomal locus by incorporating cosmid DNA templates into synthetic nuclei using Xenopus egg extracts. These organelles form higher-order chromatin structures and are capable of DNA replication, protein transport, and cell cycle events. Nuclear assembly in the presence of stage-specific erythroid proteins correctly recapitulates tissue-specific chromatin structure and long-range promoter-enhancer interactions within the 40 kb chromosomal locus resulting in ß-globin gene activation. We find that the programmed transcriptional state of a gene, as encoded by its chromatin configuration and long-range interactions, is stable to DNA replication. That is, active genes are still expressed and inactive genes remain silent after the passage of a replication fork. However, transcriptional reprogramming of ß-globin genes can occur during DNA replication by active chromatin structural remodeling in the presence of erythroid-specific DNA binding proteins. In this case, replication provides a transient opportunity to alter nucleosomal placement by competition with transcription factors. Our results also demonstrate that enhancer function

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does not require DNA replication but once promoter-enhancer communication is established, it is stable to transient disruption by replication. Studies conducted with individually cloned ß-globin genes indicate that the interaction of two erythroid-specific DNA binding proteins, GATA-1 and NF-E4, with a specialized TATA box (-30 GATA) in the promoter is sufficient to generate actively transcribed nucleosomal templates. These proteins function by displacing a nucleosome from this critical control region and recruiting the distal enhancer in a developmentally-regulated manner.

Future Directions:

Using ß-globin and TCR genes reconstituted into chromatin or incorporated into synthetic nuclei, we plan to 1) decipher the mechanism of enhancer-dependent transcription and analyze how enhancers switch between individual promoters at different stages of development; 2) determine how large chromosomal domains become tissue-specifically activated by LCR elements; and 3) examine the transcriptional regulation of a complex gene locus during the cell cycle.

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"Participation of chromatin structure in regulated transcription"

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Eukaryotic cells control expression of their genetic information in response to internal and external stimuli by signal transduction to the cell nucleus where the activity of transcription factors is modulated by ligands or by changes in phoshorylation. For each particular gene, a specific array of DNA *cis*-acting elements in the enhancer and promoter regions determines the combinatorial interaction of transcription factors. In addition, the organization of the eukaryotic genome in chromatin influences the accessibility of the regulatory DNA sequences and influences the interactions among DNA-bound factors. To study these questions, we have chosen a viral promoter and a cellular enhancer.

The MMTV promoter responds to steroid hormones and is organized into phased nucleosomes. The proviral genome is repressed in the absence of hormones, but its transcription is rapidly activated in response to glucocorticoids or progestins. The MMTV promoter contains a complex hormone responsive unit (HRU) composed of 5 receptor binding sites, upstream of a binding site for the transcription factor nuclear factor I (NFI), two octamer motifs, and the TATA box. In cells carrying MMTV sequences, the HRU is organized into a phased nucleosome, and the orientation of the double helix on the surface of the histone octamer is compatible with binding of the hormone receptors to two of the five sites, but precludes binding to the NFI site and to the octamer motifs [1]. Hormone treatment leads to a rapid alteration in chromatin structure that makes the dyad axis of the regulatory nucleosome more accessible to digestion by DNaseI [1]. Simultaneously, all four receptors binding sites, the NFI site, and the octamer motifs are occupied, while the histone octamer remains in place [1]. Since a full loading of the MMTV promoter with transcription factors can not be achieved in on free DNA [2, 3], the nucleosomal organization, not only keeps the promoter silent prior to hormone induction, but enables simultaneous binding of all relevant factors. This hypothesis is supported by the results obtained in yeast strains carrying the MMTV promoter and expressing hormone receptors and NFI. In these strains, the MMTV sequences are regularly organized in chromatin, and transcription from the MMTV promoter is dependent on hormone, receptors and NFI. Nucleosome depletion in this yeast strains enhances MMTV transcription in the absence of hormone but decreases the magnitude of the hormonal induction, as expected if nucleosomes would fulfil a double function: repression in the absence of inducer and facilitation of optimal expression after induction.

The uteroglobin gene is expressed in a variety of secretory epithelial cells. In the uterus it is induced in response to the sequential action of estrogens and progesterone. Whereas the promoter contains a functional estrogen responsive element (ERE) [4], the elements mediating progesterone induction are located 2.5 kb upstream of the transcription start site in the context of a complex enhancer region [5]. In primary cultures of rabbit endometrial cells,

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this region is regularly organized in chromatin and does not show evidence for bound factors prior to hormonal stimulation. After hormonal induction, DNaseI hypersensitive sites appear on the enhancer [5], and clear protection ovef a binding site for NF-Y is detected by genomic footprinting. A short fragment containing the NF-Y binding site exhibits enhancer function in transfection experiments and mutation of the a single base that eliminates NF-Y binding *in vitro* also inactivates enhancer function. Thus binding of hormone receptors to the HREs of the uteroglobin enhancer recruits NF-Y, in a process reminiscent of the hormone-dependent recruitment of NFI to the MMTV promoter. How the recruitment of factors to the enhancer mediates activation of the uteroglobin promoter remains to be established.

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ASSEMBLY AND FUNCTION OF MULTICOMPONENT ENHANCER COMPLEXES

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Cis-acting regions that control transcription, recombination or replication are typically composed of multiple binding sites for DNA-binding proteins, which allows for both diversity and accuracy in the regulation of DNA transactions. The binding of specific proteins to these regulatory sequences is complicated by large genomes that can sequester DNA-binding proteins non-specifically and by the multiplicity of proteins with similar or even identical sequence recognition properties. Therefore, the binding of proteins to their genetic target sites *in vivo* has been proposed to require the formation of three-dimensional nucleoprotein complexes in which multiple protein-DNA and protein-protein interactions augment the specificity of factor recruitment and the stability of the final complexes (Echols, 1986).

High mobility group (HMG) domain proteins have been identified as chromosomal nonhistone proteins that are typically unable to stimulate transcription on their own, but act in concert with other regulatory proteins. HMG-domain proteins change the conformation of DNA and have been proposed to function as "architectural" components in the assembly of higher-order nucleoprotein complexes (reviewed in Maniatis & Tijan, 1994; Wolffe, 1994). We have studied the role of lymphoid enhancer-binding factor (LEF-1) in the assembly and function of a specific multiprotein complex at the T-cell receptor (TCR) α enhancer. LEF-1 recognizes a specific sequence in the TCRα enhancer and participates in the synergistic regulation of this enhancer by multiple proteins (Travis et al., 1991; Waterman et al, 1991; Carlson et al, 1993; Giese & Grosschedl, 1993). We find that TCRα enhancer function requires a specific arrangement of binding sites for LEF-1, Ets-1, PEBP2α (CBFα) and an ATF/CREB protein. Ets-1 cooperates with PEBP2α to bind adjacent sites at one end of the enhancer, forming a ternary complex that is unstable by itself. Stable occupancy of the Ets-1 and PEBP2α binding sites in a DNase I protection assay depends on both a specific helical phasing relationship with the nonadjacent ATF/CREB-binding site at the other end of the enhancer and on LEF-1. The HMG domain of LEF-1 which was previously found to bend the DNA helix in the center of the TCRα enhancer, can in part be replaced with the HMG domain of the distantly related SRY protein, which also bends DNA (Giese et al, 1992; 1995). Taken together with the observation that Ets-1 and ATF-2 can associate in vitro, these data suggest that LEF-1 can coordinate the assembly of a specific-higher order enhancer complex by facilitating interactions between proteins bound at nonadjacent sites.

Enhancers and locus control regions have been found to mediate transcriptional activation in the context of chromatin that limits factor access (reviewed in Felsenfeld, 1992; Grosveld et al., 1994; Kornberg and Lorch, 1995). We have studied the immunoglobulin μ heavy chain gene to assess the role of enhancer sequences in mediating accessibility in chromatin. By replacing an RNA polymerase II promoter with a prokaryotic T7 promoter we attempted to study factor access independent of transcription by endogenous polymerases and protein-protein contacts (Jenuwein, et al., 1993). Toward this end, we linked various μ enhancer fragments to the T7 promoter and introduced the gene constructs into the mouse germ line. The accessibility of the T7 promoter was subsequently examined by adding purified T7 RNA polymerase to nuclei from immortalized transgenic pre-B cells and by analyzing nascent T7-specific transcripts. A 95-bp μ enhancer core element was found to be necessary and sufficient to confer accessibility on the T7 promoter, independent of its chromosomal position. However, these minimal enhancer sequences were not sufficient to induce DNase I hypersensitive sites and extended accessibility. Additional μ sequences that mediate association with the nuclear matrix (Cockerill et al., 1987) were found to be required to induce DNase I hypersensitive sites. The nuclear matrix attachment regions (MAR)

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flank the μ enhancer on either side and are important for position-independent expression of the rearranged μ gene in germline transformation assays (Forrester et al, 1994). Thus, multiple *cis* acting components are required to confer the correct developmental expression upon the μ gene.

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FUNDACION JUAN MARCH EX-LIBRIS

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SIXTH SESSION

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Target sequences for growth factor-like signals in Drosophila

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During Drosophila embryogenesis, an induction takes place between two inner germ layers: the endoderm is induced by the visceral mesoderm to which it adheres (1). The two extracellular signals which mediate this induction are *decapentaplegic* (*dpp*), a TGF-ß-like protein, and *wingless* (*wg*), a Wnt-1 protein. These signal proteins are produced in the visceral mesoderm, and their localised expression in this cell layer depends on the homeotic gene *Ultrabithorax* (*Ubx*). They are secreted from the visceral mesoderm and induce localised expression of the homeotic gene *labial* (*lab*) in the endodermal cell layer (2). Subsequently, *lab* specifies differentiation of a particular cell type in the larval midgut, the so-called copper cells (3). Thus, subdivision of the larval gut into functionally distinct sections depends on this induction by the visceral mesoderm.

The two extracellular proteins *dpp* and *wg* also signal back to the visceral mesoderm: they are required for maintenance of *Ubx* expression in this cell layer. They are thus part of an indirect autoregulatory loop of *Ubx* (4). Since *wg* is expressed in a cell group adjacent to that expressing *Ubx* and *dpp*, this implies that the indirect autoregulatory mechanism depends on cell-cell communication. It is worth noting that induction of *lab* in the endoderm is also autoregulatory, and that there is tight linkage between autoregulatory target sequences and signal response elements in this gene (5). This intimate connection between autoregulation of homeotic genes and their controls by extracellular signals indicates that the autoregulatory process in both cases does not merely ensure maintenance of expression, but that it also allows reassessment of position (1).

Our aim is to identify target transcription factors which mediate the response to the two signals in the visceral mesoderm and in the endoderm. To do this, we have started to pin down response sequences for *dpp* and *wg* in the upstream flanking sequence of *Ubx* and *lab*. In the case of *Ubx*, *dpp* response elements are clearly separable from *wg* response elements, and the two signals act independently, as well as synergistically, to stimulate *Ubx* expression (6). The same appears to be true for *lab* (5). Our preliminary evidence suggests that binding sites for the same proteins function in both genes, and therefore in both cell layers of the embryonic midgut, to mediate the response to *dpp* and to *wg* signalling, respectively. The *dpp* response sequence confers transcriptional activation upon *dpp* signalling, whereas the *wg* response sequence apparently confers transcriptional repression in the absence of *wg* signalling. Furthermore, our results suggest that, in both cases, an activator binding site overlaps a repressor binding site: *dpp* signalling targets a transcriptional activator which competes with a constitutive repressor, whereas *wg* signalling appears to target a transcriptional repressor competing with a constitutive activator. This indicates that the response elements for both signals consist of sensitive transcriptional switches.

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Transcriptional control of *ac/sc* expression in proneural clusters.

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The peripheral nervous system of *Drosophila melanogaster* provides a model to study the determination of specific cell types in precisely defined positions. Indeed, over one thousand bristles and other types of external sensory organs (SOs) appear on the cuticle of this insect either in remarkably constant positions or in "density" types of arrangements that cover constant areas of the fly's body. These patterns are prefigured in the imaginal discs, anlagen of the adult epidermis, by the pattern of expression of the proneural *achaete* (*ac*) and *scute* (*sc*) genes, two members of the *ac-sc* complex (AS-C). These genes are simultaneously expressed by groups of cells, the proneural clusters, located at constant positions in discs. Their products -transcription factors of the basic-helix-loop-helix family- allow cells to become sensory organ mother cells (SMCs), a fate normaly restricted to only one or a few SMCs per cluster by negative cell-cell interactions mediated by the products of the neurogenic genes. Thus, the pattern of expression of *ac/sc* in proneural clusters is one of the agents that specify the positions where SMCs are born. Evidence will be presented showing that the complex pattern of proneural clusters is constructed piecemeal, by the action on *ac* and *sc* of site-specific, enhancer-like elements distributed along most of the AS-C DNA (approximately 90 kb). In transgenic flies, fragments of AS-C DNA containing these enhancers drive a reporter *lacZ* gene in only one or a few proneural clusters. The data indicate that coexpression of *ac* and *sc* is accomplished by activation of both genes by the same set of position-specific enhancers. Remarkably, nearly equal relative levels of *ac* and *sc* proteins accumulate in most cells of different proneural clusters, suggesting that the *ac* and *sc* promoters interact equally efficiently with enhancers upstream of *ac*, in between both genes (which are separated by 25 kb of DNA), and downstream of *sc*, a so far almost unique case.

Expression of *lacZ* driven by the isolated enhancers is independent of the *ac* and *sc* endogenous genes, which indicates that the enhancers respond to local

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combinations of factors (prepattern) different from the *ac* and *sc* products. Most prepattern factors are unknown, one exception being the products of the *iroquois* (*iro*) and at least two other closely related genes located at the 69CD chromosomal subdivision. *iro* encodes a 717 amino acid polypeptide with an homeodomain, which is highly divergent from that of Antennapedia and most closely resembles those of *Drosophila* extradenticle and human PBX proteins, a very acidic motif, glutamine stretches and gly/ser-rich motifs as its most salient features. In wing imaginal discs, these genes are expressed in a complex pattern which covers regions that comprise one or more proneural clusters. The *iro* protein binds in vitro to at least the enhancer sequence that directs expression of *ac/sc* in the vein L3 and TSM proneural clusters. Site-directed mutagenesis of the binding site, abolishes enhancer activity. In mutants that remove expression of *iro* and related genes in the L3 region, the corresponding proneural cluster is abolished. Ectopic expression of *iro* at this site restores the proneural cluster. Taken together, these and other results indicate that the *iro* and *iro*-like homeoproteins are necessary for *ac/sc* activation at several sites and for wing vein formation. They are members of a prepattern of factors intimately related to the patterning of the imaginal discs.

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FUNCTIONAL ANALYSIS OF *Krox-20* DURING CNS AND PNS DEVELOPMENT

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In the hindbrain region of the developing CNS, antero-posterior patterning involves a transient segmentation process which leads to the formation of morphological bulges called rhombomeres (r). The rhombomeres constitute cell lineage restriction units and participate in the establishment of the metameric organisation. Like Drosophila compartments, they also constitute domains of specific gene expression. The *Krox-20* gene encodes a zinc finger transcription factor and is expressed within the developing hindbrain in two transverse domains which prefigure and then coincide with r3 and r5. We have inactivated *Krox-20* by recombination in ES cells and inserted the *lacZ* coding sequence in its locus. Analysis of mutant embryos have indicated that ß-galactosidase activity faithfully reflects *Krox-20* expression and that homozygous inactivation of the *Krox-20* gene leads to partial or complete deletion of r3 and r5. We will present the results of recent studies on the consequences of these deletions during late embryogenesis and on the involvement of *Krox-20* in the activation of downstream genes. We have also found that *Krox-20* is expressed in Schwann cells and that its activity is required for myelination in the peripheral nervous system. We will present recent data concerning the regulation of the expression of *Krox-20* in Schwann cells and its implications for Schwann cell development.

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POSTERS

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THE PROMOTER REGION OF THE HUMAN *C4BPB* GENE.

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The human complement regulator C4b-binding protein (C4BP) is an oligomeric protein present in plasma in three isoforms with different composition of two types of polipeptides, the α-chain (70kDa) and the β-chain (45kDa). These polypeptides have distinct and independent functions in the C4BP molecule. The α-chain has the binding site for C4b, whereas the β-chain binds and inactivates the anticoagulant regulator protein S. The α and β chains most likely originated from a common ancestor. They are structurally related and are encoded by closely linked genes within the RCA gene cluster. Both *C4BPA* and *C4BPB* genes exhibit a hepatic-restricted expression in humans and respond to acute phase modulators. Different lines of evidence suggest a complex regulation of the expression of these genes during acute phase responses, which results in the modulation of the relative proportion of the C4BP isoforms.

We have previously reported the characterization of the human *C4BPA* promoter. To get further insights in the molecular mechanisms that control the expression of C4BP in humans, we have analyzed the promoter region of the *C4BPB* gene: Our data show that the *C4BPB* promoter is contained within the first 126 nucleotides upstream the major transcription start site and that this region is sufficient to confer hepatic-specific expression to a CAT reporter gene. We also described that a small region of 36 nucleotides (-126 to -90) is responsible for more than 90% of the promoting activity and that this region includes three functional binding sites for ubiquitous and hepatic-enriched factors. Two of these sites interact with the HNF3 family of transcription factors and the third one binds NFI. Each of these sites has a positive contribution to the activity of the *C4BPB* promoter. A comparative analysis of the structure of the promoter regions of both human *C4BPA* and *C4BPB* genes will be presented.

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Analysis of the Paramyosin and miniparamyosin expression in *D. melanogaster*. J. J. Arredondo, M. Maroto, R. Marco, and M. Cervera. Departamento de Bioquímica e Instituto de Investigaciones Biomédicas. Universidad Autónoma. Arzobispo Morcillo 4. Madrid 28029. Spain

The PM/mPM gene encodes two proteins, paramyosin and miniparamyosin with very different mobility, 107 and 55 kDa respectively. The paramyosin and miniparamyosin mRNAs arise from two overlapping transcriptional units; the miniparamyosin transcription initiation site is located inside a PM intron, 8 kb downstream from the one used for paramyosin transcription. The existence of two different promoters controlling the expression of the paramyosin and miniparamyosin, as well as the conserved and non-conserved features of their sequences suggest a very complex regulation of these two muscle proteins. In fact, while the expression of paramyosin is similar to many other *Drosophila* muscle proteins, miniparamyosin appears late in development and in fact is only present only in adult musculature. Moreover, both PM and mPM show several isoforms, have different localization in the sarcomere and the relative amount of myosin, paramyosin and miniparamyosin changes with the muscle type. The promoters study has been made using two different approaches: 1). Comparison of the promoter sequences from two different Drosophilidae: *D. melanogaster* and *D. virilis*, evolutively separated more than 50 millions of years. This comparison will reveal "putative" functional sequences. Specificaly, in this case: a) The existence of conserved sequences defining the transcriptional factor binding sites for *nau* and *Dmef2* (homologues in *D.m.* of MyoD and MEF-2). The conservation of a sequence in both organisms would be indicative of possible functionality and will help us in the selection of sequences for a posterior *in vivo* or *invitro* study of these motives. b) Identification of novel sequences, common to all muscle sarcomeric promoter genes. 2). Analysis of the functional properties of these "putative" sequences: The studies have been made *in vitro* by band shift and CAT assays of the above selected sequences and *in vivo* by transforming the germinal line of *Drosophila* . We are studing the expression of the βgalactosidase gene under control of the minimal promoter plus the selected sequences.

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MOLECULAR INTERACTIONS BETWEEN TRANSCRIPTION FACTOR USF AND THE LONG TERMINAL REPEATS OF HIV-1

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We have purified to homogeneity transcription factor USF from HeLa cells and obtained its 43-kDa subunit as a recombinant GST-fusion product.
Both proteins bind to the Long Terminal Repeat of HIV-1, footprinting over nucleotides from -173 to -157 upstream of the transcription start site and generating strong DNase I hypersensitivity sites at the 3' sides on both strands. The protected region contains a conserved E-box sequence, which is the target site for factors of the b-HLH-Zip family of nuclear proteins.
As detected by methylation protection studies, the factor forms symmetric contacts with the guanines of the palindromic CACGTG core of the recognized sequence. Its binding ability is abolished by the mutation of this core sequence, and strongly reduced by the cytosine methylation of the central CpG dinucleotide.
Upon binding, both recombinant and purified USF bend the LTR DNA template, as detected by circular permutation assays.
The role of USF in the control of transcription initiation from the LTR was tested by *in vitro* transcription assays. Upon addition of the protein, transcription is increased from constructs containing an intact binding site, while the responsiveness is abolished in constructs with a mutated sequence. Furthermore, addition of a decoy plasmid which contains multiple repeats of the target sequence results in downregulation of transcription from the LTR.
These results suggest that USF is a positive regulator of LTR-mediated transcriptional activation.

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Identification of cis-active promoter elements responsible for autoregulation of the human Pit-1/GHF-1 promoter.

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We examined the regulation of the human Pit-1/GHF-1 promoter activity in pituitary (GH3) and non-pituitary (HeLa) cells.
The human Pit-1/GHF-1 gene was isolated from a human placental genomic library. A 1.5 Kbp EcoRI fragment containing the proximal promoter was sequenced and analyzed.

Footprinting analysis of promoter fragments with a recombinant human GHF-1/Pit-1 protein revealed the presence of 7 Pit-1/GHF-1 binding sites at positions +14, -56, -333, -379, -409, -468 and -492 relative to the transcription start site. The sites at positions -468 (sequence ATGA<u>G</u>AAA) and -492 (ATGAAT<u>C</u>A) differed from the consensus Pit-1/GHF-1 binding sequence (ATGNWWWW) by one nucleotide and represented new high affinity Pit-1/GHF-1 binding sites. Only the sites at position +14 and -56 have been reported in the rat GHF-1 gene promoter.
An OTF site was identified at position -784 as well as a TPA-responsive element (TRE) located at position -491 and overlapping with the Pit-1/GHF-1 binding site located at position -492.

Pit-1/GHF1-LUC constructs containing progressive 5' deletions of the promoter were transfected into GH3 and HeLa cells. A very low promoter activity was observed in HeLa cells. However, cotransfections with RSVrGHF1 could restore the promoter activity indicating that the promoter activity was Pit-1/GHF-1 protein dependent. A promoter fragment from -103 to +14 containing the TATA box and one Pit-1/GHF-1 binding site was sufficient for a full activity of the promoter in GH3 cells. The addition of the Pit-1/GHF-1 binding site located in the 5' untranslated region resulted in a marked decrease in promoter activity indicating that this site could be a negative modulator of the transcriptional activity as it is the case for the rGHF1 gene.
Eventhough the TRE site was shown to bind the AP1 protein in footprinting experiments, no TPA induction but rather a moderate inhibition of the Pit-1/GHF-1 promoter activity was observed in transfected GH3 cells. However, a 4 to 7 fold TPA induction was observed in transfected HeLa cells in the absence of the human Pit-1/GHF-1 protein. This indicated that there is a competition between AP1 and Pit-1/GHF-1 for the binding on the composite TRE/GHF1 site. The role of the TRE site in the presence of the human Pit-1/GHF-1 protein remains to be elucidated.

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The role of the OTF site was also examined. In transient transfection experiments, the addition of the OTF site to the proximal promoter resulted in a slight decrease in promoter activity in GH3 cells or in HeLa cells cotransfected with RSVrGHF1 indicating that this site might modulate the promoter activity. The proteins binding to the OTF site were partially identified. The recombinant human Pit-1/GHF-1 protein was shown to bind to this site with a low affinity in footprinting experiments and a complex containing both Oct-1 and Pit-1/GHF-1 was identified in bandshift assays. Additional experiments are needed to determine the function of the OTF site in the regulation of the human Pit-1/GHF-1 promoter activity.

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TRANSCRIPTION FACTORS INVOLVED IN THE LEUKOCYTE-RESTRICTED AND THE REGULATED EXPRESSION OF LFA-1 AND p150,95 INTEGRINS

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The ß2 or leukocyte-restricted Integrins, which include the LFA-1, Mac-1 and p150,95 (CD11a-c/CD18) heterodimers, play a key role in cell-cell interactions during immune and inflammatory reactions, as demonstrated by the existence of an immune disorder termed Leukocyte Adhesion Deficiency. LFA-1 is the only integrin expressed on all leukocyte lineages and is involved in the cellular adhesion events required for CTL and NK-mediated cytotoxicity, T and B lymphocyte responses, granulocyte and monocyte antibody-dependent cell cytotoxicity, and leukocyte extravasation into inflamed tissues. On the other hand, p150,95 is almost exclusively expressed on cells of the myeloid lineage and on activated B lymphocytes, and its expression is regulated during cell activation and differentiation by transcriptional mechanisms acting on the CD11c gene. The p150,95 integrin mediates CTL-mediated cytotoxicity, leukocyte adhesion to endothelium, and is a receptor for fibrinogen and the complement component iC3b. We have previously demonstrated that the proximal region of the *CD11a* and CD11c promoters are sufficient to direct the tissue-restricted and developmentally-regulated expression of LFA-1 and p150,95 (López-Cabrera et al., J. Biol. Chem. 268:1187,1993; Nueda et al., J. Biol. Chem. 268:19305, 1993).

Experiments designed to identify the DNA elements and transcription factors responsible for the unique pattern of p150,95 expression have demonstrated the presence of two Sp1-binding sites at -70 and -120 in the CD11c proximal promoter. Sp1 was demonstrated to transactivate the CD11c promoter in SL2 cells and mutations at the Sp1 proximal site (Sp1-70) greatly decreased the Sp1 transactivation capacity, whereas mutation of the distal Sp1 site (Sp1-120) had only a minor effect. More importantly, mutation of either Sp1 site led to a 3-fold reduction in the basal CD11c promoter activity in myeloid U937 cells, while the opposite effect was observed in the CD11c-negative HeLa cell line, indicating the involvement of both Sp1 sites in the tissue-specific expression of CD11c, a fact further confirmed by *in vivo* footprinting.

To identify transcription factors involved in the developmental regulation of the CD11c gene, we have used the U937 myelomonocytic cell line, whose PMA-driven monocytic differentiation induces the transcription of the CD11c gen. EMSAs demonstrated the presence of an AP-1-binding site within the CD11c gene promoter (AP1-60) and identified c-jun, junB, c-fos and Fra-1 as the members of the AP-1 family interacting with the AP1-60 site in differentiated U937 cells. The actual involvement of AP1-60 in DNA-protein interactions was also determined by means of *in vivo* footprinting experiments, and its functionality was demonstrated by transactivation of the CD11c proximal promoter by a c-jun expression vector in the AP-1-deficient F9 murine teratocarcinoma cell line. Site-directed mutagenesis of the AP1-60 site inhibited the basal promoter activity as well as the induction of the CD11c promoter activity during the PMA-driven U937 cell differentiation, demonstrating the implication of the AP-1 complex in the inducibility of CD11c during myeloid differentiation.

In addition, the combination of *in vivo* footprinting, functional analysis of serial 5'deletion mutants and the construction of U937 stable transfectants containing the CD11c promoter ligated to the luciferase gene reporter has allowed us to establish the importance of members of the bHLH family of proteins in the myeloid-restricted expression of CD11c. Thus far, we have identified three discrete regions of myeloid-specific interactions by EMSA and *in vivo* footprinting, which overlap CANNTG sequences. The identity of the specific factors responsible for these complexes is being pursued at the present moment.

The importance of the bHLH proteins in the tissue-specific expression of the leukocyte integrins and their functional relationships with ubiquitous transcription factors (Sp1, AP-1) will be discussed in the light of the functional activity of distinct promoter constructs harbouring distinct deletions and mutations.

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HORMONAL REGULATION OF THYROPEOXIDASE PROMOTER GENE TRANSCRIPTION.

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The mechanisms for hormonal regulation of rat thyroperoxidase (rTPO) gene transcription in rat thyroid cells (FRTL-5) has been well investigated. Previously we have demonstrated that the thyrotropin(TSH), via cAMP, and the insulin/insulin like grow factor (IGF-I) stimulate the activity of the minimal rTPO promoter element that confers thyroid-specific expression. This effect is mainly mediated by the thyroid-specific transcription factor TTF-2, however the fact that others thyroid-specific (TTF-1 and Pax-8) and ubiquitous (UFB) transcriptions factors will be able to recognise and bind to cis sequences located in the promoter, stated us to study the role that these factors play in the hormonal regulation of the rTPO promoter.

First we assayed by electrophoretic mobility shift assay the binding of nuclear proteins to DNA. The nuclear protein fraction was extracted of FRTL-5 thyroid cells, maintained in presence or depleted of TSH and/or IGF-I. As DNA, were used synthetic oligonucleotides with homologous sequences to the binding sites of the transcription factors TTF-1, Pax-8 and UFB. The results show that the binding of these factors to DNA is not hormonally regulated. However we believe that, in some way, these factors have to be involved in the observed regulation, since mutations in their binding sites have shown a decreased responsiveness to TSH and IGF-1.

One possible role of these factors could be participate with TTF-2 in mediating such regulation. In order to confirm our hypothesis, we made several constructions containing different combinations of the binding sites of all factors linked to the coding region of the reporter luciferase gene. All constructions were transfected into FRTL-5 cells and tested for their ability to respond to TSH and/or IGF-I. The results obtained indicate that TTF-1/Pax-8 binding site is actuating simply as amplifier element of hormonal response. However the distance between TTF-1/UFB and TTF-2 binding sites must be conserved. The combination of both binding sites is able to confer hormonal response in an heterologus promoter, while this response is abolished by incrementing the distance between both sites by only 5 pb.

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REGULATION OF E-CADHERIN PROMOTER IN MOUSE EPIDERMAL KERATINOCYTES

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E-cadherin (E-CD) and P-cadherin (P-CD) are two members of the calcium dependent cell-adhesion molecules expressed in normal epidermis, P-CD in basal proliferative cells, and E-CD in basal and suprabasal cell layers. During mouse skin carcinogenesis there is a down-regulation of the E-CD expression, and both, E and P-CD are completely switched off in the last stage of tumor progression (1).

In order to understand the molecular mechanisms involved in the regulation of the expression of the cadherin genes, we are analysing the E-CD promoter in a collection of keratinocyte cell lines ranging from nontumorogenic to highly tumorogenic exhibiting different levels of E-CD expression: MCA3D, PDV and E24 (E-CD +), HaCa4 and CarB (E-CD -).

Transient transfection assays with the -178 bp promoter/CAT construts and with different deletions (2) have shown low levels or complete absense of promoter activity in E-CD negative cells (HaCa4 and CarB) while variable levels of activity are detected in E-CD positive keratinocytes (MCA3D, PDV and E24). These studies have also indicated the positive involvement of several putative regulatory elements: the E-pal palindromic region (-90 to -70), a putative AP2 binding site (-71 to -64), a CAAT box (-65 to -60) and a GC rich region (-58 to -24).

Footprinting analysis with nuclear extracts of the varios cell lines have shown the existence of nuclear factors in the E-CD positive keratinocytes which interact with the GC rich region and the CAAT box, the AP2 binding site and the central region of the E-pal element. Interestingly, these factors appear to be absent or inactivated in E-CD negative keratinocytes.

Bandshift analysis and cotransfection assays with the E-CD promoter and several transcription factors (i.e. helix-loop-helix) are being performed to find out the nature of the nuclear proteins implicated in epithelial specific E-CD gene transcription.

Similar analysis are also being performed with the P-CD promoter (3) in the different cell lines.

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Natural killer (NK) cells are a subset of granular lymphocytes that have cytolytic activity against a variety of tumor and viral-infected cells, and also appear to mediate the rejection of bone marrow allografts. NK cells are able to lyse a variety of virally infected and neoplastic cells in an MHC-unrestricted manner. Although a variety of NK cell surface proteins have been identified, only a few seem to have a role in the cytolytic process, among them the cell receptor NKR-P1. This molecule plays a crucial role both for target cell recognition and for delivery of stimulatory or inhibitory signals linked to the NK cytolytic machinery.

The NKR-P1 protein was first characterized in rat A-LAK (Giorda et al, *Science* **249**: 1298, 1990) cells, and several isoforms occurs in both rat and mouse (Giorda and Trucco, *J. Immunol.* **147**: 1701,1991). These molecules are dimeric type II transmembrane proteins, each containing an extracellulár C-type lectin-like domain, a stalk region and a cytoplsmic tail with tyrosine and serine residues which are potential phosphorylation sites. The human homolog of NKR-P1 has also been cloned (Lanier et al, *J. Immunol.* **153**: 2417, 1994), and the oligo-saccharide ligands from NKR-P1 protein defined and proven able to activate NK cell cytotoxicity (Bezouska et al, *Nature* **372**: 150, 1994).

We are currently pursuing two lines of work to understand the expression of NKR-P1 genes in mouse:

a) In several genetic backgrounds (such as BALB/c and C57BL/6) the levels of expression of the three NKR-P1 genes differ considerably. Nucleotide sequence analysis of the promoter regiòns in the different strains suggest that differences in the level of expression do not result from alteration in the upstream regions of these genes, but may be caused by the expression of strain-specific transacting factors instead. To characterize and isolate such factors, we have developed a transient cell transfection assay, and our studies of the proximal promoter region of the genes suggest the presence of a specific transcriptional regulator activity in C57BL/6 background.

b) We plan to achieve NK cell lineage ablation in transgenic mice. K. O. NKR-P1 mice cannot be easily generated because it will involve the simultaneous inactivation of all the genes present in the NKR-P1 family. To bypass the problem, we are characterizing the control region that confers specific expression of NKR-P1 to NK cells. We will use such region to direct specific expression of a toxin gene to NK cells. As the initial set of experiments, we have generated transgenic mice expressing n-lacZ under the control of NKR-P1 3.5 Kb upstream region. Those experiments and related ones will help us to identify the regulatory region required *in vivo* for high level of expression of NKRP1 in NK cells. We'll then generate a next set of transgenic mice expressing diphteria toxin (DT) specifically in NK cells, our goal being the generation of mice in which only NK cells will be missing. Such transgenic animals should be optimal not only for understanding the transcriptional mechanisms that control a specific cell marker, but also for the study of the NK cells' role in normal immune reactions and its implications in tumorigenesis.

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TSH stimulates MAP kinase in human thyroid and rat astrocytes.

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Not only thyrotropin (TSH) stimulates the function of the thyroid, but also its growth and differentiation. Most of the effects of TSH are attributed to the stimulation of cAMP production, via G_S. However, only a part of the mitogenic effect of TSH is reproduced by forskolin in human thyroid. Moreover, in WRT cells, a dominant negative mutant of Ras decreases partially the mitogenic potential of TSH. As Ras has been shown located on a signalling pathway to stimulate MAP kinase by growth factors, the question of whether MAP kinase is subjected to regulation by TSH in thyroid remains unanswered.

We have found that the stimulation of human thyroid follicles by 10 nM TSH increased the MAP kinase activity in cytosolic fractions by 8-9 fold at 20 min. Only one isoform of MAP kinase was immuno-detected at 42 kDa (Erk2). The TSH-stimulated MAP kinase activity correlated with the intensity of its phosphorylation on tyrosine residues, and the appearance of a shift in its electrophoretic mobility. This TSH effect was decreased specifically by anti-TSH receptor antibodies. No intracellular cAMP elevating agent (neither forskolin, cholera toxin, nor permeant cAMP analogues) was able to reproduce the stimulation of MAP kinase by TSH, suggesting that cAMP-dependent protein kinase was not implicated. Pertussis toxin did not inhibit this TSH effect which therefore probably does not implicate $G_{i/o}$ proteins.

Furthermore, we have found the expression of TSH receptor in another type of cells, primary cultured rat astrocytes. In these cells, TSH stimulated MAP kinase (mostly Erk2, but also Erk1), but neither the cAMP production, nor the PtdIns(4,5)P_2 signalling pathway, and was not mitogenic.

We have demonstrated that MAP kinase belongs to a new signalling pathway of TSH. MAP kinase has been shown to be stimulated within mitogenic processes, but also within cellular differentiation. We do not know, as yet, whether the mitogenic effect of TSH involves MAP kinase in human thyroid. Moreover, TSH controls the expression of thyroid specific genes, such as thyroglobulin or thyroperoxydase. Their expression needs the binding of the transcription factors TTF1 and Pax8. TTF1 is a phosphoprotein presenting potential sites of phosphorylation by proline-directed kinases. Then, the question is raised whether TTF1 could be phosphorylated by MAP kinase, with a modification of its transcriptionnal activity. TTF1 has also been found bound to the TSH receptor gene, the transcription of which is regulated by TSH in thyroid. Does TSH regulate the expression of its receptor in astrocytes too, and could MAP kinase and/or TTF1 be involved in this regulation?

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AML1 (PEBP2/CBF) transcription factors regulate the expression of the CD36 gene in myeloid cell lines.

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CD36 is a cell surface glycoprotein composed of a single polypeptide chain, which interacts with a large variety of ligands, including thrombospondin, collagens type I and IV, oxidized and acetylated LDL, fatty acids and erythrocytes parasitized with Plasmodium falciparum. Its expression is restricted to platelets, monocyte/macrophages, adipocytes, microvasculature endothelial cells and some epithelial cells. CD36 and the structurally related glycoproteins CLA-1 and LIMPII constitute a novel gene family.

CD36 may play a dual functional role in monocyte/macrophage cells . On one hand, CD36 may participate via binding to oxLDL in foam cell formation and development of atherosclerosis lessions. On the other hand, CD36 cooperates with thrombospondin and the $\alpha_v\beta_3$ vitronectin receptor in the recognition of apoptotic cells (such as neutrophils, T cells and eosinophils), resulting in cell clearance by phagocytosis.

We have recently elucitated the genomic organization of the CD36 gene and characterized the transcription initiation site of the gene. In order to study the molecular mechanisms that control gene transcription of the CD36 gene in monocyte/macrophages we are currently analysing its promoter region.

Transient expression experiments in myeloid cell lines with a seric of deletions of the CD36 promoter coupled to the luciferase reporter gene demostrated that as little as 158 base pairs located upstream from the transcription initiation site were sufficient to direct gene transcription of the reporter gene. A detailed analysis of the -158/+30 proximal promoter region revealed the presence of a binding site for members of the AML1 (PEBP2/CBF) transcription factor family, which are very frequently translocated or inverted in some myeloid leukemias. Disruption of the AML1 binding site markedly diminished the promoter activity, indicating an essential role of the AML1 family in the basal transcription of the CD36 gene. Like CD36, the myeloid genes encoding myeloperoxidase, elastase and CSF-receptor are also controlled by AML1 transcription factors.

The involvement of AML1 in the regulation of CD36 expression raises the possibility that an impaired expression of CD36 might contribute to some of the pathological manifestations observed in patients suffering acute myelogenous leukemia.

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INITIATOR-MEDIATED, NEURON-SPECIFIC EXPRESSION OF THE RAT FE65 GENE

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The FE65 gene encodes a nuclear protein of unknown function that is expressed in several areas of the rat nervous system during development, and in the adult animal, particularly in somatic and visceral ganglia (1). FE65 mRNA is particularly abundant in brain, whereas is barely detectable in other tissues (2). Analysis of the promoter region of the FE65 gene identified two regions: the first one (position -163-55) includes a site for the Sp1 factor, whose removal greatly decreases the transcriptional efficiency in both neural and non-neural cells, and which forms similar complexes with nuclear proteins from rat pheochromocytoma PC12 and rat fibroblasts Rat2 cell lines. The second element (position -55+44) encompasses the transcription start site and it is able to efficiently drive transcription only in neural cells (3). This element shows sequence similarity to the *consensus* sequence of the initiator binding protein YY-1 (4), with the homology centered at the transcription start site. The gel shift pattern of the second element is different in PC12 and in Rat2 cell lines. At least four different complexes are detected in extracts prepared from PC12 cells; among these, the complex with lower mobility and the one with higher mobility are common to PC12 and Rat2 cells; the slower is more abundant in the pheochromocytoma cells, while the faster is the main complex of the fibroblasts. Both complexes are specifically competed by the YY-1 *cis*-element. Furthermore, similar complexes are formed with the labeled YY-1 probe. Factors present exclusively in PC12 extracts form two different complexes with intermediate mobility, not competed by the YY-1 *cis*-element. Nuclear extracts from bovine brain show a shift pattern in which the YY-1 factor is not involved, while complexes with a mobility similar to the PC12-specific complexes are formed. Purification of the factors involved in the brain-specific expression of the promoter is in progress. Experiments are also in progress in order to establish the role of YY-1 and of the factors so far identified in the determination of the neuron-specific expression of the FE65 gene.

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Workshop on

SELECTIVE GENE ACTIVATION BY CELL TYPE SPECIFIC TRANSCRIPTION FACTORS

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