Instituto Juan March
de Estudios e Investigaciones

32 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Chromatin Structure and Gene Expression

Organized by

F. Azorín, M. Beato and A. P. Wolffe

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INTRODUCTION

F. Azorín, M. Beato and A. Wolffe

"Chromatin Structure and Gene Expression"

26 to 29 September1994

Preface

During the past decade there has been considerable advance in our understanding of gene regulation in eukaryotes. The present picture envisions the combinatorial participation of regulatory elements in enhancer and promoters regions of DNA that act as targets for regulatory proteins and transcription factors. This picture often ignores the fact that the natural template for the transcriptional machinery is not free DNA but chromatin, a highly ordered superstructure composed of equal amounts of DNA and structural proteins. The organisation of DNA in chromatin introduces important constrains in terms of accessibility and topology. There is a growing appreciation that mechanism exists in the nucleus which deal with these constrains and adapt the chromatin structure to the requirements of the transcriptional process. The very efficiency of these mechanisms may explain the fact that they have not been recognised earlier. However, modern genetic and molecular approaches are beginning to unravel the complexity of the cellular machinery which deals with chromatin dynamics.

The complexity of the various families of transcription factors, the relatively poor definition and partial overlapping of their corresponding binding sites on DNA, and the multiplicity of arrays of these sites in various enhancers and promoters, has given rise to the notion that the interactions among transcription factors are promiscuous and permissive, and that, in principle, anything goes. However, this impression may be a consequence of the use of relatively unphysiological assays (transient transfections, overexpression of transactivators, *in vitro* DNA binding methods). As more and more systems are analysed in terms of their *in situ* chromatin structure, it is becoming progressively apparent that a full understanding of the interactions taking place during transcriptional control in eukaryotic cells will only be accomplished when the organisation of regulatory DNA elements in chromatin is taking into account. It is likely that the structural organisation of DNA in chromatin sets the stage for directing the three dimensional array of factors which enables the multiplicity of protein-protein interactions required for regulated gene expression.

A Workshop on the same topic was sponsored by the Fundación Juan March in 1990 and was considered successful by most participants. We believe that recent progress in the field, in particular the new technical advancements in genomic footprinting, chromatin assembly and cell-free transcription, as well as the accumulating genetic evidence from *Drosophila* and yeast, justified a second workshop four years later. Therefore, we thought that is was timely to organise a workshop focusing on the more functional aspects of chromatin structure in relation to gene expression.

The aim of the Workshop was to present recent findings and discuss novel mechanisms in order to define the state of our present knowledge as a starting point for future research. We were fortunate that the leading specialists in the fields of structure, dynamics, biochemistry and genetics of chromatin accepted our invitation to come to Madrid and participate in an interdisciplinary discussion. In addition we were lucky to have received numerous applications for participation in the Workshop, out of which we could only select 23, due to space limitations. We want to thank the invited speakers and the participants for coming to Madrid and for their excellent contributions to the success of the meeting. We want also to apologise to those experts in the chromatin field who were not invite due to limitations of budget and space. The small size of this type of meetings, which is the secret of their success, is also a source of frustration for the organisers, since it forces them to leave out many excellent experts. Finally we want to thank the Fundación Juan March, and in particular Andrés González, for their highly professional organisation, for their hospitality, and for the friendly treatment given to all participants during their stay in Madrid.

I. NUCLEOSOME STRUCTURE AND PHASING; HIGHER ORDER STRUCTURE

Nucleosome positioning, Nucleosome dynamics and transcription through nucleosome

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Fully defined systems based on Simpson's tandemly repeated nucleosome positioning sequence $(207)_{18}$ have been used to study nucleosome positioning, nucleosome dynamics and transcription through nucleosome. It has been shown that nucleosome cores position themselves on a major site flanked by minor sites ±10 bp, ±20 bp. At low ionic strengths we observed a temperature - dependent redistribution of histone octamers between this cluster of positions, all of which have the same rotational setting of the DNA duplex. This dynamic behavior of nucleosome cores was suppressed by the addition of histone H1.

A transcription template was constructed in which $(207)_{18}$ was inserted between the T7 and SP6 transcription promoters of pGEM-37. It was found that nucleosome cores partially inhibit transcription by T7 RNA polymerase. Whereas complete transcripts were produced with high efficiency from short nucleosome templates, the production of full-length transcripts from long nucleosomal arrays was relatively inefficient. Extensive cross-linking of the histone octamers had little effect on the transcriptional efficiency of nucleosomal arrays showing that the dissociation or splitting of the histone octamers was not an essential prerequisite for transcription through nucleosomes. However the addition of histone H1 significantly reduced transcriptional efficiency.

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MULTIPLE NUCLEOSOMES AND CHROMATIN STRUCTURES IN YEAST PROMOTERS

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We limit our experimental approach to the analysis of nucleosome localization(s) in yeast to the following aspects: i) the selection of the DNA sequences onto which the nucleosome core particles nucleate; ii) the stability of the interaction; the role of the rotational and of the translational information of the DNA sequence for both i and ii.

These aspects can be meaningfully studied in vivo taking advantage of two techniques recently developped by our group: permeabilization of living yeast cells to macromolecules by nystatin (1); nucleotide-level mapping of the in vivo position(s) of nucleosomes (2).

We have analyzed the in vivo organization of chromatin in the H. polymorpha MOX promoter, one of the strongest regulatory RNA polII promoters known. The results show that the 4 nucleosomes analyzed localize in alternative positions along a unique rotational phase and that the linker regions can be occupied by alternative nucleosomes.

This so far unreported in vivo organization implies the facilitated movement of nucleosomes among quasi-isoenergetic proximal locations, has deep implications for our vision of the kinetics of transcription in chromatin (i.e., for understanding the structural and thermodynamic rules that govern nucleosomal movements and their interaction with moving RNA polymerase II), substantiates the notion that DNA rotational information is the major determinant of nucleosome positioning, offers a quantitative picture of the conformational flexibility in the organization of eukariotic promoters.

Data have been obtained in the same methodological frame on in vivo nucleosomes on the DNA topoisomerase I gene promoter, on the Alcohol dehydrogenase II promoter, on ARS 1, on the 5S gene.

The rotational information has been altered by insertion of ½ helical period and the consequences on nucleosome distribution have been analyzed both in vivo and in vitro. The results confirm the relevance of the rotational information and teach how to modify nucleosome positions in vivo.

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Nucleosome structure in yeast and the fate of nucleosomes during transcription.

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Whether nucleosomes unfold or dissociate during transcription is controversal and might be gene specific or depend on the RNA-polymerase, the transcription rate, the local tortional stress, the DNA-sequence, or on the chromosomal location. To study this topic, we have constructed artificial yeast genes in which the same sequence was transcribed by the RNA-polymerase-I (POLI-URARIB) in the ribosomal locus or by RNA-polymerase-II (GAL-URARIB) in the leu2-locus. In GAL-URARIB, nucleosomes were maintained to a large extent, but positions were rearranged during pol-II transcription. Regeneration of the inactive structure after glucose repression occurred rapidly presumably without histone synthesis. The data are consistent with a local dissociation of nucleosomes at the site of the polymerase II followed by a rapid reassembly behind it (1). Preliminary results with toposisomerase I and topoisomerase II mutant strains show that lacking topoisomerase I or II activities do not promote nucleosome loss during transcription, nor do they prevent regeneration of the inactive structure after repression. When the same sequence was transcribed by RNA-polymerase I, nucleosomes were lost as in the transcribed ribosomal RNA genes. Hence, loss of nucleosomes was not sequence dependent. We favor a hypothesis that heavy transcription of many closely spaced genes leads to a local depletion of histones and prevents efficient reassembly of nucleosomes behind the polymerase.

While the structure of isolated or reconstituted nucleosome cores is known in great detail, nucleosomes in their chromosomal context might have altered properties. To address this topic, the chromatin structures of the genomic URA3 gene and of a whole minichromosome (YRpTRURAP) which contains the URA3 gene and the ARS1 origin of replication, were mapped by nuclease digestions at nucleotide resolution. The structures of the chromosomal and extrachromosomal URA3 gene were nearly identical confirming that the URA3 gene behaves as a structural and functional unit. Properties of the URA3 and ARS1 nucleosomes will be discussed.

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STRUCTURE AND PROPERTIES OF d(GA·TC)$_n$ DNA SEQUENCES.

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Alternating d(GA·TC)$_n$ DNA sequences are very abundant in eukaryotic genomic DNA. They are frequently found at the regulatory regions of many eukaryotic genes and a role in transcriptional regulation has been demonstrated in some cases. This type of DNA sequences show a remarkable degree of structural polymorphism. Depending on the precise environmental conditions they can exist under a vareity of different structural conformations which include triple-stranded and hairpin forms. In particular, d(GA·TC)$_n$ sequences form an intramolecular pu·pu·py triplex in the presence of some transition metal-ions (Zn, Co, Cd, Mn). Increasing the metal-ion concentration results in the formation of a pu·pu hairpin. This pu·pu hairpin is stabilized by the formation of G·A pairs. The structural details of these conformations will be discussed. We will also address the question as to what extent the structural properties of these sequences are biologically relevant. It is known that, at least in *Drosophila*, a nuclear protein exist -the GAGA factor- which specifically recognize double-stranded d(GA·TC)$_n$ sequences. In addition, we have partially purified from mouse cells, a nuclear protein of about 65 Kd which recognizes single-stranded d(TC)$_n$ sequences but not single-stranded d(GA)$_n$ sequences. This protein shows also a strong affinity for single-stranded d(T)$_n$ and, though to a much lesser extent, for single-stranded d(C)$_n$ and d(A)$_n$ sequences. The possible contribution of these proteins to the induction and stabillization of the different structural conformers of d(GA·TC)$_n$ sequences will also be discussed.

Chromatin Structure, DNA Structure and Regulated Expression of *Drosophila hsp26* in Different Chromosomal Environments.

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Our goal is to understand the contribution to gene regulation made by the organization of the 100Å chromatin fiber - the nucleosome array - and the contribution made by higher order packaging. We have analyzed the inducible activity and structural features of the *Drosophila hsp26* gene, examining both the sequence determinants of *hsp26* packaging in a euchromatic environment, and alterations in *hsp26* packaging in a heterochromatic environment. The *hsp26* promoter region includes two DNase I hypersensitive sites (DH sites), which encompass the heat shock regulatory elements (HSEs) and the TATA box; these DH sites are separated by a precisely positioned nucleosome (1). Analysis of a large number of *hsp26* transgenes with alterations (deletions, rearrangements, and point mutations) in the 5' regulatory region indicates that (a) formation of the appropriate DH sites is necessary but not sufficient for gene expression, and that (b) the $(CT)_n$ elements play a major role in establishing this chromatin structure, apparently through binding of the GAGA factor (2). The heat shock elements, while essential for induction of transcription, play only a minor role in establishing the chromatin structure. Similarly, a mutation in the TATA box that essentially eliminates inducible expression has only a minor effect on chromatin structure (3). The proximal $(CT)_n$ element includes both $(CT)_{3.5}$ and an inverted repeat of $(CT)_{4.5}$ that can shift to H-form DNA under conditions of supercoiling and acid pH. To test the significance of the potential H-form DNA, a number of transgenes with substitutions in this region have been analyzed *in vivo*. Loss of the inverted repeat has little effect on *hsp26* activity, as does its replacement with a potential Z-form DNA sequence. Replacement with an $(CCTTT)_n$ inverted repeat decreases activity. The results indicate that the potential H-form DNA does not play a major role in *hsp26* regulation (4).

When a P-element construct containing a marked copy of *hsp26* and a *white* gene as a visible marker is mobilized in the *Drosophila* genome, flies recovered showing position effect variegation (PEV) of the eye color marker invariably have P-element inserts in/near the centromeres, along the fourth chromosome, or at/near the telomeres (5). These regions are associated with heterochromatin protein 1 (HP1) (6); mutations in HP1 result in suppression of PEV (7). Some mutant alleles of HP1 suppress variegation of the transgenes in the chromocenter and fourth chromosome, but none suppress variegation of transgenes at the telomeres (5). *Su-var* $(2)1^{01}$, a mutation associated with increased histone acetylation, also suppresses variegation of transgenes at the chromocenter and along the fourth chromosome (8). The *hsp26* transgenes in variegating sites show reduced accessibility to the HSEs as measured by restriction enzyme digestion; those at the chromocenter/fourth chromosome, but not those at the telomeres show reduced expression on heat shock. Those *hsp26* transgenes studied in greater detail showed a normal nucleosome array across the uninduced gene. The evidence points to altered packaging as a means of gene inactivation at the chromocenter/fourth chromosome, but suggests a different source of variegation at the telomeres (5). Double immunofluorescence staining of the polytene chromosomes shows that HP1 and GAGA factor have very different distribution patterns, with few areas of overlap. This suggests a partitioning of the eukaryotic genome into active and inactive packaging formats. The observation of suppressors and enhancers of PEV that have effects based on gene dosage suggests a competition. This competition may involve two type of proteins, both those that interact directly with DNA, such as GAGA factor, and those that do not bind DNA but presumably act through multiprotein complexes, such as HP1.

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CHROMATIN STRUCTURE AND GLOBIN GENE EXPRESSION. G. Felsenfeld, E. Bresnick, J. Boyes, J. Chung, D. Clark and V. Studitsky. Laboratory of Molecular Biology, NIDDK, National Institutes of Health, Bethesda, MD 20892-0540 USA.

We have used the globin gene family in chicken erythroid cells as a model system in which to examine regulation of gene expression at the levels of individual genes and gene clusters, in order to determine the roles of both local trans-acting factors and long range chromatin structural elements. Far upstream of the chicken β-globin locus are several nuclease hypersensitive sites, some of which are involved in locus control activity. However the HS at the 5' end of this group (HS4) has unusual properties, which led us to investigate its possible role as a boundary element to the transcriptionally active domain. To approach this problem we studied the ability of the DNA sequence containing HS4 to block enhancer/LCR - promoter interactions. We found (1) that when 1.2 kb fragments containing this sequence were placed on either side of an LCR element in tests constructions, and stably integrated into a human erythroid cell line, stimulation of a test gene by the LCR element was inhibited. Insulating properties were also observed in *Drosophila* transformed with *white* gene constructs flanked by copies of the chicken element. Our recent work has extended these observations and identified other potential insulator/boundary elements.

We are also interested in the problem of how an RNA polymerase molecule can transcribe a DNA template within the eukaryotic nucleus, since that DNA is packaged as chromatin and might be expected to be inaccessible for transcription. In earlier work (2) we established systems with which to investigate this problem, and we were led to two possible models: in one, the octamer was displaced into solution and then recaptured on the same DNA molecule, and in the other it was transferred to another part of the plasmid by collision with naked DNA, without ever being released from the template. New experiments now allow us to distinguish between these models (3). Our data strongly support the internal transfer model, and have led us to new experiments that give more precise information about the transfer mechanism.

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BINDING OF CHICKEN HISTONE H1 TO DNA IS INDIFFERENT TO METHYLATION AT CpG SEQUENCES

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The major DNA modification in vertebrates is methylation of cytosines in the dinucleotide CpG. CpG methylation often causes repression of transcription (1,2). Methylation of CpG can inhibit binding of some transcription factors (3). Some evidence strongly suggests that repression is mediated by proteins which bind to DNA containing methyl CpG (2). Methylation-mediated repression could arise from alteration of chromatin structure due to the presence of methyl CpG. So, transfection experiments have shown that preferential repression of methylated templates requires their interaction with nuclear components and assembly of chromatin (1).

Although these results could be explained by known methyl CpG-binding proteins, it is possible that an ubiquitous component of chromatin interacts differentially with methylated DNA. The linker histone H1 is an attractive candidate for mediator of the effects of methylation on chromatin. H1 intervenes in chromatin condensation (4). It is depleted in CpG islands, which are non methylated and contain the promoters of many genes (5), is concentrated in inert chromatin (4), and inhibits transcription in vitro (6). If H1 were able to recognize methylated DNA, it might contribute to the effects of methylation on chromatin structure and gene expression. Methylated CpGs are preferentially located in nucleosomes containing H1 (7). Jost and col. described MBDP-2, a 40 KDa protein with preference for methylated DNA sequences from the vitellogenin gene. MDBP-2 was later reported to share sequences with H1 (8). However, there are discrepancies as to whether H1 has higher affinity for methylated DNA (9,10). Levine et al. observed that transcription of methylated templates was inhibited by H1 at a lower H1/DNA ratio (10), but this result might be explained by the effect of methylation on promoter strength (11).

We have studied the interaction of chicken histone H1 with methylated and non methylated DNA using various assays. H1 was a gift from J. Thomas (12). When gel retardation assays were performed, the behaviour of methylated and non methylated probes was identical. This was true for probes with different densities of methyl-CpG, including the vitellogenin gene promoter used by Jost and col. (8). Filter binding assays with H1 and labelled plasmid DNA in the presence of increasing amounts of competitor DNA showed that methylated DNA was only about 20% more effective as

competitor than non methylated DNA. Southwestern assays with H1 bound to nitrocellulose membranes showed identical signals for methylated and non methylated probes, both being equally displaced by competitor *E. coli* DNA. These results indicated that H1 has no significant preference for binding to methylated DNA.

On the other hand, Higurashi and Cole (9) have suggested that the DNA complexed with H1 adopts a distinct conformation when it is methylated. They observed that in the complexes only methylated MspI sites (CmCGG) became protected against nuclease cleavage. Nevertheless, we have observed that this apparent protective effect of H1 does not extend to other methylated CpGs. Indeed, H1 does not preferentially protects TCGA sites against TaqI digestion when these sites are methylated. Furthermore, MspI shows a slower digestion rate of methylated sites even on naked DNA. Therefore, the protection of methylated MspI sites in H1-DNA complexes can be explained without invoking differential interactions between H1 and methylated versus non methylated DNAs.

We conclude that H1 is unlikely to be a primary mediator of the biological effects of DNA methylation. This role could be accomplished by methyl CpG-binding proteins such as MeCP1 and MeCP2 (2,13). Expression of the PGK1 gene can be inhibited by methylation in extracts and cell lines containing MeCP1. On the contrary, methylated genes are not efficiently repressed in cell lines which lack MeCPs - even though they contain H1 (2).

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K.E. van Holde: "New Insights Into the Higher Order Structure of Chromatin"

The higher order structure of the chromatin fibre has long been a subject of debate and hypothesis. Definitive evidence has been difficult to obtain due to the inadequacies of experimental methods. Using the new technique of scanning force microscopy (SFM) we have been able to investigate fibers under much milder conditions than heretofore. We conclude that at low ionic strength the fiber exists as an irregular helix of ca. 30 nm diameter.[1] This structure can be predicted as a consequence of linker stiffness and length heterogeneity, if the lysine-rich histones fix linker angles.[2] Evidence for the latter hypothesis comes from studies of the interaction of linker histones with crossed-DNA.[3,4] Removal of these histones leads to a relaxation of this constraint and collapse of the pseudo helix.[2]

On the basis of this model, we predict that the condensed 30 nm fibre obtained at high salt has *no* regular structure, and that elaborate models for this structure are unjustified.

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SEQUENCE-DIRECTED NUCLEOSOME POSITIONING ON THE CHICKEN β^A-GLOBIN GENE REFLECTS CHROMATIN STRUCTURE

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Positioned nucleosomes contribute to both the structure and function of the chromatin fibre and play a decisive role in controlling gene expression[1-3]. A factor which determines nucleosome positioning is the DNA sequence itself[3-5]. Using a recently developed *in vitro* technique[6], we have mapped core histone octamer binding sites over a 4.3 kb stretch of DNA containing the entire chicken adult β-globin gene and its enhancer.

A plasmid containing the chicken gene, its enhancer and flanking sequence was reconstituted with core histones and digested with micrococcal nuclease to produce a population of core particle DNAs. These were mapped by monomer extension[6]. Briefly, the DNAs were 5' end-labelled and used as primers for extension on any one of a variety of single-stranded phagemids containing overlapping sections of the 4.3 kb globin fragment. Extension was carried out in the presence of a restriction enzyme which cleaves the nascent double-stranded DNA at a unique location to produce fragments whose lengths reveal the distance from the restriction site to boundaries of the core particle DNAs. Each restriction band thus defines a location at which a core histone octamer was bound to the 4.3 kb globin DNA sequence. Fragment sizing and quantitative densitometry yielded a binding site map for the whole region.

In our analysis, the relative intensity of an extension product is determined by the abundance of a particular binding site in the core particle DNA population, which we have argued reflects the relative affinity of the core histone octamer for that sequence[6]. Our data indicate the range of affinities to be greater than 2 orders of magnitude.

The strongest octamer binding sites are located 5' and 3' to the β-globin gene and in its large second intron but are notably absent from the coding regions. These strong binding sites display a notable periodicity along the globin DNA, a feature which is confirmed by auto-correlation analysis. 5' of the gene, binding sites are located approximately every 200 bp, a periodicity repeated, in phase, within the second intron and towards the 3' end of the gene. A 200 bp periodicity is close to the 212 bp repeat length of inactive chicken erythrocyte chromatin, suggesting that these strong binding sites may contribute towards folding the chicken β-globin gene into an inactive higher order chromatin structure. Because sequence within the second intron of the gene constitutes part of the phased repeating pattern, this would indicate a function for these transcribed sequences in determining chromatin structure.

Our analysis also reveals a number of short range periodicities relating overlapping, alternative core histone binding sites, the most notable being 20 and 40 bp. Although these short range periodicities are multiples of approximately 10 bp, a 10 bp periodicity *per se* is infrequent. 20 and 40 bp periodicities could suggest that particular aspects of core particle structure, possibly the sites at which the DNA is distorted, are features particularly demanding of DNA sequence in relation to precise translational positioning.

The manner in which DNA is packaged into a higher order chromatin fibre is likely to be sensitive to the spatial distribution of nucleosomes along the length of the nucleic acid. The precise structure of the higher order fibre in specific regions of the genome will, therefore, at least in part be a reflection of the underlying DNA sequence. Given that coding sequence has other priorities, the capacity to direct the precise packaging of DNA, and consequently

influence its functional capacity, would need to reside within sequences which flank and interrupt genes.

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CHROMOSOMAL PROTEINS HMG-14/-17 ENHANCE THE TRANSCRIPTIONAL POTENTIAL OF NASCENT CHROMATIN THROUGH THEIR C-TERMINAL DOMAIN.

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Chromosomal proteins HMG-14 and HMG-17 bind specifically to nucleosome core particles. Assembly of these proteins into nascent nucleosomes during, but not after, chromatin assembly on replicating DNA enhances the transcriptional potential of the resulting chromatin template. We have used various peptides and recombinant mutant protein to define the transcription activation domain of the HMG-14/-17 proteins. Mutant proteins whose binding to core particles is impaired, fail to enhance the transcriptional potential of chromatin. Mutants with intact nucleosome binding domains, but mutated C-terminal domains, bind to nucleosomes but fail to enhance the transcriptional potential of chromatin. Synthetic peptides containing the entire nucleosomal binding domain of either HMG-14 or HMG-17 bind to nucleosome cores. In the presence of these peptides the HMG-14/-17 proteins fail to enhance the transcriptional potential of chromatin. These results suggest that these peptides can act as independent functional modules and compete with the intact protein for binding to chromatin. In addition, the results suggest that chromosomal proteins HMG-14/-17 enhance the transcriptional potential of nascent chromatin through their C-terminal domain. The amino acid residues necessary for binding to nucleosomes and for transcriptional activation were mapped with peptides and proteins containing deletion and point mutations. The results lead to the following conclusions: 1) The enhancement of the transcriptional potential of chromatin by HMG-14/17 depends on proper binding of the protein to its target. 2) The binding of the protein to chromatin can be disrupted by single point mutations suggesting stringent structural and ionic requirement for proper binding. This mode of binding may be readily disrupted by regulatory factors. 3) The nucleosomal binding function and the transcriptional activation function of the protein are located in distinguishable domains 4)The transcriptional activation domain is located in the C-terminal region of the proteins, which has a net negative charge. 5) The nucleosomal binding domain can act as an independent functional module but the transcription activation domain cannot.

Thus, the HMG-14/-17 proteins are modular proteins built of distinct functional motifs. Therefore, in chromatin these proteins may participate in more than one interaction.

Is there a Locus Control Region in the mouse tyrosinase gene?. An experimental approach using yeast artificial chromosomes and transgenic mice.

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This past year a number of groups, including our laboratory, have succeeded in generating transgenic mice with yeast artificial chromosomes (YACs). We were able to microinject a 250 kb YAC spanning the mouse tyrosinase locus into mouse fertilized oocytes from an albino strain. The transgenic mice were phenotipically undistinguishable from pigmented wild type mice. Moreover, the expression of the YAC-borne transgene was found to be copy number dependent and position independent, features which have been associated with the existence of a Locus or Dominant Control Region in a gene (1). This faithful expression of the tyrosinase transgene was not observed before when artificial minigenes, including only 5 kb of promoter and 5´-upstream sequences, were used for the generation of transgenic mice. A new DNaseI hypersensitive site (HS) has been identified at -12 kb upstream of the tyrosinase gene. An artificial minigene, including this HS, has been used for the generation of transgenic mice. The expression of the transgene was also found to be copy number related (2), suggesting an important role for this HS. In order to test the *in vivo* relevance of this HS in the regulation of the mouse tyrosinase gene we have constructed a series of deletions, in yeast, based in the original 250 kb YAC. This YAC contained 150 kb of 5´upstream sequences. Three different YACs have been created: a first one extends up to the HS and leaves it intact, a second one carries a big deletion which includes the HS site and, finally, a third one which represents the 250 kb YAC without a functional HS, deleted by homologous recombination in yeast. These YACs are being microinjected at present. We expect that the analysis of these transgenic mice will offer some light on the role of this HS as a major cis-regulator sequence in the mouse tyrosinase gene.

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In vitro reconstitution of structural and dynamic aspects of chromatin

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We use a chromatin reconstitution system derived from *Drosophila* embryos (1) to investigate structural and dynamic aspects of chromatin in vitro. With this system we demonstrate ATP-dependent mobility of nucleosomes and are currently characterizing the underlying mechanisms for this flexibility.

We reconstituted the chromatin structure of the Drosophila *hsp26* promoter. Through the combined action of components endogenous to the reconstitution extract and the *GAGA*-factor we obtain a structure that resembles the one described by Thomas and Elgin for the in vivo situation (2): A localised nucleosome flanked by bound *GAGA*-factors, with proximal and distal heat shock elements (HSEs) being fully accessible to the heat shock factor. The reconstitution is facilitated by ATP hydrolysis analogous to the one described for the *hsp70* promoter (3). A localised nucleosome within a promoter might organize the DNA in a way to facilitate interaction between distally bound activators and factors at the start site of transcription. Current studies focus on the functional implications of this structure and the mechanisms responsible for nucleosome positioning.

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II. LINKER HISTONES AND HMGs

HISTONE MODULATED TRANSCRIPTION: STRUCTURAL AND DEVELOPMENTAL ASPECTS, Alan P. Wolffe, Geneviève Almouzni, Philippe Bouvet, Stefan Dimitrov, Jeffrey J. Hayes, Nicoletta Landsberger, Dmitry Pruss, and Kiyoe Ura, Laboratory of Molecular Embryology, National Institute of Child Health and Human Development, NIH, Bethesda, MD. 20892.

A chromatin environment provides many advantages to the eukaryotic transcriptional machinery in regulating gene expression. Nucleosome formation can alternately either repress (1) or potentiate transcription (2). The three dimensional folding of DNA in chromatin has an important structural and regulatory role. We have characterized the influence of individual histones and their domains on nucleosome structure, nucleosome positioning and the capacity of nucleosomes to repress transcription (3-7). These in vitro experiments have been extended to chromatin function in vivo. Replication coupled chromatin assembly is required for the general repression of basal transcription in Xenopus oocyte nuclei (8). In spite of complete chromatin assembly, certain transcription factors (eg GAL4/VP16, HSF) activate transcription from the initially repressed state. These transcription factors have two functions: the relief of repression (chromatin disruption) and the activation of the transcription process (recruitment of the basal transcription machinery). We have examined the relative importance of these two functions during vertebrate development as the structural components of chromosomes are modified. These modifications include the replacement of linker histones eg B4, H1, H1° and alterations in histone acetylation (9-12). Some of the consequences of removal or overexpression of the linker histones for gene expression in the developing embryo have been determined.

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DIFFERENT POST-SYNTHETIC CHEMICAL MODIFICATIONS SWITCH THE ROLE OF IBR/IBF FROM ACTIVATOR TO REPRESSOR OF HISTONE *H5* GENE TRANSCRIPTION

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Mature and immature chicken erythrocytes contain a glycosylated protein IBR (*i*nitiation *b*inding *r*epressor) that represses transcription of the *H5* histone gene by binding to sequences spanning the transcription start site. Although IBR was not detected in other tissues or species, a non-glycosylated factor (IBF, *i*nitiation *b*inding *f*actor) that recognizes the same sequence was found in a variety of cells from different vertebrate species, including erythrocyte precursors. The sequence of a cDNA clone revealed that IBR is the chicken homologue of the recently cloned human NRF1 [nuclear respiratory factor 1, 99.4% identity] and highly homologous to sea urchin P3A2 and Drosophila EWG proteins. Antibody reactivity, the sequence of a partial cDNA clone, peptide mapping, northern and southern analysis, and binding site selection, strongly suggest that IBR and IBF are differently modified isoforms. In vivo and in vitro assays indicated that IBF increases the rate of *H5* gene transcription, suggesting that unique modifications of IBR and IBF are responsible for their opposite transcriptional effects. Although the role of IBR/IBF phosphorylation in transcription has not yet been evaluated, we have demonstrated that casein kinase II efficiently phosphorylates the recombinant protein at one or several putative sites located in the N-terminal region. IBR and IBF form dimeric complexes in solution and bind as such to full or half cognate sites, suggesting that the modifications do not affect complex formation.

The HMG boxes of HMG1: structure and interaction with DNA

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HMG1 is an abundant chromosomal protein which binds to DNA with no apparent sequence-specificity. It has been implicated in a broad range of functions in which it may play an architectural role. It has three domains: two homologous DNA-binding domains now termed "HMG boxes" and an acidic C-terminal tail containing ~ 30 contiguous aspartic or glutamic acid residues. Removal of the acidic domain of HMG1 shows that the AB didomains are able to manipulate DNA structure by looping, compaction and changes in DNA topology, and that the acidic tail down-regulates these effects by modulation of the DNA-binding properties (Stros *et al.*, 1994).

The HMG-box sequence motif was first identified in the RNA polymerase I transcription factor, UBF, and has since been identified in several relatively abundant proteins that bind DNA with little or no sequence specificity, as well as in sequence-specific transcription factors. All HMG box proteins so far tested bind to four-way junctions and bend DNA. In many cases these properties are retained by the isolated boxes. The HMG-box thus emerges as a common structural motif which recognises DNA distortions and which may have additional sequence preferences present to varying degrees in different HMG box proteins (for a review see Grosschedl *et al.*, 1994).

The structure of the HMG-box in the B-domain of HMG1 has been determined by NMR spectroscopy (Weir *et al.*, 1993; Read *et al.*, 1993) and the A-domain is currently under study. The HMG box is a novel DNA-binding motif with a distinctive L-shape. The two arms, at an angle of about 80°, enclose a concave surface which is implicated in DNA-binding. An important determinant of the structure of the molecule is a close-packed cluster of aromatic and hydrophobic residues in the 'joint' between the two arms, and the most conspicuous feature of this is a highly conserved tryptophan residue. Recent results for a tryptophan mutant of the A-domain have provided insights into aspects of structure-specific recognition by the HMG-box.

In proteins with multiple HMG boxes, are the individual boxes functionally distinct? Various reports suggest that the tryptically generated A and B boxes of HMG1 might have different properties, but some of the results were difficult to evaluate since the proteolytic fragments were not precisely defined. We have therefore made a systematic study of the DNA-binding properties of the recombinant A and B domains of HMG1, and of an HMG box that binds sequence-specifically, and compared their ability to bind to DNA and to distort double-stranded DNA. Subtle differences suggest that the highly versatile HMG-box has been adapted to a variety of different functions; multiple HMG-boxes may thus confer a diversity of DNA-binding features in a single protein.

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Structure and function of HMG-D

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HMG-D is one of two Drosophila homologues of the vertebrate HMG 1/2 class of proteins (Wagner et al., 1992; Ner et al., 1993). HMG-D contains a single HMG box domain which is followed by a stretch rich in alanine and lysine residues and a short acidic tail. The global fold of the HMG domain is essentially identical to that of rat HMG1 box 2 (Weir et al., 1993; Jones et al., 1994).

Unlike its mammalian counterparts HMG-D binds selectively to a particular flexible DNA sequence, of which examples are located within the scaffold associated region (SAR) of the *fushi tarazu* pair-rule gene. The region of principal contacts with the DNA has been determined by NMR spectroscopy. When binding to a single site the protein exchanges rapidly with the DNA. However, the binding of HMG-D to DNA is cooperative resulting in distortion of the double helix between as well as within its binding sites.

HMG-D is particularly abundant in the Drosophila embryo and is associated with the mitotic chromosmes at a time when no somatic histone H1 is present (Ner and Travers, 1994). From nuclear cycle 7 in the developing embryo H1 gradually accumulates and the size of the mitotic chromosomes decreases. Concomitantly the ability to detect HMG-D in these chromosomes decreases. We have consequently suggested that HMG-D, either by itself or in concert with another protein(s), is required for the formation of more 'loosely' condensed form of chromatin whose function is to facilitate the rapid nuclear cycles characteristic of early embryogenesis.

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Chromosomal domains: SARs and Boundary Elements:

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The role of scaffold associated regions (SARs) in gene experession, chromosome condensation and chromosome banding phenomena will be briefly reviewed. The genome of eukaryotic chromosomes is thought to be organized into functional domains defined by so-called DNA boundary elements (BE). BE's are proposed to insulate one domain from the enhancer or repressor activities of flanking domains. The SCS and SCS' elements, which flank the Drosohila 87A7 hsp70 heat shock locus, were experimentally identified as BE's by Schedl's group.

To approach a molecular understanding of such elements, we initiated a search for SCS/SCS' binding proteins. A sequence-specific activity with a high affinity for a subfragment (named D) of SCS' was detected by band-shift assays and the target sequence identified by footprinting. This protein activity, called BEAF32 (Boundary Element Associated Factor 32 kD) was purified by DNA affinity chromotography using the DNA target. The amino acid sequence of a number of tryptic peptides of BEAF32 was determined and, using degenerate PCR primers, an 800 bp fragment from the 3' end of the cDNA was amplified and used to screen for a full length cDNA clone. This clone encodes a novel protein of 283 amino acids containing one atypical leucine zipper. The identity of the cloned protein with the purified activity derived from Drosophila was established by a number of criteria such as Western blot, bandshift and antibody supershift using both the cloned protein and Drosophila BEAF32. In situ hybridization to polytene chromosomes mapped the BEAF32 gene to band 51D1 (2R) and Southern blot analysis revealed a single copy for this gene.

The protein appears to bind DNA as a dimer, as established by cross-linking studies. Immuno-staining of embryos at different developmental stages identifies BEAF32 as an abundant and general nuclear protein, first detectable in blastoderm nuclei. In line with its proposed role, immuno-staining of polytene chromosomes localizes BEAF32 to most interbands and boundaries of developmental puffs as well as the SCS' side of the 87A7 heat shock puff.

BEAF32 clearly associates with the SCS' element in vivo as established by experiments using formaldehyde crosslinking and immunoprecipitation. Using an hsp27-CAT reporter gene and a variety of constructs, we confirmed the enhancer blocking activity of SCS' and extended this to its D subfragment after stable transformation. Insertion of these elements at -170 strongly block the distal ERE (ecdysone response element; -579 to -455) and to a lesser extent block the more proximal HSE (heat-shock element; -370 to -270) of the hsp27 promoter.

III. HISTONE MODIFICATIONS, CHROMATIN ASSEMBLY AND TRANSCRIPTION

Core Histone Acetylation Mapping At Active Gene Loci.

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The acetylation of lysine residues in the N-terminal tails of core histones has been shown to occur at high levels in the chromatin of both transcriptionally active and poised globin genes of chicken erythrocytes by immuno-precipitation of acetylated chromatin with an antibody specific to ε N-acetyl lysine [Hebbes et al., (1988) EMBO J. **7**, 1395; Hebbes et al., (1992) Nucleic Acids Res. **20**, 1017]. The distribution of core histone acetylation across the chicken β-globin locus has been mapped in 15 day chicken embryo erythrocytes by immuno-precipitation of mononucleosomes followed by hybridisation probing at several points across the locus. A continuum of acetylation was observed, covering both genes and intergenic regions. Using the same probes, the generalised sensitivity to DNase I was mapped by monitoring the disappearance of intact genomic restriction fragments from Southern transfers. Close correspondence between the 33 kb of DNase I sensitive chromatin and the extent of acetylation indicates that one role of the modification could be the generation and/or maintenance of the open conformation at this active tissue specific locus [Hebbes et al., (1994) EMBO J. **13**, 1823].

Using the same immuno-precipitation techniques with chromatin fragments derived from human K562 cells and cDNA gene probes, we have shown that house-keeping genes also carry acetylated histones but apparently at a lower level. Since most housekeeping genes are associated with CpG islands and moreover it has been shown that bulk 'island chromatin' is **very** highly acetylated [Tazi & Bird, (1990), Cell **60**, 909], the reduced acetylation was unexpected. A possible explanation is that the acetylation of housekeeping genes is concentrated within their CpG islands and cDNA probes show acetylation levels averaged across the whole gene. Some tissue specific genes also have CpG islands and it could be that high levels of acetylation are present at all islands, irrespective of transcriptional status. Using genomic fragments from the chicken thymidine kinase (TK) and protamine (P) genes (both of which have a CpG island) and 15 day erythrocytes as chromatin source, preliminary results indicate that there is just a moderate level of acetylation across most of the active TK gene but an **elevated** level within its CpG island. The inactive protamine gene is not observed to carry acetylated histones. These results suggest that the high level of histone acetylation at CpG islands is not a property of islands *per se* but related to the transcriptional competence of the gene. For housekeeping genes, the high levels of histone acetylation at their CpG islands seem not to be maintained along the length of the genes, in contrast to the uniform high levels of acetylation throughout active tissue specific gene loci such as globins.

Acetylated isoforms of histone H4 and functional chromatin domains

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Histone acetylation is a common and ubiquitous post-translational modification whose effects on chromatin structure and function remain unclear. One reason for this has been the difficulty of defining the functional consequences of modifying *individual* core histones and different lysine residues on a single histone molecule. There is a strong possibility *a priore* that these will be different. Certainly in the case of protein phosphorylation, a related post-translational modification, it seems that the effects are more often mediated by residue-specific modifications than by an overall shift in net charge. In order to test the functional significance of acetylation of a specific histone at defined lysine residues, we have prepared antisera that can distinguish isoforms of histone H4 acetylated at lysines 5, 8, 12 or 16 (the four residues acetylated *in vivo*). The extreme evolutionary conservation of H4 and its acetylatable lysines means that these antisera can be used across a wide variety of species and we have been able to use various approaches to study the association between specific acetylated isoforms of H4 and functional domains in chromatin. Immunofluorescence microscopy of polytene and metaphase chromosomes from the fruit fly Drosophila, has produced clear evidence for the significance of lysine-specific acetylation. H4 acetylated at lysine 16 (H4Ac16) is found almost exclusively at defined regions along the transcriptionally hyperactive male X chromosome while H4 in centric heterochromatin is underacetylated at all lysines except lysine 12 [1]. These patterns of acetylation are found in different cell types and marking of the male X with H4Ac16 persists through mitosis [2]. H4Ac16 may bring about increased transcription on the male X (dosage compensation) by interacting with a complex formed by the protein products of the four MSL genes [3]. In mammals, both immunofluorescence microscopy [4,5] and immunoprecipitation of chromatin fragments and DNA analysis have been used to show that H4 in both constitutive (centric and telomeric) and facultative (Xi) heterochromatin is usually underacetylated. Low levels of H4 acetylation may facilitate its interaction with proteins involved in heterochromatin formation. Conversely, coding DNA is associated with H4 having a moderate level of acetylation that is relatively unaffected by changes in transcriptional activity. While the details may differ from one species to another, our results are consistent with the general proposition that the acetylation of histone H4 serves as a relatively stable (though not immutable) marker of functionally different genomic domains. Recent evidence suggests that those changes which do occur are associated with longer-term shifts in genomic function during differentiation and development.

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BIOCHEMICAL ANALYSIS OF TRANSCRIPTIONAL REGULATION WITH RECONSTITUTED CHROMATIN TEMPLATES

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The control of gene expression is a problem of fundamental and far-reaching importance. Accordingly, a significant amount of effort has been devoted to the analysis of the regulation of transcription by RNA polymerase II. These studies have generally focussed upon the identification, purification, cloning, and mutational analysis of promoter- and enhancer-binding factors and components of the basal transcriptional machinery. Yet, while the characterization of the factors that are involved in the intrinsic transcription process is essential, it is also important to consider the structure and assembly of the chromatin template in the analysis of gene regulation. Biochemical studies of transcription by RNA polymerase II have shown that the use of reconstituted chromatin templates, which probably provide a better model for the structure of the native template than naked DNA, has led to the ability to recreate in vitro a variety of phenomena that are observed in vivo. Moreover, the data support a model for the mechanism of transcriptional activation by promoter- and enhancer-binding proteins in which these factors function both to relieve chromatin-mediated repression ("antirepression") and to facilitate the intrinsic transcription reaction ("true activation"). To investigate the mechanisms by which sequence-specific transcription factors are able to counteract chromatin-mediated repression, we have employed an S-190 extract from *Drosophila* embryos for the reconstitution of chromatin for subsequent transcriptional and structural analyses. Recent experiments in this area will be presented. We have also devoted effort toward elucidation of the mechanisms by which chromatin is assembled by either DNA replication-independent or DNA replication-coupled pathways. In these studies, we hope to gain a better understanding of the relationship amongst DNA replication, chromatin assembly, and transcription, which may lead to a more unified view of gene regulation in a broader context of activities that occur within the nucleus.

TRANSCRIPTION FACTOR / NUCLEOSOME INTERACTIONS IN VITRO: MULTIPLE MECHANISMS INDUCE FACTOR BINDING.

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Biochemical studies of the interactions of regulatory transcription factors with nucleosome cores have illustrated several parameters which restrict the binding of individual transcription factors to nucleosomal DNA. These include a differential intrinsic affinity of different factors for their recognition sites on nucleosomes, the location of the binding sites within the nucleosome core, and inhibition from the core histone amino termini. However, nucleosome-mediated repression of factor binding can be overcome by the cooperative binding of multiple factors, relief of inhibition from the core histone amino termini (i.e. by histone acetylation), and through the function of accessory protein complexes which stimulate factor binding by interacting with histones or nucleosomal DNA.

Cooperative binding.

Transcription factor binding to nucleosomes is inherently cooperative. Cooperative binding of GAL4-derivatives to nucleosomal DNA overcomes inhibition of binding from the core histone amino termini. This cooperativity is also observed with unrelated transcription factors. GAL4-derivatives, USF and NFkB in various combinations all bind to nucleosomes cooperatively. Thus, this mode of binding is a general feature and may play a role in the binding of multiple factors to complex regulatory elements in chromatin.

Histone acetylation.

Factor binding to nucleosomes is also enhanced by removal of the core histone amino termini resulting in a non-cooperative mode of binding similar to that observed on naked DNA. We have utilized antibodies to acetylated histones (kindly provided by B. Turner and C. Crane-Robinson) to perform immunoblots of factor bound and unbound nucleosome cores. These data demonstrate an enrichment of acetylated histones in the factor-bound population confirming the role of acetylation of the amino termini in inducing the binding of transcription factors to nucleosomes.

Histone chaperones.

The binding of transcription factors to nucleosomes can be induced by accessory protein complexes. For example the pentameric histone-binding protein, nucleoplasmin, stimulates nucleosome binding by GAL4, USF and SP1. Analysis of the proteins in ternary complexes formed upon the binding of 5 dimers of GAL4 to nucleosome cores in the presence of nucleoplasmin indicate a depletion of H2A/H2B. This suggest that nucleoplasmin increases the affinity of these factors by mediating the removal of H2A/H2B dimers from factor/nucleosome complexes. Consistent with this hypothesis, crosslinking of the histone octamer to prevent H2A/H2B dissociation abolishes stimulation of factor binding by nucleoplasmin.

The SWI/SNF complex.

The purified yeast SWI/SNF protein complex stimulates the binding of GAL4 derivatives to nucleosomal DNA. This stimulation is independent of transcription activation domains but does require ATP hydrolysis. Stimulation is abolished by a mutation in the SWI2 subunit which cripples the ATPase activity of the complex. The SWI/SNF complex can interact directly with nucleosomal DNA resulting in an ATP-dependent disruption of rotational orientation of DNA on the surface of the nucleosome core. The stimulatory activity of SWI/SNF is augmented by small concentrations of nucleoplasmin which also appears to facilitate release of SWI/SNF-nucleosome interactions. These data are consistent with a model whereby SWI/SNF interacts with transcription factors and nucleosomal DNA utilizing the energy of ATP hydrolysis to disrupt the underlying nucleosome. Displacement of H2A/H2B dimers from the disrupted nucleosomes would provide a permanent increase in the affinity of the transcription factor.

Conclusion.

The existence of multiple mechanisms to enhance transcription factor binding to nucleosomal DNA suggest that in vivo nucleosomes might be very susceptible to factor-induced disruption and displacement. These observations and the clear examples of factor-induced nucleosome disruption in vivo challenge previous notions that, once formed, nucleosomes present an insurmountable barrier to the transcription apparatus.

Template Topology Requirements for Transcription *in vivo*

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Several lines of evidence support the proposal that transient torsional stress is necessary for transcription to proceed *in vivo* (reviewed in Freeman and Garrard, 1992). First, the facts that transcription itself generates twin domains of DNA supercoiling (Giaever and Wang, 1988) and that topoisomerases are recruited to these regions (Fleischmann et al., 1984), suggests that relaxation must kinetically lag behind stress generation. Indeed, recent studies using psoralen cross-linking support the idea that transient torsional tension exists *in vivo* within the dihydrofolate reductase, ribosomal, and heat shock inducible genes (Ljungman and Hanawalt, 1992; Jupe et al., 1993). Second circular, but not linearized DNA templates are transcriptionally active *in vivo* (Harland et al., 1983; Pruit and Reeder, 1984; Ryoji and Worcel, 1984; Weintraub et al., 1986). Third, nicking genomes *in vivo* with X-rays leads to inhibition of transcription (Luchnick et al, 1986). Finally, adenovirus DNA molecules containing mutant terminal protein, which can no longer bind thightly to the nuclear matrix, are defective in transcription (Schaack et al., 1990).

A requirement for transient torsional stress in transcription could be exhibited at the level of initiation or elongation (or both). Several experiments support a role for negative DNA supercoiling in transcriptional initiation (Schultz et al., 1992; Pavin and Sharp, 1993; Dunaway and Ostrander, 1993; Usheva and Shenk, 1994; Goodrich and Tjian, 1994). Although earlier *in vitro* transcription experiments did not support the notion that topological anchorage may be necessary for RNA polymerase traversal through nucleosomal templates, more recent studies indicate that elongation through arrays of nucleosomes does not efficiently occur *in vitro*, and that nucleosomes are potent road blocks (Izban and Luse, 1991; reviewed in Freeman and Garrard, 1992). *In vivo*, however, nucleosome arrays provide no apparent barrier to RNA polymerase passage (Bjorkroth et al., 1988), consistent with a requirement for torsional stress which is missing from *in vitro* systems.

To further investigate in a definitive way whether transient torsional stress is required for transcription of chromatin templates *in vivo*, we have developed a model system in which yeast episomal template plasmids can be linearized at predefined positions. The technique uses yeast HO endonuclease a sequence-specific double-strand DNA nuclease involved in the yeast mating-type switching (Jensen and Hurkowitz, 1984), for targeting DNA cleavage (Sugawara and Haber, 1992). Our goal is to see if template linearization by HO endonuclease will abolish transcription from a reporter *lacZ* gene. We reasoned that if torsional stress is required for transcription, a double stranded break in the DNA template may permit dissipation of local torsional stress and cause inhibition of transcription of the reporter gene.

The experimental design is outlined as follows: There are two yeast *CEN*-based episomal plasmids used in this study. One carries a HO endonuclease gene under the control of yeast *GAL10* promoter. The other is the template plasmid containing a *CUP1-lacZ* fusion gene and an HO cut site at a preselected position. We first arrest yeast harboring those two plasmids at the G1 phase of the cell cycle and then induce synthesis of HO endonuclease by adding galactose. When most of the templates are linearized by HO endonuclease, copper ions are added to induce *lacZ* expression. The levels of *lacZ* transcript are measured by an RNase protection assay. The results from these template linearization experiments will be presented and discussed.

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IV. INDUCIBLE SYSTEMS

CHROMATIN STRUCTURE AND THE REGULATION OF THE *PHO5* PROMOTER

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We are investigating the regulation of the *PHO5* gene which encodes a strongly regulated acid phosphatase in *S. cerevisiae*. In these studies we are focussing on the contribution of histone-DNA interactions to gene regulation and the interplay between transcription factors and nucleosome structure (1,2).

The chromatin structure of the *PHO5* promoter undergoes a massive transition upon induction of the gene in the course of which four nucleosomes are disrupted. A trans-activating basic-helix-loop-helix protein, Pho4, is primarily responsible for the activation of *PHO5* and the chromatin transition. We are attempting to define domains of trans-activators which are capable of triggering such chromatin changes. In Pho4, the DNA binding domain by itself cannot interact with a binding site when it is contained in a nucleosome as shown by dimethyl sulfate *in vivo* footprinting experiments with truncated Pho4 variants. Binding to a nucleosomal site requires the simultaneous disruption of the nucleosome. The domain required for this function largely overlaps with the trans-activation domain. Along the same line, VP16 can also disrupt nucleosomal structure at the *PHO5* promoter when fused to the Pho4 DNA binding domain. We have, however, generated fusions with heterologous protein domains which can disrupt nucleosomes but give no trans-activation at all.

PHO5-LacZ fusion constructs were generated in which the MMTV B nucleosome was incorporated. These constructs were assayed for LacZ activity, and their chromatin organization was analyzed in yeast strains expressing the glucocorticoid receptor (3). Addition of glucocorticoid to the cells resulted in strong activation of the promoter with little detectable change, however, in the structure of the MMTV B nucleosome.

We have also investigated the role of several proteins of the Snf/Swi and Sin/Spt families (4) in the chromatin organization and the regulation of the *PHO5* promoter. These proteins were identified in yeast as pleiotropic regulators of transcription, and there is evidence that they exert their effects via chromatin. Our results demonstrate that some of these proteins are required for setting up a repressive chromatin structure at the *PHO5* promoter and that the disruption of the corresponding genes leads to partially constitutive *PHO5* expression.

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CHROMATIN ORGANISATION OF THE MMTV PROMOTER AND ITS CHANGES DURING HORMONAL INDUCTION

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Eukaryotic gene regulation is achieved by the combinatorial interaction of transcription factors orchestrated by the array of DNA *cis*-acting elements in enhancer and promoter regions. The compaction of the eukaryotic genome in nuclear chromatin influences the accessibility of these DNA sequences for recognition by DNA binding proteins. Restricted access is probably effected at each level of chromatin organisation. The wrapping of the DNA around the octamer of histones already hinders access to those sequences which are oriented with the relevant sites pointing toward the histones. The additional compaction due to the cooperative binding of linker histones is likely to have a more general effect upon accessibility of DNA sequences which are not exposed in the surface of the fibre. To study the contribution of chromatin organisation to DNA binding and gene expression, inducible systems are particularly well suited as they permit to correlate the influence of the inducer on gene expression and chromatin structure. In most cases induction is accompanied by changes in chromatin structure often manifested in a localised hypersensitivity to cleavage by DNase I (Elgin, 1988). We have chosen to study the structure and function of the MMTV promoter which in organised into phased nucleosomes, and becomes hypersensitive to DNaseI after hormone induction (Zaret and Yamamoto, 1984; Richard-Foy and Hager, 1987).

The MMTV proviral genome is repressed by unknown mechanism in the absence of steroid hormones, but its transcription is rapidly activated in response to glucocorticoids or progestins. The MMTV promoter contains a complex hormone responsive region (HRR) upstream of a binding site for the transcription factor nuclear factor I (NFI), two octamer motifs and the TATA box. In transiently transfected cells the HRR precludes access of NFI-VP16 to the MMTV promoter, probably due to its organisation in chromatin. This repression is relieved upon hormone induction. In cells carrying MMTV sequences either as minichromosomes or integrated in the chromosomal context, the HRR, the NFI site and the octamer distal motif are organized into a phased nucleosome (Truss et al., 1993). High resolution analysis of the nucleosomal DNA by genomic footprinting shows that its rotational setting in the living cell is identical to that reported for reconstituted nucleosomes (Piña et al., 1990). The orientation of the double helix on the surface of the histone octamer is compatible with binding of the hormone receptors to two of the four sites on the HRR, but would precludes binding of NFI and OTF1 (Piña et al., 1990). 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 and restriction enzymes (Truss et al., 1994). As demonstrated by genomic footprinting, this structural alteration of the nucleosome is accompanied by occupancy of the four receptors binding sites of the HRR, the NFI, and the octamer motifs, while the histone octamer remains in place. This means that all relevance factors are bound to the surface of the rearranged nucleosome, whereas they can not bind simultaneously to MMTV promoter in free DNA (Brüggemeier et al., 1990; Möws et al., 1994). Therefore, the array of the MMTV promoter sequences on a positioned nucleosome, not only keeps the promoter silent prior to hormone induction, but enables binding of a full complement of factors, which will not be possible on free DNA. The exact nature of the structural alteration in the regulatory nucleosome is presently unknown, but it is only induced by agonistic receptor ligands and is independent of transcription elongation. The possible participation of the SWI/SNF complex as well as the potential role played by the linker histones and by modifications of the core histones in hormone induced chromatin remodelling are discussed in connection with studies on MMTV promoter expression in yeast.

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DEVELOPMENTAL STUDIES ON CHROMATIN ASSEMBLY AND REPRESSION OF TRANSCRIPTION

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The interaction of basal general factors and regulatory proteins with DNA together with chromatin organization are required to control the level of transcription by RNA Polymerase II.

To investigate these issues, Xenopus oocytes have been used to generate chromatin templates *in vivo* . Two chromatin assembly pathways can be reproduced in vivo, independent or dependent on DNA synthesis. As seen with in vitro systems the later one appears more efficient. We have analyzed transcription from different Polymerase II promoters under these circumstances. We find that chromatin assembly coupled to DNA synthesis is repressive for basal transcription. However, activation of transcription can be obtained regardless of the assembly pathways with Gal4 VP16 on a minimal reporter construct harboring Gal4 binding sites. The timing of requirement for Gal4 VP16 demonstrates that this activator is dominant over chromatin. This observation excludes a disruptive involvement of DNA replication in the process of activation. We propose that the replication coupled chromatin assembly may represent a general mechanism to direct repression of basal transcription.

In the embryo we made use of à similar approach to examine which parameters are important for the absence of transcription during the early development of Xenopus laevis. We find that it is possible to bypass this negative control on transcription by two means. The first one makes use of DNA amounts similar to those found at the time of zygotic activation together with additional recombinant basal transcription factor (TBP). The second one needs a simple addition of the activator Gal4/VP16. We will discuss these results in a developmental context.

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A HUMAN HOMOLOG OF *S. CEREVISIAE SNF2/SWI2* AND *BRM* GENES CO-OPERATES WITH NUCLEAR HORMONE RECEPTORS IN TRANSCRIPTIONAL ACTIVATION.

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Genetic studies in Saccharomyces cerevisiae suggest that particular nuclear factors function by interacting with chromatin and may form a new class of proteins assisting some transcriptional activators to overcome nucleosome repression at the level of the promoter. At least one of these factors, SNF2/SWI2, is conserved in *Drosophila* since it is similar in both size and structure to the brahma (or brm) gene product. We have shown that a counterpart of the yeast SNF2/SWI2 and the *Drosophila* brm gene products also exists in mouse and human cells. The human protein, designated hbrm, is a 180 kD nuclear protein, which can function as a transcriptional activator when fused to a heterologous DNA binding domain. In addition, intact hbrm protein is able to co-operate with the glucocorticoid receptor, the retinoic acid receptor and the estrogen receptor but not with other factors like AP-2 or the homeoprotein HNF-1 in transcriptional activation. Mapping of the transcriptionally active regions of hbrm has shown that this protein requires other regions than it's conserved helicase domain for full activity and we find that hbrm is likely to activated transcription by several different mechanisms. In particular, we have shown that a region located at the carboxy-terminal end is necessary for activation of an MMTV promoter integrated in the genome whereas it is completely dispensable for activation of the same promoter in transient transfections. On the other hand, a region at amino-terminal end of the protein appears to be very important in transient experiments and has only moderate effect when the promoter is integrated in the genome.

SPREADING THE SILENCE: MECHANISMS OF HERITABLE GENE INACTIVATION IN DROSOPHILA

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Cell identities in multicellular organisms are established on the basis of intricate regulatory networks which eventually converge in stably inherited differential gene expression programs. Mechanisms of "cellular memory" are of fundamental importance for a cell to remember and maintain its determined state during differentiation. On the basis of their reciprocal phenotypes two classes of genes that are part of such a mechanism have been identified in *Drosophila* : the trithorax-Group (trx-G) and the Polycomb-Group (Pc-G). These two groups guarantee the maintenance of respectively the active and the repressed state of the homeotic genes in the appropriate segments. They do not play any role in the establishment of a given expression pattern, but they rather fix a determined state, thus dispensing the cell from reproducing at every generation the full complexity of a particular regulatory cascade. Several parallels between the Pc-G and a class of genes involved in heterochromatin formation suggest a common regulatory mechanism. An interesting possibility being that Pc-G proteins inactivate homeotic genes by generating heterochromatin-like structures, thus confining repressed genes in transcriptionally inactive compartments of the nucleus.

Indeed, for the Polycomb protein (Pc) we could show, by applying a novel in vivo formaldehyde-crosslinking method, that it covers large domains of the homeotic bithorax complex encompassing regions with inactive genes, while regions devoid of Pc correspond to transcriptionally active domains. Thus, Pc is a structural determinant of chromatin repressed domains. To test whether this special Pc-G chromatin is rendering genes inaccessible for DNA binding factors we have applied the formaldehyde-fixation method to map a possible interaction of the Abd-B homeoprotein whithin the BX-C. We do not find any binding of Abd-B in this region. However, we find a binding of Abd-B to the empty spiracle gene which we used as a positive control.

In order to test the interaction of an activator protein with the Pc-G silenced genes we generated gene constructs flanked by Pc-G regulated elements (PRE) of the BX-C. These constructs were tested in transformed fly lines. We find that the PREs can induce mosaic expression of Gal-4 driven reporter gene and of a distantly located white marker gene. At the integration site of the transgene in polytene chromosomes we can show with immunostaining that the repressor Pc and the activator Gal-4 have reciprocal patterns. Indeed, the dotted pattern observed seem to indicate an "all-or-none" type mechanism with a clonal propagation of either the repressed or the active state on the polytene chromosome fibers.

What determins the target specific binding of Pc is still unknown. We show that mutations in the conserved regions of Pc result in a dramatic rearrangement of the protein in the nucleus and correspondently other Pc-G members lose their ability to bind restricted regions of the nucleus. We have also evidence that Pc binds to a RNA moiety. The interaction with the RNA gives the protein an abnormal mobility in 2D-gel system. We were able to isolate the RNA part and a first characterization of it will be discussed.

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V. CHROMATIN GENETICS AND IMPRINTING

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DIFFERENT HISTONE FUNCTIONS IN YEAST HETEROCHROMATIN AND EUCHROMATIN

The silent mating type loci and chromosomal regions adjacent to telomeres of Saccharomyces cerevisiae have features similar to heterochromatin of more complex eukaryotes. Transcriptional repression at these sites depends on the Silent Information Regulators *SIR3* and *SIR4* an well as histones H3 and H4. We demonstrate that the Sir3 and Sir4 proteins interact with specific silencing domains of the H3 and H4 N termini in vitro. Certain mutations in these factors, which affect their silencing functions in vivo, also disrupt their interactions in vitro. By immunofluorescence we show that deletion of the N termini of H3 and H4 interferes with the correct subnuclear localization of Sir3 and with perinuclear positioning of yeast telomeres. Based on the observed interactions, we propose a model for heterochromatin-mediated transcriptional silencing in yeast, which may be a paradigm for eukaryotic organisms as well.

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THE YEAST SWI/SNF COMPLEX CONTAINS A SUBUNIT OF THE YEAST TRANSCRIPTION FACTOR TFIIF

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Both genetic and biochemical experiments have established a role for chromatin in the repression of transcription (1). In addition, there is considerable evidence that many non-histone chromatin components assist in the establishment or maintenance of chromatin structure and contribute to this repression (2). There is also much evidence that this hierarchy of condensation is reversed and chromosomal DNA unfolded for transcription. For example, positioned nucleosomes at the yeast *PHO5* and *SUC2* promoter are disrupted under derepressing conditions (3-5). Importantly, this disruption requires neither DNA replication nor transcription from the promoter to occur, suggesting that these processes are not required for these chromatin transitions (4, 5).

Genetic studies from several labs identified the components of a multi-protein SWI/SNF complex which may help mediate these chromatin transitions. The products of the *SWI1/ADR6*, *SWI2/SNF2*, *SWI3*, *SNF5*, and *SNF6* genes are all required for proper control of the *HO* gene, the *SUC2* gene, the *GAL1* gene, the *ADH2* gene, and Ty elements (6-14). Additional genetic data suggest that these SWI and SNF proteins form a complex that is recruited to promoter regions through interactions with gene-specific DNA-binding proteins (8,15). *SWI2/SNF2* contains a domain homologous to certain DNA-dependent ATPases, and a recombinant form of this domain exhibits DNA-dependent ATPase activity (17, 18).

Several lines of evidence suggest a connection between SWI and SNF proteins and chromatin structure (2). Selections for extragenic suppressors of both *swi* and *snf* mutants yielded mutations in genes encoding histones and putative non-histone chromatin components (19-21). In addition, chromatin structure on the *SUC2* promoter is altered in *snf2* and *snf5* mutants (3).

We have purified this complex to homogeneity from yeast extracts and have shown that it contains the five SWI and SNF proteins discussed above, and five additional polypeptides (termed SWP's for SWI/SNF associated polypeptides) (22). None of these additional proteins are SRB proteins or TBP (TATA-binding protein)(23). A potent DNA-dependent co-purifies with the complex, and the activity is associated with the SWI2/SNF2 component (22).

A similar profile of proteins has been purified by C. Peterson, J. Workman and colleagues (24). They have demonstrated that the yeast SWI/SNF complex can disrupt nucleosomes, and assist GAL4 derivatives in their binding to nucleosomal templates (24). The human SWI/SNF complex (hSWI/SNF) has recently been purified by M. Green, R. Kingston and colleagues (25). This complex consists of seven polypeptides; the largest of which is a SWI2/SNF2 homolog. The other polypeptides have not been identified. They have also shown that hSWI/SNF can disrupt nucleosomes, and assist either an activator or TBP in binding to nucleosomal DNA (25, 26).

We have found that the complex does not have a significant effect on the level of basal transcription on naked DNA templates *in vitro* (22). In addition, GAL4-VP16, GCN4, and a LexA-glucocorticoid receptor fusion protein can activate transcription in the absence of SWI/SNF complex under these conditions.

We have recently sequenced all five of the remaining members of the SWI/SNF complex, and have determined that the SWP29 component of the complex is identical to TFG3, a component of the yeast transcription factor TFIIF. Yeast TFIIF consists of three proteins, the two largest of which show striking similarity to mammalian RAP74 and RAP30, respectively (27). We have shown that TFIIF forms a stable stoichiometric complex with RNA polymerase II and the yeast mediator of activated transcription. These results suggest that a physical link between the SWI/SNF complex and the basal transcription machinery may exist.

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Cis- and trans-repression of transcription by a chromatin insulator.

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The gypsy element of *Drosophila* causes mutant phenotypes by affecting the expression of adjacent genes in a temporal- and tissue-specific manner. These effects are due to the presence of *su(Hw)* binding sites in the transcribed untranslated region of gypsy (Corces and Geyer, 1991) . The *su(Hw)* protein bound to these sequences represses the function of transcriptional enhancers controlling the expression of the mutant gene. Only enhancers located distally with respect to the promoter from the location of the *su(Hw)* binding region are affected by the presence of this protein, whereas all other enhancers are functionally normal (Geyer and Corces, 1992). The *su(Hw)* protein contains a leucine zipper region and two acidic domains that mediate this repressive effect on enhancer function (Harrison et al., 1993). To explain this striking polarity in the repressive effect of *su(Hw)* protein on gene expression it has been proposed that *su(Hw)* might establish boundaries between chromatin domains that specify levels of gene activity (Wolffe, 1994). The leucine zipper and acidic domains might interact with other proteins to establish these boundaries and create a chromatin insulator that represses enhancer function on one side of the boundary while allowing normal gene expression on the other.

One good candidate for a protein that interacts with *su(Hw)* is the product of the *modifier of mdg4* [*mod(mdg4)*] gene. Mutations in this gene have been reported to modulate the phenotype of gypsy-induced mutations in genes such as *cut, forked, scute, yellow*, etc. For example, mutations in *mod(mdg4)* enhance the phenotype of the gypsy-induced y^2 allele to that of a null in which all tissues of the fly show mutant pigmentation. We have carried out an analysis of genetic interactions between *mod(mdg4)* mutations and alleles of *su(Hw)* affecting specific domains of the protein. Mutations in *mod(mdg4)* fail to enhance the phenotype of *yellow* mutations not resulting from gypsy insertion, suggesting that sequences present in gypsy are important to mediate the effect of *mod(mdg4)* mutations on *yellow* expression. Furthermore, a y^2 derivative lacking the *su(Hw)* binding region fails to respond to *mod(mdg4)*, and a *yellow* gene containing only the *su(Hw)* binding region in the same location as gypsy is inserted in y^2 responds to *mod(mdg4)* mutations in the same fashion as the y^2 allele. These results indicate that the effect of *mod(mdg4)* on *yellow* expression is carried out through the same sequences that interact with *su(Hw)*, suggesting that the *mod(mdg4)* protein either binds to the *su(Hw)* binding region or interacts directly with this protein. The later possibility is supported by the fact that, in the presence of a null deletion allele of *su(Hw)*, mutations in *mod(mdg4)* fail to enhance the phenotype of y^2. This result indicates that the effect of *mod(mdg4)* on *yellow* expression requires an intact *su(Hw)* protein, suggesting that the two proteins interact or, if *mod(mdg4)* binds to DNA, it requires first binding of the *su(Hw)* protein. Mutations affecting the Leu zipper of *su(Hw)* suppress the ability of *mod(mdg4)* mutations to enhance the *yellow* phenotype, suggesting that perhaps the two proteins interact through this domain of *su(Hw)*. In addition, mutations in the *su(Hw)* acidic domains decrease the effect of *mod(mdg4)* mutations on *yellow* expression, suggesting that these regions of *su(Hw)* are also important.

These results suggest the following model to explain the effects of *su(Hw)* and *mod(mdg4)* on gene expression. The *su(Hw)* protein exerts a polar effect on enhancer function

by establishing a chromosomal insulator, i. e. a chromatin boundary that separates higher order domains of gene expression. This polarity in the function of the insulator requires the *mod(mdg4)* protein; in its absence, the effect of *su(Hw)* looses its polarity and becomes bi-directional, affecting all enhancers of the *yellow* gene and explaining the enhancement of the y^2 phenotype by mutations in *mod(mdg4)*. Therefore, the role of the *mod(mdg4)* protein is to give polarity to the *su(Hw)* insulator, i. e. to indicate activation of gene expression on one side of the boundary and repression on the other.

If the *su(Hw)* protein can repress gene expression by establishing a chromosomal insulator, the mechanism of this effect might be similar to the inhibition of gene expression by heterochromatin in the phenomenon of position-effect variegation. In fact, the *su(Hw)* protein affects enhancer function in a distance-dependent manner similar to that observed with the effect of heterochromatin on adjacent genes. For example, the effect of *su(Hw)* on the wing enhancer of the *yellow* gene increases as the distance between the *su(Hw)* binding region and the wing enhancer decreases. In some cases, heterochromatic sequences present in one chromosome can repress the expression of a paired gene located in the other homolog, as is the case for the *brown-dominant* mutation. Under normal circumstances, the polar effect of the *su(Hw)* insulator cannot be transmitted to the enhancers in the gene located in the other homolog, since the phenotype of $y^2/+$ flies is wild type. This indicates a clear difference in the structure and mode of action of the insulator versus heterochromatin. Nevertheless, in the absence of *mod(mdg4)* protein in the $mod(mdg4)^{u1}$ mutation, the *su(Hw)* protein can repress the expression of the *yellow* gene located in the other homolog. This effect requires the presence of the *su(Hw)* binding region in one of the chromosomes and is enhanced by other gypsy sequences. The *trans*-inactivation of *yellow* expression is abolished by chromosomal rearrangements that disrupt pairing, suggesting that close apposition between the *su(Hw)* binding site and the target gene in the other chromosome is required for repression, but is not affected by mutations in *zeste*. These results again point to a role for the *mod(mdg4)* protein in modulating the repressive properties of the *su(Hw)* protein. In the absence of *mod(mdg4)* protein, the *su(Hw)* insulator looses its directional characteristics (as described above) and becomes a generalized repressor that causes effects similar to those of heterochromatin: not only the insulator inhibits gene expression upstream and downstream of its location, but it can also inhibit enhancer function in *trans* by affecting the gene located in the other homolog.

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Long-Range Analysis of a Chromosomal Domain Containing Reciprocally Imprinted Genes.

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Genetic imprinting confers parent of origin dependent expression on imprinted genes; expression of some genes occurs after maternal inheritance while expression of others occurs when paternally inherited. This unique mode of gene regulation is presumably the result of germ line-specific heritable epigenetic modifications. However, this does not exclude further post-zygotic modifications of imprinted loci partly because the monoallelic expression of imprinted genes show both temporal and tissue-specificity.

The location of all the imprinted genes so far examined is within unique asynchronous early/late DNA replication domains in which the paternal chromosome is early replicating. Within these domains are imprinted genes that show expression of either the maternal or paternal allele. Differential epigenetic modifications are utilised to mark these parental loci. DNA methylation is an important component of this epigenetic marking process. Studies have shown that the imprinted expression of a number of genes is deregulated in embryos with a null mutation for the DNA methyltransferase gene.

The focus of our studies is to analyse the organisation within a higher order functional domain of a cluster of imprinted genes found on the distal region of chromosome 7 where four imprinted genes have so far been identified; these are *Igf-2*, *H19*, *Ins-2* & *Mash-2*. Detailed studies on the region containing *Igf-2* and *H19* have been carried out. These genes are in the same transcriptional orientation, closely linked within 130 kb and reciprocally imprinted with expression of the paternal *Igf-2* and maternal *H19* gene. They also show remarkable similarities in their temporal tissue-pecific expression during development. While the analysis of the promoter region of *Igf-2* did not reveal any significant parent of origin dependent differences, a region 3kb upstream of *Igf-2* did show greater degree of methylation on the active paternal allele. However, the most obvious

difference was seen around the *H19* gene where the promoter and the first exon of the inactive paternal allele were more methylated as well as significantly less sensitive to DNase I. Further studies have shown that the methylation of the paternal *H19* gene does not occur in sperm but it occurs post-zygotically sometime after implantation. This illustrates step-wise modification of an imprinted locus in the germ line and subsequently during development.

The main regulatory elements known in the region so far are two enhancers located about 5kb downstream of the *H19* gene. It has been postulated that these enhancers play a significant role in controlling expression of both the *H19* and *Igf-2* by the so called enhancer competition for promoter model. However, these enhancers are apparently insufficient for full expression of *H19* and especially do not confer expression in the mesoderm. To gain further insight in to the regulation of expression and imprinting in this region, we have carried out a detailed long range comparative analysis of the 130kb region containing the two genes. The critical boundaries of differential methylation and DNase I hypersensitivity is apparently confined within and proximate to the imprinted gene. However, we have recently identified a novel intergenic region that is conserved in mammals, GC-rich, unmethylated in embryos and contain major DNase I hypersensitive sites. Further studies are in progress to determine whether additional genes and/or regulatory elements are located within this region.

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POSTERS

DNA resolution and DNA inversion mediated by the β recombinase of the *Streptococcus pyogenes* plasmid pSM19035 requires a chromatin-associated non-specific DNA-binding and DNA-bending protein

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Genetic evidence suggests that the β protein encoded by the *Streptococcus pyogenes* plasmid pSM19035 is involved both in the resolution of plasmid multimers into monomers and in DNA inversion. The recombinase binding site has been localized to an 85-bp region (crossing over site, *six* site) that can be divided into two discrete subsites (I and II). These subsites, which are about 34-bp in length, are separated by about 16-bp, and contain two 13-bp imperfectly conserved sequences (half-sites) with dyad axis symmetry. The highly purified β recombinase is unable to mediate *in vitro* DNA recombination unless a host factor is provided. In the presence of a *Bacillus subtilis* host factor, DNA resolution was obtained when the two *six* sites were directly oriented, whereas DNA inversion was the product when the *six* sites were in inverse orientation. The factor has been identified as the *B. subtilis* Hbsu protein. Hbsu, which belongs to the family of chromatin-associated proteins, is a non-specific DNA-binding and DNA-bending protein. The *Escherichia coli* HU protein is the best characterized member of the family. The β recombinase, in the presence of highly purified *B. subtilis* Hbsu, *E. coli* HU or mammalian HMG-1 protein, is able to catalyze *in vitro* intramolecular recombination between two specific recombination sites on a supercoiled molecule. *In vitro*, the *E. coli* IHF protein could partially substitute for Hbsu in promoting β-mediated recombination, while Fis and H-NS failed to show such stimulating effect. The data presented provide a direct evidence that the chromatin-associated Hbsu protein promotes synaptosome assembly on β mediated recombination with a Kapp of 10^{-8}, whereas the nucleosomal organization is formed with a Kapp of 10^{-6}. The β protein shares a significant homology with the Tn*3*/ family of site-specific recombinases. In the case of β mediated recombination the chromatin-associated protein could work as an accesory effector to compensate for the absence of the third binding site (DNA resolvases) or as an enhancer and Fis protein (DNA invertases) as it is commonly observed in case of other enzymes of the Tn*3* family.

Analysis of the function of a cruciform present *in vivo* on yeast chromatin by site-directed mutagenesis. A. Aranda, M. del Olmo and J.E. Pérez-Ortín. Departamento de Bioquímica y Biología Molecular. Facultades de Ciencias. Universitat de València. c/ Dr. Moliner 50. E-46100 Burjassot. Spain. 346-3864385.

Inverted repeats potentially able to form cruciforms have been found in many different organisms, specially in promoters and regions of transcription termination in bacteria and viruses. Their preferential location has been interpreted as a proof of implication in the regulation of gene expression. The existence of cruciforms in superhelical DNA *in vitro* has been demonstrated in many cases using specific nucleases and other methods. However the existence of cruciforms *in vivo* in eukaryotes is less certain. We have showed that an imperfect $(dA\text{-}dT)_{12}$ tract placed at the 3' end of the *FBP1* gene is part of a hypersensitive site in chromatin (1) which acts, probably, as a boundary positioning nucleosomes at both sides. This tract exists as a cruciform in naked DNA depending on the supercoiling state (2). We have introduced a mutation in the sequence of the cruciform and we have demonstrated by cruciform cutting endonucleases that this mutation abolishes the formation of the cruciform. Now our studies are centering on the effect of this mutation on the sensitivity to nucleases. The sensitivity to micrococcal nuclease disappears in yeast cells in the position of the cruciform. We have also analyzed the nucleosome positioning by indirect end-labeling.

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Chromatin repress MMTV transcription in yeast by restricting accessibility of NFI

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Transcription of the mouse mammary tumour virus (MMTV) is induced by glucocorticoids and progestins in a process involving several transcription factors including the corresponding hormone-receptor and the nuclear factor I (NFI). The chromatin structure of the MMTV promoter plays an essential role in its function. It is proposed that the organization of the promoter region in chromatin repress transcription of MMTV by restricting NFI accessibility and that the alteration of that structure by hormone-receptor binding releases the repression imposed by chromatin. To test this model in an organism lacking NFI and with available genetic tools, we have introduced the MMTV transcriptional system in the yeast *Saccharomyces cerevisiae*.

MMTV promoter, that is well organized on chromatin in yeast, behaves in this organism like ,in animal cells: it is up to 100 fold induced by steroids, depending on the presence of hormone-receptor and NFI in the cell; and it is silent in the absence of hormone, even when NFI or a NFI-VP16 chimera are expressed. A first conclusion from this picture is that histone H1, that is lacking in *Saccharomyces*, is neither required for MMTV repression nor for its hormonal induction.

In the recombinant yeast strain UKY403, containing histone H4 under GAL control, basal transcription in the presence of NFI or NFI-VP16 increases when the cell is partially nucleosome depleted after switching H4 synthesis off, demonstrating *in vivo* that chromatin repress MMTV transcription by hindering NFI accessibility. A similar conclusion was obtained by using deleted MMTV promoters, lacking the sequence able to stably positioning a nucleosome on the NFI-binding site.

CHROMATIN STRUCTURE OF THE HUMAN UROKINASE GENE

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Urokinase-type plasminogen activator (uPA) is a serine protease involved in the degradation of the extracellular matrix. Urokinase is thus involved in processes like metastatic cell migration, trophoblast implantation, wound healing, etc.

The uPA gene coding region is 6.6 kb long and is composed of 11 exons and 10 introns, with a long 3' untranslated region. The 5' flanking region (2.3 kb long) has been functionally divided into 3 regions: an enhancer, an RRBE (rel-related binding element) region and a silencer. The enhancer contains consensus sequences for transcription factors such as AP1, ATF1, PEA3 and a COM (cooperativity mediator) region, which seems to contain the binding sites for 4 new factors. The RRBE region contains a consensus sequence for NFkB, to which either a proper NFkB element binds or, alternatively, a p65/c-rel heterodimer. The silencer region appears to contain a consensus sequence for the transcription factor TCF-1, which belongs to the HMG-box containing transcription factor family. The gene is highly regulated in different human cell lines, depending on a variety of exogenously added factors, such as phorbol esters and hormones.

We have studied the chromatin structure of the human uPA gene in two cell lines: HeLa cells, which have a very low basal level of uPA expression and PC3 cells, which are high producers of uPA. Both micrococcal nuclease and DNase I digestions indicate that the 5' flanking region, beyond the 2.3 kb of known sequence, may contain other regulatory elements. In PC3 cells we find protected sites from MNase cleavage at positions -3210 and -3660. The latter is the 5' boundary of a MNase protected region, extending for approximately 500 bp (until position -4160). Further 5' we find another sensitive region, spanning positions -4160 to -4500). Finally another protected region spans positions -4500 to - 5300.

As mentioned above, the 5' sequenced flanking region contains several regulatory elements and therefore it appears to be tightly packed in DNA-protein interactions. In HeLa cells MNase detects a nucleosome-size protected region, located in the silencer.

Two ovelapping restriction fragments spanning positions +438 to +3786 show both nucleosome-like structures and other protein-DNA interactions, which are possibly non-nucleosomal. Interestingly, in some positions of this region (i.e. the 8th intron) the boundaries to MNase cleavage change between HeLa and PC3 cells, perhaps as a consequence of sublte changes in the chromatin structure.

Our results indicate that other regulatory elements, either functional or structural, may be present in the 5' flanking region, beyond the known sequence. Furthermore the data suggest that:
1) at least part of the silencer may be packed in a nucleosome-size structure;
2) part of the coding region undergoes changes in the location of the primary MNase cleavage sites, perhaps as a consequence of subtle changes in its chromatin structure.

MECHANISM OF NUCLEOSOME DISSOCIATION DURING TRANSCRIPT ELONGATION IN A SHORT LINEAR CHROMATIN TEMPLATE

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We have used a short linear DNA template (239 bp) containing a nucleosome positioning sequence (NX1) downstream of the T7 RNA polymerase promoter to study the mechanism of transcription elongation through a nucleosome. Transcription reactions have been performed at ionic strength approaching the physiological conditions. Under these conditions we have observed that transcription causes nucleosome dissociation and histone redistribution within the template. We have examined the role of the different elements that, in principle, could induce nucleosome dissociation during transcription. The high affinity of histones for single-stranded DNA observed in titration experiments performed using the purified (+) and (-) strands of the NX1 fragment suggests that nucleosome dissociation is not due to the formation of segments of single-stranded DNA by RNA polymerase in the elongation process. Furthermore, our results show that although RNA can interact with core histones, the RNA synthesized is not involved in the nucleosome dissociation detected during chromatin transcription. Our results demonstrate that core histones released by transcription are bound to free DNA or chromatin present in the transcription system. Taking into account the dynamic properties of excess histones bound to chromatin (with or without histones H1-H5) and the fact that chromatin is present at a high concentration in the cell nucleus, we suggest a mechanism for nucleosome transcription in which histones dissociated by the polymerase are transiently bound to chromatin and finally reform nucleosomes in DNA regions depleted of histones due to the previous passage of the polymerase.

INTERNAL STRUCTURE OF SMALL FRAGMENTS OF FOLDED CHROMATIN

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We have carried out an electron microscopic study of the structure of small chromatin fragments isolated from chicken erythrocytes. In the presence of 1.7 mM Mg^{2+}, the diameter of the circular structures produced by the small chromatin fragments remains essentially unchanged (~33 nm) when the number of nucleosomes in these fragments increases from 10 to 36. In contrast, the results obtained in unidirectional shadowing experiments show that under the same conditions the height of the chromatin fragments increases with the number of nucleosomes. These observations indicate that the electron microscope images studied in this work correspond to a top view of small chromatin fragments. Rotary-shadowed chromatin fragments show three parts: (a) a contour with a heavy deposition of platinum, (b) an annular zone between the central region and the periphery, and (c) a central hole. The heterogeneous ring generated by the deposition of platinum in the periphery suggests that nucleosomes form a one-start helix (5-7 nucleosomes per turn) that apparently can be left- or right-handed. The annular region (thickness of about 11 nm) shows spokes probably due to flat faces and core DNA of radially oriented nucleosomes. The central hole (8-12 nm) is clearly seen in many images but it is not empty because some deformed fragments show coated material (probably linker DNA) that protrudes from this central depression. We have observed that these structural elements directly detected in short chromatin fragments are also present in long chromatin fibers. This allows us to conclude that these elements are basic structural components of the 30 nm chromatin fiber.

Chromatin organization of the intergenic spacers and transcription regulation of the rRNA genes in yeast

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A detailed restriction analysis of the psoralen cross-linked DNA revealed that in *Saccharomyces cerevisiae* the transcriptionally active rRNA genes are organized in clusters containing two to three gene copies. A tagged integrated chromosomal rDNA unit at the ribosomal locus (one copy per cell) shows that after DNA replication the activation of the newly replicated ribosomal RNA gene copies is random.

Since is was possible to determine the chromatin structure of a 200 bp rDNA enhancer fragment in the psoralen gel retardation assay, the nucleosomal structure and therefore the functionality of the enhancers between two contiguous active / active, active / inactive, inactive / active and inactive / inactive gene copies was studied in detail. The data reveal that the enhancer structure and the activity of corresponding upstream gene always correlate: active gene —> nucleosome-free enhancer or inactive gene —> nucleosome-packed enhancer. However, this clear correlation is not found in the gene localized downstream of the enhancer, which suggests that an enhancer acts mainly on the most proximal upstream promoter and not on the downstream gene copy.

The nucleosome-free enhancer sequence persists in a strain containing a disruption of the gene for the 135 kd subunit of the RNA polymerase I and seems to be independent of transcription. In this mutant the rRNA coding sequences are packaged in nucleosomes. These data suggest that the preactivation of the rRNA gene copies is not sufficient for the disruption of the nucleosomes on the coding region. The advancing of the RNA polymerase I molecules is required for the establishment of the active ribosomal chromatin devoid of nucleosomes.

Frequently the RNA polymerase I promoter sequences also appear as nucleosome-free elements in front of transcriptionally inactive gene copies.

DEVELOPMENTAL ANALYSIS OF EPIGENETIC MODIFICATIONS IN THE IMPRINTED *IGF2* AND *H19* GENES.

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The mouse *Igf2* gene encodes a fetal growth-factor and is predominantly expressed from the paternal allele. The closely linked *H19* gene is expressed from the maternal allele and codes for a transcript which possibly down-regulates cellular proliferation. We are interested in the precise structure of allelic epigenetic modifications in these genes and have analysed DNA methylation (Feil et al., *Development*, in press) as well as other features of chromatin such as DNAse-I sensitivity, association of Topoisomerase-II, and positioning of nucleosomes.

While for the *H19* promoter allelic nuclease sensitivity has already been demonstrated (Ferguson-Smith *et al.*, 1993; *Nature* **362**, 751), we have not detected allele-specific chromatin features in *Igf2* so far, and the promoters are equally accessible on both parental alleles. However, two regions in the *Igf2* gene, one ~3 kb upstream of the gene (Sasaki *et al.*, 1992; *Genes & Dev.* **6**, 1843) and one in the 3' part of the gene, were found to be more methylated on the expressed paternal allele. Genomic sequencing (Frommer *et al.*, 1992; *PNAS USA* **89**, 1927; Feil *et al.*, 1994; *NAR* **22**, 695) of individual chromosomes in the upstream region showed that the parent-specific methylation is highly mosaic, and that individual sperm chromosomes carry different methylation patterns into the egg. Interestingly, in the 3' region of the *Igf2* gene the level of methylation on the expressed paternal allele is highly tissue-specific and correlates with expression of the gene. This could possibly indicate that these sequences are involved in the silencing of the maternal allele. That allelic methylation is found in the corresponding sequences in the imprinted human *IGF2* gene (Schneid *et al.*, 1993; *J. Med. Genet.* **30**, 353) also argues for the functional importance of these sequences.

We sought to examine allelic methylation of *Igf2* and *H19* in situations in which imprinting of these genes is altered. In adult choroid plexus, a brain epithelium in which *Igf2* is expressed from both alleles and *H19* is not expressed, both genes adopt a paternal type methylation pattern on both parental alleles. To understand when allelic methylation patterns in the *Igf2* and *H19* genes arise we examined monoparental androgenetic and parthenogenetic ES cells and differentiated derivatives. Analysis of these ES cells showed for both genes that the methylation patterns on maternal and paternal chromosomes are very similar, and teratomas derived from these ES cells show the appropriate parental-allele specific methylation.

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CLONING AND STRUCTURAL ANALYSIS OF THE MOUSE HMG1 LOCUS.

The HMG1 protein is a well conserved DNA binding protein present in large amount in all vertebrate nuclei. A closely related protein, HMG2, is also present in the nucleus, although at a lower abundance.
HMG1 binding appears to be sequence independent but requires the presence of specific DNA structures containing sharp bends or kinks. Two homologous domains (HMGboxA and HMGboxB) have been shown to be directly responsible of the DNA binding activity. The function of the C-terminal acidic tail is unknown.
Its ubiquity and sequence conservation point to a fundamental role for HMG1, but up to now it has not been shown to be essential for any known biological process.
HMG1 cDNA sequences have been cloned from bovine and trout testis, CHO cells, rat liver, calf and pig thymus, human placenta and mouse embryonic carcinoma cells; in this poster I describe the cloning and characterization of the mouse HMG1 genomic locus.
The presence of several sequences homologous to HMG1 have been previously reported in human and pig genomes. Southern-blot analysis with different regions of HMG1 cDNA confirmed the presence of many related genes or pseudogenes in the mouse genome too: clearly it was not possible to screen a mouse genomic library with any fragment coming from the HMG1 cDNA.
Recently Shirakawa and Yoshida reported the cloning of the functional gene coding for human HMG2 protein, closely related to HMG1. We supposed that genes which belong to the same family and which show a high degree of sequence conservation might have the exon-intron boundaries in the same position. We designed 3 couples of oligonucleotides on the basis of the HMG1 cDNA sequence in such a way as to amplify presumptive introns, which were present in the same position in the HMG2 gene. By PCR, performed on genomic DNA from NIH3T3 cells, we amplified two fragments corrisponding to intron 3 and intron 4 of the HMG1 gene. λ-FIX mouse genomic library was screened with both intron 3 and intron 4 and four positive phages were isolated which contained the HMG1 genomic locus.
The gene has five exons and four introns; the first untranslated exon falls in a region of very high C and G content and CpG dinucleotides, a feature indicative of CpG islands.
The gene we have isolated corresponds to the HMG1 active gene: the 2.5 Kb DNA segment upstream of the HMG1 transcription start site drives the expression of the APH gene to confer G418 resistance to NIH3T3 cells.
The mapping of HMG1 gene on mouse chromosomes is in progress.

IN VIVO BINDING OF TRIMETHYL-PSORALEN DETECTS DNA STRUCTURAL ALTERATIONS ASSOCIATED WITH EXPRESSED REGIONS IN THE HUMAN β-GLOBIN CLUSTER.

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In order to increase the knowledge about the mechanisms that regulate expression of human β-like globin genes, we have used a novel technique to analyze the chromatin structure in living cells. The approach allows us to detect specific DNA regions *in vivo* where nucleosome folding or unconstrained DNA supercoiling in erythroid cells differs from that in non erythroid cells. In this method we use trimethyl-psoralen (TMP) as a probe for altered chromatin conformation. TMP intercalates between both DNA strands and binds covalently to them when irradiated with long-wavelength U.V. light. Variations on nucleosome folding or unconstrained DNA supercoiling affect the rate of TMP binding to DNA. Our results show that TMP binds to DNA with a higher affinity over the regions in the locus that are actively expressed, including both the promoter and the transcribed region. This higher affinity detected when comparing erythroid cells with non erythroid cells does not extend to other regions inside the β-globin cluster, even though in erythroid cells the entire locus is preferentially sensitive to DNase I. Our data suggest that the observed effect is likely due to nucleosome displacement. Alternatively, it could result from localized DNA supercoiling, but not from widespread torsional stress across the entire β-like globin locus as hypothesized previously.

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Mitotic displacement of transcription factors from the metaphase chromosome: implications for replication-independent remodeling of chromatin structure.

During mitosis genomic DNA is packaged into condensed chromosomes to facilitate its accurate segregation to daughter cells. At the same time, nuclear transcription becomes inhibited during mitosis. Such mitotic repression of transcription could benefit the cell in that ultimely transcription, which might interfere with the correct segregation of the replicated genome, is prevented.

Different mechanisms have been proposed to explain the mitotic repression. In particular we are interested in knowing the relationship between the transcriptional silencing during mitosis and nucleosome/chromatin modifications and/or chromatin condensation.

Our results show that the mitotic displacement of transcription factors from the metaphase chromosome is concomitant whit the inhibition of transcription. Sp1, C/EBP, G-string factor and HSF are displaced from the heat shock promoter in prophase and they reassociate in early telophase. The implications for replication-independent remodeling of chromatin structure will be discussed.

Transcriptional restrictors: A novel structural component of activation at distance.

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Bacterial promoters dependent on the alternative sigma factor $\sigma 54$ are one of the few cases of transcriptional control at distance known in the prokaryotic world. These promoters include distant enhancer-like upstream activating sequences (UAS) which are the target sites for specific regulators. In addition, one class of these promoters include target sequences for the histone-like protein, IHF, which bends DNA more than 140º. It is currently believed that the role of IHF in these promoters is to provide an structural aid to improve contacts between RNAP and activators, by looping-out the intervening DNA. We present data which show that protein-induced or sequence-directed DNA bends within these promoters are not only structural aids for full transcription activity, but also supressors *in vivo* of cross-activation by others regulators of the same famuily of proteins. We have coined the term *restrictor* to describe this role, i.e., a structural element which constrains the induction range and the specificity of the promoter *in vivo* within defined limits out of the whole activity potentially available in the system. The increase number of histone-like proteins described in eukaryotic cells prompted us to suggest similar roles in eukaryotic transcription.

Functional Characterization of the chromo domain of HP 1.

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The non-histone heterochromatin associated protein 1, or HP 1 (James,T.C. and Elgin, S. C. R., 1986 Mol. Cell. Bio. 6,3862-3872.) contains a region of high homology with another Drosophila protein, *polycomb(Pc)* , a repressor of homeotic genes in Drosophila. In *Pc* this domain is responsible for directing the protein to the nucleus and binding to chromatin. Furthermore this region is highly conserved in C. elegans, Drosophila, mealybug, mouse and humans.

To study the function of this domain in HP 1, site-directed mutagenesis was carried out within this region. Five different mutants were generated and introduced into the Drosophila germ line by P element transformation. The different mutants were analyzed for their ability to localize a ß-galactosidase fusion protein to the nucleus and their binding to heterochromatin. Also the capability of these mutants to genetically complement mutations of HP 1 was tested.

Besides these mutants, we have generated a fusion cDNA by a modification of the process of PCR splicing by overlap extension. This cDNA contains the chromo domain of Pc and the rest of HP 1. Using P element transformation we generated flies containing such cDNA. Again, we analyze the cellular localization of the fusion protein, its localization in polytene chromosomes and its ability to complement mutations in HP 1.

Another chimera containing only the chromo domain of HP 1 fused to ß-galactosidase was evaluated with regards to its localization to the nucleus and binding to polytene chromosomes.

The results of the above experiments, together with proposed explanations will be presented in the poster.

Competition between chromatin and transcription complex assembly regulates gene expression during early development

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Early development in Xenopus is characterized by transition of gene expression. During oogenesis transcription is very active providing the accumulation of RNAs necessary for the early development. Fertilization of the egg triggers a series of rapid cell cycles during which the genome is entirely repressed. At the twelfth cell cycle, a transition called the midblastula transition (MBT), involves the coordinated initiation of transcription, cell motility and the onset of an asynchronous and slower cell cycle.

We analyzed this regulation using a reporter plasmid containing the c-myc promoter which is under the same developmental control when injected into oocytes and embryos. We find that the repression of transcription before the MBT can be relieved simply by incubating the reporter plasmid with TATA bindind protein (TBP) before injection. In oocytes, this preincubation does not change the efficiency of transcription. It is necessary to potentiate the promoter with TBP to induce premature transcription as injection of the embryo with the same amount of exogenous TBP is not sufficient to induce premature transcription. However, we observe that repression of gene activity normally occurring before the MBT soon becomes dominant over this activation. We show that the succession of rapid cell cycle events is not responsible for this represion and that inactivation correlates with chromatin assembly. Moreover, titration of chromatin components relieves repression of TBP-dependent transcription and also permits the establishment of stable transcription. Finally we have also measured the basal transcription activity of unfractionated oocyte and embryo extracts and we show that the activity is the same in both extracts.

Our data suggest that the large excess of histones present in the early embryo is the primary factor that represses gene activity during early development. This excess of histones is progressively titrated by the accumulation of newly synthesized DNA in the embryo and at each cell cycle DNA replication provides a new opportunity for the competition between histone assembly and transcription factors assembly to occur. In post-MBT embryos the natural titration of the excess of histones displaces the equilibrium in favor of the binding of transcription factors.

NUCLEOSOME POSITIONING ON THE HUMAN C-FOS PROMOTER.

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The human c-fos oncogene expression is largely controlled at the level of transcription. The gene can be induced in various tissues by a number of external stimuli including serum, growth factors, phorbol esters and cAMP. Induction by serum is mediated by a Serum Response Element (SRE, located at -300) which is recognized by a ternary complex composed of the Serum Response Factor (SRF) and members of a family of accessory proteins, the Ternary Complex Factors (TCFs). cAMP induction of transcription is mediated by a cAMP Responsive Element (CRE) centered at -60. A number of genes encode CRE-binding proteins among which are found both activators like CREB (CRE binding protein) and repressors of cAMP induced transcription like CREM (CRE modulator) α and β.

We have investigated the chromatin structure present on the human c-fos promoter after in vitro reconstitution with purified histones. Using DNase I, OH radical and Exonuclease III analysis, we have detected a favoured nucleosome positioning in the proximal region of the promoter. Although the rotational setting is well defined, different translational positions are observed. In contrast, the analysis of the distal region of the promoter does not reveal any preferential incorporation of a nucleosome . The CRE element located at -60 is included in the nucleosome positioned on the proximal promoter region. We are currently investigating the role of this positioned nucleosome in the regulation of transcription mediated by cAMP on this promoter.

CONCLUDING REMARKS

G. Felsenfeld

CONCLUDING REMARKS - Gary Felsenfeld

Although we have some understanding of the organization of chromatin around transcriptionally active genes, we know relatively little about how these active structures are generated, or about the inactive structures from which they are derived. The work presented at this conference shows that we are finally making substantial progress in answering these questions.

Chromatin structure at the level of the nucleosome is fairly well understood, but as we ascend the hierarchy of structures, there is increasing controversy. Laemmli's analysis of the role of SARs in the organization of the mitotic chromosome thus seems an important step forward. The binding of scaffold proteins to the SARs and their interaction with each other appears to give rise to the central 'scaffold', which one can now imagine derives from the combination of DNA and protein rather than from a pre-organized protein structure. At the other extreme of structure, the polynucleosome filament and the '30 nm fiber' are again the subject of scrutiny. Scanning force microscopy has been used by van Holde to examine the extended polynucleosome filament stable at low ionic strength, and to show that when histone H1 is bound, local constraints lead to an open helical structure made irregular by variations in linker DNA length. It is not yet clear how this relates to the compact 30 nm fiber observed at physiological ionic strength; although the mass per unit length and other characteristics of the isolated fiber are well established, its relationship to the structure present *in vivo* remains to be determined.

A number of regulatory mechanisms discussed at this meeting may involve the higher orders of chromatin structure. Attention is focussing on the boundaries of chromatin 'domains', which may be marked by multiple elements with distinct characteristics and with functions only partially understood. These include the SAR (or MAR) elements, as well as elements capable of functioning as insulators that block the action of enhancers on promoters when placed between them. Such insulator activity was described by Felsenfeld for DNA sequences from the 5' and 3' ends of the chicken beta-globin locus. Evidence that these sequences are part of domain boundaries came from the work of Crane-Robinson, who showed that levels of histone acetylation and DNase I sensitivity, both markers that distinguish heterochromatic from active chromatin regions, show abrupt changes near these sequences. New insight was provided by Laemmli into the function of the *scs/scs'* insulating elements from *Drosophila*, first identified by Schedl and his collaborators. The identification and cloning of BEAF32, a 32 kD protein that binds to *scs'*, and is associated with interbands and puff boundaries, is a major advance toward determining the mechanism of insulator action. Another step in this direction is provided by Corces' data concerning the mode of action of the *Drosophila* transposable element, gypsy, which provides directional inactivation (insulation) associated with the binding of the *su(Hw)* protein. The new evidence implicates the

product of a second gene, *mod(mgd4)*, in the directionality of the activity. Mutations in this gene result in bidirectional inactivation by wild type *su(Hw)*. This result makes it unlikely that the insulating effect can be explained in terms of 'tracking' mechanisms, and suggests that compartmentalization of some kind is involved. The complex behavior of gypsy also reminds us that it is unlikely that insulating and boundary effects arise from the action of a single protein.

The organization of chromatin into domains may serve to protect regions of the genome that are transcriptionally active from those that are inactive, and *vice versa*. It has also been suggested that a domain presents the possibility of topological isolation, and therefore of action at a distance through control of the extent of supercoiling. It does not seem likely that supercoiling distributed evenly over a large domain could give rise to significantly large superhelical densities, but small topologically isolated subdomains might allow high densities, as suggested by Felsenfeld for transcription through histone octamers in model systems, and by Garrard for transcription in yeast (although by quite different mechanisms).

A major theme of this meeting has been the identification of mechanisms for activating promoters packaged in chromatin. This brings up the old issue of whether packaging in chromatin is in some sense irreversible at least during the lifetime of the cell, or whether chromatin structure is labile and disruptible. The former leads to pre-emptive models of regulation, in which gene activation involves competition of the transcription factors and the histones for sites on DNA, presumably during replication, the winner being the first component to bind. The latter implies a dynamic model in which favorable free energy states may be achieved by displacement or rearrangement within the nucleus. This is in principle always possible, since every structure can be disrupted if enough energy can be expended.

Work described at this meeting suggests that in this respect the nucleosome and the 30 nm fiber are not unassailable fortresses. Studies of the PHO5 gene (Hörz) and of the binding of glucocorticoid receptor to chromatin (Beato) extend the work on these systems, which has already established that chromatin structure around promoters can be altered and genes activated *in vivo* without DNA replication. Further illustrative of the change in viewpoint about the inviolability of chromatin structure is Wolffe's description of the relative roles of histone H1 and TFIIIA in suppression of Xenopus oocyte 5S RNA expression. This seems much more like a competitive model than it does like the preemptive model proposed years ago to explain suppression of this gene during development, and reminds us that regulation of expression of the H1/H5 family of histones (Ruiz-Carillo) is likely in turn to affect expression of entire families of genes.

Nucleosomes can also participate in gene activation without major disruption. Beato suggests that the DNA wrapped around the nucleosome core actually allows for

accommodation of multiple transcription factors that are not all capable of binding simultaneously on linear DNA. One is reminded of a second role for the histone octamer suggested by Elgin in the case of the *Drosophila hsp 26* gene, that of bending the DNA in regions adjacent to it to bring into contact distant bound transactivating proteins.

These studies in vivo of the generation of active promoters have been paralleled by direct investigations in vitro of competition between transcription factors and histones for promoter binding sites. Here we are dealing in many cases with relatively simple equilibrium considerations. In systems investigated by Workman, short DNA carrying binding sites for transcription factors is complexed with histone octamers, and challenged with transcription factors under a variety of conditions. Consistent with equilibrium models, the nucleosome can be disrupted by a sufficiently high concentration of factors, and by lower concentrations of factors in the presence of competitors such as nucleoplasmin that are capable of binding histones. Such mechanisms are dependent on the size of the factor binding site, and its rotational position relative to the nucleosome surface. Though these may not affect significantly the overall free energy change of the dissociation reaction, they will affect its activation energy. Because each gene is unique in the disposition of its factor binding sites and its nucleosomes, it is becoming increasingly important to know for as many systems as possible the detailed positions of nucleosomes over promoters and the genes themselves. The approaches described by Thoma and by Di Mauro show us the proper way to do this. Of course the placement of nucleosomes on DNA is not entirely fixed: histone octamers are capable of limited spontaneous movement under appropriate ionic conditions (Bradbury) , and octamers can transfer out of the path of an advancing RNA polymerase without losing contact with DNA (Felsenfeld).

The relevance of simple competition models to the situation *in vivo* is certainly open to question, but that question is to a certain extent rendered irrelevant by the exciting discovery of ATP dependent mechanisms for disrupting nucleosome structure over promoters. The genetics and biochemistry (Cairns) of the SWI/SNF complex in yeast are opening the way to an understanding of this class of activation mechanisms. In extensions of the model systems described above, Workman showed that the yeast SWI/SNF complex can displace single histone octamers in an ATP-dependent reaction. What is lacking so far in this system is the gene specificity of SWI/SNF action that must certainly prevail *in vivo*. At a higher level of complexity, Kadonaga described the resistance to transcriptional activation of reporter genes packaged in 30nm fibers (core histones + histone H1), and the overcoming of this resistance by the action of a partly purified complex that appears to be distinct from SWI/SNF, but is ATP dependent.

The other underlying message of this meeting is that chromatin is not to be viewed as a static structure that is a passive participant in biological processes. As

Grunstein made clear, the histones are active participants in the biochemical processes leading to repression at *S. cerevisiae* silent mating type loci. In particular, histones H3 and H4, already shown by genetic analysis to be essential to that process, are now demonstrated by biochemical methods to interact specifically with the SIR3 and SIR4 proteins. In combination with detailed information about the sites of interaction between these histones and SIR proteins, and the localization of the complexes on the nuclear periphery, we have the beginning of a credible model for the inactivation process, and possibly a more generally applicable guide to the nature of heterochromatinization.

Chromatin reactivity of course includes reactions like histone acetylation. Variations in levels of acetylation have been known for some time to be associated with levels of transcriptional activation, and Crane-Robinson's studies (see above) with general antibodies to ε-N-acetyl lysine confirmed this idea. A somewhat more cautious conclusion emerges from Turner's work with individual antibodies to the various acetylated H4 species; he finds, at least for H4, that although heterochromatic regions are underacetylated, the active genes he has examined do not contain hyperacetylated H4. Despite this caution, there seems good reason to believe that acetylation and other modifications to histones are important in modulating chromatin activity. Other chromatin proteins also must play a role in modifying DNA and chromatin structure within the nucleus. Travers and Thomas both described studies of members of the HMG1 family of proteins that help us to understand how these proteins bind to DNA; work by Bustin on the very different HMG14/17 family also addressed problems of DNA binding and its relationship to function. Finally, we must not forget the ability of DNA containing special sequences to assume non- B conformations under superhelical stress, making it a potential target for regulation by proteins designed for that task (Azorín). It is important to continue searching for the presence of non-B DNA forms within the eukaryotic nucleus.

Although there is now reason to believe that chromatin structure at the level of the nucleosome or the 30 nm fiber may be fairly labile in the cellular environment, some 'higher order' structures may still be formed in a functionally irreversible way for the life of the cell or even of the lineage. Position effect variegation, parental imprinting and X chromosome inactivation are candidates for this category. The analysis of chromatin structure in the neighborhood of variegated genes, described by Elgin, is beginning to yield valuable information about the organization of centromeric and telomeric heterochromatin, and recent work (Orlando) on the function of polycomb group proteins in homeotic gene inactivation provides an important insight into mechanisms that may be related to the formation of heterochromatin. In the case of parental imprinting (Surani) there is ample reason to propose a role of DNA methylation in marking the DNA, but whether it is the only signal, or whether some other mechanism is responsible for transmitting marking information from one generation to the next, is much more uncertain. Efforts to identify some selective interaction between proteins such as histone H1 and methylated DNA (Campoy,

Thomas) seem important, because it is likely that DNA and the protein components of chromatin respond to mutual modification.

This conference has demonstrated the renewed vitality of chromatin studies. Chromatin is now viewed as an important and equal participant in the process of transcription (and of replication). Like other large complexes within the cell it is subject to chemical modification and structural change brought about by the action of other complexes designed specifically for that purpose, and to further the vital reactions of the cell. We can be certain that as the details of these interactions become clear, chromatin will be seen to be involved intimately in the biological activity of DNA within the nucleus.

List of Invited Speakers

Workshop on

CHROMATIN STRUCTURE AND GENE EXPRESSION

List of Invited Speakers

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Workshop on

CHROMATIN STRUCTURE AND GENE EXPRESSION

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27 Workshop on Ras, Differentiation and Development
Organized by J. Downward, E. Santos and D. Martín-Zanca. Lectures by A. Balmain, G. Bollag, J. Downward, J. Erickson, L. A. Feig, F. Giráldez, F. Karim, K. Kornfeld, J. C. Lacal, A. Levitzki, D. R. Lowy, C. J. Marshall, J. Martín-Pérez, D. Martín-Zanca, J. Moscat, A. Pellicer, M. Perucho, E. Santos, J. Settleman, E. J. Taparowsky and M. Yamamoto.

28 Human and Experimental Skin Carcinogenesis
Organized by A. J. P. Klein-Szanto and M. Quintanilla. Lectures by A. Balmain, F. F.

Becker, J. L. Bos, G. T. Bowden, D. E. Brash, A. Cano, C. J. Conti, G. P. Dotto, N. E. Fusenig, J. L. Jorcano, A. J. P. Klein-Szanto, E. B. Lane, H. Nakazawa, G. Orth, M. Quintanilla, D. R. Roop, J. Schweizer, T. J. Slaga and S. H. Yuspa.

29 **Workshop on the Biochemistry and Regulation of Programmed Cell Death.**
Organized by J. A. Cidlowski, H. R. Horvitz, A. López-Rivas and C. Martínez-A. Lectures by J. C. Ameisen, R. Buttyan, J. A. Cidlowski, J. J. Cohen, M. K. L. Collins, T. G. Cotter, A. Eastman, D. R. Green, E. A. Harrington, T. Honjo, H. R. Horvitz, J. T. Isaacs, M. D. Jacobson, P. H. Krammer, A. López-Rivas, C. Martínez-A. S. Nagata, G. Núñez, R. W. Oppenheim, S. Orrenius, J. J. T. Owen, M. C. Raff, L. M. Schwartz and J. Yuan.

30 **Workshop on Resistance to Viral Infection.**
Organized by L. Enjuanes and M. M. C. Lai. Lectures by M. Ackermann, R. Blasco, L. Enjuanes, M. Esteban, P. A. Furth, F. L. Graham, L. Hennighausen, G. Keil, M. M. C. Lai, S. Makino, J. A. Melero, K. Olson, J. Ortín, E. Paoletti, C. M. Rice, H. L. Robinson, J. F. Rodríguez, B. Roizman, J. J. Rossi, P. Roy, S. G. Siddell, W. J. M. Spaan, P. Staeheli and M. Del Val.

31 **Workshop on Growth and Cell Survival Factors in Vertebrate Development**
Organized by M. C. Raff and F. de Pablo. Lectures by Y.-A. Barde, H. Beug, Y. Courtois, A. Efstratiadis, F. Giraldez, J. B. Gurdon, T. M. Jessell, N. Le Douarin, G. Martin, A. McMahon, D. Metcalf, E. Mitrani, F. de Pablo, P. H. Patterson, M. C. Raff, M. M. Rechler, D. B. Rifkin, J. P. Thiery and M. L. Toribio.

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