Instituto Juan March
de Estudios e Investigaciones

47 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Switching Transcription in Development

Organized by

B. Lewin, M. Beato and J. Modolell

R. Axel
P. A. Baeuerle
M. Beato
A. Bird
N. Brockdorff
P. Chambon
J. E. Darnell
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E. Hafen
I. Herskowitz
T. Hunter
H. Jäckle
B. Lewin
R. Losick
T. Maniatis
J. Modolell
K. Nasmyth
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INTRODUCTION

Benjamin Lewin

Integration of Information in Signaling to Transcription

Benjamin Lewin
Cell

The general theme of this meeting is the universality of the concept that cells respond to their environment—whether unicellular organisms in culture or animal cells *in vivo*—by a signaling process in which reception at the cell membrane is followed by transduction of the message to effect changes in transcription. The results of the process are very different in each case, but the basic design of the process in terms of a pathway of interaction, and sometimes even the individual components of the system, may be very similar.

The initiation of the process in the neural context is typified by Axel's data on olfaction. Here an olfactory receptor (presumably) binds to ligand and initiates a process that culminates in the conversion of the olfactory signal into a spatial activation at a particular glomerulus. The receptors appear also to be necessary for axonal guidance to make the contacts that determine the specificity of contacts.

This contrasts with the superficially very different system of yeast mating type, in which the interaction between pheromone and its receptor initiates a series of structural changes that culminate in cell fusion. The general message of Herskowitz's paper is that we can now begin to trace the individual interactions by which the stimulus at the cell surface is converted into changes in the cytoskeleton. Nasmyth showed that a process of differential transcription in mother and daughter cells may have its roots in the preferential distribution of a transcription factor between the mother and bud, in a process that appears to rely on the cytoskeleton.

The pathway of signaling may involve a linear cascade, in which successive phosphorylation events culminate in the activation of a transcription factor, as in examples provided by Treisman, or may require degradation of components that inhibit the pathway, as characterized by Baeuerle and by Goldstein.

One important question is at which level different sources of information are integrated. In some cases, the integration occurs effectively at the cell surface. An extreme case is perhaps represented by the steroid receptors, where, as Chambon reported, information is now becoming available about how the ligand causes the receptor to switch into an active form. Another case is represented by the JAK-STAT pathway, where, as Darnell reported, the JAK kinase and STAT transcription factor assemble into a complex with the receptor; activation results in the translocation of the STAT to the nucleus, where a characteristic set of genes are activated. Specificity here resides in the STAT that is recruited to the receptor.

A contrast is represented by signaling to NF-κB, where, as Maniatis reported, a complex of multiple transcription factors must assemble at the enhancer; the system is activated only in the presence of all the factors, so the formation of the enhanceosome in effect provides for integration of information on the DNA. Another example of integration on DNA is provided by early *Drosophila* development, where, as Jaeckle reported, we can now account for the anterior-posterior patterning along the embryo in terms of the levels and availabilities of individual transcription factors.

The events involved in activation may involved structural rearrangements of various types, ranging from the bending or unbending of DNA to the development of complexes that rely upon nucleosome structure, as characterized by Beato, that involve large scale changes in the state of chromatin, as characterized by Pirrotta and Brockdorff, or changes in the state of modification of DNA, as analyzed by Bird.

We see therefore that, when a signaling pathway is initiated, it may use integration of information at various levels, that various mechanisms may be employed to pass the signal from one stage to the next, that there may be significant crosstalk between pathways, but that a particular signal culminates in the activation of a defined pattern of gene expression.

FIRST SESSION: REGULATION OF TRANSCRIPTION

Chairman: Benjamin Lewin

Controlling the transcriptional activity of NF-κB. Tom Maniatis, Harvard University, Department of Molecular and Cellular Biology, Cambridge, MA 02138, USA.

NF-κB and other members of the rel family of transcriptional activator proteins are required for the regulation of a large number of genes that play an essential role in immune and inflammatory responses. In most cell types NF-κB is sequestered in the cytoplasm in an inactive state by virtue of its association with the inhibitor protein IκBα or another member of the IκB family. A variety of extracellular signals and stress responses can lead to the phosphorylation and subsequent degradation of IκBα, resulting in the translocation of NF-κB into the nucleus. Once in the nucleus, the activity of NF-κB can depend on the enhancer context of its binding site, and can be controlled both positively and negatively through specific interactions with members of the high mobility group (HMG) family of proteins. My laboratory has been investigating the mechanisms involved in these regulatory processes.

The ubiquitin-proteasome pathway is required for the activation of NF-κB

We have found that the signal-induced site-specific phosphorylation of IκBα targets the protein to the ubiquitin-proteasome pathway. First, we showed that highly specific inhibitors of the proteasome block the signal-induced degradation of IκBα and the accumulation of the phosphorylated protein. Second, in collaboration with Zhijian Chen at ProScript Inc. and Dean Ballard at Vanderbilt University, we found that phosphorylation of IκBα targets the protein to the ubiquitin-proteasome pathway. We showed that IκBα is ubiquitinated in vivo and in vitro following phosphorylation, and single amino acid substitutions in serine residue 32 or 36, which were previously shown to abolish phosphorylation and degradation of IκBα in vivo, prevent ubiquitination in vitro. Remarkably, ubiquitinated IκBα remains associated with NF-κB, and the bound IκBα is degraded by the 26S proteasome. Thus, ubiquitination provides a mechanistic link between phosphorylation and degradation of IκBα. Third, we found that lysine residues 21 and 22 serve as the primary sites for signal-induced ubiquitination of IκBα. Conservative Lys-Arg substitutions of both Lys-21 and Lys-22 produce dominant-negative mutants of IκBα in vivo, and

they prevent signal-dependent degradation of IκBα in vivo and ubiquitin conjugation in vitro. Thus, site-specific ubiquitination of phosphorylated IκBα at Lys-21 and/or Lys-22 is an obligatory step in the activation of NF-κB. We propose that phosphorylation of IκBα at serine residues 32 and 36 leads to a specific interaction between IκBα and a specific ubiquitin ligase (E3), which ubiquitinates the nearby lysine residues at positions 21 and 22. The ubiquitinated IκBα remains bound to IκBα and is degraded by the 26S proteasome.

Control of NF-κB activity in the nucleus

Our studies of the virus-inducible enhancer of the human interferon-β (IFN-β) gene have revealed that the transcriptional activity of NF-κB on this enhancer requires the high mobility group protein HMG I(Y) and interactions with transcription factors bound to adjacent DNA sequences. These interactions lead to the formation of a specific higher order nucleoprotein complex that includes at least three distinct transcription factors and the high mobility group protein HMG I(Y). Both the in vitro assembly and in vivo transcriptional activity of this complex require a precise helical relationship between individual transcription factor binding sites. In addition, HMG I(Y), which binds specifically to three sites within the enhancer, is required for cooperative binding of transcription factors in vitro and transcriptional synergy between these factors in vivo.

The structural role of NF-κB and HMG I(Y) in the IFN-β enhanceosome was investigated by DNA bending measurements. These studies revealed that the NF-κB binding site in the IFN-β enhancer (called PRDII) contains an intrinsic DNA bend of 20° toward the minor groove and that HMG I(Y) binding reduces the angle of this bend to 7°. Remarkably, NF-κB binding completely reverses the intrinsic bend in PRDII (to 22° toward the major groove). The combination of NF-κB and PRDII results in a bend of 26° toward the major groove. This DNA conformation differs from that in a complex formed between NF-κB and a κB site which does not function in the context of the IFN-β gene enhancer. These and other observations suggest that the binding of NF-κB and HMG I(Y) to PRDII promotes DNA conformational changes required for the formation of a functional enhanceosome.

NF-κB: A PARADIGM OF A SIGNAL-TRANSDUCING TRANSCRIPTION FACTOR

Patrick A. Baeuerle, Institute of Biochemistry, Albert-Luwigs-University, D-79104 Freiburg, Germany.

Inducible transcription is governed by transcriptional activator proteins that bind in some cases *de novo* to *cis*-regulatory elements. One such activator is NF-κB which activates a plethora of genes in response to primarily pathogenic conditions. Among the target genes of NF-κB are those encoding inflammatory cytokines, chemokines, hematopoetic growth factors, MHC class I molecules, cell adhesion molecules and NO synthase. The stimuli of NF-κB include viral and bacterial infections, chemical and physical stresses and inflammatory cytokines. With this panel of activators and target genes, NF-κB is a central genetic switch in the early pathogen response of higher eukaryotic organisms.

We have extensively analyzed the processes underlying the activation of NF-κB following pathogenic stimulation of cells. In unstimulated cells, the transcription factor is complexed to an inhibitory subunit, IκB. In the course of activation, human IκB-α is rapidly phosphorylated on serines 32 and 36. This covalent modification does not release IκB but renders the protein susceptible to increased ubiquitin conjugation and subsequent degradation by the proteasome. The rapid decay of IκB ultimately releases active NF-κB which can migrate into the nucleus and initiate mRNA synthesis upon binding to regulatory sites in genes. Novel aspects of this signal transducing process will be discussed. A main focus is the involvement of the endoplasmic reticulum in the activation of NF-kB in response to overexpression of membrane proteins. Intracellular calcium release appears to be uniquely required for this novel signalling pathway.

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SREBP: STEROL SENSOR AND bHLH-ZIP TRANSCRIPTION FACTOR COMBINED IN A SINGLE MEMBRANE-BOUND MOLECULE

Joseph L. Goldstein and Michael S. Brown
University of Texas Southwestern Medical Center, Dallas TX U.S.A.

An efficient regulatory mechanism maintains constant levels of unesterified cholesterol in animal cells despite wide fluctuations in the external supply. Cells have two regulated sources of cholesterol: 1) plasma lipoproteins via the low density lipoprotein (LDL) receptor; and 2) endogenous synthesis from acetyl coenzyme A. When demand is high, cells increase transcription of the genes for the LDL receptor and several enzymes of cholesterol synthesis, including 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) synthase. When sterols accumulate, these genes are repressed. The sterol sensing mechanism uses two closely related transcription factors designated sterol regulatory element binding proteins -1 and -2 (SREBP-1 and -2). Both SREBP-1 and -2 (1147 and 1141 amino acids) are intrinsic membrane proteins of the endoplasmic reticulum and nuclear envelope. In the absence of sterols a protease cleaves both SREBPs, releasing a NH_2-terminal fragment of ~500 amino acids that contains a basic helix-loop-helix leucine zipper motif and a transcriptional activating domain. This fragment translocates to the nucleus where it activates the LDL receptor and HMG CoA synthase genes. When sterols accumulate, the protease no longer acts, the SREBPs remain bound to the endoplasmic reticulum, and transcription declines. In addition to the sterol-regulated proteolysis, SREBP-1 and -2 are cleaved when cells are induced to undergo programmed cell death. This apoptosis-induced cleavage is not inhibited by sterols and is mediated by CPP32, a member of the interleukin-1 converting enzyme (ICE) family of cysteine proteases and the closest mammalian homologue to *ced-3*, the death gene in *C. elegans*. The current data indicate that CPP32 and the sterol-regulated protease cleave SREBPs at different sites and both proteolytic events are subject to independent regulation.

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ON THE ORIGIN OF CpG ISLANDS.

Bird, A., McQueen, H., Cross, S., Tweedie,S. and Macleod, D.
Institute of Cell and Molecular Biology, University of Edinburgh, Ki
Edinburgh, EH9 3JR, U.K.

CpG islands are DNA sequences that are on average 1kb in size and predominantly found at the 5' end of genes. Their distribution in the asymmetric, with high densities on some mammalian and avian chr very low densities on other chromosomes. CpG islands are GC-rich genomic DNA, and, unlike the rest of the genome, have CpG at the They lack methylation in germ cells and, in the vast majority of case as well. In mouse and man CpG island genes include all sequenced genes and many genes that show a tissue-restricted pattern of expre attempt to find the mechanism by which CpG islands remain free of undertook a detailed examination of the mouse adenine phosphorib (*aprt*) gene. This housekeeping gene has a CpG island which extend promoter and includes the first two exons.*In vivo* footprinting across showed that three GC-boxes are clustered at the 5' edge of the CpG i occupied, most probably by Sp1. No other footprints are detected w region. Deletion or mutagenesis of the Sp1 sites caused *de novo* meth island in a transgenic mouse assay. Thus the peripherally located Sp necessary to keep the *aprt* island methylation-free. We have extende CpG islands associated with genes that are silent in most cell types. suggest a common origin for CpG islands, based upon transcription

SECOND SESSION: CHROMATIN STRUCTURE AND EXPRESSION

Chairman: James E. Darnell

"Hormone induced chromatin remodelling at the MMTV promoter"

Sebastián Chávez, Karin Eisfeld, Christian Spangenberg, Mathias Truss & Miguel Beato

I.M.T, E.-Mannkopf-Str. 2, 35037 Marburg, F.R.Germany.

Regulation of eukaryotic genes is specified by a particular array of DNA *cis*-acting elements in the enhancer and promoter regions which determines the combinatorial interactions of sequence specific and general transcription factors. Moreover, the organization of the eukaryotic genome in chromatin is likely to influence the accessibility of the regulatory DNA sequences and could modulate the interactions among DNA-bound proteins. To approach these questions we have chosen to study the regulated transcription from the Mouse Mammary Tumor Virus (MMTV) promoter. The MMTV provirus is repressed in the absence of hormones and activated by the addition of glucocorticoids or progestins. The hormonal effects are mediated by a complex hormone responsive unit (HRU) composed of five receptor binding sites, upstream of a binding site for the transcription factor nuclear factor I (NFI), two octamer motifs, and the TATA box. In several mammalian cells, and even in yeast 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 allows binding of the hormone receptors to only two of the five binding sites [1, 2]. Moreover, the positioned nucleosome also precludes access to the NFI binding site and to the distal octamer motif. In reconstituted nucleosomes the NFI binding site is unaccessible no matter the rotational orientation of the relevant major groove, and efficient binding is only observed when the binding site is placed outside of the nucleosome. The difference in the ability of hormone receptors and NFI to recognize their binding sites in nucleosome reflects the different sector of the DNA double helix contacted by the two proteins.

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 five receptors binding sites, the NFI site, and the octamer motifs are occupied, while the nucleosome remains in place [1, 2]. Since a full loading of the MMTV promoter with transcription factors can not be achieved in on free DNA [3, 4], it seems that the nucleosomal organization, not only is not an obstacle for hormone induction, but may be required for 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, nucleosome depletion 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.

For factor binding to take place on previously unaccessible sites in chromatin a remodelling of the nucleosome is necessary. The nature of this remodelling is unknown but there is experimental evidence pointing to alterations in the core histones. Moderate hyperacetylation of the lysine-rich histone tails enhances transcription from the MMTV promoter in the absence of hormone and potentiates hormone induction. A potential role for dissociation of histone H2A/H2B dimers is suggested by the observation that whereas NFI and OTF1 cannot bind to the MMTV promoter wrapped around a histone octamer, they bind efficiently to the same sequences on the surface of tetramer a of histone H3 and H4. Nucleosome remodelling *in vivo* likely requires ATP-dependent enzymatic activities, such as those encompassed in the SWI/SNF complex, which could be recruited to the MMTV promoter via direct interactions with the hormone receptors [5].

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The establishment of stable chromatin states in the regulation of homeotic genes in Drosophila.

V. Pirrotta
Department of Zoology. University of Geneva. 30 quai E. Ansermet, CH1211 Geneva, Switzerland

Drosophila homeotic genes have specific domains of expression established in the early embryo by the segmentation genes, ultimately depending on maternal antero-posterior cues. When these early genes cease functioning, the correct expression domain is maintained by a generalized chromatin-based repressive mechanism used by all homeotic genes and by a large set of other genes, including many responsible for the development of the nervous system. This repressive system, which depends on the Polycomb Group (Pc-G) of genes, establishes a complex that initiates at specific targets, the Polycomb Response Elements or PREs, and spreads over a large chromatin region, maintaining the gene repressed in cells in which it was initially repressed but allowing expression in cells in which the gene was originally active. The repressed state persists over many cell divisions, providing a cellular memory of transient developmental cues that were present at some earlier stage of development.

We have studied these regulatory mechanisms in the expression of the Ultrabithorax gene, which contains a principal PRE and a number of weaker response elements that contribute to the establishment of Pc-G repression over a large region. In vivo, transposons containing a PRE can cause a partial (variegation) or total repression of neighboring genes strongly resembling the repressive effects caused by the vicinity of heterochromatin. The formation of chromatin complexes that include known Pc-G proteins can be detected on polytene chromosomes at the site of insertion of the transposon. The PRE generates a set of strong DNase hypersensitive sites mapping within the PRE sequence. These can be reproduced in vitro with nuclear extracts but efforts to demonstrate complex formation in vitro have not succeeded so far, probably because the interaction of individual components with DNA is very weak and requires a high degree of cooperativity in addition to some components that are present only transiently. The PRE itself appears to be composite of multiple sequence elements which appear to interact with different subsets of Pc-G proteins.

Our current model is that complex formation requires the cooperative interactions of a number of Pc-G proteins, some of which may interact individually with DNA sequences at the PRE but too weakly to result in stable binding by themselves. We suppose that once a nucleating complex is formed, the cooperative interactions extend the complex over an larger region region and are prevented from doing so when a gene is active but not when the gene is inactive at the time of complex formation. The Pc-G proteins are a heterogeneous lot and include some with functional homologies to Drosophila heterochromatin proteins (Suvar products) which are thought to form extended chromatin complexes in much the same manner. Several Pc-G proteins have mammalian homologues and experiments by others have shown that the mammalian Pc homologue is functionally equivalent to the Drosophila Pc while Drosophila Pc-G proteins can be recognized by a silencing mechanism in mammalian cells.

Insertion of a transposon carrying a PRE can often silence not only genes contained in the transposon but also genes flanking the insertion site in the Drosophila genome. Genetic evidence suggests that the genome contains boundary sequences that normally prevent silencing complexes from extending to inappropriate regions. We have shown that boundary elements such as the scs, scs' and su(Hw) binding sites contained in the gypsy transposon not only block interactions between enhancer and promoter but prevent the spreading of Pc-G complexes. These experiments have also demonstrated that the formation of an effective silencing complex requires interactions of a PRE with other cooperating sites in flanking sequences or in homologously paired sequences on the homologous chromosome.

Molecular and genetic similarities with Pc-G silencing suggest a relationship with mechanisms such as heterochromatic and telomeric silencing, X chromosome inactivation and genetic imprinting in mammals. It may be possible to interpret these phenomena as initiated as chromatin complexes related to those formed by Pc-G proteins which then induce changes in histone acetylation and CpG methylation. The latter may be necessary to insure continued silencing in organisms that, unlike Drosophila, have long lives and a large number of cell divisions intervening between embryo and adult.

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X chromosome inactivation and the Xist gene.

Graeme Penny, Graham Kay, Steven Sheardown, Sohaila Rastan and Neil Brockdorff.

Comparative Biology Group, MRC Clinical Sciences Centre, RPMS, Hammersmith Hospital, London W12 ONN, UK.

X chromosome inactivation (X inactivation) is the genetic silencing of a single X chromosome in XX female mammals and provides the mechanism whereby X-linked gene dosage is equalised relative to XY males. The inactive X chromosome (X^i) is heterochromatic and forms the highly condensed Barr body, often seen to be localized at the periphery of the cell nucleus. X inactivation occurs early in female embryogenesis and there is normally an equal probability that either the maternally derived or the paternally derived X chromosome will be inactivated in a given cell. The random nature of X inactivation gives rise to the classical variegated phenotypes observed in females bearing X-linked mutations, for example the tortioseshell cat. The pattern of variegation reflects the fact that the initiation of X inactivation occurs in early embryogenesis and also that the inactive state is clonally inherited in subsequent cell divisions. The regulation of X inactivation is attributable to a single cis-acting master switch locus, the X inactivation centre (Xic), which is located on the X chromosome. The Xic is required in cis for X inactivation to occur, suggesting that it is the effector of facultative heterochromatin formation (propagation). The Xic is also required for the initiation of X inactivation early in embryogenesis. The first step in initiation requires that a cell determine how many, if any, X chromosomes to inactivate (counting), and subsequently, which X chromosome(s) to inactivate (choice). Experimental observations suggest that the Xic is the element that is numerated, and also that alleles at the Xic (Xce alleles) can influence the choice of which X chromosome(s) will be inactivated. It is not known whether these distinct functions of the Xic, counting, choice and propagation, are genetically seperable or inextricably linked. The onset of overt X inactivation correlates closely with the differentiation of cells from totipotent/pluripotent early embryonic lineages, and this may indicate that factors not present in undifferentiated cells are required to respond to a determined state of the Xic (on or off), in order to complete the process of X inactivation.

Studies in recent years have focused on the X inactive specific transcript (Xist) gene, a candidate for the Xic. Xist maps to the critical minimum Xic interval on the human and mouse X chromosome, and is expressed exclusively from the inactive X chromosome. The Xist gene produces a large transcript (approximately 15 kb) which does not encode a protein, and is localised entirely within the nucleus. In situ hybridization to Xist RNA indicate that it is present in close association with the Barr body. These findings have led to the proposal that Xist is the in cis effector of X inactivation, functioning either as an RNA or through an alternative mechanism involving modulation of local chromatin structure. In the context of this model, the initiation of X inactivation would involve the decision as to which, if any, Xist genes are switched on. The hypothesis that Xist is involved in the initiation of X inactivation is supported by the observation that Xist expression is first seen prior to the onset of overt X inactivation in early mouse embryos. The Xist gene (and all of the minimal Xic region) does not seem to be required for the clonal maintenance of the inactive state.

We have recently analysed XX ES cells with a targeted deletion of a single Xist allele. XX ES cells provide a useful model system for studying X inactivation as they can be induced to undergo X inactivation by differentiating cells, either in vitro, or in vivo. The phenotype of the Xist knockout cells suggest that the mutation results in failure to X inactivate in cis (propagation). Initiation of X inactivation (counting and choice) does not appear to be affected. I will present this data and discuss the implications with respect to models for the function of Xist in X inactivation.

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For an up to date review see " Rastan,S., Current Opinion in Genetics and Development **4**, 292-297 (1994)."

The Molecular Logic of Smell

Richard Axel, M.D.

How is the diversity and specificity of olfactory sensory perception accomplished? The initial step in olfactory discrimination requires the interaction of odorous ligands with a large family of seven transmembrane domain receptors on olfactory sensory neurons. Discrimination among odorants, however, will require that the brain determine which of the numerous receptors have been activated. A given sensory neuron expresses only one receptor such that individual neurons are functionally distinct. Thus, the problem of distinguishing which receptor has been activated can therefore be reduced to a problem of distinguishing which neurons have been activated. By analogy with other sensory systems, the brain may therefore determine which of the functionally distinct neurons have been activated by virtue of the spatial segregation of sensory neurons or their projections in the brain. We have demonstrated that neurons expressing a given receptor project their axons to common glomeruli within the olfactory bulb. Moreover, the position of specific glomeruli is bilaterally symmetric and is constant in different individuals within a species. These experiments support a model in which exposure to a given odorant may result in the stimulation of a spatially-restricted set of glomeruli such that individual odorants would be associated with specific topographic patterns of activity within the olfactory bulb of the brain.

Yeast Cell Types: At the Interface of Gene Regulation, Cell Biology, and Development

Ira Herskowitz
University of California, San Francisco

The cell types of *S. cerevisiae* play different roles in the life cycle of the organism: **a** and α cells are specialized for mating whereas **a**/α cells are specialized for meiosis and spore formation [*1*]. Three aspects of yeast cell specialization are relevant to general issues in cell biology and in development of multicellular organisms. Transcriptional regulatory proteins play important roles in each of these facets.

(1) *Cell specialization* results from the action of regulatory proteins ("endogenous programming") and from cell-cell interactions. The mating type locus codes for regulatory proteins, which include two homeodomain proteins with known targets [*1,2*]. The final differentiation of **a** and α cells results from the action diffusible peptide mating pheromones. This pathway is understood in considerable detail and includes a MAPK/ERK cascade which includes a transcriptional regulator, STE12 [*3*].

(2) *Cell cycle arrest* in G1 is also induced by the mating pheromones. It results from transcriptional and post-translational activation of the FAR1 protein, which then binds to the cyclin-dependent kinase, Cdc28/Cln, to arrest the cell cycle [*4,5*]. FAR1 is a prototype cyclin-dependent kinase inhibitor (CKI), of which several are now known in mammalian systems, including one (p21) which is a transcriptional target of the tumor suppressor protein p53 (see *6*].

(3) *Cell polarity and the plane of cell division* are regulated in a cell-type-specific manner: **a** and α cells exhibit an axial pattern of bud-site selection; **a**/α cells exhibit a bipolar pattern. The choice of bud site is determined by a group of proteins that include a small GTP-binding protein (Bud1/Rsr1) which is proposed to organize another group of proteins (Cdc24 etc.) that ultimately organizes the cytoskeleton [*7-9*]. The cell-type-specific difference in budding pattern results from repression of *AXL1* by **a**1-α2 (*8*). The timing of bud site selection probably results from activation of cyclin-dependent kinases and transcriptional activation of the *BUD2* gene (*10*).

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THIRD SESSION: SIGNALLING TO TRANSCRIPTION

Chairman: Tony Hunter

Ligand-dependent transactivation by nuclear receptors

Bertrand LeDouarin, Elmar von Baur, Christina Zechel, David Heery, Hinrich Gronemeyer, Régine Losson and Pierre Chambon
Institut de Génétique et de Biologie Moleculaire et Cellulaire, CNRS/INSERM/ULP, Collège de France, BP 163, 67404 Illkirch-Cedex, France. Tel. (33) 88 65 32 13/15/10 -Fax (33) 88 65 32 03

Nuclear receptors (NRs) bound to specific response elements transduce the effects of cognate ligands to control transcription from target gene promoters. Their ligand-dependent activation function, AF-2, presumably acts on the transcription machinery through intermediary factors/mediators. We have isolated and characterized mouse nuclear proteins which interact in a ligand-dependent manner with several NRs in yeast and mammalian cells. In all cases these interactions require the conserved amphipathic α-helix which constitutes the core sequence of the AF-2 activating domain (AF-2 AD). Interestingly, the precise amino-acids of the AF-2 AD core, which are indispensable for interaction between the various NRs and the putative intermediatory factors that we have studied, vary depending on the nature of both the receptor (RAR, RXR, TR and ER) and the mediator. Mechanisms which may underlie the mediation of the ligand-dependent activation fonction of NRs by intermediatory factors will be discussed in the light of the crystal structure of RARs and RXRs.

SIGNALLING GENES FROM THE CELL SURFACE: THE JAK-STAT PATHWAY.
James E. Darnell, Jr., The Rockefeller University, New York, N.Y. 10021

Binding of IFN-α [interferon alpha] and IFN-γ [interferon gamma] to their cell surface receptors promptly induces tyrosine phosphorylation of latent cytoplasmic transcriptional activators, which we designate STATs (signal transducers and activators of transcription). IFN-α activates both Stat1 [a 91 kD protein] and Stat2 [a 113 kD protein] while IFN-γ activates only Stat1. After phosphorylation the proteins form homo- or heterodimers and then move into the nucleus and directly activate genes induced by IFN-α or IFN-γ. Somatic cell genetics experiments have demonstrated a requirement for tyrosine kinase 2 [Tyk2] in the IFN-α response pathway and Jak2, a kinase with similar sequence, in the IFN-γ response pathway.

In addition to activation by the IFNs it has now been found by us and other investigators that over 25 polypeptide ligands activated the four known Jak kinases and one or more of the six known STAT proteins. Both cytokine receptors that employ JAKs and receptors with intrinsic tyrosine kinases can activate the STATs. Recent work has turned to the molecular events underlying specificity in the pathway beginning with a demonstration that binding to phosphotyrosine in the receptor-kinase complex (most likely on the receptor chain) through interaction with the -SH2 group of a Stat molecule is the first specific step in activation. The STATs have different affinities for different DNA sites based on amino acids in the 400-500 segment of the moleucule and also can heterodimerize and multimerize with other DNA binding proteins extending their potential as specific activators. Finally, the mechanism of activation of transcripiton differs with Stats 1 and 3 having serine residues in their COOH terminal regions that must be phosphorylated for the Stat to be maximally activated while Stat2 activation operates through different sequence motifs.

Since the pathway is activated by such a large number of cytokines and growth factors, it may be that a large fraction of the polypeptide-induced immediate transcriptional responses obligatorily pass through this pathway and are further modified by serine phosphorylation.

We have begun to focus on the biologic outcome of STAT family activation in two different lines of experiments: 1) the role of Stats 1 and 2 in interferon-induced growth restraint and 2) the demonstration in Drosophila that STAT molecules exist and can be shown to be required in development. Successful experiments in this realm are obviously necessary to understand the possible broader importance of this new signalling pathway.

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SIGNALLING PATHWAYS TO THE FOS PROMOTER

Caroline Hill, Judy Wynne, Mary Ann Price, Richard TREISMAN
Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK

The c-fos promoter has been extensively studied as a model growth factor-regulated promoter. Its conserved upstream regulatory region contains three known promoter elements, including the SRE (Serum Response Element; Treisman, 1990), the SIE (sis-inducible element; Wagner *et al.*, 1990) and an AP1/ATF site. At the SRE a ternary complex forms which contains SRF (Serum Response Factor; Norman *et al.*, 1988) and an Ets domain protein, TCF (Ternary Complex Factor), which can only bind the SRE via interaction with SRF (reviewed in Treisman, 1994). The TCFs, which bind an Ets motif adjoining the SRF binding site, are regulated by MAP kinase phosphorylation in response to extracellular signals that activate the ras-raf-ERK pathway. The SIE binds cytokine- and growth factor-regulated transcription factors of the STAT family (Sadowski *et al.*, 1993). Genomic footprinting studies of cultured cells indicate that the SRE and AP-1/ATF sites are constitutively occupied in vivo, whereas SIE occupancy occurs only following stimulation by polypeptide growth factors (Herrera *et al.*, 1989). In addition to the upstream region, further promoter elements are located in the vicinity of the TATA box, and some of these are also responsive to extracellular signalling pathways.

Only some of the different signalling pathways that target the c-fos upstream regulatory region have been characterised. For example, although the activation of the SRE via TCF by the ras-raf-ERK pathway is well established, whole serum can also activate the SRE independently of TCF by an uncharacterised pathway (Graham and Gilman, 1991). In addition the role of the SIE in signal transduction and transcriptional activation remains poorly understood. To investigate the roles of the SIE and SRE in transduction of different signals to the promoter we have used transient transfections of c-fos promoter mutants in fibroblasts. Results will be presented that demonstrate that the SRE and SIE can cooperate in promoter activation, but that activation of STAT DNA binding per se is not sufficient for activation of c-fos transcription.

Analysis of the promoter mutants also shows that agents that act through heterotrimeric G protein-coupled receptors, such as lysophosphatidic acid (LPA) can, like serum, activate the SRE in the absence of TCF. TCF-independent serum-regulated activation by SRF can be blocked by mutations within the DNA binding domain that do not impair DNA binding. We have proposed that SRF adopts an "active" conformation upon binding DNA, and that this allows its interaction with a non-TCF accessory factor activated by a serum-induced signalling pathway (Hill *et al.*, 1994). We have investigated the nature of this SRF-linked signalling pathway in more detail. We show that activated forms of the rho-

family GTPases RhoA, Rac1 and CDC42hs can activate the SRE independently of TCF. Functional RhoA is also required for activation of SRF in response to extracellular stimuli such as serum and LPA. In contrast, functional Rho is not required for activation of SRF by activated forms of Rac1 and CDC42hs. These results establish SRF as a target for nuclear signalling pathways mediated by rho-family GTPases.

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HOW ASYMMETRIC DIVISION GOVERNS CELL FATE

Richard Losick, Department of Molecular and Cellular Biology, The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138, USA.

My presentation will be concerned with how cell fate is determined by asymmetric division in the bacterium Bacillus subtilis. Early during the process of sporulation, the formation of an asymmetrically positioned septum partitions the developing cell or sporangium into unequal-sized progeny called the forespore (the small cell) and the mother cell. The dissimilar developmental fates of the progeny are governed by transcription factors σ^E and σ^F. These factors are produced in the predivisional sporangium but do not become active until after the formation of the polar septum, when σ^F becomes active in the forespore and σ^E in the mother cell. Evidence will be presented that:

1. Activation of σ^E is controlled by an intercellular signal transduction pathway operating at the level of the proteolytic processing of an inactive proprotein precursor (pro-σ^E) to σ^E. The conversion of pro-σ^E to mature σ^E in the mother cell is mediated by a membrane-bound receptor/protease, SpoIIGA. A secreted, signal protein (SpoIIR) produced in the forespore under the control of σ^F is believed to bind to SpoIIGA on the outside surface of the mother-cell membrane, thereby activating the intracellular proteolytic domain of the receptor/protease.

2. Activation of σ^F is governed by the proteins SpoIIE, SpoIIAA, and SpoIIAB. SpoIIAB is an inhibitor of σ^F and holds it in an active complex in the predivisional sporangium and in the mother cell. In the forespore SpoIIAB switches partners to SpoIIAA, thereby releasing free and active σ^F. Partner switching is governed by the relative levels of ATP and ADP and by SpoIIAB-mediated phosphorylation of SpoIIAA, which prevents SpoIIAA from binding to SpoIIAB. SpoIIE is a membrane protein that is located at the polar septum and is responsible for the dephosphorylation of SpoIIAA~P, thereby counteracting the SpoIIAB kinase. *In toto*, these findings link the chain of biochemical events from the formation of the polar septum to the cell-specific activation of σ^F.

3. The formation of the polar septum is governed by the sporulation transcription factor Spo0A. Spo0A induces a switch in the site of polymerization of the tubulin-like cell division protein FtsZ from the midcell position (used during binary fission) to potential division sites near both poles of the sporangium. A septum is then formed at one pole, subsequent events under the control of σ^E shutting down the use of the second, potential, polar division site.

FOURTH SESSION: CYCLIC CONTROL OF GENE EXPRESSION

Chairman: Richard Treisman

CONTROLLING S-PHASE IN THE CELL CYCLE

Paul Nurse, Jaime Correa-Bordes, Daniel Fisher and Hideo Nishitani
Cell Cycle Laboratory, Imperial Cancer Research Fund,
44 Lincoln's Inn Fields, London WC2A 3PX. UK.

The major controls acting during the cell cycle regulate the onset of S-phase and mitosis. In fission yeast a single cyclin dependent kinase (CDK), $p34^{cdc2}/p56^{cdc13}$ cyclin B, can promote both S-phase and mitosis. The protein kinase activity associated with this oscillates in level once per cell cycle. In late G1, the kinase is activated to a low level to bring about S-phase, and is then maintained at this level during the rest of S-phase and G2 when it has an inhibitory role preventing initiation of a further round of S-phase. In late G2, activation to a high level brings about mitosis, and a fall to a low level brings about exit from mitosis. This fall in activity relieves the inhibition preventing initiation of S-phase and so when kinase activity rises again in late G1, S-phase can once again take place. The CDK inhibitor $p25^{rum1}$ acts in G1 to prevent $p34^{cdc2}/p56^{cdc13}$ activity rising to a high level which could initiate mitosis from this inappropriate stage of the cell cycle.

The initiation of DNA replication during S-phase is brought about by $p65^{cdc18}$. The *cdc18* gene is periodically transcribed in late G1 and as a consequence $p65^{cdc18}$ protein level oscillates in level peaking during late G1 and S-phase. Over-expressing *cdc18* to a high level drives cells into S-phase from any stage of the cell cycle indicating it has a key triggering role in initiating DNA replication.

Programming a Mitotic Cell Lineage

Anita Sil and Ira Herskowitz. Department of Biochemistry & Biophysics, University of California, San Francisco, California 94143-0448 USA

The ability of cells of Saccharomyces cerevisiae to undergo mating-type interconversion is a classic example of asymmetric determination of cell fate (Strathern & Herskowitz, 1979). Haploid cells that have experienced previous cell divisions (mother cells) produce the HO endonuclease and switch mating type whereas their genetically identical, newly born progeny (daughter cells) do not (Nasmyth, 1983). These properties of mitotically growing yeast cells generate a stem cell lineage consisting of the most recent daughter cells. Using a novel colony morphology assay for mating type switching, we have identified a mutant, ash1-1, in which daughter cells transcribe the HO gene and switch mating type. The wild-type gene, ASH1 (Asymmetric Synthesis of HO), appears to be required to repress transcription of the HO gene in daughter cells. The Ash1 protein has a Zn-finger domain related to those of the GATA family of transcriptional regulators. Strains deleted for ASH1 are viable; mother and daughter cells switch mating type at the same high frequency in the ASH1 deletion mutant. Overexpression of ASH1 inhibits transcription of HO in mother cells. The Ash1 protein is predominantly localized to the daughter nucleus after nuclear division but before cytokinesis. These observations indicate that Ash1 protein is a cell-fate determinant that is localized asymmetrically in the daughter nucleus, where it serves to inhibit HO transcription. Thus, Ash1 protein is necessary for production of the stem cell lineage.

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THE BASIS OF DIFFERENTIAL GENE EXPRESSION: HOW YEAST CELLS CONTROL THE MOTHER CELL-SPECIFIC EXPRESSION OF THE *HO* GENE

Ralf Jansen , Nicoletta Bobola, Tae-Ho Shin and Kim Nasmyth, IMP, Dr. Bohr-Gasse 7, A-1030 Vienna, Austria.

The generation of cellular heterogeneity through asymmetric cell divisions is a crucial aspect during development of multicellular organisms. However, only little is known of the molecular mechanisms involved. A well understood example of differential (asymmetric) gene activation occurs in the budding yeast *S. cerevisiae* where the *HO* gene is expressed only in mother cells but not in daughter cells. *HO* encodes an unstable endonuclease that initiates mating-type switching in budding yeast.
So far, only two genes have been implicated in the mother-specific expression of *HO*, the transcription factor *SWI5* and the repressor *SIN3*. However, *SWI5* and *SIN3* are symmetrically segregated to mother and daughter cells and not sufficient for asymmetric *HO* activation.

In a new screen we have identified 5 additional genes (*SHE1-5*) needed for mother-cell specific *HO* expression. Their function is specific to *HO* since *she* mutants express other Swi5-regulated genes normally. All *SHE* genes have been cloned. Surprisingly, *SHE1* (which is identical to the minimyosin *MYO4*) and *SHE5* code for yeast cytoskeletal proteins. The She1/Myo4, 3 and 5 proteins are localized to the bud that becomes the daughter cell. Yet, the function of the *SHE* gene products seem to be mother-cell specific. Our results could be explained by a model in which the She proteins (with Myo4p as a motor) transport a putative repressor of *HO* expression (or factors controlling the activity of such a repressor) from the mother to the daughter cell.

A putative *HO* repressor has been isolated by two different approaches. In both independent screens the *ASH1* gene was isolated, mutations in which lead to a very efficient *HO* expression in both mother and daughter cells. This points to a role of *ASH1* as a repressor of *HO* in daughters. Ash1 protein has been shown to be located only in daughter cell nuclei. It is cell cycle regulated and present in daughter cell nuclei upon entry of the Swi5 transcription factor (necessary for *HO* expression) into nuclei of both mother and daughter cell. Ash1 might therefore block the function of Swi5 in daughter nuclei. The asymmetric distribution of Ash1p is dependent on functional *SHE* genes. However, our data indicate that Ash1p is not transported by the She proteins but its expression or production is controlled by a yet unknown factor moved by the She proteins. A likely candidate for this factor, *ASH3* is currently analyzed.

The regulation of cell cycle transitions by protein phosphorylation

Tony Hunter Molecular Biology and Virology Laboratory, 10010 North Torrey Pines Road, La Jolla, California 92037

The cyclin-dependent kinase (Cdk) inhibitor (Cdi) p27 accumulates in G0 in Swiss 3T3 cells and is bound to residual cyclin/Cdk2 complexes (1). Inhibition of all cyclin/Cdk function may be required for cells to withdraw from the cell cycle. When cells enter the cycle, p27 levels drop 3-4 fold, and concomitantly p27 binds cyclin D/Cdk4, as these complexes accumulate in G1 upon induction of cyclin D1 expression. p27 switches to cyclin A/Cdk2 as cells enter S phase, concomitantly with the loss of cyclin D/Cdk4 complexes. This occurs despite the continued presence of both subunits, and may be due to accumulation of a p16 family Cdk inhibitor. In Swiss 3T3 cells the level of p27 is significantly lower than those of cyclin D1 and Cdk4 in G1 phase, and it seems unlikely that p27 normally acts as G1 threshold device in these cells. However, p27 may play a role in cell cycle arrest in G1 under specific conditions. For instance, cyclin/Cdk2 complexes bind p27 and are inhibited when cells are irradiated with UV, even though p27 unlike p21 is not induced by UV, and when cells are treated with lovastatin, apparently as a result of the loss of cyclin D under these conditions. p27 is also the cause of G1 arrest when surface IgM is crosslinked on WEHI-231 pre B cells, which causes a large increase in p27. An increase in p27 also contributes to the failure to progress through G1 arrest when suspended fibroblasts are treated with serum. Finally, the adenovirus transforming protein E1A can override the inhibitory action of p27 on cyclin/Cdk2 complexes through a direct interaction with p27, and this mechanism may be important in the ability of adenovirus to stimulate quiescent cells to progress into S phase.

The NIMA protein-serine kinase is essential for entry into mitosis in Aspergillus nidulans, and acts on a pathway separate from the Cdc2 pathway. We have shown that there is a NIMA-like pathway in mammalian cells that acts independently of Cdc2 to regulate entry into M phase (2). However, no true mammalian homologue of NIMA has been identified. We have used a two hybrid screen in an attempt to identify NIMA-interacting proteins that may be components of the mammalian NIMA pathway. In this fashion we have identified Pin1, which is the homologue of the budding yeast Ess1. Pin1/Ess1 contains an N-terminal WW domain and a C-terminal prolyl isomerase domain. Overexpression of Pin1 arrests HeLa cells in G2, and partly blocks the ability of NIMA to drive these cells prematurely into M phase. Pin1 colocalizes with NIMA in nuclear substructures that contain spliceosomes. In yeast, depletion of Ess1 leads to premature entry of cells into M phase from G2. Pin1 appears to be a regulator of the G2/M transition, acting as a negative regulator of the M phase-promoting NIMA pathway.

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TRANSITIONS IN CHROMOSOMAL TRANSCRIPTION AND REPLICATION DURING EARLY DEVELOPMENT

Marie-Noëlle Prioleau, Olivier Hyrien, Robin Buckle, Chrystelle Maric, and Marcel Méchali. Institut Jacques Monod, Molecular Embryology - 2 place Jussieu, 75251 Paris Cedex 05, France.

The early embryonic development is characterized by a major transition in gene expression with the onset of zygotic transcription, which in most cases is preceded by a generalized transcriptional repression. In *Xenopus* this repression occurs during 12 cell cycles after fertilization, and activation of transcription occurs at the 13th cell cycle (the midblastula transition, or MBT). We have analyzed the mechanism underlying such regulation and the possible relationships between chromatin transcription and replication during development.

We examined the requirement and interactions of basal as well as trans-activating factors with chromatin during the early development. We showed that gene expression during this period is regulated by a dynamic competition between nucleosome assembly and transcription factor assembly (Prioleau *et al.*, 1994, Cell *77*, 439-449). A dominant repression by chromatin assembly is caused by the large excess of maternal histones stored in the egg, until their titration by the amount of DNA synthesized in the embryo at the MBT. The transcriptional step which is repressed is the assembly of the basal transcription complex. In contrast, activating proteins have access to the DNA during this period. This specific regulation leads to a repressed but already committed chromatin structure during early development, and may be of general importance in the control of early embryonic events by providing a molecular basis for determination (Prioleau et al EMBO J., in press).

We have also examined replication origin spacing and specificity in relation to gene expression during development. Prior to the MBT, in the absence of transcription, chromosomal replication in the rDNA locus occurs at sites bound to the nuclear matrix, at regularly spaced but apparently random sites. (Hyrien and Méchali, 1993, EMBO J., *12*, 4511-4520). Resumption of RNA synthesis at the blastula-gastrula stage is accompanied by a major change in the replication initiation. Origins become localized in the spacer region containing promoter elements whereas Initiation of replication is repressed in the transcription units. A circumscribed zone of initiation of DNA replication therefore emerge during development, when the chromatin is remodeled for gene transcription (Hyrien et al, 1995, Science, in press).

Notch as a switch that assigns cell fates during Drosophila development. A. Martinez Arias. Dpt. Zoology. University of Cambridge. Cambridge CB2 3EJ. UK

The *Notch* gene of *Drosophila* encodes a receptor with a well characterized role in the process of lateral inhibition in which the product of the *Delta* gene functions as the ligand. On the other hand, the *wingless* gene encodes a secreted glycoprotein that allows cells to adopt fates during development.

The function of *Notch* is required for all aspects of *wingless* signalling during *Drosophila* development. A detailed genetic analysis of the interaction between *Notch* and *wingless* indicates that there is a direct interaction between the molecules encoded by these genes. In this interaction Wingless functions as a ligand that i) antagonizes lateral inhibition and ii) utilizes Notch to signal. These observations lead to a model in which Notch functions as a switch in the assignation of cell fates during development. When and where Delta is bound to Notch cell fate decissions are suppressed, when and where Wingless is bound to Notch the same decissions are implemented.

Evidence for this model will be presented and discussed.

Negative control of MyoD activity

Norbert Kraut, Amy Chen and Harold Weintraub, Fred Hutchinson Cancer Research Center, 1124 Columbia Street, A3-025, Seattle/WA 98104, U.S.A.

MyoD is a muscle-specific transcriptional activator that stimulates transcription through binding sites in muscle genes, while E12 is a B-cell activator. Both MyoD and E12 are basic helix-loop-helix (bHLH) proteins that bind to similar E-boxes (CANNTG). Although the muscle creatine kinase (MCK) and immunoglobulin heavy chain (IgH) enhancers contain identical E-boxes, they retain exclusive specificity for being activated by either MyoD or E12, respectively. The IgH enhancer is strongly activated by E12 but not at all by MyoD. We have shown previously that the IgH enhancer contains a *cis*-acting negative element consisting of 4 bp (2 bp flanking each side of the μE5-E box), that inhibits the activity of MyoD, but not E12, presumably through an interaction with a *trans*-acting negative regulator (Weintraub et al., *Genes & Dev.* 8: pp. 2203-2211, 1994). The results explain how various members of the bHLH family have the competence to bind to the same site yet give cell type dependent activation.

We are currently further characterizing the mechanism of this novel negative repression system at the IgH enhancer. We are trying to isolate the putative MyoD-repressor molecule that interacts with the μE5-site by performing genetic screens in yeast and mammalian cells. In mammalian cells, MyoD does not activate a reporter containing multimerized μE5-IgH enhancer E-boxes, whereas MyoD can transactivate this reporter in yeast, suggesting that yeast cells lack the repressor that interacts with the μE5-IgH site. Therefore, the first approach is aimed at providing the repressing activity lacking in yeast cells by complementation with cDNAs from a mammalian library derived from a cell type containing this activity. The second approach towards identification of the MyoD μE5-repressor involves a "negative-positive" selection strategy, using repressor-containing mammalian cells transfected with a vector containing multimerized μE5 E-boxes in front of a hygromycin/thymidine kinase (HyTK) fusion gene reporter in the presence of MyoD. After negative selection for clones that are gancyclovir-resistant and hygromycin-sensitive (as a consequence of the HyTK-reporter not being expressed in the presence of MyoD), the repressor will be inactivated by introduction of an antisense library from 3T3 cells. Positive selection for hygromycin-resistant clones will include those colonies in which the repressing activity has been inactivated and MyoD is able to stimulate transcription of the HyTK-reporter.

As might be expected, the activity of MyoD and its highly related sister proteins, Myf-5, Myogenin, and MRF-4, is highly controlled and subject to negative regulation at various levels. Using a yeast two-hybrid screen, we have identified Sclero-1a, a novel protein whose C-terminus is required for specific interaction with myogenic bHLH proteins. Two other proteins, Sclero-1b and -1c which contain different C-termini generated by alternative splicing fail to interact with

myogenic bHLHs. Furthermore, only Sclero-1a can strongly reduce the transactivation capability of myogenic factors, but not other bHLH proteins, in transient cotransfections when assayed on synthetic reporter constructs. This observation correlates with the finding that Sclero-1a can specifically inhibit Myf-5-induced myogenesis in 10T1/2 cells. Interestingly, Sclero-1a is localized in the cytoplasm and when coexpressed with Myf-5 or MyoD, these proteins are colocalized in the cytoplasm, indicating that Sclero-1a can inhibit the nuclear translocation of myogenic bHLH proteins. In summary, our data suggest that Sclero-1a can negatively regulate myogenic gene activation and consequently myogenesis by specifically retaining myogenic factors in the cytoplasm.

We are now beginning to study the role of Sclero-1 during mouse embryogenesis. By *in situ*-hybridization, we have found that Sclero-1 is expressed within somites in the sclerotome, but not in the dermomyotome, and we are currently in the process of generating Sclero-1 mutations by homologous recombination.

TRANSCRIPTIONAL SWITCH MECHANISMS MEDIATED BY bHLH ACTIVATOR AND REPRESSOR PROTEINS. Michael Caudy; Cornell University Medical College; New York, N.Y..

Basic Helix-Loop-Helix (bHLH) transcription factors function as developmental switches for controlling yes or no decisions between alternative cell fates during development. For example, in Drosophila and mammals a highly conserved family of bHLH activator and repressor proteins control the switch between neuronal and non-neuronal (ectodermal) cell fates. We are taking a combined genetic and biochemical approach to understanding the precise mechanisms by which bHLH activator and repressor proteins function in these regulatory processes.

The Drosophila neuronal bHLH activator proteins consist of Daughterless (Da), the four proteins encoded by the achaete-scute complex (AS-C), and Atonal. The bHLH repressor proteins consist of Hairy (H), Deadpan (Dpn) and the seven proteins encoded by the Enhancer of split complex (E(spl)). Biochemical analysis has shown that the bHLH activator and repressor proteins recognize distinct classes of DNA sites (Class A and Class C, respectively)[1]. Genetic analysis has shown that Hairy represses neuronal precursor determination by binding to a single Class C site in the promoter of the achaete (ac) proneural activator gene and repressing ac transcription[1,2]. Because the activator and repressor proteins bind to different sites and are not competing for binding sites in vivo indicates that the repressor proteins ("Hairy-related proteins") have intrinsic transcriptional repression (TR) domains by which they "actively" repress transcription. Because all fifteen known Hairy-related proteins contain a WRPW (Trp-Arg-Pro-Trp) tetrapeptide motif at their carboxyl termini, the WRPW domain was proposed to be the intrinsic TR domain in these repressor proteins[1]. We recently have confirmed that hypothesis by showing that the WRPW domain is sufficient to provide full repression activity to a heterologous DNA binding protein[3].

Recent work by others[4] has shown that the WRPW motif is recognized by the non-HLH protein Groucho, and that Groucho functions genetically as a co-repressor for Hairy-related proteins in fly segmentation, sex determination and neuronal precursor determination. Based on these results, Groucho was proposed to be a transcriptional co-repressor for Hairy-related proteins[4]. We have tested and confirmed this hypothesis showing that Groucho protein directly bound to the ac promoter (via fusion to a Gal4 DNA binding domain) directly represses ac transcription in cultured cells in the absence of binding by bHLH repressor proteins[3]. We also have found that the WRPW domain alone is sufficient to form a stable complex with Groucho as a GST-WRPW fusion protein efficiently co-precipitates Groucho from Drosophila Schneider cells[3]. Together these results show that Hairy-related bHLH repressor proteins function to recruit Groucho (or Groucho-like) transcriptional co-repressors to specific DNA sites in target genes.

We have found that Hairy-related proteins also bind to novel, very high affinity DNA sites present in the early promoter of Sex lethal, a known target for bHLH proteins in vivo[5]. Sex lethal provides an interesting model target gene for bHLH proteins in that it can accurately switch on or off in response to a two-fold difference in the ratio of bHLH activator and repressor proteins. The novel repressor site in Sex lethal consists of a pair of adjacent Class C hexamer sites which are spaced such that the centers of the sites are separated by one turn of DNA. Biochemical analysis has shown that pairs of bHLH repressor dimers bind cooperatively to these adjacent sites. The level of cooperativity is very high - greater than 200 fold - and it appears likely that these sites contribute to the very sensitive response of Sex lethal to the level of bHLH activator and repressor proteins. Moreover, very similar "double sites" are present in a number of potential neuronal target genes in Drosophila and mammals, strongly suggesting that cooperative binding by repressor proteins to these sites is important for precise regulatory switching of these target genes during development. We currently are testing whether these sites are functional in vivo.

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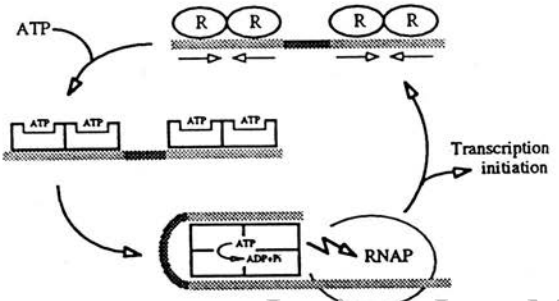
Mechanics of the prokaryotic enhancer of σ^{54}-promoters : ATP binding to the activator XylR governs a protein multimerization cycle that controls transcription initiation

JOSÉ PÉREZ-MARTÍN and VÍCTOR DE LORENZO. Centro de Investigaciones Biológicas del CSIC, Velázquez 144, Madrid 28006. Fax (+341) 5627518. E-mail CIBLP70@CC.CSIC.ES

Prokaryotic promoters dependent on the alternative sigma factor σ^{54} are one of the very few examples of transcriptional activation at a distance known in the bacterial world. Typically, these promoters require the binding of cognate regulatory proteins to UAS located >100 bp from the binding site of the σ^{54} RNApol and thus, activation involves the looping out of the upstream nucleoprotein complex to make productive contacts with the enzyme [1]. Transcription initiation is coupled also to ATP hydrolysis by the activator, a feature rarely seen in other prokaryotic promoters [2]. Such an arragement of control elements provide an optimal model to address general questions on the mechanism by which distant DNA sequences and their bound proteins control activity of distinct promoters.

We have studied *in vitro* the series of events that take place at the enhancer of the *Pu* promoter of *Pseudomonas* [3,4] prior to the engagement of the RNA polymerase in transcription initiation. This promoter bears two phased upstream binding site for the cognate regulator of the system, the toluene-sensing activator XylR. Exposure to the aromatic effector releases the intramolecular represion [5] caused by the N-terminal domain of the protein on a surface involved in activation (not in DNA binding) so that deletion of such domain gives rise to a constitutive form of the regulator. Occupation of the two phased UAS by active XylR showed a remarkable cooperativity dependent on ATP binding, but not ATP hydrolysis. Furthermore, in the presence of ATP or ATP analogs (but not of ADP), the UAS catalysed formation of an XylR oligomer, as detected with cross-linking agents under various conditions. These experiments suggested also that XylR undergoes a major conformational change upon ATP binding. This was confirmed in a series of limited proteolysis assays in the presence or absence of UAS DNA and ATPγS. Furthermore, a XylR variant mutated within the ATP-binding motif (G268N, Walker A domain) was [a] still able to bind DNA, but without any ATP-dependent cooperativity, [b] it was unable to multimerize and [c] it did not show any significant confomational change as detected with limited proteolysis. To examine the meaning of XylR multimerization at the UAS, we carried out a collection of ATPase assays with purified XylR, along with phased and offset binding DNA sequences. The results indicated that UAS-mediated multimerization of the activator at the *Pu* promoter enhancer triggers, in fact, ATP hydrolysis, as summarized in this scheme :



That ATP hydrolysis is followed by a return to the non-multimerized state is supported by the properties of mutant R453H. Unlike the G258N variant (that seems to be *frozen* in the non-multimerized state), R453H behaved as if arrested in the multimerized state. This mutant bound ATP but did not not hydrolyse it, appears as a multimer regardless nucleotide addition, and showed a protease digestion pattern virtually identical to that of the wild-type activator with ATPγS. All these results supports a model of ATP-dependent mutimerization of XylR at the *Pu* enhancer that preceeds any involmement of the polymerase in transcription initiation. Under this scheme, the one point of contact of the cycle with open complex formation is the ATP hydrolysis event which, on the basis of observations made in other σ^{54} systems, is believed to be channeled into activation of the sigma factor [6].

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FIFTH SESSION: DEVELOPMENTAL CONTROL

Chairman: Paul Nurse

GENETICS OF PHOTORECEPTOR CELL DEVELOPMENT IN *DROSOPHILA*

E. Hafen, D. Brunner, B. Dickson, M. Domínguez, K. Dücker, N. Oellers, T. Raabe, A. van der Straten, Zoological Institute of the University of Zürich, Zürich, Switzerland

The decision between cone cell fate and R7 cell fate in the developing eye of *Drosophila* depends on the activity of the sevenless (sev) receptor tyrosine kinase (RTK). The signal from the sev RTK is transduced by a set of highly conserved signaling components including Drk, Sos, Ras1 and Raf. Using a combine biochemical and genetic approach we have identified the major binding site of the SH2 adapter Drk on the sev receptor. A mutant sev receptor lacking this binding site is still able to induce R7 cell development albeit with lower efficiency. Genetic experiments suggests that signaling from this receptor is independent of Drk and Sos providing evidence for an alternative signaling pathway. To identify loci required for signal transduction downstream of the Raf kinase we have performed several genetic screens for dominant suppressors of the rough eye phenotype caused by the constitutive activity of Raf in the R7 equivalence group. One of the loci identified in these screens is *rolled*, which encodes the *Drosophila* homolog of MAP kinase. A gain of function mutation in *rolled* causes the recruitment of multiple R7 cells in the eye suggesting that the activation of MAP kinase is not only necessary but also sufficient to specify R7 cell fate. We have shown that MAP kinase transmits the signal at least in part by phosphorylating two ETS transcription factors, pointedP2 and yan. In contrast to the general requirement of the cytoplasmic signaling components, the *phyllopod* (*phyl*) gene, identified as a dominant suppressor of activated Raf, is only required for the development of the R1, R6 and R7 photoreceptor cells and therefore exhibits a novel photoreceptor subtype specificity. *phyl* appears to be an early response gene of the sev pathway. Ectopic expression of *phyl* under the *sev* regulatory sequences is sufficient to specify R7 development in the R7 equivalence group. Hence *phyl* represents a cell-type selector gene in the sev pathway.

GENE REGULATION IN THE *DROSOPHILA* EMBRYO

Herbert Jäckle, Abteilung Molekulare Entwicklungsbiologie, Max-Planck-Institut für biophysikalische Chemie, Postfach 2841, D-37018 Göttingen, Germany.

Segmentation during *Drosophila* embryogenesis is controlled by a cascade of zygotic gene activities that derive from polarly localized maternal factors which provide positional information along the entire longitudinal axis of the egg. The zygotic target genes can be subdivided into three distinct classes depending on the mutant phenotype and the spatial patterns of wildtype gene expression: Gap genes are expressed in broad contiguous domains that fail to develop in the corresponding gap mutant embryos; pair-rule genes are expressed in a repetitive banded pattern corresponding in position to alternating segment equivalents that fail to develop in pair-rule mutant embryos; segment polarity genes are expressed in the equivalents of anterior or posterior portions of each segment which are absent in the corresponding mutant larvae.

The cascade of zygotic segmentation genes is initiated by maternal gene products forming anterior-posterior and posterior-anterior gradients in the early embryo. After local activation in response to the maternal gene products, the gap gene expression patterns are spatially controlled by mutual interaction among the gap genes themselves. The gap-gene protein products, mostly zinc finger-type proteins, form broad and overlapping protein gradients along the longitudinal axis of the embryo. These local protein gradients contain the information for the control of zones of expression of the other gap genes which in turn provide spatial cues for generating the periodic pattern of subordinate pair-rule and homeotic gene expression.

Our results show that most of the genetic interactions within the segmentation gene cascade depend on direct DNA-protein interactions which are modulated through homo- and heterodimer formation of the gap-gene proteins that cause regulatory switches such as turning an activator into a repressor. The implication of such interactions on pattern formation in the early embryo will be discussed.

The Iroquois Complex, a group of genes that control the expression of proneural and vein forming genes.

Juan Modolell, José-Luis Gómez Skarmeta, Ruth Diez-del-Corral, Elisa de la Calle and Dolors Ferrés Marcó.

Centro de Biología Molecular Severo Ochoa, CSIC and UAM, Canto Blanco, 28049-Madrid, Spain.

The mesothorax and wings of *Drosophila melanogaster* provide a model to study the determination of specific cell types in precisely defined positions. Indeed, several hundred bristles and other types of external sensory organs (SOs) appear in the epidermis of these parts of the fly's body, either as landmarks in remarkably constant positions or in "density" types of arrangements that cover well defined areas. Similarly, the wings display a very constant pattern of veins. The pattern of SOs is prefigured in the imaginal wing discs, the anlagen of the adult mesothorax and wing epidermis, by the pattern of expression of the proneural *achaete* (*ac*) and *scute* (*sc*) genes, two members of the *ac-sc* complex (AS-C)[1]. These genes are simultaneously expressed by groups of cells, the proneural clusters, located at constant positions in the 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. The pattern of veins is also prefigured in the wing imaginal discs, in this case by the expression of the gene *veinlet* (=*rhomboid*) (*ve/rho*), which is essential for vein formation[2].

It has been shown[3] 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. The coexpression of *ac* and *sc* is accomplished by activation of both genes by the same set of enhancers. The action of the enhancers is independent of *ac* and *sc*. It has been proposed[4,5] that the enhancers respond to local combinations of activators and repressors (prepattern), but most of these have not yet been characterized. An exception is the products of the novel group of genes that constitute the *iroquois* (*iro*)

complex. We have characterized two of its genes, *araucan* (*ara*) and *caupolican* (*caup*). They encode highly related polypeptides with a homeodomain that most closely resembles that of *Drosophila* Extradenticle and human Pbx proteins. Ara and Caup also have strongly acidic motifs, glutamine stretches, putative MAPK phosphorylation domains and the central part of a Notch-like EGF repeat. These domains and motifs indicate that Ara and Caup are most likely transcriptional activators. In wing imaginal discs, *ara* and *caup* are expressed in similar patterns which cover relatively broad regions and which include one or more proneural clusters. *ac/sc* expression at these clusters depends on Ara/Caup. The interaction between Ara/Caup and *ac/sc* is most likely a direct one since Ara binds at least to a conserved sequence essential for the function of the enhancer that directs expression in the vein L3 and TSM proneural clusters. In the wing disc, *ara* and *caup* are expressed in the territories of veins L3 and L5 and are required for the differentiation of these veins. They probably act at an early step of vein determination since they are necessary for the expression of *ve/rho*[6]. These and other results indicate that *ara* and *caup* are members of a combinatory of genes that governs the patterning of both SOs and wing veins.

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Cdx1 functions in axis specification via direct regulation of Hox genes

Peter Gruss, Thomas Knittel, Vasanta Subramanian, and Barbara Meyer
Max Planck Institute of Biophysical Chemistry
Department of Molecular Cell Biology
37077 Göttingen, Germany

There is ample evidence that the mammalian Hox genes are involved in the anterior/posterior specification of the developing embryo. The Hox gene activity has to be highly precise in order to assure the distinct spatial character of "segments" along the body axis. In order to understand parts of this regulatory network we pursue two directions of research. We mapped minimal fragments required for the establishment of the anterior boundary of a model Hox gene. Using transgenic mice we could show that a 470 based pair long control element located 1.6 kb upstream of the transcription start sight was sufficient to direct expression of a reporter protein in a pattern reflecting the anterior boundary of the endogenous Hoxa-7 gene. This activity was confirmed in an orientation independent manner and thus designated as the Hoxa-7 enhancer. Sequence analysis of the Hoxa-7 enhancer showed the presence of several putative binding sites for a caudal related protein. We consequently asked the question if a caudal related protein in the mouse, (*Cdx1*) is involved in the regulation of Hox gene activity. The *Cdx1* gene exhibits a gastrulation specific expression pattern in ectodermal and mesodermal cells of the primitive streak and thus also on the basis of this expression would be a good candidate involved in the regulation of Hox gene activity. We tested this hypothesis by inactivation of *Cdx1* using homologous recombination. Homozygous mutant mice exhibited homoeotic transformations of vertebrae. In general, the phenotypic alterations of the *Cdx1* -/- mice have to be interpreted as anterior transformation of vertebrae. Examination of Hox gene expression in *Cdx1*-/- mice demonstrate a posterior shift in their expression domains, that correspond to the observed changes in axial identities. In vitro binding of *Cdx1* on Hox regulatory sequences and transactivation experiments on Hoxa-7 promoter constructs in P19 cells strongly suggest *Cdx1* as a direct regulatory of Hox genes.
Together these data point to a novel regulatory mechanism of Hox gene activity.

POSTERS

HMG1 cooperates with HOX proteins in DNA binding and transcriptional activation

Vincenzo Zappavigna[1], Luca Falciola[1], Manuela Helmer Citterich[2], Fulvio Mavilio[1] and Marco E. Bianchi[1,3,4]

[1]DIBIT, Istituto Scientifico San Raffaele, v. Olgettina 58, 20132 Milano, Italy; [2]Dipartimento di Biologia, Università Tor Vergata, v. Raimondo, 00173 Roma, Italy; [3]Dipartimento di Genetica e di Biologia dei Microrganismi, Universitá di Milano, v. Celoria 26, 20133 Milano, Italy.

High mobility group protein 1 (HMG1) is a non-histone, chromatin-associated nuclear protein which has been proposed to play a role in the regulation of eukaryotic gene expression. We show that HMG1 interacts with proteins encoded by the HOX gene family by establishing protein-protein contacts between the HMG-box domains and the HOX homeodomain. The functional role of these interactions was studied using the transcriptional activation of the human HOXD9 protein as a model. HMG1 significantly enhances HOXD9-mediated transcriptional activation in a co-transfection assay. This synergy requires the DNA-binding activity of the homeodomain, and is implemented by the activation domain of HOXD9. The sequence-specific DNA-binding activity of HOXD9 is enhanced in vitro by HMG1 in a dose-dependent fashion and a DNA/HMG1/HOXD9 ternary complex can assemble on a HMG1-Sepharose matrix. Interaction with HMG1 increases the target specificity of HOX protein in cultured cells: HMG1 cooperates with HOXD9, but not with HOXD8, on a HOXD9-activated element, while HOXD8 activity is enhanced on a HOXD8-activated element. Specific recognition of the HOXD9 target and synergy with HMG1 can be transferred to HOXD8 by homeodomain swapping. We propose that HMG1 might be a general co-factor in HOX-mediated transcriptional activation. HMG1 might modulate functional specificity by facilitating access of HOX proteins to specific DNA targets, and/or by introducing architectural constraints in the assembly of HOX-containing transcriptional complexes.

M-PHASE SPECIFIC PHOSPHORYLATION OF THE POU TRANSCRIPTION FACTOR GHF-1 BY A CELL CYCLE REGULATED PROTEIN KINASE INHIBITS DNA BINDING.

Carme Caelles*, Hanjo Hennemann and Michael Karin.

Department of Pharmacology. Program in Biomedical Sciences. School of Medicine. University of California, San Diego. La Jolla, California 92093-0636.

GHF-1 is a member of the POU family of homeodomain proteins. It is a cell type specific transcription factor responsible for determination and expansion of growth hormone (GH) and prolactin (PRL) expressing cells in the anterior pituitary. It was previously suggested that the cAMP responsive protein kinase A (PKA) phosphorylates GHF-1 at a site within the N-terminal arm of its homeodomain, thereby inhibiting its binding to the GH promoter. These results, however, are inconsistent with the physiological stimulation of GH production by the cAMP pathway. As reported here, cAMP agonists or PKA do not inhibit GHF-1 activity in living cells and although they stimulate the phosphorylation of GHF-1, the inhibitory phosphoacceptor site within the homeodomain is not affected. Instead, this site, Thr220, is subject to M-phase specific phosphorylation. As a result, GHF-1 DNA binding activity is transiently inhibited during M-phase. This activity is regained once cells enter G1, a phase during which GHF-1 phosphorylation is minimal. Thr220 of GHF-1 is the homolog of the mitotic phosphoacceptor site responsible for the M-phase specific inhibition of Oct-1 DNA binding, Ser382. As this site is conserved in all POU proteins, it appears that all members of this group are similarly regulated. A specific kinase activity that is distinct in its substrate specificity and susceptibility to inhibitors from the Cdc2 mitotic kinase or PKA was identified in extracts of mitotic cells. This novel activity could be involved in regulating the DNA binding activity of all POU proteins in a cell-cycle dependent manner.

*Present address: Instituto de Investigaciones Biomédicas, C.S.I.C. Arturo Duperier, 4. 28029-Madrid.

TISSUE-SPECIFIC TRANSCRIPTION REGULATION OF THE HGH/HPL LOCUS IN PITUITARY AND PLACENTA.

V. Vila, O. Jiménez, J. Cosano, M. Palma, M. de la Hoya, A. Güell and José Luis Castrillo

Centro de Biología Molecular "Severo Ochoa" (CSIC-UAM).
Universidad Autónoma de Madrid. Cantoblanco.
Madrid-28049 (SPAIN).

The human GH/PL locus (66 Kb) contains the HGH-N gene (transcribed exclusively in the pituitary) and the placental lactogen HPL-3,4 genes (expressed only in placental tissue). The purposes of this proyect are: 1) The study of the tissue-specific transcription of the PL genes in placenta, and 2) The study the extintion (inhibition of gene expression) of the GH-N gene in placenta and PL genes in pituitary.

The HGH 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. The goal of our work is to investigate how chromatin structure and transcription factors associated will regulate the tissue-specific gene expression of the HGH/HPL locus.

From a human genomic library we have isolated and characterized four cosmids spanning 100 Kb from chromosome $17q_{22-24}$. It contains the whole HGH/HPL locus in addition of 5´ and 3´ flanking regions whose tissue-specific control elements are under investigation.

We present here the mapping of the whole 100 Kb region, the deletional analyses of GH and PL promoters and the cloning of PLA-1, a new POU transcription factor expressed in placental tissue.

Squenching or competition as mechanisms of of I-Aβ gene repression.

Jorge LLoberas[1], Richard A. Maki[2] and Antonio Celada[1]

Departament de Bioquimica i Fisiologia (Immunologia), Facultat de Biologia, Universitat de Barcelona, Avd. Diagonal 645, Barcelona 08028, Spain.[1] and La Jolla Cancer Research Foundation, 10901 N. Torrey Pines Rd., La Jolla, CA 92037, USA.[2]

The induction of class II MHC gene expression is mediated by three DNA elements in the promoters of these genes (W, X and Y boxes). The Y box contains an inverted CCAAT box sequence and the binding activity to the CAAT box is mediated by factor NF-Y, which is composed of subunits NF-YA and NF-YB. We have found that transfection of either dbp A or dbp B (mYB-1) or both, inhibits I-Aβ gene expression. Although the genes for some members of the Y-box family of binding proteins have been isolated by screening an expression library using the Y-box sequence, in our conditions no binding of dbp A or dbp B to the Y-box of the I-Aβ or I-Eα promoter was detected. This suggested that repression of I-Aβ gene expression by dbp A and dbp B was not due to competition for binding to the Y-box sequence. The results suggest two other mechanisms by which dbpA and dbpB can inhibit transcription from the I-Aβ promoter. When dbp A was added, the binding of NF-YA to DNA increase, which could be explained by interaction between these two proteins whose purpose is to increase the binding affinity of NF-YA to DNA. However, this complex was unable to stimulate transcription from the I-Aβ promoter. Thus, dbp A competed for the interaction between NF-YA and NF-YB by

binding to NF-YA. When dbp B factor was added together with NF-YA and NF-YB the binding of NF-YA/NF-YB complex was reduced. This suggested that dbp B may compete with NF-YB for interaction with NF-YA. These results provide an example of how dbp A and dbp B may regulate transcription of promoters that utilize NF-Y as a transcription factor.

Reference:

Jorge LLoberas, Richard A. Maki and Antonio Celada. Repression of Major Histocompatibility Complex I-Aβ gene expression by dbp A and dbp B (mYB-1) proteins. Molecular and Cellular Biology. (In press).

ARAUCAN AND *CAUPOLICAN*, TWO HOMEOBOX GENES MEMBERS OF THE *IROQUOIS* COMPLEX, TRANSCRIPTIONALLY REGULATE THE PRONEURAL *ACHAETE* AND *SCUTE* GENES OF *DROSOPHILA MELANOGASTER*.

J.L. Gómez-Skarmeta, R. Diez del Corral, E. de la Calle, D. Ferrés Marcó and Modolell, J.; Centro de Biología Molecular "Severo Ochoa", C.S.I.C. and Universidad Autónoma, 28049 Madrid, Spain.

achaete (*ac*) and *scute* (*sc*), two proneural genes members of the *achaete-scute* complex (AS-C) of *Drosophila melanogaster*, play a key role in the development of the adult pattern of sensory organs. Both genes encode bHLH proteins which are thought to confer to cells the ability to become neural precursors. *ac* and *sc* have similar complex patterns of expression that delimit the regions where sensory mother cells emerge. The expression patterns of these genes are constructed piecemeal by the action on both genes of site-specific enhancers distributed along most of the AS-C DNA. These enhancers are thougth to respond to site-specific combinations of factors, the products of the prepattern genes.

Flies homozygous for a viable *iroquois* (*iro*) allele (iro^1) lack sensory organs on the lateral notum. Moreover, in iro^1 mutant imaginal discs, the *ac*/*sc* expressing cell clusters that will give rise to these sensory organs are missing. These data indicate that iro^1 may be a mutation affecting a putative prepattern gene. We have cloned 50 kb of DNA spanning the region between a *lacZ* enhancer trap line (rF209) that not fully complements iro^1 and the position of the breakpoint associated with this mutation. This region contains two homeobox genes, *araucan* (*ara*) and *caupolican* (*caup*), with very similar expression patterns that cover many *ac*/*sc* proneural clusters, including those affected by the iro^1 mutation. We have denominated this DNA region the *iroquois* complex.

We will present the molecular cloning of the *iroquois* complex, the expression pattern of *ara* and *caup*, and evidence indicating that both genes activate the transcription of *ac* and *sc* by a direct action on enhancers located of the AS-C.

Control of *Drosophila* adult pattern by *extradenticle*.

Sergio González-Crespo and Ginés Morata. Centro de Biología Molecular. Consejo Superior de Investigaciones Científicas. Universidad Autónoma de Madrid. 28049 - Madrid. Spain.

The homeobox gene *extradenticle (exd)* acts as a cofactor of the homeotic genes in the specification of larval patterns during embryogenesis. To study its role in adult patterns, we have generated clones of mutant *exd*$^-$ cells and examined their effect on the different body parts.

In some regions *exd*$^-$ clones exhibit homeotic transformations similar to those produced by known homeotic mutations such as *Ultrabithorax (Ubx), labial (lab), spineless-aristapedia* (ss^a) *or Antennapedia (Antp)*. In other regions the lack of *exd* causes novel homeotic transformations producing ectopic eyes and legs. Moreover, *exd* is also required for functions normally not associated with homeosis, such as the maintenance of the dorso-ventral pattern, the specification of subpatterns in adult appendages or the arrangement of bristles in the mesonotum and genitalia.

Our findings indicate that *exd* is critically involved in adult morphogenesis, not only in the homeotic function but also in several other developmental processes.

Developmental and synaptic activation of NF-κB in cerebellar granule neurons in vitro.

Luisa Guerrini and Francesco Blasi, Dep. of Genetics and Microbial Biology, Via Celoria 26, University of Milano, 20133 Italy.

Neuronal proliferation, migration and differentiation are regulated by the sequential expression of particular genes at specific stages of development. The molecular control of neuronal differentiation can be followed in the cerebellar granule cells since these neurons develop in the post-natal period and undergo sequential stages of maturation and migration in a well-characterized, spatially restricted pattern, following a regular timetable. Under appropriate culture conditions granule cells undergo both biochemical and morphological differentiation *in vitro* (1). The neurotransmitter glutamate has been shown to play an important role in this developmental process. While the steps of this process are well known, the genes involved in these sequential events are only now beginning to be identified (2) and virtually nothing is known as to the molecular mechanisms regulating their expression.

Recently the presence of the transcription factor NF-κB in several areas of the rat brain has been described (3, 4). We show by immunohistochemistry that the RelA subunit of the transcription factor NF-κB is present in several areas of the mouse brain, including the cerebellum. In cerebellar granule cell cultures derived from early post-natal mice (3-7 days PN), the RelA subunit of NF-κB is present in both astrocytes and granule neurons in an inactive state in the cytoplasm. Translocation to the nucleus, resulting in a DNA-binding form of NF-κB, can be obtained in both cell types using cell type-specific receptor stimulation. In granule neurons, a neurotransmitter such as glutamate can activate NF-κB with rapid kinetics. The use of different antagonists of the glutamate receptors indicates that the effect of glutamate occurs mainly via NMDA-receptor activation, possibly as a result from an increase in intracellular [Ca_i]. The effect of glutamate appears to be developmentally regulated. Indeed, NF-κB is found in an inducible form in the cytoplasm of neurons of 3 to 7 day old mice, but is constitutively activated in the nuclei of neurons derived from older pups (8-12 post natal). Activation may occur through an autocrine activation loop. Overall, these observations suggest the existence of a new pathway of trans-synaptic regulation of gene expression.

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TRANSCRIPTIONAL REGULATION OF LFA-1 AND p150,95 INTEGRINS: AP-2, Sp1 AND AP-1 DRIVE A LINEAGE AND DEVELOPMENTALLY REGULATED PROTEIN EXPRESSION.

Cristina López-Rodríguez[1] and Angel L. Corbí[2].
[1]Unidad de Biología Molecular, Hospital Universitario de la Princesa, Diego de León 62, 28006 Madrid; and [2]Instituto López-Neyra, Ventanilla 11, 18001 Granada, SPAIN.

The β2 or leukocyte restricted Integrins, wich 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 leukocite 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).

The transcriptional regulation of the CD11a gene is strongly regulated at the level of its proximal promoter. Over this region, 5'deletions and *in vitro footprinting* experiments signaled the presence of a putative AP-2 transcrption factor binding site (-100). Such activity was assesed by means of transactivation experiments and, what is more relevant, using both especific AP-2 antisera and competitor consensus oligonucleotides. The delectionally disruption of this AP-2 site strongly affected the promoter activity. Such reduction resulted much more relevant at CD11a expressing cells, illustrating the **relevance of the interaction between the AP-2 protein and the CD11a promoter to account for the leukocyte specific expression of the LFA-1 integrin.**

Experiments designed to identify the DNA elements and transcription factors responsible for the unique pattern of p150,95 expression have demonstreated the presence of two Sp1 binding sites at -70 and -120 in the Cd11c proximal promoter. Sp1 was demonstrated to transactivete the CD11c promoter in SI2 cells, where mutation of the most proximal Sp1 site greatly decreased the Sp1 transactivation capacity, whereas mutation of the distal one 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 any reduction was observed in the CD11c-negative HeLa cell line, indicating the **involvement of both Sp1 sites in the tissue-especific 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 gene. Mobility shift assays demonstrated the presence of an AP-1-binding site within the CD11c promoter (AP-1-60) and identified c-jun, junB, c-fos and fra-1 as the menbers of the AP-1 family interacting with AP-1-60 site indifferentiated U937 cells. The actual involvement of AP-1-60 in DNA-protein interactions was also determined by means of *in vivo footprinting* experiments, andits functionallity was demonstrated by transctivation 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 AP-1-60 site inhibited the basal promotor activity as well as the induction of the CD11c promoter activity diring 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, mobility band shift assays and the construction of U937 stable transfectans containing the CD11c promoter ligated to the luciferase gene reporter has allowed us to establish the importance of menbers of the Ets, Oct and bHLH families of trancription factors in the myeloid-restricted expression of CD11c. The idetity of the specific factors responsible for these complexes is being pursued at the present moment.

Transcription activation mechanism of phage Φ29 regulatory protein p4: Interaction with *Bacillus subtilis* RNA polymerase.

Mario Mencía, María Monsalve, Fernando Rojo and Margarita Salas.
Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Cantoblanco, 28049-Madrid, Spain.

Bacillus subtilis phage Φ29 regulatory protein p4 activates transcription from the late A3 promoter by stabilizing the σ^A-RNA polymerase at the promoter as a closed complex (Nuez et al., 1992). Activation requires a proper interaction between both proteins (Mencía et al, 1993). Protein p4 induces a severe bend in the DNA upon binding (Rojo et al., 1990). Previous evidences suggested that the carboxyl-end of the protein contains residues involved in the activation process and also in the maintenance of the p4-induced DNA bending (Mencía et al., 1993). To precisely determine which residues are involved in interacting with the RNA polymerase to activate transcription, and which are responsible for the DNA bending, we have performed an exhaustive mutagenesis study of this region of the protein.

The results confirm our previous proposal that Arg 120 mediates the interaction with RNA polymerase that leads to the activation of transcription. At the same time, the mutagenesis study has allowed to identify several basic residues that contribute to maintain the DNA bending induced at both sides of the binding

site, most likely through electrostatic interactions with the phosphates of the DNA backbone. The analysis of simple, double and triple mutants showed that the degree or stability of the induced bend relies on the additive contribution of all the basic residues of the p4 carboxyl-end. These residues are located in the vecinity of Arg 120, that seems both to mediate the interaction with RNA polymerase required to activate transcription and to contribute to the maintenance of the DNA bending.

Since protein p4 binds to the A3 promoter at a region spanning positions -58 to -104 relative to the initiation site, it is likely to contact the C-terminal domain of the alpha subunit of RNA polymerase to activate transcription. We tested this hypothesis by purifying the *B. subtilis* RNA polymerase alpha subunit and assaying the ability of protein p4 to stabilize it at the A3 promoter, as it does with the RNA polymerase holoenzyme. DNase I footprinting and gel retardation assays showed that protein p4 can indeed drive the RNA polymerase alpha subunit to the A3 promoter. Protein p4 mutants in residue Arg 120 were unable to stabilize the alpha subunit at the promoter. This interaction seems to be specific of *B. subtilis* RNA polymerase. Therefore, the mechanism by which protein p4 activates transcription at the A3 promoter requires an interaction between p4 residue Arg 120 and the alpha subunit of *B. subtilis* RNA polymerase.

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B type cyclins regulate G1 progression in fission yeast in opposition to the $p25^{rum1}$ CDK inhibitor.

Cristina Martín, Karim Labib and Sergio Moreno.
Instituto de Microbiología Bioquímica. Departamento de Microbiología y Genética. CSIC/Universidad de Salamanca. Edificio Departamental. Campus Miguel de Unamuno. 37007 Salamanca. Spain.

In yeasts the process of commitment to cell division, termed Start, defines a period in late G1 beyond which cells can no longer undergo other developmental fates, such as sexual differentiation. At Start, yeast cells monitor the extracellular microenviroment (presence or absence of nutrients, sexual pheromones, etc.) and either make a commitment to progression through the mitotic cell cycle, appropiate for cells growing in rich medium, or undergo cell cycle arrest in G1 as a prelude to conjugation and meiosis, appropiate for nutritionally starved cells. Before cells can pass Start, they must grow sufficiently to attain a critical cell mass, and small cells in G1 cannot undergo S-phase until they reach this mass.

The $p34^{cdc2}$ kinase is essential for G1 progression past Start, but until now its regulation has been poorly understood. We will show that the cig2 B-type cyclin has an important role in G1 progression, and demonstrate that $p34^{cdc2}$ kinase activity is periodically associated with cig2 in G1. Cells lacking cig2 are defective in G1 progression, and this is particularly clear in small cells that must regulate Start with respect to cell size. We also find that the cig1 B-type cyclin can promote G1 progression. Whilst $p25^{rum1}$ can inhibit cig2/cdc2 activity *in vitro*, and may transiently inhibit this complex *in vivo*, cig1 is regulated independently of $p25^{rum1}$. Since cig1/cdc2 kinase activity peaks in mitotic cells, and decreases after mitosis with similiar kinetics to cdc13 associated kinase activity, we suggest that cig2 is likely to be the principal fission yeast G1 cyclin. cig2 protein levels accumulate in G1 cells, and we propose that $p25^{rum1}$ may transiently inhibit cig2 associated $p34^{cdc2}$ activity until the critical cell size required for Start is reached.

Silencing of homeotic genes in Drosophila

Jürg Müller, Bea Christen and Mariann Bienz. MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, England

In Drosophila, *Polycomb-group* gene products keep homeotic genes stably silenced in cells where these genes must remain inactive during development. Polycomb-group proteins are present and act in most cells and therefore cannot, on their own, be responsible for this localised silencing. The spatial specificity for the silencing process is provided by gap proteins, which themselves are transiently expressed in characteristic patterns in the early embryo. In particular, the gap protein hunchback binds directly as a repressor to specific control regions of the homeotic gene *Ultrabithorax* to prevent its activation by more generally distributed factors[1,2]. Early repression by *hunchback* is then converted into stable and heritable silencing which affects enhancers anywhere in the gene and which is maintained in a *Polycomb-group* gene-dependent fashion even after the decay of the hunchback protein[3,4]. As Polycomb-group proteins bind to the chromatin associated with homeotic genes[5], but do not appear to bind to DNA directly, this led to a model, in which hunchback protein directly or indirectly recruits Polycomb-group proteins to DNA[6]. In an attempt to identify Polycomb-group proteins which physically interact with hunchback, we used a yeast two-hybrid interaction screen. We isolated cDNAs of four different genes, the products of which show a specific interaction with hunchback protein. None of these genes corresponds to a known *Drosophila* gene and they do not map to known loci of *Polycomb-group* genes. However, one of these genes is uncovered by a deficiency which, when present homozygous, results in ectopic expression of homeotic gene products. We are currently testing whether this *Polycomb*-like phenotype of the deficiency is due to the lack of the gene identified in our screen. The protein encoded by this gene appears to be a homologue of CHD-1[ref.7], a murine DNA-binding protein. In addition to the conservation in the DNA-binding domain, both proteins also contain a chromodomain and a helicase domain.

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Biochemical characterization of Rolled^{Sem}, an activated form of *Drosophila* MAP kinase

Nadja Oellers and Ernst Hafen
Zoologisches Institut, Universität Zürich, Winterthurerstr.190,
CH-8057 Zürich, Switzerland

In *Drosophila* the specification of R7 photoreceptor cell fate in each ommatidium of the developing eye is dependent on the components of the mitogen-activated protein kinase (MAPK) cascade. In the R7 precursor cells the Sevenless (Sev) receptor tyrosine kinase (RTK) is activated by its ligand, the Boss protein and the signal is then transduced via Drk, Sos, Ras1 and the protein kinases Raf, Dsor1 and Rolled (Rl) to the nucleus.
The *rl* gene encodes a *Drosophila* homologue of vertebrate MAP kinases. A gain-of-function mutation in this gene, *rl^{Sevenmaker}* (*rl^{Sem}*), has been shown genetically to activate not only the Sev pathway but also the Torso and the *Drosophila* EGF receptor (DER) pathway. Genetic analyses have shown that *Dsor1*, encoding a *Drosophila* homologue of MAP kinase kinase (MAPKK) acts upstream of *rl* in these pathways and *pointedP2* (*pntP2*) and *yan*, encoding two members of the ETS-family of transcription factors, act downstream of *rl* in the Sev pathway.
In order to investigate the biochemical properties of the first identified gain-of-function mutation in a MAP kinase gene, *rl^{Sem}*, we examined the kinase activity of the corresponding mutant protein, immunoprecipitated from transfected COS cell lysates and *Drosophila* larvae extracts. We demonstrate that Rl^{Sem}/MAPK, expressed in mammalian COS cells or purified from bacteria is two to three fold more active than wild-type Rl/MAPK using PntP2 and Yan as natural substrates as well as myelin basic protein (MBP) as an artificial MAPK substrate. However, we could observe a more pronounced kinase activity when the proteins were immunoprecipitated from *Drosophila rl* and *rl^{Sem}* larvae extracts. In these assays Rl^{Sem}/MAPK exhibited a five to nine fold higher kinase activity than wild-type Rl/MAPK, which is comparable to the kinase activity of larvae expressing either an activated Sev receptor or an activated Raf kinase.
We also could demonstrate that Dsor1/MAPKK is able to phosphorylate and activate Rl/MAPK *in vitro*.

RESPONSE TO ALKALINE AMBIENT PH INVOLVES PROTEOLYSIS OF THE *ASPERGILLUS NIDULANS* PACC TRANSCRIPTION FACTOR

Margarita Orejas[1], E.A. Espeso[1], J. Tilburn[2], D.A. Sarkar[2], H.N. Arst Jr.[2] and M.A. Peñalva[1]

[1]Centro de Investigaciones Biológicas (C.S.I.C.). Madrid, Spain

[2]Department of Infectious Diseases and Bacteriology, Royal Postgraduate Medical School. London, U.K.

In common with many microorganisms, *Aspergillus nidulans* grows over a very wide pH range. Survival effectively requires, in adition to efficient pH homeostasis system, a regulatory system tailoring the syntheses of molecules functioning beyond the cell boundaries such as permeases, secreted enzymes and exported metabolites to the pH of the growth enviroment (Caddick et al., 1986).

We have investigated how alkaline ambient pH effects conversion of PacC from its inactive form to a functional activator/represor. Previous work established that the PacC transcription factor mediates pH regulation in response to a signal provided by the products of the six *pal* genes at alkaline ambient pH (Tilburn *et al.*, 1995). In the presence of this signal, PacC becomes functional, activating transcription of genes expressed at alkaline pH and preventing transcription of genes expressed at acidic pH.

We detect two different forms of PacC in extracts (both with PacC binding specificity) differing postranslationally (Orejas *et al.*, 1995). Under acidic growth conditions or in acidity-mimicking *pal* mutants, the full length form predominates. Under alkaline ambient pH or in *pacC*^{c} mutants (indepenent of the signal transduction pathway and resulting in an alkalinity-mimicking phenotype), a proteolysed version containing the N-terminal ~ 40% of the protein predominates. This specifically cleaved shorter version is functional. Thus, PacC proteolysis is an essential and pH-sensitive step in the regulation of gene expression by ambient pH. Our results support a model in which the C-terminal region negatively modulates PacC function, rendering the full-length inactive form and that a pH-sensitive step in conversion of PacC to a functional form is removal of the C-terminal ~ 60% of the protein.

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KINETICS OF MMTV-LTR HORMONE RESPONSE IN FIBROBLASTS.

Susanna Boronat and Benjamin Piña.
Centre d'Investigació i Desenvolupament. Consejo Superior de Investigaciopnes Científicas.
c/Jordi Girona, 18. 08034 Barcelona, Spain.

We have analysed the hormonal response of different MMTV-based constructs either stably integrated or transiently transfected into fibroblasts. Stably transfected MMTV constructs are strongly stimulated by dexamethasone (Dex), but their response diminishes after two days of continuous treatment with the hormone, independently of the nature of the gene driven by the MMTV promoter. This deactivation is not observed in other hormone responsive resident genes (like the metallothionein, MT, gene), nor it is observed when the same MMTV constructs are assayed in transient transfection experiments.

The analysis of chromatin structure over our integrated MMTV constructs during the activation/deactivation process shows a clear DNaseI hypersensitivity site appearing upon Dex treatment at the expected position (about nucleotide -150). This hypersensitive site fades after 4 days of continued hormone treatment, concomitantly with the decrease of transcription of the plasmids. This is consistent with a loss of GR function upon continuous hormone exposure, resulting on a simultaneous decay in transcriptional activation and a loss of the structural changes on chromatin occurring upon GR binding.

In order to investigate the affinity of the GR for different HREs, we analysed the response of the integrated MMTV constructs and of the metallothionein (MT) gene to different Dex concentrations. Our results show a 10 fold higher Km for the integrated MMTV-HRE (about $2x10^{-8}M$) than for the MT-HRE (about $2x10^{-9}M$). Both the bibliographic data on relative affinities of the corresponding DNA sequences *in vitro* and the fact that the dose-response curves for transiently transfected MMTV-CAT and MT-CAT plasmids are absolutely identical indicate chromatin structure as the main responsible for this apparent low affinity of the MMTV-LTR.

Although the deactivation effect could be due to the presence of a specific repressor in hormone-treated cells, our current data favors a mechanism where deactivation correlates with a loss of GR receptor function upon continuous hormone administration. During this process, the behavior of different genes and reporters would be related to their differential accessibility to the hormone-receptor complex, which can be affected by chromatin structure. Specific nucleosome positioning has been shown for at least some homone-regulated genes. If nucleosome positioning modulates accessibility of the different DNA sequences to transcriptional factors *in vitro* as it does *in vitro*, it is conceivable that it may determine whether or not a given gene becomes activated by a given hormone concentration in a given cell type. As chromatin structure changes in different ways depending on the cell differentiation stage, it may represent a fine-tune mechanism of modulating hormone response of specific genes.

DITHIOCARBAMATES TRIGGER DIFFERENTIATION, APOPTOSIS AND INDUCTION OF CD11C GENE THROUGH AP-1 IN THE MYELOID LINEAGE.

Julián Aragonés[1], Cristina López-Rodríguez[1], Angel Corbí[2], Pablo Gómez del Arco[3], Manuel López-Cabrera[1], Manuel O. de Landázuri[1], and Juan Miguel Redondo[3*].
From Servicios de Inmunología y Biología Molecular de Hospital de la Princesa. Madrid [(1)].Instituto López Neyra, CSIC. Granada [(2)]. Centro de Biología Molecular, CSIC-UAM. Cantoblanco, Madrid[(3)].

It has recently been shown that the alteration of the cell-redox status affects the transcription factor expression and activity. Dithiocarbamates (DTCs) are potent antioxidant agents that can switch the expression of genes dependent on the activation of the transcription factors AP-1 and NFκB. In this study, we show that these agents triggered the expression of genes involved in myeloid differentiation of the promonocytic U-937 cell line. DTCs promoted differentiation-associated changes that included the surface up-regulation of β2-integrins (CD11a-c/CD18), cell growth arrest concomitant with transferrin receptor (CD71) down-modulation, induction of the non-specific esterase enzyme, and a rapid drop in the mRNA levels of c-myc. In addition, depending on the time of analysis, DTCs could either trigger apoptosis or prevent that induced by etoposide. A further analysis, focused on the molecular mechanisms leading to the activation of CD11c expression, revealed that the pyrrolidine derivative of DTC (PDTC) increased CD11c mRNA levels and augmented its gene promoter activity. Transfection experiments with reporter constructs harboring different promoter regions of CD11c gene, indicated the presence of a functional DTC-responsive region located between positions -160 and +40 of the promoter. Gel retardation assays revealed that the PDTC-induced DNA-protein complexes were restricted to members of the Fos and Jun families that bound to an AP-1 site located at position -60 from the transcription start site. A role for this site was confirmed by *in vitro* mutagenesis experiments that indicated the functional importance of this site for the CD11c gene transcriptional activation in response to the DTC. The effect of DTCs on myeloid cell differentiation and apoptosis supports a possible role for these agents in the therapy of some bone marrow-derived

trkB EXPRESSION IS REGULATED BY THYROID HORMONE AND THYROID HORMONE RECEPTOR DURING BRAIN DEVELOPMENT.

P.M. Pombo, D. Barttino & A. Rodríguez-Peña.
Instituto de Investigaciones Biomedicas, CSIC. C/ Arturo Duperier,4. Madrid 28029. Tfno 34-1-58584632. Fax 34-1-585 4587. e-mail "Arodriguez.iib.biome.uam.es".

We have previously shown that unliganded thyroid hormone receptor induces trkB expression in different neuroblastoma cell lines. RNAse protection assays to detect trkB expression in transient trasfection experiments with the expression vector for thyroid hormone receptor (TR) suggested that this effect could be direct. Thus, we have isolated 7 Kb genomic fragment up stream of the coding sequence. Transient transfection experiments with this 7Kb fragment linked to the reporter gene CAT showed that thyroid hormone receptor induced the expression of the reporter gene and the addition of thyroid hormone (T3) reduced its expression to basal levels. Deletion analysis of this fragment showed that the element(s) responsible for the receptor activation were located -3.2 Kb upstream. On the other hand, the repression by T3 of this promoter is due to element(s) included in the shortest construct tested (-700 bp-CAT).

We have studied the in vivo implication of these results. Although we have no means to manipulate the levels of thyroid hormone receptor, and therefore we cannot test the effect of the TR expression, we can deplete of thyroid hormone fetus and neonatal rats. In agreement with our in vitro data, rat brain with thyroid hormone deficiency showed an increase in the trkB mRNA levels when compared with aged matched controls. Run-on assays with nuclei from hypothyroid and control cerebral cortices showed that this effect is at the transcriptional level.

Multiple roles of *Pax-2* in the development of central nervous system, sense organs and urogenital system

MIGUEL TORRES

MPI for Biophysical Chemistry
Göttingen (Germany)

Urogenital system development in mammals requires the coordinated differentiation of two distinct tissues, the ductal epithelium and the nephrogenic mesenchyme, both derived from the intermediate mesoderm of the early embryo. The former give rise to the genital tracts, ureters, and kidney collecting duct system, whereas mesenchymal components undergo epithelial transformation to form nephrons in both the mesonephric (embryonic) and metanephric (definitive) kidney. *Pax-2* is a transcriptional regulator of the paired-box family and is widely expressed during the development of both ductal and mesenchymal components of the urogenital system. We report here that *Pax-2* homozygous mutant newborn mice lack kidneys, ureters and genital tracts. We attribute these defects to dysgenesis of both ductal and mesenchymal components of the developing urogenital system. The Wolffian and Müllerian ducts, precursors of male and female genital tracts respectively, develop only partially and degenerate during embryogenesis. The ureters, inducers of the metanephros are absent and therefore kidney development does not take place. Mesenchyme of the nephrogenic cord fails to undergo epithelial transformation and is not able to form tubules in the mesonephros. In addition we show that the expression of specific markers for each of these components is de-regulated in *Pax-2* mutants. These data show that *Pax-2* is required for multiple steps during the differentiation of intermediate mesoderm. *Pax-2* is also expressed during the Central Nervous System development in midbrain, eye and inner ear. We observe malformations all these three structures suggestive of a role of Pax-2 in morphogenetic movements undergoing in these structures. Recently a human *PAX-2* mutation has been associated with a familial case of congenital eye and kidney malformations. Therefore, *Pax-2* mouse mutants provide an animal model for human hereditary diseases.

Conserved protein binding motifs in E1A$_{12S}$ and E2F1 define a new class of activation domain

Didier Trouche and Tony Kouzarides*

Wellcome/CRC Institute, Tennis Court Road, Cambridge (UK)

Activation domains of transcription factors are classified according to their "richness" in a particular residue. This classification has grouped activation domains which, for certain functions, have common characteristics. Here we show that two "acidic" activation domains, one from E1A and the other from E2F1 show, what we term, "specific" sequence identity, mostly in non-acidic residues. The conserved residues are colinear along the two activation domains and represent common binding sites for the RB tumour suppressor protein and the p300/CBP family of co-activators. The binding of RB and CBP to these two activation domains results in the same functional consequences: both domains are stimulated by CBP and repressed by RB. We conclude that the activation domains of E1A$_{12S}$ and E2F1 belong to a novel functional class, characterized by specific protein binding sites. These results highlight the existence of "specific" sequence similarity between activation domains, which reflects the conservation of functional protein interaction sites rather than the mere preponderance of a particular residue.

Retinoid-dependent in vitro transcription.

Rafael Valcárcel, Herbert Holz, Custodia García Jiménez and Hendrik G. Stunnenberg. EMBL. Meyerhofstrasse 1, D-69117, Heidelberg. GERMANY.

Abstract

Retinoids play an important role in development, differentiation and homeostasis. Their effect is mediated by two classes of ligand-inducible transcription factors: retinoic acid (RAR) and retinoid X (RXR) receptors. Transcription from the retinoid-inducible RARβ2 promoter is achieved in a reconstituted cell-free transcription system upon addition of RXRα/6H-RARα heterodimer isolated from vaccinia virus infected cells. The ligand-dependent transcriptional activation function AF-2 of RARα, but not of RXRα, is essential for transactivation. Recombinant RXRα/RARα is partially active in vitro. However, full activation requires the addition of either all-trans RA, 9-cis RA or other RAR-specific agonists, whereas an RARα-specific antagonist abolishes transactivation. None of these ligands affected the DNA binding ability of the heterodimer. Thus, we provide the first biochemical evidence that the transcriptional activity of RXRα/RARα is governed by ligand. The results indicate that the major role of RXR in transactivation of the RARβ2 promoter is to serve as an auxiliary factor required for binding of RAR which in turn is directly responsible for transcriptional activity. At present we are looking for the factors that mediate positive and negative interactions with the basal transcription machinery and trying to study how they function.

List of Invited Speakers

Workshop on

SWITCHING TRANSCRIPTION IN DEVELOPMENT

List of Invited Speakers

Richard Axel	HHMI-HHSC 1014, Columbia University, 701 West 168th Street, New York, NY. 10032 (USA). Tel.: 1 212 305 69 15 Fax : 1 212 923 72 49
Patrick A. Baeuerle	Institute of Biochemistry, Albert-Ludwigs-University, D-79104 Freiburg (Germany). Tel.: 49 761 203 52 21 Fax : 49 761 203 52 57
Miguel Beato	I.M.T.,E.-Mannkopff-Str.2, 35037 Marburg (F.R. Germany). Tel.: 49 6421 28 62 86 Fax : 49 6421 28 53 98
Adrian Bird	Institute of Cell and Molecular Biology, University of Edinburgh, Ki Edinburgh EH9 3JR, (U.K.). Tel.: 44 031 650 56 70 Fax : 44 031 650 53 79
Neil Brockdorff	Comparative Biology Group, MRC Clinical Sciences Centre, RPMS, Hammersmith Hospital, London W12 0NN (U.K.). Tel.: 44 181 259 82 78 / 82 98 Fax : 44 181 259 83 03
Pierre Chambon	Institut de Génétique et de Biologie Moleculaire et Cellulaire, CNRS/INSERM/ULP, Collège de France, B.P. 163, 67404 Illkirch-Cedex (France). Tel.: 33 88 65 32 13 /15 /10 Fax : 33 88 65 32 03
James E. Darnell	The Rockefeller University, New York, NY. 10021 (USA). Tel.: 1 212 327 87 91 Fax : 1 212 327 88 01
Joseph L. Goldstein	University of Texas Southwestern Medical Center, Dallas, TX. (USA). Tel.: 1 214 648 21 41 Fax : 1 214 648 88 04
Peter Gruss	Max-Planck-Institute of Biophysical Chemistry, Department of Molecular Cell Biology, 37077 Göttingen (Germany). Tel.: 49 551 201 13 61 Fax . 49 551 201 15 04
Ernst Hafen	Zoological Institute of the University of Zürich, Zürich (Switzerland). Tel.: 41 1 257 48 71 Fax : 41 1 361 31 85

Ira Herskowitz	Department of Biochemistry & Biophysics, University of California, San Francisco, CA. 94143-0448 (USA). Tel.: 1 415 476 49 77 Fax : 1 415 476 09 43
Tony Hunter	Molecular Biology and Virology Lababoratory, 10010 North Torrey Pines Road, La Jolla, CA. 92037 (USA). Tel.: 1 619 453 41 00 Fax : 1 619 457 47 65
Herbert Jäckle	Abteilung Molekulare Entwicklungsbiologie, Max-Planck-Institut für biophysikalische Chemie, Postfach 2841, D-37018 Göttingen (Germany). Tel.: 49 551 20 11 482 Fax . 49 551 20 11 755
Benjamin Lewin	Cell Editorial Offices,1050 Massachusetts Ave., Cambridge, MA. 02138 (USA). Tel.: 1 617 661 70 57 Fax : 1 617 661 70 61
Richard Losick	Department of Molecular & Cellular Biology, The Biological Laboratories, Harvard University, Cambridge, MA. 02138 (USA). Tel.: 1 617 495 49 05 Fax : 1 617 496 46 42
Tom Maniatis	Harvard University, Department of Molecular and Cellular Biology, Cambridge, MA. 02138 (USA). Tel.: 1 617 495 18 11 Fax : 1 617 495 35 37
Juan Modolell	Centro de Biología Molecular "Severo Ochoa", CSIC, UAM, Cantoblanco, 28049 Madrid (Spain). Tel.: 34 1 397 50 73 Fax : 34 1 397 47 99
Kim Nasmyth	IMP, Dr. Bohr-Gasse 7, A-1030 Vienna (Austria). Tel.: 43 1 797 30/0 Fax : 43 1 798 71 53
Paul Nurse	Cell Cycle Laboratory, Imperial Cancer Research Fund, P.O. Box 123, 44 Lincoln's Inn Fields, London WC2A 3PX (U.K.). Tel.: 44 171 269 32 64 Fax : 44 171 269 36 10
Vincenzo Pirrotta	Department of Zoology, University of Geneva, 30 quai E. Ansermet, CH1211 Geneva (Switzerland). Tel.: 41 22 702 67 86 Fax : 41 22 702 67 76
Richard Treisman	Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX (U.K.). Tel.: 44 171 269 32 71 Fax : 44 171 269 35 81

List of Participants

Workshop on

SWITCHING TRANSCRIPTION IN DEVELOPMENT

List of Participants

Marco E. Bianchi	DIBIT, Istituto Scientífico San Raffaele, Via Olgettina 58, 20132 Milano (Italy). Tel.: 39 2 26 43 47 80 Fax : 39 2 26 43 47 67
Carme Caelles	Instituto de Investigaciones Biomédicas, CSIC, Arturo Duperier 4, 28029 Madrid (Spain). Tel.: 34 1 585 46 00 Fax : 34 1 585 45 87
José-Luis Castrillo	Centro de Biología Molecular "Severo Ochoa", (CSIC-UAM), Universidad Autónoma, Cantoblanco, 28049 Madrid (Spain). Tel.: 34 1 397 80 74 Fax : 34 1 397 47 99 or 397 83 44
Michael Caudy	Cornell University Medical College, New York, NY. 10021 (USA). Tel.: 1 212 746 61 60 Fax : 1 212 746 81 75
Antonio Celada	Departament de Bioquimica i Fisiologia (Immunologia), Facultat de Biologia, Universitat de Barcelona, Avd. Diagonal 645, 08028 Barcelona (Spain). Tel.: 34 3 402 15 55 Fax : 34 3 411 03 58
Margarita Cervera	Departamento de Bioquímica, Instituto de Investigaciones Biomédicas, CSIC, Arturo Duperier 4, 28029 Madrid (Spain). Tel.: 34 1 397 54 09 Fax : 34 1 585 45 87
José Luis Gómez Skarmeta	Centro de Biología Molecular "Severo Ochoa", (CSIC), Universidad Autónoma, Cantoblanco, 28049 Madrid (Spain). Tel.: 34 1 397 50 72 Fax : 34 1 397 47 99
Sergio González Crespo	Centro de Biología Molecular "Severo Ochoa", (CSIC), Universidad Autónoma, Campus de Cantoblanco, 28049 Madrid (Spain). Tel.: 34 1 397 50 70 Fax : 34 1 397 47 99
Luisa Guerrini	Department of Genetics and Microbial Biology, Via Celoria 26, University of Milano, 20133 Milano (Italy). Tel.: 39 2 26 60 52 37 Fax : 39 2 26 64 55 1

Norbert Kraut	Fred Hutchinson Cancer Research Center, 1124 Columbia Street, A3-025, Seattle, WA. 98104 (USA). Tel.: 1 206 667 45 04 Fax : 1 206 667 58 94
Santiago Lamas	Centro de Investigaciones Biológicas, CSIC, c/Velázquez 144, 28006 Madrid (Spain). Tel.: 34 1 564 45 62 Ext. 4302 Fax : 34 1 562 75 18
Mª Cristina López-Rodríguez	Unidad de Biología Molecular, Hospital Universitario de la Princesa, Diego de León 62, 28006Madrid (Spain). Tel.: 34 1 402 80 00 Fax : 34 1 309 24 96
Victor de Lorenzo	Centro de Investigaciones Biológicas, CSIC, c/Velázquez 144, 28006 Madrid (Spain). Tel.: 34 1 564 45 62 Ext. 4243 Fax : 34 1 562 75 18
Alfonso Martínez Arias	Department of Zoology, University of Cambridge, Cambridge CB2 3EJ. (U.K.). Tel.: 44 1223 33 66 20 / 40 Fax : 44 1223 33 66 76
Marcel Méchali	Institut Jacques Monod, Molecular Embryology, 2 Place Jussieu, 75251 Paris Cedex 05 (France). Tel.: 33 1 46 33 67 85 Fax : 33 1 44 27 52 65
Mario Mencía	Centro de Biología Molecular "Severo Ochoa", (CSIC-UAM), Universidad Autónoma, Cantoblanco, 28049 Madrid (Spain). Tel.: 34 1 397 84 34 Fax : 34 1 397 47 99
Sergio Moreno	Instituto de Microbiología Bioquímica, Departamento de Microbiología y Genética, CSIC/ Universidad de Salamanca, Edificio Departamental, Campus Miguel de Unamuno, 37007 Salamanca (Spain). Tel.: 34 23 12 15 89 Fax : 34 23 26 79 70
Jürg Müller	MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH (U.K.). Tel.: 44 1223 40 22 74 Fax : 44 1223 41 21 42
Nadja Oellers	Zoologisches Institut, Universität Zürich, Winterthurerstr. 190, CH-8057 Zürich (Switzerland). Tel.: 41 1 257 47 88 Fax : 41 1 361 31 85

Margarita Orejas	Centro de Investigaciones Biológicas, CSIC, 28006 Madrid (Spain). Tel.: 34 1 564 45 62 Fax : 34 1 562 75 18
José Pérez Martín	Centro de Investigaciones Biológicas, CSIC, c/Velázquez 144, 28006 Madrid (Spain). Tel.: 34 1 561 18 00 Fax : 34 1 562 75 18
Benjamín Piña	Centre d'Investigació i Desenvolupament, CSIC, c/Jordi Girona 18, 08034 Barcelona (Spain). Tel.: 34 3 400 61 57 Fax : 34 3 204 59 04
Juan Miguel Redondo	Centro de Biología Molecular, (CSIC-UAM), Facultad de Ciencias, Cantoblanco, 28049 Madrid (Spain). Tel.: 34 1 397 84 13 Fax : 34 1 397 47 99
Angeles Rodríguez-Peña	Instituto de Investigaciones Biomédicas, CSIC, c/Arturo Duperier 4, 28029 Madrid (Spain). Tel.: 34 1 585 46 32 Fax : 34 1 585 45 87
Pilar Santisteban	Instituto de Investigaciones Biomédicas, CSIC, Arturo Duperier 4, 28029 Madrid (Spain). Tel.: 34 1 585 46 44 Fax : 34 1 585 45 87
Leandro Sastre	Instituto de Investigaciones Biomédicas, CSIC, Arturo Duperier 4, 28029 Madrid (Spain). Tel.: 34 1 585 46 26 Fax : 34 1 585 45 87
Miguel Torres	MPI for Biophysical Chemistry, Göttingen 37077 (Germany). Tel.: 49 551 20 15 07 Fax : 49 551 20 15 04
Didier Trouche	Wellcome/CRC Institute, Tennis Court Road, Cambridge (U.K.). Tel.: 44 1223 33 40 88 Fax : 44 1223 33 40 89
Rafael Valcárcel	EMBL, Meyerhofstrasse 1, D-69117 Heidelberg (Germany). Tel.: 49 6221 38 74 78 Fax : 49 6221 38 75 18

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