Instituto Juan March
de Estudios e Investigaciones

124 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

The Regulation of Chromatin Functions

Organized by

F. Azorín, V. G. Corces, T. Kouzarides and C. L. Peterson

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M. Beato
A. Belmont
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Introduction
F. Azorín

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The main goal of this workshop was to discuss the recent advances occurring in the field of chromatin structure and function. In eukaryotic nuclei, DNA is found in the form of chromatin, the basic structural matrix of chromosomes, which originates from the tight association of DNA with histones and non-histones proteins. Chromatin constitutes the actual framework for all biological processes involving DNA as a substrate (i.e., transcription, replication, recombination, repair). Learning about the structure and function of chromatin is equivalent to learn how the hereditary information is organized and used by the eukaryotic cell. The basic structural organization of chromatin is by now well understood but how it adapts to changing chromosomal conditions and functions is just beginning to be unravelled. Chromatin is not a uniform static structure. On the contrary, different chromosomal regions show a differential degree of condensation and chromatin structure is remodeled during development, cell cycle progression and in response to its various functions.

The basic building blocks of chromatin are nucleosome core particles. At its simplest level, the chromatin structure of all eukaryotes is organized as long arrays of these histone-DNA complexes. Over the past few years information gleaned from the high resolution crystal structures of the histone octamer and of the nucleosome core particle have revolutionized our understanding of histone-histone interactions and DNA organization within the nucleosome. In addition, biophysical analyses of nucleosomal arrays have provided valuable insights into the dynamic nature of chromatin, as well as leading to the identification of histone domains and histone modifications that govern the folding of nucleosomal arrays into higher order chromatin structures. Even as we learn more about how chromatin is assembled, folded, and stabilized, several new types of enzymes have been identified over the past few years that use the energy of ATP hydrolysis to disrupt chromatin structure. These "chromatin remodeling machines" are all complex, multi-subunit enzymes that appear to play key roles in gene transcription, development, and in the control of cell proliferation. Moreover, many enzymes are known to covalently modify histone tails. These include histone acetyl-transferases, deacetylases and methylases. The identification of so many enzymes raises a number of questions (a) how is their role distinct mechanistically; (b) is there regulation of enzymatic activity by, for example, post-translational modification or protein-protein interaction and (c) what is their biological function in the cell, relative to cell proliferation and differentiation. The workshop provided an up-to-date view into the

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mechanistic and regulatory properties that govern the in vivo and in vitro activities of several of these "chromatin remodelling and modifying enzymes".

The contribution of the structure of the chromatin fiber and the arrangement of the chromatin within the nucleus in the control of gene expression was also reviewed. The association of chromatin with specific proteins that seem to form large multiprotein complexes might alter chromatin structure and therefore transcriptional activity. A similar effect could be the result of the association of chromatin with specific RNAs, or protein-mediated interactions with homologous sites located in a different chromosome. Recently, a combination of molecular and cell biological approaches has allowed the establishment of a correlation between nuclear organization and gene expression. The composition and organization of this structural framework are beginning to emerge. Sequences involved in organizing the DNA within the nucleus have been isolated and proteins that interact with these sequences are being characterized. A general characteristic of eukaryotic chromatin is the presence of highly condensed heterochromatic chromosomal regions. Heterochromatin regions are usually located near the centromeres and telomeres and they appear to have the same properties in nearly all plant and animal species. Despite extensive data on heterochromatin, its biological significance has remained elusive and it was only until recently that specific heterochromatin elements and functions are begininig to be understood, mainly through the contributions of studies performed in yeasts (*S. cerevisiae* and *S. pombe*) and in *Drosophila*. Different aspects of heterochromatin structure and function were also addressed during the workshop.

Fernando Azorín

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Session 1: Chromatin structure and remodeling
Chair: Craig L. Peterson

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DNA conformation in the nucleosome core particle at high resolution

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The fundamental building block of chromatin is the nucleosome core comprising B-form DNA wrapped in $1^2/_3$ superhelical turns around the histone octamer protein complex. The crystal structure of the nucleosome core particle containing a 147 bp DNA (NCP-147) has been determined at 1.9 Å resolution and refined to an R-value of 20.8% (R_{free}=27.5%). The weakly diffracting NCP crystals necessitated merging data from 44 crystals collected at an intense synchrotron source to yield a complete data set to this resolution. The DNA of the NCP-147 is considerably better ordered than previous constructs containing 146 bp[1,2], which has allowed for the first time a detailed understanding of the conformational properties and solvation of DNA in chromatin as well as a highly accurate picture of the direct and water-mediated histone-DNA contacts. The nucleosomal DNA is not bent uniformly, but rather displays a variety of specific effects dependent on major/minor groove orientation with respect to the histone octamer, on sequence-dependent DNA bending and on the configuration of each histone-DNA binding site. For example, whereas bending into the major groove is always continuous, bending into the minor groove can also be discontinuous or kinked depending on the local DNA sequence. Our analysis reveals general mechanisms for DNA-wrapping on the nucleosome core and provides insight into the basis of sequence-dependent positioning and stability of the nucleosome.

Two nucleosome core particle structures containing different 146 bp DNA constructs (NCP-146[1,2] and NCP-146b) contain distinct regions in which the DNA is relatively unbent and overtwisted between flanking histone-DNA binding sites, yielding an effective advancement of the DNA setting by 1 bp. Such alternative fitting may increase binding stability by 'fine tuning' DNA-positioning requirements of adjacent segments of the DNA in a single nucleosome core. Comparison with empirical double-helix twist values suggests that this phenomenon is commonplace in chromatin. NCP-146 and NCP-146b also serve as structures containing trapped-intermediate DNA implicated in a "twist diffusion" mechanism for histone octamer movement along the DNA double helix. Comparison of NCP-146b with NCP-147 (43% DNA sequence identity) suggests how the nucleosome core can accommodate a vast spectrum of different DNA sequences within a relatively narrow range of binding energies.

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Sir3-dependent assembly of supramolecular chromatin structures *in vitro*

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Higher order chromatin structure is generally described in terms of folding of the 10 nm filament into the 30 nm fiber. The core histone tail domains are essential for the formation of 30 nm fibers, whereas linker histones stabilize the highly folded 30 nm state. Because of the extensive involvement of the core histone tail domains in chromatin folding, we have focused our attention on structural changes that occur to the chromatin fiber stemming from interactions with chromatin-associated proteins that bind the tail domains. One such protein is the yeast silencing protein, Sir3p. In the present study, baculovirus-expressed recombinant Sir3p (rSir3p) has been purified to near homogeneity, and its binding to naked DNA, mononucleosomes, and nucleosomal arrays characterized *in vitro*. At stoichiometric levels rSir3p interacts with intact nucleosomal arrays, mononucleosomes, and naked DNA, as evidenced by formation of supershifted species on native agarose gels. Proteolytic removal of the core histone tail domains inhibits, but does not completely abolish, rSir3p binding to nucleosomal arrays, suggesting that both the tail domains and DNA mediate rSir3p-chromatin interactions. Furthermore, the linker DNA of the nucleosomal arrays remain freely accessible to restriction endonuclease cleavage after assembly of the supershifted complexes. These results indicate that rSir3p cross-links individual nucleosomal arrays into supramolecular assemblies whose physical properties transcend those of typical 10 nm and 30 nm fibers. Based on these data we propose that Sir3p functions, at least in part, by mediating reorganization of the canonical chromatin fiber into specialized higher order chromosomal domains that are functionally repressive to transcription.

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Chromatin remodeling machines: contending with chromatin condensation

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Regulation of eukaryotic gene expression requires ATP-dependent chromatin remodeling enzymes, such as SWI/SNF, and histone acetyltransferases, such as SAGA. Recently we have shown that SWI/SNF remodeling controls recruitment of SAGA's HAT activity to many genes in late mitosis and that these chromatin remodeling enzymes play a key role in controlling late mitotic gene expression. In these initial studies we proposed that SWI/SNF and SAGA are globally required for mitotic gene expression due to the condensed state of mitotic chromatin (Krebs et al., 2000). We have begun to identify mutations that allow mitotic gene expression in the absence of SWI/SNF or SAGA. We find that inactivation of the HMG1-like protein, SIN1, or Sin mutations in histones H3 and H4 alleviate the need for chromatin remodeling enzymes in mitotic gene expression. We have used recombinant wildtype and sin- histone octamers to investigate how sin- histones alter the structure of nucleosomal arrays. Our preliminary results indicate that Sin- alleles of histone H4 do not alter the assembly of nucleosomes or disrupt the ability of the histone octamer to organize nucleosomal DNA. However, nucleosomal arrays that harbor a sin- histone H4 allele are defective for salt-dependent compaction of nucleosomal arrays. These results support our view that one key role for chromatin remodeling enzymes *in vivo* is to promote localized decondensation of chromatin domains rather than the disruption of individual nucleosomes.

To date, the majority of the mechanistic studies of chromatin remodeling enzymes have been performed with mononucleosome substrates or nucleosomal arrays. In each case, these substrates are presented to the enzymes as relatively de-condensed structures. To determine how SWI/SNF-like enzymes contend with stably folded chromatin, we have monitored the activity of multiple ATP-dependent chromatin remodeling enzymes on nucleosomal arrays that harbor a linker histone, H5. Such chromatin arrays are stably folded into ~30 nM structures in buffers containing divalent cations. Surprisingly, the remodeling activity of ySWI/SNF, hSWI/SNF, xMi-2, or xACF remodeling complexes are dramatically inhibited by H5 incorporation. Furthermore, the linker histone-dependent inhibition of chromatin remodeling is not due to chromatin folding, since H5 still blocks remodeling of arrays assembled with trypsinized core histones. We also find that phosphorylation of linker histone with recombinant Cdc2/cyclin B kinase alleviates the repression of remodeling activity. We propose that linker histones inhibit ATP-dependent chromatin remodeling by constraining the topology of nucleosomal DNA, and furthermore, that alleviation of this repression will require the prior action of a CDK kinase.

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Regulation of chromatin structure by SWI/SNF and Polycomb

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The polycomb-group (Pc-G) genes are required to maintain repression of homeotic genes during development. Mutations in these genes are suppressed by mutations in genes of the SWI/SNF family of ATP-dependent remodeling complexes. These genetic studies raise the possibility that the stable maintenance of gene expression during development involves, in part, a competition between the Pc-G and SWI/SNF remodeling complexes.

We have purified complexes of the SWI/SNF family from human cells and have reconstituted the chromatin remodeling activities of these complexes following overexpression of cloned subunits. We use these subunits to investigate the mechanism and regulation of ATP-dependent remodeling. We find that remodeling by the BRG1 protein creates multiple different remodeled states from a single nucleosome. These experiments suggest to us the hypothesis that SWI/SNF remodeling complexes function in regulating gene expression by continuously interconverting nucleosomes between distinct remodeled and non-remodeled states. Thus, they appear analagous in mechanism to protein chaperones. This provides a window of access for binding by regulatory fractors to 'lock in' specific states.

An epitope-tag strategy was used to purify a complex from Drosophila that contained the products of the Pc-G genes *polycomb* (PC*)*, *posterior sex combs* (PSC), *polyhomeotic* (PH) and dRING1. The complex, termed PRC1 (Polycomb Repressive Complex 1),contains between 20 and 30 proteins and migrates at greater than 2 MD on sizing columns. PRC1 blocks the ability of SWI/SNF to remodel nucleosomal templates but does not block access of proteins such as MNase and restriction enzymes. PRC1 inhibits transcription by both RNA polymerase II and T7 RNA polymerase. Microsequencing of the components of PRC1 demonstrates that several proteins known to be involved in other complexes, including TAFs, are components of PRC1. A complex from HeLa cells similar to PRC1 has been purified, and is smaller (approximately 7 proteins) but has the same repressive activities as PRC1 purified from Drosophila embryos. Human homologs to PC, PH, PSC and dRING1 make up the bulk of human PRC1. Many of the activities of PRC1 can be recapitulated with a complex reconstituted from cloned subunits of PC, PH, PSC and dRING1, implying that this set of proteins constitutes the functional core of PRC1.

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Mechanism of SWI/SNF remodelling *in vitro*

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Manipulation of chromatin structure by ATP-dependent remodelling activities is an important step in gene regulation. These complexes appear to be recruited to promoters to create the chromatin setting necessary for transcription and other nuclear processes. Recent work with mononucleosomes shows that remodelling can change DNA wrapping around the histone octamer core, and slide DNA along the histone octamer surface. We are attempting to dissect the detailed mechanism of this process by both functional and structural investigations.

- SIN point mutants of core histones confer SWI/SNF independence on the activation of certain genes. Comparison of wild-type and SIN mutant-containing nucleosomes *in vitro* show the latter are more translationally mobile. This suggests that SWI/SNF-deficient cells compensate for the absence of the chromatin remodelling activity by tolerating increased fluidity in nucleosome positioning.

  The same elevated mobility is also exhibited for ATP-dependent remodelling of nucleosomes containing SIN mutant histones, suggesting that thermal and catalysed nucleosome mobilisation occurs by a similar mechanism. As well as highlighting structural features responsible for stabilising nucleosomes, the results suggest the rate-limiting step for catalytic remodellers could be at specific DNA-protein interactions.

- The common sequence feature of all ATP-dependent remodellers is an essential helicase-like motif. Using alignments with more distantly related sequences in the helicase-like superfamily, and with three dimensional structures of family members, we have developed a general model for the organisation of this core SNF2 helicase-like ATPase domain.

Taken together with other work within our laboratory on the enzymatic activity of SWI/SNF, these results support and extend a general mechanism by which ATP-dependent remodelling complexes alter nucleosome structure via topological stress.

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Session 2: Histone modifications
Chair: Tony Kouzarides

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Snf1 is a histone H3 Ser-10 kinase required for Gcn5-mediated histone acetylation of the *INO1* promoter

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Gene expression is regulated through modification of chromatin structure by regulatory multisubunit protein complexes. Previously we showed the intimate functional connection between histone H3 phosphorylation and acetylation on regulation of transcription. Using conventional chromatographic techniques, we have isolated a predominant histone H3 kinase complex from yeast. Mass spectrometric sequencing has identified Snf1 as the catalytic subunit of this complex. The nuclear kinase Snf1 was previously known to function as a transcription factor, to induce transcription of several genes, including *INO1*, that are also regulated by the histone acetyltransferase Gcn5. We have characterized Snf1 activity, both *in vivo* and *in vitro*, to begin to understand its role as a histone kinase, and how it functions in concert with Gcn5.

As either a recombinant protein or when purified from extracts, Snf1 specifically phosphorylates Ser-10 of free and nucleosomal histone H3. Snf1 up-regulates *INO1* following derepression of the inositol biosynthetic pathway, and an S10A substitution in histone H3 causes reduced transcription. The level of phosphorylated histone H3 increases in concert with rising *INO1* transcription, but not in a strain disrupted for Snf1. Analysis of histone H3 modifications directly at the *INO1* promoter shows that dual Ser-10 phosphorylation and Lys-14 acetylation occurs, and is dramatically lowered by disruption of either Snf1 or Gcn5 or the substitution S10A in histone H3. Strikingly, histone H3 Lys-14 acetylation at the *INO1* promoter is greatly reduced by loss of Snf1 or substitution of H3 S10A. These results show that phosphorylation of histone H3 Ser-10, mediated by Snf1, is required for acetylation of Lys-14. While the S10A substitution mutation also lowered Lys-14 acetylation at the *HO* promoter, the *snf1-* disruption had little effect. Thus, phosphorylation is linked to acetylation at multiple promoters, resulting in gene activation, but there are distinct kinases required at different promoters. Our results identify the first linked histone kinase/acetyltransferase pair required for gene activation. Finally, we believe, based on our results and recent results describing a role of histone H3 Lys-9 in gene silencing, that Lys-9 methylation/Ser-10 phosphorylation acts a critical switch in histone H3 for the silenced vs. activated state of gene transcription.

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Histone methylation and transcriptional silencing

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The modification of histones, most notably by acetylation, can regulate the transcriptional activity of a gene. However, the effects of distinct modifications such as lysine methylation are less understood. Several lysines are known to be methylated on histone H3 and histone H4, but only one lysine methylase is known, Suv39H1. This enzyme is able to methylate lysine 9 of histone H3. We can show that this "methyl marker" on K9 of H3 generates a binding site for heterochromatin protein 1 (HP1), a mediator of transcriptional silencing at heterochromatin sites. Thus, methylation is defined as a process which sets up the heterochomatically repressed state and HP1 is the protein that executes this silencing.

This model for repression is not unique to hereochromatin but also takes place at enchromatic sites. We have evidence that the Retinoblastoma protein RB recruits the SuvarH1 methylase to the E2F-regulated cyclin E promoter. This recruitment results in transcriptional repression via methylation of a specific nucleosome. RB also mediates the recruitment of HP1 which then binds specifically to the methylated nucleosome on the cyclin E promoter. We propose that histone methylation / HP1 association is a commonly used mechanism for transcriptional silencing.

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Transcriptional regulation by histone deacetylases

Eric Verdin

Histone deacetylases (HDACs) play key roles in regulating eukaryotic gene expression and chromatin structure. A highly conserved domain homologous to the yeast transcriptional repressors RPD3 and HDA1 is found in class I and class II HDACs. This domain is considered necessary and sufficient for enzymatic activity. We have studied the mechanism of HDAC enzymatic activity and have observed that the isolated HDAC domains of HDAC4 and HDAC5, two representative members of the class II HDACs, do not possess intrinsic enzymatic activity. We found that the catalytic domains of HDAC4 and HDAC5 independently interact with HDAC3, a class I HDAC, to form enzymatically active complexes. We further demonstrate that the transcriptional corepressors N-CoR and SMRT can simultaneously bind to both HDAC4 and HDAC3. In the case of HDAC6, another class II HDAC, no interaction with HDAC3 and no interaction with N-CoR and SMRT was observed. However, the protein contains two tandemly arranged HDAC domains. Separation of the two HDAC domains resulted in a loss of enzymatic activity. We propose that dimerization (homo for HDAC6 or hetero for HDAC4,5 with HDAC3) of HDAC domains is necessary to generate an enzymatically active HDAC protein.

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Dynamic interplay of transcription factors on MMTV chromatin

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Hormonal induction of the MMTV promoter is mediated by a regulatory unit including five binding sites for steroid hormone receptors upstream of a binding site for the ubiquitous transcription factor NF1amd two binding sites for the octamer transcription factor OTF-1. All receptor binding sites and the NF1 binding site are needed for efficient hormone induction, but the corresponding factors do not synergize on free DNA. *In vivo* the MMTV promoter is located on a phased nucleosome, which allows binding of the hormone receptors to only two of their five cognate sites and precludes binding of NF1. Hormone treatment results in rapid simultaneous occupancy of all five receptors binding sites and the NF1 site on the surface of a nucleosome-like particle (1). In *S. cerevisiae* expressing hormone receptors and NF1 as well as in *Drosophila* embryo extracts the functional synergism between hormone receptors and NF1 depends on positioned nucleosomes, but surprisingly does not require the proline-rich transactivation domain of NF1 (2, 3). On a truncated MMTV promoter, which does not exhibit well positioned nucleosomes, NF1 can access the promoter in the absence of added hormone receptors and the two proteins synergize in a process which depends on the proline-rich transactivation domain of NF1. In *Drosophila* extracts promoter-bound hormone receptors recruit the ATP-dependent NURF complex, which remodels the wild type MMTV chromatin and facilitates stable NF1 binding (3). In yeast ISWI1 and ISWI2 are not essential for MMTV induction and the SWI/SNF complex is most likely involved in hormone induced remodeling of MMTV chromatin. In none of the systems studied, namely mammalian cells in culture, *Drosophila* extracts or yeast, do changes in histone acetylation play a role in the initial steps mediating the synergism between hormone receptors and NF1. Once bound to DNA, intact NF1 or just its DNA binding domain stabilize an open conformation of the nucleosome by precluding its folding back to the closed conformation (3). The open nucleosome conformation favors full receptor binding, topological changes of the minichromosomal DNA (3), and the appearance of a nuclease hypersensitive site in yeast chromatin. Mechanistically this open conformation could be generated by partial dissociation of histones H2A/H2B dimers, since MMTV promoters sequences assembled on a tetramer of histones H3 and H4 bind hormone receptors and NF1 with high affinity (4).

As the *Drosophila* embryo extracts used for our experiments are free of linker histones, we studied the role of exogenous histone H1 on transcription of the MMTV promoter. Addition of histone H1 leads to an increase in the nucleosome spacing and a to a more homogeneous population of positioned nucleosomes, which makes the hormone responsive region less accessible for DNase I and micrococcal nuclease. As expected, this tighter chromatin structure lead to a reduction of the basal transcription observed when progesterone receptor or NF1 were added separately. Unexpectedly, however, the synergism between hormone receptor and NF1 was potentiated in the presence of histone H1, and the absolute level of transcription was also enhanced. On the truncated MMTV template without well positioned nucleosomes, histone H1 had a repressive effect and did not enhance the synergism between hormone receptors and NF1.

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Taken together these findings show that positioned nucleosomes participate in hormonal induction by mediating the reciprocal synergism between receptors and NF1 in a process involving ATP-dependent nucleosome remodeling. Our results with NF1 variants exemplify how the same transcription factor can act in different ways depending on the precise nucleosomal organization of the promoter sequences. In a promoter without positioned nucleosomes it cooperates with hormone receptors via its transcription activation domain, while on a promoter with well positioned nucleosomes it acts as an architectural factor that stabilizes an open nucleosome conformation.

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Activity of the catalytic subunit of the mammalian SWI/SNF complex is regulated by acetylation

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Following the identification of nuclear histone acetylases, several non-histone proteins have been identified as substrates for acetylation. Many of these substrates including p53, E2F1 and EKLF, are involved in the regulation of transcription. In the current project, we have examined possible acetylation of Brm, the catalytic subunit of the mammalian SWI/SNF complex that is directly involved in ATP-dependent chromatin remodeling. Examination of the Brm amino-acid sequence revealed several putative acetylation sites and in vitro acetylation experiments showed that the protein could be efficiently acetylated by P/CAF and to a lesser extend by CBP. Antibodies directed against acetylated lysines demonstrated that the protein is also acetylated in vivo. To test the effect of acetylation on Brm activity, we transfected mouse NIH3T3 fibroblasts with a Brm expression vector Then, we selected for clones stably expressing Brm in the presence or in the absence of sodium butyrate, an inhibitor of histone deacetylases. Under normal conditions, no or very few clones are obtained because of the growth arresting effect of Brm. In the presence of sodium butyrate, the number of clones was increased about 10-fold. Removal of the histone deacetylase inhibitor from the culture medium lead to rapid decrease of the number of cells expressing Brm. Adding back the inhibitor reactivated the expression of the SWI/SNF protein. A similar result was obtained when using tricostatin A, another histone deacetylase inhibitor. NIH-3T3-derived clones expressing a Brm protein mutated in two consensus acetylation sites located immediately downstream of the bromodomain, were no longer sensitive to histone deacetylase inhibitors. Taken together, these observations suggest that histone deacetylase inhibitors directly affect acetylation of Brm and that acetylation of the Brm protein leads to inactivation of its growth-arresting properties.

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Session 3: Chromatin regulators
Chair: Renato Paro

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Chromosomal elements conferring epigenetic inheritance

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During the development of an organism, mechanisms of pattern formation program cells for particular functions and structures. The program code is based on a cell-specific differential repression or activation of master control genes. Once established such codes need to be maintained over many cell divisions during the growth phases and eventually also during adulthood, a process termed "cellular memory". In this context, the stable and mitotically heritable inactivation of transcription (Silencing) becomes an important developmental function necessary to generate and to maintain defined gene expression patterns in determined cells. The proteins of the Polycomb group (PcG) control the permanent inactivation of program coding factors like the HOX genes. PcG proteins appear to inactivate their target genes by generating heterochromatin-like structures. Conversely, the proteins of the trithorax group (trxG) counteract the silencing role of the PcG and maintain the active expression state of the target genes. Thus, the maintenance of gene expression patterns is controlled at the levels of higher order chromatin structures. Chromatin components such as histones, through their modifications, and other chromatin proteins seem to be able to generate an "epigenetic code" that influences the processing of the underlying genetic information. We are studying and trying to decipher this epigenetic code in the fruit fly *Drosophila melanogaster* by assessing the molecular functions of the PcG and trxG proteins in the chromatin-based control of HOX gene expression.

Chromatin-based silencing mechanisms appear to have many evolutionary conserved features. Thus, their analysis in a genetically well tractable system like Drosophila serves as a paradigm for other epigenetic phenomena like the mammalian X-chromosome inactivation or genomic imprinting. In addition, there is increasing evidence that the missregulation of elements of cellular memory in adulthood can result in tumorigenesis in mammalian organisms. We would like to understand and to identify the common denominator involved in these apparently diverse processes. What creates and forms particular chromatin structures that maintain defined gene expression patterns? Is the DNA-encoded genetic information overlaid by an epigenetic code, and how is this code transmitted from one cell generation to the next?

I. Cellular Memory Modules

Using chromatin-IP (X-CHIP) methodology developed in the lab, we have identified several chromosomal elements of the bithorax complex (BX-C) through which the PcG and trxG proteins exert their functions. We have shown in a particular transgenic set-up that chromosomal elements, such as *Fab-7* controlling the Abdominal-B gene in the BX-C, are able to maintain a decision made early during embryogenesis through many mitotic cell divisions during development. *Fab-7* is switched from a silenced (PcG-controlled) to an activated (trxG-controlled) chromatin state by a pulse of embryonic transcription. Once activated the expression of a nearby reporter gene becomes independent of the primary transcription factor. Surprisingly, in the transgenic system, an active *Fab-7* state can be transmitted meiotically in a substantial portion of the progeny.

While we initially established the system using *Fab-7*, we have now demonstrated a similar maintenance function for two other elements of the BX-C, MCP and BXD. Thus, our

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results indicate that in genetic complexes containing master control genes like the homeotic genes, defined chromosomal elements act as "cellular memory modules " (CMMs) capable of memorizing active or repressed gene expression patterns over developmental time.

II. Epigenetic Marking and Switching of CMMs

PcG proteins show an extended binding profile at CMMs. At the molecular level we could demonstrate a highly specific binding of PC protein through its C-terminal part with *in vitro* reconstituted nucleosomes. Surprisingly, we found that also in the activated state PcG proteins are bound to the CMM. Conversely, trxG proteins are still bound to a repressed CMM. Thus, the interplay between PcG and trxG members appears to be vital for the proper function of CMMs. What defines a CMM, like*Fab-7*, to be in an active or in an inactive mode and how this tag is maintained over many cell generations, are questions we are currently addressing. We could demonstrate that hyperacetylated histone H4 tags an activated CMM, and this epigenetic mark is transmissible mitotically.

We are now assessing how CMMs become switched from an apparent silenced default state to an activated state. After embryogenesis the epigenetic state of CMMs become fixed and cannot be switched anymore. Thus, a specific event occurring during this early stage appears to switch a CMM. After passing a specific developmental threshold, the epigenetic state is fixed and clonally inherited in subsequent cell generations. TSA treatment of transgenic embryonic cells does not relieve silencing of CMM-controlled reporter genes. However, we have evidence that early transcription through the element might be important to change the overall chromatin structure and thus the epigenetic state of the CMM. We are testing how histone modifications at CMMs contribute as primary epigenetic tags and how such modifications can be inherited at DNA replication and at mitosis.

In a complementing approach, we study the role of CMMs in determination and trans-determination processes in *Drosophila* imaginal discs. So far we found that several segmentation genes possess CMMs that could be switched during development. We further showed that reduction or removal of Polycomb in imaginal discs increases the frequency of transdetermination, again suggesting a role of CMMs and Polycomb in keeping the activity state of regulator genes.

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Steps in the assembly of Polycomb complexes

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Polycomb complexes are assembled at their target site, the Polycomb Response Element (PRE) and maintain the silent state of promoters in the surrounding chromatin for distances of tens of kilobases. We do not know how the Polycomb complex prevents transcription and, although a number of PREs have been identified in different genes subject to Polycomb regulation, the mechanisms that recruit Polycomb Group (PcG) proteins and establish the repressed state is not understood. Immunoprecipitation experiments and biochemical purification of PcG complexes have shown that two different kinds of complexes exist in mammalian cells or in Drosophila embryos: one containing the ESC and EZ proteins and another containing PC, PSC and PH proteins, among others. Since none of these proteins bind directly to DNA, some other DNA binding proteins must, separately or in combination, act as specific recruiters of the PcG proteins. Two such potential recruiters are the GAGA factor and the PHO protein, the homologue of the mammalian YY1 factor.

We have studied the steps in the assembly of a repressive complex using fusion proteins in which the LexA DNA binding domain is fused to different PcG proteins to target them to a reporter gene. While LexA-PC, -PSC, -PH, -SUZ2 can recruit a silencing complex, LexA-PHO or -GAGA factor cannot and LexA-ESC can only do so if present at the earliest stages of embryonic development. Immunoprecipitation experiments using overnight embryonic extracts confirm that PC, PSC, PH and SUZ2 are associated together but not with EZ, ESC or PHO. At the same time, PHO, ESC and EZ are associated in these extracts. Nevertheless, the silencing established by the LexA fusion proteins requires the presence of a full complement of endogenous PcG proteins, implying that they must all be recruited to the reporter gene containing LexA binding sites. When we look at nuclear extracts prepared from very early embryos we find in fact that PC co-precipitates with EZ, ESC, PHO and GAGA factor. At this stage, however PSC is not yet involved and does not co-precipitate with the other proteins.

A separate line of investigation suggests a possible mechanism for PSC recruitment. In the polytene chromosomes of late third instar larvae, PSC is found at a set of about 80 sites, most of which correspond to the sites of PC or PH. In slightly earlier larvae, however, a much more general distribution of PSC is observed, at virtually all interband regions. This localization is not due to the more decondensed state of interbands since in puff regions PSC is still detected in a narrow band within the puff and co-localizes with promoter complexes such as TFIID. Overexpression of PSC in late larvae produces the same result. Dissection of the PSC protein shows that this generalized distribution is due to the RING domain, while the HTH domain is responsible for interaction with other PcG proteins. Preliminary results indicate that the RING domain of PSC co-precipitates with TFIID.

These results, together with other observations suggest a model in which the assembly of the repressive complex at the PRE occurs in steps. Two separate kinds of recruitment events occur at the PRE: one that assembles the ESC/EZ complex and may depend on PHO but probably also other DNA-binding proteins. A second, independent recruitment brings in PC, which then

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interacts with the ESC/EZ complex. A third kind of recruitment brings PSC to the promoter region, probably through direct interaction with promoter complexes. The PSC at the promoter then interacts with the PC complex at the PRE, possibly after events mediated by the ESC/EZ complex, and results in the functional silencing complex.

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The MCP silencer of the *Drosophila Abd-B* gene requires both Pleiohomeotic and GAGA factor for the maintenance of repression

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Silencing of homeotic gene expression requires the function of cis regulatory elements known as Polycomb Response Elements (PREs). The MCP silencer element of the *Drosophila* homeotic gene *Abdominal-B* has been shown to behave as a PRE and to be required for silencing throughout development. Using deletion analysis and reporter gene assays, we defined a 138 bp sequence within the MCP silencer that is sufficient for silencing of a reporter gene in the imaginal discs. Within the MCP138 fragment, there are four binding sites for the Pleiohomeotic protein (PHO) and two binding sites for the GAGA factor (GAF), encoded by the *Trithorax-like* gene. PHO and the GAF proteins bind to these sites *in vitro*. Mutational analysis of PHO and GAF binding sequences indicate that these sites are necessary for silencing *in vivo*. Moreover, silencing by MCP138 depends on the function of the *Trithorax-like* gene, and on the function of the PcG genes, including *pleiohomeotic*. Deletion and mutational analyses show that, individually, either PHO or GAF binding sites retain only weak silencing activity. However, when both PHO and GAF binding sites are present, they achieve strong silencing. We present a model in which robust silencing is achieved by sequential and facilitated binding of PHO and GAF.

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Large scale chromatin organization in relation to gene silencing and gene activity

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The interphase nucleus is highly compartmentalized, which is instrumental in the regulation of gene expression. Two major compartments can be distinguished. One contains compact chromatin and little or no RNPs (ribonucleoparticles: RNA-protein complexes). The other compartment is the interchromatin space, i.e. the nuclear space that is not filled with compact chromatin. This notion is based on electron microscopy and light microscopy observations, mainly on nuclei of higher eukaryotes (e.g. see Verschure et al. (1999) and Cmarko et al. (1999)) and is an extension of the interchromosome domain (ICD) model formulated by Thomas Cremer and coworkers (Cremer et al., 1993). What can be concluded from these studies is that essentially all chromatin, being 'heterochromatin' or 'euchromatin', is in a state of relatively high compaction. Mostly in the interchromatin space, the nucleus harbours a variety of other subnuclear compartments (e.g. nucleoli, nuclear bodies) each apparently facilitating the expression of specific classes of genes. For instance, Cajal (coiled) bodies are subnuclear domains that are associated with the histone gene cluster and with a variety of snRNA genes, whereas splicing factor compartments (or 'speckles') are storage sites for splicing factors, supplying genes with co-transcriptionally acting splicing components (e.g. see Misteli and Spector (1998), Schul et al. (1998a)). Together, large scale chromatin folding and subnuclear domains seem to a create a specific local micro-environment that inhibits, permits, or boosts expression of nearby genes.

In the past few years we have investigated the relationship between gene expression and nuclear structure in general, and large scale organization of chromatin in particular. To this end, we have analysed and compared in dual labelling studies the spatial distribution of chromosomes and chromatin (Cmarko et al., 1999; Verschure et al., 1999), a variety of components of the gene expression machinery (including sites of transcription (Wansink et al., 1993), transcription factors (Grande et al., 1997; Van Steensel et al., 1995) and hnRNP proteins (Mattern et al., 1999)), and a number of nuclear domains (Grande et al., 1996; Schul et al., 1996; Schul et al., 1998b; Schul et al., 1999).

Recently, we have extended these studies by asking the question where Polycomb group (PcG) proteins are located, which are involved in epigenetic gene silencing (D.Cmarko, A.P. Otte, P.J. Verschure, R. van Driel and S. Fakan, submitted). Unexpectedly, we found that these silencing factors are highly concentrated in the same perichromatin compartment that contains transcriptionally active genes. No PcG proteins were found inside compact chromatin domains. This supports the notion that PcG proteins act locally in the genome, rather than inactivating large chromatin domains. Evidently, PcG-silenced genes and transcriptionally active loci occur interspersed in the nucleus.

Based on these and other results, the following picture is emerging. Transcriptionally active loci are localized exclusively at the surface of the compact chromatin domains, i.e. in

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the perichromatin area. In this way, components of the transcriptional machinery, present predominantly in the interchromatin compartment, have immediate access to the regulatory sequences. Newly synthesized RNA is deposited directly into the interchromatin space, which contains all factors required for processing, packaging and transport through the interchromatin channels, for instance to the nuclear pores. Also epigenetically silenced loci seem located exclusively in the perichromatin compartment. These results raise the question what sequences are inside the compact chromatin domains. Does it contain genes that are silenced by mechanisms not involving PcG proteins, or are all coding sequences concentrated at the surface of the chromosomal fibre and is its interior made up of non-coding (junk) DNA? Experiments have been initiated to address this question. In my presentation I will give an overview of the of our recent experiments and present ideas concerning the functional organization of the interphase nucleus.

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Large-scale chromatin structure and dynamics

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Over the past several years, we have developed a method for labeling specific chromosome sites using the lac repressor/operator interaction. This method allows direct *in vivo* visualization of chromosome dynamics. In addition it can be combined with gene amplification to create labeled, amplified chromosome regions, ranging in size from chromosome bands to entire chromosome arms, with homogeneous folding patterns. Using immunogold labeling, the folding motifs of these specifically labeled chromosome regions can be analyzed by electron microscopy reconstruction methods. Here we review some of the progress made using this technology. We demonstrate large-scale chromatin fibers, representing a level of chromatin folding beyond the 30 nm chromatin fiber, within mammalian interphase nuclei. The compaction and orientation of these fibers is generally stable over a period of hours. Over a shorter time of seconds, rapid but constrained mobility is observed and over longer periods, a reproducible pattern of conformational changes occurs during cell cycle progression, including changes associated with DNA replication initiation. Despite the general, long-term immobility of chromosome regions, several examples of long-range motion within the nucleus of specific chromosome regions have been observed which are linked to distinct stages of the cell cycle. Targeting of transcriptional activators to these labeled chromosome regions results in dramatic unfolding of large-scale chromatin structure, which may be related to targeting of HAT and remodeling complexes to these chromosome regions; changes in intranuclear positioning are also observed. The mechanism underlying this large-scale chromatin remodeling is now being investigated for several activators. Mitotic chromosome condensation is accompanied by highly reproducible but dynamic positioning of specific sequences within the condensed chromosome. EM reconstructions of labeled chromosome regions of varying size during chromosome condensation / decondensation are in progress to reveal the underlying folding motifs for this large-scale chromatin structure.

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Session 4: Heterochromatin
Chair: Fernando Azorín

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Subcompartments and dynamics of chromatin domains in the yeast nucleus

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The higher-order organization of the eukaryotic nucleus is proposed to play a role in facilitating nuclear functions. We have examined nuclear organization in the budding yeast, *S. cerevisiae*, and tested its functional importance using yeast mutants and established assays for transcriptional silencing and replication.

Yeast telomeres are sites of transcriptional silencing and are clustered in 6 to 8 foci at the nuclear periphery. Live GFP-tagging shows that telomeres, compared to other genomic domains, remain relatively immobile until late G2 phase. In G2-M they are released from the nuclear periphery as mitotic division proceeds, and they reattach to the nuclear periphery prior to cytokinesis. The end-binding protein yKu, and Sir4p both play a role in attaching telomeres through partially redundant pathways. We have tested whether the association with the nuclear envelope is altered in strains mutated for Rpd3p, a major histone deacetylase in yeast: In fact, telomeric silencing is enhanced dramatically in *rpd3* strains and a tagged telomere shows a significant enrichment at the nuclear periphery. This increased association with the nuclear envelope is dependent on the integrity of the SIR complex, such that an *rpd3 sir2* double mutant shows a wild-type distribution of telomeres.

We have analyzed the subnuclear position of early- and late-firing origins of DNA replication in intact yeast cells using fluorescence in situ hybridization and GFP-tagged chromosomal domains. In both cases, origin position was determined with respect to the nuclear envelope, as identified by nuclear pore staining or a NUP49-GFP fusion protein. We find that non-telomeric late-firing origins are enriched near the nuclear envelope in G1 phase, when the late firing character is established. Although time-lapse movies of GFP-tagged origins show that yeast chromosomal origins are dynamic in G1, they have a highly constrained movement in S phase. This constraint is imposed by active DNA polymerase complexes and is reduced in the *orc2-1* mutant, which reduces origin efficiency. Telomeres and centromeres show contrained movement throughout G1 and S phases. The functional consequences of chromosomal mobility on will be discussed.

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Dissecting fission yeast centromere architecture via silencing

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There are two distinctive regions within fission yeast centromeres; the central domain is packaged in unusual chromatin lacking regularly spaced nucleosomes whereas the flanking outer repeats are coated in normal nucleosomal chromatin(1;2). Genes placed in either region of these centromeres are transcriptionally silenced (3;4). It is likely that centromere silencing reflects the assembly of a fully functional kinetochore over this centromeric DNA. Consistent with this, mutations that alleviate centromeric silencing over the outer repeats or central domain interfere with chromosome segregation (5-8).

Like other heterochromatic regions in other organisms the outer repeat nucleosomes at fission yeast centromeres are underacetylated on the N-terminal lysine residues of histones H3 and H4 (9). Silencing over the outer repeats is dependent on the activity of a histone H3 methylase (10-12), Clr4 (a Suv39 counterpart) and Rik1 (unknown function). Both of these proteins are required to allow the recruitment of the chromo-domain protein Swi6 (a HP1 counterpart) and another chromo-domain protein Chp1 to centromeres (7;13). *In vitro*, Swi6 binds directly to histone H3 only when methylated on lysine 9 (12). We are testing *in vivo* the importance of particular H3 and H4 N-terminal residues for centromeric silencing. The *chp1*, *clr4*, *rik1* and *clr4* genes are not essential for cell viability. Note that the formation of silent outer repeat chromatin does not just require nucleosomes and the above proteins since many other uncharacterised factors are known to be required (14). The Swi6 and Chp1 proteins are only associated with the outer repeats not the central domain, thus their effects on silencing reflects their distribution over the centromere (7).

The unusual chromatin coating the central domain contains the fission yeast counterpart, Cnp1, of the centromere specific histone H3-like protein, CENP-A (6). Our analyses suggest that there is little or no normal H3 associated with the central core. It is likely that most H3 is replaced by Cnp1 in central domain chromatin. In a recent screen we have identified mutations at seven loci (*sim 1 to 7: silencing in the middle of centromeres*) that specifically alleviate silencing within the central domain but not the outer repeats (A. Pidoux and R. Allshire, unpublished). Included amongst these are mutations in the gene encoding Cnp1 (*sim2*). Chromatin IP with anti-sera raised against the Cnp1 and Sim4 demonstrates that they are associated with the central core and not the outer repeats. This validates the screen as route to identifying novel components that act via the central core. The fact Cnp1 (CENP-A) and Cnp3 (a CENP-C homologue identified in the database) associate with the central core but not the outer repeats indicates that the *bona fide* kinetochore assembles over the central domain.

What is the role of the outer repeats and 'Swi6' Chromatin? One possibility is that outer repeat chromatin provides a favourable environment for the assembly of specialised Cnp1(CENP-A) kinetochore chromatin over the central domain. Once this kinetochore chromatin has been assembled it can perhaps self-propagagate in the absence of silent outer repeat chromatin.

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Centromeric chromatin in *Drosophila*

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Centromere function requires coordination of many processes including kinetochore assembly, sister chromatid cohesion, spindle attachment, and chromosome movement. We have shown that the presence of the *Drosophila* homolog of the CENP-A centromere-specific histone H3-like proteins (CID) is correlated with centromere function, since it is localized to minichromosomes that contain centromeric DNA as well as those that lack detectable centromeric repeats (neocentromeres). Protein inactivation studies demonstrated that CID-dependent kinetochore formation is required for multiple mitotic processes, including entry into mitosis, prometaphase congression, transition from metaphase to anaphase, and normal anaphase segregation. Simultaneous localization analyses showed that CID chromatin is physically separate from proteins involved in centric sister cohesion (MEI-S332), centric condensation (PROD), kinetochore function (POLO kinase) and heterochromatin structure (HP1). CID localization is unaffected by mutations in *mei-S332*, *Su(var)2-5* (HP1), *prod*, and *polo*. Conversely, the localization of POLO, Cenp-meta, ROD, BUB1 and MEI-S332, but not PROD or HP1, was dependent on the presence of functional CID. We conclude that the centromere and flanking heterochromatin are physically and functionally separable protein domains required for different inheritance functions, and that CID is epistatic to the recruitment of outer kinetochore proteins and a centric sister cohesion protein.

CID has also provided an entry point into studies of the composition and 3D structure of true centromeric chromatin. We performed 3D deconvolution microscopy and EM Tomography (collaboration with Marc Ellisman, UCSD) on CID stained metaphase chromosomes from *Drosophila* and human cells, which revealed that CID-containing chromatin formed a conserved cylinder at the primary constriction that extends through the entire depth of the chromosome, and does not appear to contain histone H3. To determine if the centromeric chromatin is composed of intermixed standard nucleosomes and CID containing nucleosomes, or if it is composed of a continuous stretch of CID containing nucleosomes, we simultaneously localized CID and H3 at high resolution on extended chromatin fibers. CID chromatin is present in a repeated structure, and each domain is separated by longer blocks of H3 chromatin, in both humans and flies. Biochemical analyses demonstrate that H3 and CID are not mixed in mononucleosomes, but can be present on the same polynucleosome fragments. The 2D and 3D results suggest that the DNA may spiral through the cylinder, or assume a looped structure, in both cases ensuring that H3 repeat units reside in the region between sister kinetochores while CID chromatin is present on the poleward face of the chromosome. These results demonstrate that centromeric chromatin is organized into a specific, conserved higher order structure. We propose that the function of this structure may be to 'present' centromeric chromatin to the outside of the chromosome, where it is free to assemble kinetochore components and attach to the spindle.

Finally, we have used two different nucleotide incorporation methods combined with indirect immunofluorescence to evaluate replication timing of centromeric chromatin. All *Drosophila* centromeres replicated asynchronously in mid to late S phase. Our *in vitro* and *in vivo* results indicate that incorporation of CID/CENP-A into newly duplicated centromeres

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does not depend on replication timing and argue against determination of centromere identity by temporal or physical sequestration of centromeric chromatin relative to bulk genomic chromatin. These results suggest that centromere identity is propagated in a replication-independent fashion, perhaps through the activity of a post-replicative chromatin assembly or loading factor that deposits new CENP-A nucleosomes in regions that already contain CENP-A.

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The association of multi KH-domain proteins with heterochromatin

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Vigilins are highly conserved proteins that have been found in all eukaryotic organisms analyzed to date, from yeast to humans. Vigilins contain multiple (14 to 15) tandemly organized KH-domains for binding to single-stranded nucleic acids. The *Drosophila melanogaster* vigilin, DDP1, is found associated with the pericentric heterochromatin at the chromocentre in polytene chromosomes. In addition, DDP1 is also detected at several sites in the euchromatic arms co-localizing with the heterochromatin protein 1 (HP1). During embryo development DDP1 becomes nuclear after cellularization being also associated with the condensed mitotic chromosomes. *In vitro*, DDP1 shows a strong affinity for the pyrimidine-rich strand of the centromeric *Drosophila* dodeca-satellite. The molecular basis of the chromosomal association of DDP1 will be discussed on the light of its nucleic acids binding properties and the structural characteristics of centromeric satellite DNA sequences.

In *Saccharomyces cerevisiae*, disruption of the vigilin gene, *SCP160*, affects telomeric silencing and results in an increased ploidy. Expression of DDP1 complements a *Δscp160* deletion. *Δscp160* cells are hypersensitive to the microtubule destabilising drug benomyl and temperature sensitive (ts). In *D. melanogaster*, over-expression of DDP1 early during embryo development is lethal and some anucleated cells can be observed in the domains over-expressing DDP1. Over-expression of DDP1 in the eye imaginal discs results in eyes of an altered morphology and reduced size. A mutant $ddp1^{15.1}$ allele was obtain by mobilizing a P-element (EP(2)2422) inserted at the second intron in the 5'UTR of *DDP1*. $ddp1^{15.1}$ corresponds to an hypomorphic mutation that is semilethal in homozygosis and in front of a deficiency uncovering *DDP1* (65 %-70 % lethality). Female scapers homozygous for $ddp1^{15.1}$ are sterile showing an altered ovary morphology and aberrant DDP1 distribution. Ectopic expression of the cDNA encoding DDP1 partially rescues the sterility of $ddp1^{15.1}$ / $ddp1^{15.1}$ females.

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The HP1 proteins interact with partner proteins through a conserved binding model

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Higher order organization of chromatin is believed to play a crucial role in defining domains of gene expression, replication and recombination in the genome. Little is known about the factors which mediate organization of chromatin beyond the nucleosome, although proteins in the HP1 family are excellent candidates. Interestingly, the HP1 proteins have been shown to interact with a wide variety of nuclear factors, among which are proteins involved in transcriptional repression (KAP-1), nuclear substructure (lamin B receptor, SP100) and chromatin assembly (CAF-1 p150 subunit). We have previously shown that the chromoshadow domain (CSD) of the HP1 proteins directly binds to a KAP-1 polypeptide in a 2:1 stoichiometry. We have extended this analysis to other HP1 binding partners and found that each of these uses the same short motif to bind to the CSD. We also used a tryptophan substitution in the human HP1alpha CSD that does not disrupt dimerization, but lies within a presumptive ligand-binding pocket observed through structural studies. This point mutation disrupts all interactions with partner polypeptides. Thus, each HP1 partner utilizes the same surface for binding, which was confirmed by competition experiments. This raises the possibility that in the nucleus, distinct HP1 complexes are formed, each with unique activities. We went on to test the relevance of HP1 interactions in vivo, by examining the SP100 protein and its subnuclear localization in ND10 or PML bodies. We foud that HP1alpha is also localized to ND10s and this property requires a PxVxL-like motif and nearby SUMO-modification in the SP100 protein. Thus, the interaction with SP100 may be one way to modulate HP1alpha activity by sequestering the HP1alpha protein into a unique nuclear compartment.

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Session 5: Chromatin and nuclear organisation
Chair: Victor G. Corces

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The establishment and maintenance of chromatin domain boundaries

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The structure of chromatin over transcriptionally active genes is distinctly different from the structure in condensed, heterochromatic regions. At the places where two different domains are adjacent, it may be necessary to prevent the encroachment of one on the other. In some cases this may involve establishing a barrier to advancing heterochromatin. In others it may be necessary to block the action of an enhancer in one domain on promoters in another. Work during the past several years has established the existence of insulator elements: protein-binding DNA sequences that are able to block distal enhancer action, prevent the extension of a condensed chromatin region into an active one, or both. We have identified a region (5'HS4) at the 5' end of the chicken beta-globin locus, that possesses both of these properties. It is a compound element with multiple DNA binding sites that contribute separately either to enhancer blocking or position effect protection. We have identified the component responsible for positional enhancer blocking as the DNA binding protein CTCF, which contains eleven zinc fingers. We have found CTCF binding sites functioning as enhancer blocking elements within a number of loci. Notably, we have identified four CTCF sites in the *Igf2/H19* imprinted locus in mouse (seven in human), which function to block enhancer activation of the *Igf2* gene in the maternally transmitted allele. Methylation of the DNA at these sites abolishes both their ability to bind CTCF and to insulate, explaining why Igf2 is expressed from the methylated paternally transmitted allele.

In separate experiments, we have shown that 5'HS4 elements can protect against position effects, though this does not depend upon CTCF binding. When a reporter gene surrounded by these elements is stably transfected into cell lines, uniform expression is observed among lines, and the expression level remains constant over 80-100 days in culture. In contrast, uninsulated lines show variability of expression and extinction of expression over time. We show that the presence of the HS4 insulator results in maintenance of high levels of histone acetylation over the gene.

In order to understand the changes in chromatin structure that accompany developmentally regulated expression of the globin gene family, we have carried out a high resolution analysis of histone acetylation levels over the entire globin locus as well as the upstream folate receptor gene and an intervening ~16 kb region of condensed chromatin. Measurements were made on cells and cell lines representing different stages of erythroid development. The changing patterns of acetylation suggest that both local and long-range acetylation mechanisms are involved. We observe peaks of acetylation that are independent of cell type, located over 5'HS4 at the 3' end of the condensed chromatin region, and also at an element marking the 5' end of this region. This suggests mechanisms to account for the position effect protection properties of the beta-globin insulator element.

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Chromatin insulators and nuclear organization

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Chromatin insulators interfere with enhancer-promoter interactions and can buffer transgenes from chromosomal position-effects. These properties can be accounted for by assuming that insulators organize the chromatin fiber within the eukaryotic nucleus, setting up higher-order domains of chromatin organization that allow independent transcriptional regulation of genes within each domain. In support of this model, we find that individual gypsy insulator sites appear to come together at the nuclear periphery, creating rosette-like structures that might represent independent chromatin domains. These domains are disrupted by global changes in transcription that take place during the heat shock response, suggesting a correlation between the organization imposed by the gypsy insulator and gene expression.

If insulators play a functional role in transcription by controlling changes in higher-order chromatin organization, there should be proteins that control the ability of insulators to establish these domains. In an effort to identify these proteins, we have carried out a genetic screen for modifiers of the ability of the gypsy insulator to interfere with enhancer-promoter interactions. We have identified several candidates and have characterized one in detail. Mutations in this gene are embryonic lethal; hypomorphic alleles result in a non-functional gypsy insulator. The gene encodes the Drosophila homolog of the mammalian SET oncoprotein. This protein is a component of INHAT, a complex that can inhibit histone acetylation *in vitro*. In addition, SET has been shown to be an inhibitor of phosphatase 2A (PP2A). The Drosophila SET protein is present at sites of phosphorylated histone H3 and absent from sites of acetylated histones H3 and H4, but it does not overlap with gypsy insulator sites. The Mod(mdg4) protein is absent from insulator sites in SET mutants, suggesting that SET might regulate the phosphorylation of Mod(mdg4) and this in turn controls the interaction between Mod(mdg4) and other components of the gypsy insulator.

We have studied in detail the role of SET and PP2A in histone H3 phosphorylation. Phosphorylated histone H3 is widely distributed on Drosophila polytene chromosomes and is especially abundant at sites of active transcription. After heat shock, when transcription of all genes is turned off and the heat shock genes are activated, phosphorylated H3 disappears from all sites on polytene chromosomes and is abundantly present at the heat shock loci. Incubation of the salivary glands with ocadeic acid, an inhibitor of PP2A, interferes with histone H3 de-phosphorylation after heat shock. The same results are observed in flies mutant in one of several PP2A subunits, suggesting that PP2A controls histone H3 de-phosphorylation. In agreement with this conclusion, SET is observed at the heat shock puffs at the same time as phosphorylated H3, and mutations in SET interfere with accumulation of phosphorylated H3 at the heat shock puffs. These results suggest a correlation between transcriptional activation and H3 phosphorylation. Furthermore, H3 phosphorylation is controlled by an interplay between SET and PP2A.

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Protosilencers and insulators in yeast subtelomeric regions

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We have shown that the budding yeast subtelomeric repeats contain two types of cis-acting modifiers of silencing : protosilencers and insulators. Protosilencers were initially defined as DNA elements incapable of establishing silencing on their own but that can cooperate with a silencer to locally promote the formation and maintenance of a heterochromatin-like structure. The subtelomeric core X sequence, which is present at all chromosome ends, complies with the definition of protosilencers. An ACS motif and an Abf1p- binding site participate to the silencing capacity of core X, and they cooperate in an additive manner. In addition, core X was found to bring about substantial gene repression only in silencing-conducive environment, in which a low level of silencing is already detectable in its absence. We propose that protosilencers play a major role in a variety of silencing phenomenon, as is the case for core X which acts as a silencing relay prolonging silencing propagation away from telomeres. Subtelomeric insulators (also called subtelomeric antisilencing regions or STARs) behave as boundaries to telomere-driven silencing and also allow discontinuous propagation of silent chromatin. These two facets of insulator activity, boundary and silencing discontinuity, can be recapitulated by tethering various transcription activation domains to tandem sites on DNA. Importantly, we show that these insulator activities do not involve direct transcriptional activation of the reporter promoter. These findings predict that certain promoters behave as insulators and partition genomes in functionally independent domains.

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Regulated chromosomal motion and its integration into 3-dimensional nuclear structures

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There are two contrasting views for the topography of interphase chromosomes within the *Drosophila* diploid nucleus. One approach using in-situ hybridization has emphasized the regularity and surprising rather specific positioning of genetic loci within the 3-dimensional nucleus. Recent data showing remarkable homologue symmetry and chromosome structure conservation made use of a newly developed 3-dimensional in-situ hybridization bar-code developed by Michael Lowenstein; This technique allows chromosome path statements for whole chromosome arms. An alternate view comes from recent studies that utilized a technique to mark *Drosophila* chromosomes in a site specific manner, in live cells, with LacO/GFP-LacI methodology. This approach, a collaboration with Dr. Andy Belmont, has shown two distinct levels of chromosomal motion with regulation of (at least) one of the levels in the nucleus. We will present an interesting model that unifies these two seemingly conflicting views of nuclear chromosome order.

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Nuclear architecture and genomic function

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Genes are precisely positioned in linear array along the chromosomes of eucaryotic cells. In contrast, little is known about how the genome is organized and functions within the three-dimensional architecture of the cell nucleus. Recent progress in the development of fluorescent in situ labeling techniques coupled with three dimensional microscopy and multi-dimensional computer imaging analysis, however, has enabled direct analysis of global properties of genomic organization and function. These studies demonstrated that chromatin is compartmentalized into chromosome specific territories in the cell nucleus and enabled the visualization of discrete sites of DNA replication, transcription and splicing factor rich domains (1).

Using these approaches, we measured an average of 1,100 DNA replication sites (RS) following a 5 min pulse of 3T3 mouse fibroblast cells in early S-phase (2). Each early S RS takes an average of 45 min to complete, contains 1 mbp DNA and is replicated at the same time in the next round of replication. Next, the spatial properties of DNA replication (RS) and transcription (TS) sites were examined by a simultaneous dual labeling procedure in permeabilized 3T3 cells (3). While replication occurs predominantly in transcriptionally active genes in early S, we find that the over 1,000 labeled RS sites are not simultaneously active in transcription. Instead, the individual RS and TS [approx., 2,000 per nucleus] are clustered into segregated zones of replication and transcription. The individual zones that contain either RS or TS are in turn arranged into spatially separate networks that extend throughout the nuclear interior (3).

We propose that the dynamic cell cycle expression of spatially distinct functional networks of replication and transcription forms the organizational basis for the coordination of genomic expression (1). In this view, nuclear zones dedicated to replication are dezoned following replication and re-zoned for transcription and vice-versa for transcription to replication rezoning. The spatial relationships of individual replication and transcription sites, as well as their organization into nuclear zones, were strikingly maintained following extraction of cells for nuclear matrix (1,2,4). This suggests an integral role of higher order nuclear architecture in the temporal regulation, spatial positioning and coordination of DNA replication and transcription in the mammalian cell.

To further examine the relationship of higher order chromatin organization to nuclear architecture, we studied the effect of nuclear matrix isolation on chromosome territory organization (5). Normal diploid human fibroblast (NHF1) cells grown on cover slips were extracted for nuclear matrix under conditions that maintain the genomic DNA intact. Characteristic territorial organization persisted despite the extraction of over 90% of the chromatin histones. Complete disruption of the internal structure of the nuclear matrix led to a corresponding disruption of the chromosome territories and the individual DNA replication sites.

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In conclusion, the genomic functions of replication and transcription in the cell nucleus are arranged in similar but mutually exclusive higher order domains of chromatin that range from individual chromatin loops (50-250 kbp) to individual sites (approx. 1 mbp containing several loops) to higher order zones containing an average of > 20 individual sites (> 20 mbp). These different levels of chromatin functional organization are likely highly regulated and are found, following extraction for nuclear matrix, in structures indistinguishable from those imaged in intact cells. The relationship between extraction for nuclear matrix and chromosome territory intactness, provides a potentially valuable approach for further defining the properties of higher order chromatin organization and nuclear architecture.

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Concluding Comments
Gary Felsenfeld

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It is interesting to compare our present state of knowledge with what we knew seven years ago, when I made the closing comments at a Juan March Workshop that dealt with similar subjects. We knew about SWI/SNF and histone acetylation, but we hadn't figured out, quite, how histone modifications could be properly targeted to specific genes, and we certainly couldn't see how higher levels of chromatin organization could be connected to function. The difference these seven years have made is in the details. We have gone from grand overall schemes to very specific mechanisms, different in some cases for each gene.

It seemed quite right to start the meeting with Tim Richmond's description of the nucleosome core particle high resolution structure. Ultimately a lot of the phenomena described during the meeting will have to be explained in terms of the local reactions of the component core histones, and the ability of the DNA on the nucleosome surface to be deformed. These issues were raised in three papers dealing with the mode of action of SWI/SNF complexes. Craig Peterson showed that SWI/SNF action did not noticeably distort the histone octamer, but did alter either DNA twist or writhe, since topological constraint blocked SWI/SNF action. Bob Kingston showed that SWI/SNF action resulted in creation of a variety of randomly perturbed structures, only a few of which are likely to be successful intermediates in some particular pathway of activation.

Andrew Flaus showed that *Sin* point mutants suppress SWI/SNF mutations by increasing the ability of octamers to slide, so that SWI/SNF is no longer needed. The details of SWI/SNF action and of other ATP-dependent disruptors of nucleosome structure - how they perturb that structure, its precise nature and its lifetime- are still subjects of investigation.

The other major class of reactions leading to chromatin activation - those in which histones are chemically modified - may seem deceptively simpler to analyze. But as we learned from a number of speakers, the concrete simplicity of a histone modification is compensated both by the increasing diversity of the known

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modifications and by the fact that the modifications in themselves tell us little about what they do. One important discovery is that not all of the myriad modifications are independent of one another. Shelley Berger described the major yeast histone H3 kinase, showed that Snf1 is the catalytic subunit, and further showed that it works together with Gcn5 at the INO1 promoter to phosphorylate ser10 and acetylate lys 14 of H3 respectively. The phosphorylation step is required for acetylation. The prospect that different genes require different kinases suggests that phosphorylation may be the significant first step in activation, and at least in these cases acetylation is only secondary. As in a number of other examples provided at this meeting, different histone modifications may either complement or oppose each other: Serine 10 phosphorylation inhibits methylation at lys 9 and vice versa.

The role of methylation at H3 lys 9 and lys 4 was thoroughly explored by Tony Kouzarides. The picture of lys 9 methylation leading to HP1 binding and silencing was extended by the demonstration that this mechanism is not limited to condensed chromatin regions, but is also found at the cyclin E promoter, where RB brings Suv39 H1 methylase and HP1 to a nucleosome on the promoter.

Our old idea of HP1 as a marker of 'permanently' condensed chromatin is certainly destroyed, and we are faced with new questions of how, in the cases of genes which are repressed by methylation of histones, the silencing can be reversed on relatively short notice.

Another striking result is the elucidation of the mechanism of activation associated with H3 lys4 methylation, which involves the displacement of the repressive NuRD complex from the H3 tail, where it is normally bound when lys 4 is unmethylated.

We can certainly expect that HP1 will have lots of friends to help it do its job - as Mark Lechner described for the interaction of KAP-1, SP100 and probably many

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others with the chromoshadow domain. And other factors such as Fernando Azorin's DDP1, which co-localizes with HP1, are also appearing.

This is particularly interesting because it revives an old story about proteins capable of binding single stranded nucleic acids, including DNA polypyrimidine stretches that might be encouraged by superhelical stress to open from the duplex state. There is an entire nearly forgotten literature on this subject.

We are not going to understand all of this without doing the hard work of enzymology. Eric Verdin explored the structure of histone deacetylases, and showed that deacetylase activity requires the pairing of the catalytic domains of HDAC4 or 5 with HDAC3 in a complex with N-CoR/SMRT, or the internal self-pairing of HDAC6 domains.

Although we constantly refer to 'histone' acetylases and deactylases, Christian Muchardt reminded us that there are other targets- in this case BRM, which is acetylated in vivo and can be a target of PCAF in vitro. *In vivo*, inhibition of BRM deacetylation removes the growth inhibitory effects of BRM.

Miguel Beato continued his long involvement with the MMTV promoter, one of the best systems for illuminating the interaction between chromatin structure, nucleosome positioning, factor binding and activation of expression. In the end, our generalizations will have to be tested in systems like this. Of particular interest was the identification of a hormone receptor footprint, with synergistic binding of the receptor and NF1, but only on chromatin templates.

As always at a chromatin meeting, the moment comes when we move from lower orders of structure, and an illusory feeling of security, to larger scale organization. Renato Paro told us about cellular memory modules, Polycomb response elements, and the propagation of active (or inactive) states through development. The requirement for transcription through the PRE reminds us of a whole area of growing

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importance: the role of such presumably non-coding transcripts not only here, but in such diverse phenomena as globin gene activation, X chromosome inactivation, and possibly some kinds of imprinting.

Dissection of the Polycomb system by Vince Pirrotta reveals a very complex series of recruitment steps in which different Polycomb Group components are delivered to the PRE and promoter at various developmental stages. The components required for recruitment of a silencing complex resemble those described by Bob Kingston for his Polycomb Repressive Complex. Ana Maria de Busturia examined the requirements for silencing by the MCP element (a PRE of Abdominal-B) and showed that both GAGA and Pleiohomeotic binding sites are required.

I note that many models which invoked wholesale changes in chromatin structure over long distances appear to be giving way to 'discrete site' models, in which regulatory action arises from binding of factors to a relatively small number of sites. Of course we can expect that some kinds of histone modifications (at least) will propagate outward from these sites as a consequence.

We now turn to very high levels of organization - or we assume it is organization. Before doing that it is worth while to recall Jeff Hansen's admonition on the first day that we should be thinking about proteins that can mediate long range interactions. The methods that he has been developing for studying Sir3 - mediated interactions among nucleosomal arrays are going to become increasingly important.

One good thing we can say about studying chromatin organization at the level of the nucleus is that at least it can't get any more complicated (though of course there is also the interaction between nucleus and cytoplasm). But real progress is now being made, thanks to wonderful new tools, both optical and biochemical. Roel van Driel found that both transcriptionally active genes and bound Polycomb group complexes are localized in the perichromatin compartment, leading him to suggest that this kind of local perturbation is all that distinguishes 'condensed' from uncondensed chromatin.

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Andrew Belmont showed us that there is real hope of getting beyond the '30 nm fiber' and distinguishing between, for example, radial loop models and hierarchical coiling models of superstructure. Susan Gasser introduced us to chromatin dynamics - after those movies it may be harder to think of a static nucleus (John Sedat's data support the same conclusions). In fact at every level, from factor binding to replication origin location, we are going to have to consider kinetics.

Of course our ideas of what 'heterochromatin' might be are changing, and it may be that the term is no longer useful. The centromere, that paradigm of the condensed state, turns out to be an exceedingly complex set of structures. As Robin Allshire and Gary Karpen reminded us, they are not there to provide variegating signals, but first of all to help establish functioning kinetochores. Robin Allshire showed that at least one role of heterochromatin formation was to provide a stable structure for cohesin interaction in order to assure proper sister kinetochore - spindle organization.

Gary Karpen described recent studies of Drosophila centromeres, and addressed the crucial question of what makes a centromere a centromere. It has been known that centromeric function can be assumed by arbitrary DNA sequences, and that these are marked by CENP-A. The discovery of a special structure involving interspersed blocks of H3 and CENP-A containing nucleosomes, and the demonstration that there is no fixed replication timing for these central centromeric regions, should help clear up a problem that has been around for a while.

At the end of the meeting we heard about the DNA sequence elements called insulators. These have been defined operationally in terms of their ability to function in two kinds of assays: the ability to block enhancer action in a positional manner, and the ability to protect reporters against position effects. In the case of vertebrates, it has been possible in at least one case to separate the two functions and show that they arise from different DNA sites and different associated DNA binding proteins (Felsenfeld). A search by Victor Corces for mutations affecting the insulator phenotype of gypsy in

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Drosophila led to identification of a role for H3 phosphorylation. A mutation in the homolog of mammalian SET results in loss of Mod(mdg4) protein from insulator sites where complexes are usually formed with Su(Hw) protein. SET is an inhibitor of phosphatase and appears to be involved in H3 phosphorylation mechanisms at heat shock puffs following heat shock.

Eric Gilson described a remarkable system for assaying insulators in yeast telomeres. This seems a very good way to get at barrier mechanisms, though as mentioned above there are other insulator functions that may involve different mechanisms.

At the end John Sedat described a different kind of chromosome movement within the nucleus, and reminded us again that sooner or later all our mechanisms must be placed in the context of the intact nucleus, and related to the specific positioning of chromosomes and quite likely to their specific motions as well.

Ron Berezney demonstrated some of these points quite nicely with experiments showing discrete segregated zones within the nucleus for replication and transcription, zones that are connected in separate networks within the nucleus.

Looking back at where we were seven years ago, I would say that we have gained an enormous wealth of detail about the mechanisms at the level of single genes and nucleosomes, so we no longer tend to attribute everything to mysterious higher levels of organization. Although there are still plenty of mysterious things to work on, we see that they will be explained by familiar kinds of molecular modifications and intermolecular interactions.

Gary Felsenfeld

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POSTERS

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The GAGA factor of *Drosophila* interacts with SAP18, a Sin3-associated polypeptide

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The GAGA factor of *Drosophila* is a sequence-specific DNA binding protein that is involved in a variety of different nuclear processes. GAGA has been shown to regulate the expression of some developmentally regulated and stress-induced genes *in vivo* (Wilkins and Lis, 1997). GAGA is encoded by the trithorax-like (Trl) gene, which is required for the normal expression of the homeotic genes (Farkas et al., 1994). The homeotic transformations observed in some Trl alleles suggest a role for GAGA in transcription activation. However, GAGA is also found associated with some silenced polycomb response elements (PREs) of the bithorax and antennapedia complexes (Strull et al., 1997). Here, it is shown that GAGA interacts with SAP18, a polypeptide associated with the Sin3-HDAC co-repressor complex. The GAGA-dSAP18 interaction was identified in a yeast two-hybrid screen. The interaction was confirmed *in vitro* by glutathione S-transferase pull-down assays using recombinant proteins and crude SL2 nuclear extracts. The first 245 residues of GAGA, including the POZ domain, are necessary and sufficient to bind dSAP18. In polytene chromosomes, dSAP18 and GAGA co-localize at a few discrete sites and, in particular, at the bithorax complex where GAGA binds some silenced polycomb response elements. When the dSAP18 dose is reduced, flies heterozygous for the GAGA mutation Trl67 show the homeotic transformation of segment A6 into A5, indicating that GAGA-dSAP18 interaction contributes to the functional regulation of the iab-6 element of the bithorax complex. These results suggest that, through recruitment of the Sin3-HDAC complex, GAGA might contribute to the regulation of homeotic gene expression.

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The THO complex is required to transcribe long as well as G+C-rich genes of *Saccharomyces cerevisiae*

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Transcriptional activity can increase genome instability, as it has been shown for recombination between direct repeats in S. cerevisiae, immunoglobulin gene rearrangements and deletions in bacterial plasmids.

A gene linking transcription and genome instability in *Saccharomyces cerevisae* is HPR1, as hpr1 mutants show both increased levels of recombination between direct repeats and chromosome loss, as well as strong transcriptional defects. Detailed characterization of hpr1 transcriptional defects has shown that the absence of Hpr1 causes impairment of transcription elongation. The intensity of such a transcription impairment depends on the transcribed DNA sequence. There is a close correlation between the reluctance of a DNA sequence to be transcribed in hpr1 cells and the ability of such a sequence to promote recombination when inserted between direct repeats(1, 2).

Hpr1 forms the THO complex in vivo with the products of THO2, MFT1 and THP2 genes (3). The absence of any of the four proteins confers similar phenotypes of transcriptional elongation impairment and hyper-recombination, indicating that the THO complex is a functional unit in gene expression and genome stability (3).

Although the importance of the THO complex for transcription and transcription-associated recombination is clear, the way how it controls these processes remains obscure. As the relevance of the THO complex in transcription depends on the transcribed DNA sequence, the investigation of the features that make transcription of a particular DNA sequence to depend on Hpr1 can provide some clues to understand the precise function of the THO complex. We have found that transcriptional impairment in hpr1 is mainly found in long transcription units as well as in DNA sequences with a high G+C content. We discuss the relevance of these results for the understanding of RNAPII-transcription elongation and the putative role of the THO complex.

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Mechanisms additional to the repression of *Xist* accumulation by *Tsix* are required for normal random X inactivation

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One X chromosome in each female embryonic cell is chosen at random to become coated by the Xist RNA and silenced. Tsix 1, a transcript anti-sense to Xist, presents a debated action on Xist and participates in choice through unknown mechanisms 2,3. A 65 kb deletion including the terminal Xist exons and the site of initiation of Tsix, resulted in the exclusive inactivation of the deleted X in differentiated female ES cells 4. Using a cre/loxP site-specific re-insertion strategy within the deleted locus, we have examined the role of both Tsix and the terminal exons of Xist. We first show that prior to inactivation the 65 kb deleted X is associated in undifferentiated ES cells with both increased Xist expression and diffusion of the transcript away from its site of synthesis. Restoration of Tsix, but not of the Xist's terminal exons, reverted to wild-type the steady-state level of Xist expression and the association of Xist RNA with its transcription site. At the onset of inactivation in differentiated ES cells, restoration of Tsix markedly reduced Xist RNA accumulation in cis, but totally failed to promote initiation of inactivation from the unmutated X. These results demonstrate that Tsix is a repressor of the steady state level of Xist expression, point to a role for Tsix in regulating Xist RNA diffusion from its site of transcription, and indicate that mechanisms additional to in cis repression of Xist by Tsix, are required for random choice. 1. Lee, J. T., Davidow, L. S. & Warshawsky, D. Tsix, a gene antisense to Xist at the X-inactivation centre .

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The chromatin remodelling protein ACF1 is targeted to pericentromeric heterochromatin during late S phase

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ACF1 is a component of both of the ISWI-containing chromatin remodeling complexes ACF and CHRAC which are involved in chromatin assembly *in vitro* (Ito, Cell 1997; Varga-Weisz, Nature 1998). ACF1 is essential for optimal chromatin assembly activity of ACF (Ito, Genes Dev 1999). The targeting of these factors to specific sites in chromatin is poorly understood. We show that ACF1 localizes to pericentromeric heterochromatin *in vivo* in mouse cells in a cell cycle-dependent manner. Heterochromatin-targeting of ACF1 is first observed during mid-late S-phase and co-localizes with regions of replicating pericentromeric heterochromatin. Treatment of cells with histone deacetylase inhibitor, TSA, abolishes this localisation. We suggest that ACF1 may be involved in the assembly of pericentromeric heterochromatin following DNA replication and/or in facilitating replication through chromatin and the modification of histone tails may influence this. In order to investigate more fully the possible roles of ACF1 in mammalian cells, we have expressed an ACF1-GFP fusion protein in mouse fibroblast cells. We are currently creating deletion mutants of this construct to remove regions of ACF1 that are highly conserved among related proteins. These include the WAC domain which has been shown to cause localisation of the N-terminal portion of mouse ACF1 to heterochromatin and the BAZ1 domain through which ACF1 interacts with ISWI. The effects of these mutant proteins on the viability of cells and in particular the ability of the cells to establish chromatin structure, replicate and divide will provide insights into the function of the wild type protein in mammalian cells.

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Transcription factor dosage affects higher order chromatin structure and transcription of a heterochromatic gene

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The mechanisms by which higher order chromatin structure is disrupted during gene activation were studied by targeting the integration of a λ5 gene into centromeric heterochromatin in transgenic mice. FISH analysis was used to visualise sequential changes in chromatin organisation that occur during gene activation. Relocation of the transgene to the outside of the heterochromatin complex is observed in fibroblasts in the absence of transcription and is dependent on the presence of a DNase I hypersensitive site. In pre-B cells where λ5 is normally transcribed, activation of transcription occurs in a stochastic manner which resembles telomeric silencing in yeast. The transgene remains closely associated with the centromeric complex when it is transcribed, indicating that relocation away from heterochromatin is not essential for transcriptional activation. Reducing the dosage of early B cell factor (EBF) results in a reduced frequency of localisation of the transgene to the outside of the heterochromatin complex in pre-B cells and also gives reduced levels of transcription compared to the endogenous λ5 gene. These results provide evidence of direct involvement of transcription factors in modulating higher order chromatin structure.

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The role of histones in DNA double-strand break repair in *S. cerevisiae*

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Double-strand breaks (DSBs) in DNA are potentially lethal lesions in cells, and growing body of evidence indicates that in multicellular eukaryotes, these are a key event in the development of cancer. Therefore, the ability of a cell to appropriately detect and repair DNA DSBs is of great importance. Because chromatin clearly plays a central role in DNA metabolism, we sought to determine how DNA DSB repair activities interface with, and perhaps modulate, chromatin in order to maintain genomic integrity. Here we show that yeast histone H2A is phosphorylated at Ser-129 in response to DSBs by the DNA damage response kinase Mec1. The phosphorylated motif is important for the cell's ability to survive in the presence of DSBs, and our data suggest that higher order chromatin structure is modulated upon phosphorylation. The C-terminus of histone H2A has been shown to be in the vicinity of the linker histone binding site. We therefore investigated the role of histone H1 in DNA DSB repair. Loss of H1 in a strain lacking H2A Ser-129 partially rescues the DNA damage sensitivity phenotype. Furthermore, overexpression of histone H1 over-sensitizes yeast to DNA damaging agents. These data suggest a model in which the linker histone is inhibitory to successful DNA repair, and that phosphorylation of histone H2A Ser-129 may function to facilitate DNA repair at least in part by disrupting interaction of the linker histone with the nucleosome.

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A global role for chromatin remodeling enzymes in mitosis

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Proper regulation of eukaryotic gene expression requires ATP-dependent chromatin remodeling enzymes such as SWI/SNF and histone acetyltransferases such as GCN5. These enzymes, when recruited to promoters by transcriptional activators, function to establish a local chromatin structure permissive to transcription. However, how these enzymes "remodel" chromatin to facilitate transcriptional activation is not well understood. We have shown that yeast SWI/SNF and Gcn5p are required for proper expression of a large set of genes in mitosis, including a cytokenesis factor EGT2, the cdk inhibitor SIC1, and a DNA replication factorCDC6 . Surprisingly, we also found a requirement for SWI/SNF and Gcn5p for induction of the GAL1 gene in cells arrested in mitosis, whereas induction of GAL1 in interphase is independent of both enzymes. Our results suggest that SWI/SNF and Gcn5p are globally required to overcome novel constraints to gene expression conferred by mitotic chromatin. Consistent with these transcriptional defects, we found that swi/snf and gcn5 strains exhibit deficiencies in the transition through mitosis. gcn5 strains accumulate in G2/M and are hypersensitive to overexpression of the B type cyclin Clb2p. When synchronized using a nocodazole block/release regiment, swi/snf strains show a delay in SIC1 and CDC6 expression and extension of G1 phase. We recently determined that deletion of SIN1, an HMG1 homolog in yeast, alleviates the Clb2p hypersensitivity of gcn5 strains and rescues the mitotic exit delay of swi/snf strains, suggesting that Sin1p is a key determinant of mitotic chromatin structure that confers the requirement for SWI/SNF and Gcn5p.

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The site of HIV-1 integration in the human genome determines basal transcriptional activity and response to Tat transactivator

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After viral entry into the host cell, the HIV genome is reverse transcribed, transported to the nucleus and integrates in the cellular genome. Because of the heterogeneity of chromatin, the provirus integration site could have dramatic effects on its transcriptional activity. Here, we have used an HIV-1-derived retroviral vector in which the green fluorescent protein (GFP) is under the control of the HIV promoter to generate viral particles and infect Jurkat cells. We selected 34 Jurkat clonal cell lines each containing a single integration of the HIV-1 vector. The promoter activity of the integrated LTR was examined quantitatively by flow cytometry with the GFP reporter. In the absence of the viral transactivator Tat, basal promoter activity was low in 80% of the clones. However, the remaining clones exhibited very significant basal expression with a 75-fold difference in expression level between the highest and lowest expressing clones. We demonstrate that differences in expression levels are due to the integration site and are not controlled by DNA methylation or histone acetylation. In the presence of Tat, transcription was activated in each clone, and an inverse correlation was observed between basal transcriptional activity and inducibility by Tat. The extent of the chromatin remodeling at the HIV promoter in each clone correlates with its transcriptional level and responds to the Tat transactivator. These observations demonstrate that the chromatin environment influences basal HIV gene expression and that the HIV Tat protein activates transcription independently of the chromatin environment. Furthermore, we propose that the absence of transcription associated with some proviral integration sites could lead to viral latency and persistence of the virus in the immune cells. Data supporting this hypothesis will be also presented.

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The function of bromodomains in the transcriptional co-activator TFIID

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The multi-subunit co-activator TFIID is generally required for Pol-II dependent gene expression. Little is known about the interactions between any of the general transcription factors and chromatin, however. The acetylation of histone proteins in chromatin, for example, has long been known to often correlate with an enhanced transcriptional activity, but a precise role and mechanistic explanation for this modification in transcriptional activation has remained elusive. Recently, we have shown that the double bromodomain module from the TAF250 subunit of TFIID specifically recognizes di-acetylated histone substrates [Science 288, 1422 (2000)], but cannot detectably bind to unacetylated histones. Our data suggested that the conserved double bromodomain module of the TAF250 protein may play an important part in the recognition of hyperacetylated nucleosomal DNA by TFIID. The bromodomains of TFIID may thus provide a mechanistic link between histone hyperacetylation and increased transcriptional activation. We will report on our progress in deciphering the precise role of the identified interactions between the bromodomains of TAF250 (and of other proteins) and acetylated chromatin. Specifically, we will report on our site-directed mutagenesis and substrate-specificity studies, as well as on our in-vitro reconstituted transcription assays on chromatin templates.

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Regulation of methionine adenosyltransferase gene expression by histone acetylation and DNA methylation in rat hepatocytes. Role of hepatocyte growth factor in liver regeneration

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Methionine adenosyltransferase (MAT) catalyzes the synthesis of S-adenosylmethionine, the main donor of methyl groups in the cell. MAT is the product of two genes: MAT1A and MAT2A. MAT1A is expressed in adult liver, while MAT2A is expressed in proliferating fetal hepatocytes, during liver regeneration and replaces MAT1A upon neoplastic transformation of the liver. We examined MAT1A promoter methylation status by means of methylation sensitive restriction enzyme analysis. Our data indicate that MAT1A promoter is hypomethylated in liver and hypermethylated in kidney and fetal rat hepatocytes. Immunoprecipitation of mononucleosomes from liver and kidney tissues with hyperacetylated H4 antibody and subsequent Southern blot analysis with a MAT1A promoter probe demonstrated that MAT1A expression is linked to elevated levels of chromatin acetylation and, by contrast, MAT2A shows a low level of acetylation. In human HepG2 lines, in which MAT1A is not expressed, the locus is also hypermethylated and its expression is reactivated after treatment with 5-aza-2'-deoxycytidine or the histone deacetylase inhibitor trichostatin. In a model of rat hepatocytes in culture we also demonstrate that MAT2A transcription is induced time- and dose-dependently by hepatocyte growth factor (HGF), a key factor in liver regeneration. In addition we show for the first time that HGF stimulates the acetylation of histones (H4) associated with MAT2A promoter. HGF effects were blocked in the presence of the tyrosine kinase inhibitor genistein. These effects of HGF may be responsible for the induction of MAT2A expression and acetylation of histones associated with MAT2A promoter that we also demonstrate in a model of liver regeneration.

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The role of histone acetylation on cell transformation

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Inside of the eukaryotic cells, the DNA is associated with histones to be packed into nucleosomes. The N-termini ends of histones can suffer different postranscripcional modifications such as acetylation (van Holde, 1988). Histone acetylation was described many years ago and it was correlated with transcriptional activation (Hebbes, et al., 1988, Turner and O'Neill, 1995). However, it has been recently when the mechanisms underlying this stimulation have been underscore, due to the identification of enzymes with histone acetyltransferase activity (HAT), such as CBP/P300, P/CAF (Bannister et al., 1996; Yang et al., 1996), etc. On the other hand, HAT enzymes are capable to acetylate other targets different than histones, (p53, Gu and Roeder, 1997), showing the relevance of this postranscriptional modification. In the present work we analyze the role of the histone tail acetylation on the process of cell transformation mediated by the SV40 virus protein, T antigen and by cellular oncogenes.

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ISWI chromatin remodelling activities are required for cell cycle regulated expression of *CLB2* and *SWI5*

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Isw1 and Isw2 are ATP dependent helicases that act individually or together to remodel nucleosomes at the promoters of a number of transcription units throughout the yeast genome (Kent 2001). Isw2 is required for meiosis and regulates nucleosome positioning in an Ume6-dependent manner (Goldmark 2000). Cells lacking Isw1 also show a defect in meiosis. Haploid cells lacking Isw1, Isw2 or both factors have an elongated cell morphology and invade rich medium. However, this invasive growth in independent of FLO11 suggesting that it is not controlled by the nutrient dependent MAPK and PKA signaling pathways. Misregulation, particularly reduced expression, of CLB2 results in an elongated cell morphology. Here we show the Isw2 is required for nucleosome positioning and expression of CLB2 and that this is mediated by the SFF transcription factor containing the forkhead proteins, Fkh1 and Fkh2.

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A new boundary activity maps at the locus control region of the mouse tyrosinase gene: insulation from chromosomal position effects in transgenic *Drosophila melanogaster* and mice

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Yeast artificial chromosome (YAC) type transgenes encompassing the mouse tyrosinase gene are expressed faithfully in mice, in a position-independent and copy-number dependent manner, totally rescueing the albino phenotype of recipient animals (1). The analysis of YAC-tyrosinase deletion derivatives in transgenic mice revealed the presence of a locus control region (LCR) at -12 kb (2). The absence of this LCR results in variegated tyrosinase expression, and abnormal developmental patterns of tyrosinase trangenes in skin, iris and retina (3, 4). An enhancer activity has been identified within the LCR by in vitro analysis. A fragment containing the DNaseI hypersensitive site (HS) located within this LCR efficiently transactivates luciferase reporter constructs in cells irrespective of the promoter used but in a cell-specific manner, whereas surrounding LCR sequences behave transcriptionally neutral. The enhancer activity is completely abolished when any of the two putative binding boxes for nuclear factors present in the HS are deleted (5). We have investigated the putative boundary activity of these sequences in experiments conducted in transgenic flies and mice. The mouse tyrosinase LCR insulates the expression of transgenic heterologous constructs from chromosomal position effects in *Drosophila melanogaster*. These experiments have been carried out using the white minigene reporter assay, with previously described insulator sequences as positive controls and an unrelated fragment from the tyrosinase locus as a negative control. Comparable experiments have been done in transgenic mice that are currently being analysed.

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Mechanisms of transcriptional activation by Rap1p

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Synergistic activation of yeast minimal promoters by Rap1p is allosterically modulated by the exact sequence of the DNA binding sites, being stronger for UASrpg sequences (ACACCCATACATTT) than for Telomere-like sequences (ACACCCACA CACCC). These differences were orientation-dependent, and did not apply for single sites (Idrissi et al., 1998, J. Mol. Biol. 284, 925-935). The results shown here are summarized as follows:

1.- Single and tandemly repeated UASrpg showed similar occupacy *in vivo*, despite their 10 to 20-fold difference in activation strength.

2.-Single sites were selectively repressed by histone repression as well as by a secondary control in the RNA polymerase II mediator complex.

3.-Functional differences between telomeric and UASrpg sites increased in SWI/SNF complex mutants.

4.-Deletions in the Rap1p C-terminal moiety affected differently UASrpg- and telomeric-based constructs.

5.- UASrpg and telomeric sequences are differently represented among Rap1p-regulated promoter families, suggesting a link between the activation mechanism and the coordinated regulation of the corresponding genes.

On the basis of these results, we propose that Rap1p activates transcription synergistically from UASrpg sites by an orientation-dependent activation domain, abd that this function is hindered by the C-terminal portion of Rap1p when complexed with telomeric sequences. The physiological significance of this allosteric modulation of Rap1p activation potential is discussed.

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Glucocorticoid receptor dependent chromatin remodeling and activation of the Mouse Mammary Tumor Virus promoter requires the DNA binding, but not the activation domain, of nuclear factor 1

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The mechanism underlying the synergism between transcription factors in eukaryotic gene expression is not fully understood. Here we show that on the mouse mammary tumor virus (MMTV) promoter the synergism between glucocorticoid receptor (GR) and nuclear factor 1 (NF1) does not involve the proline-rich transactivation domain (PRD) of NF1. In contrast, replacing the native hormone responsive region by a single receptor binding site generates a more accessible promoter and makes the synergism with GR dependent on the PRD of NF1. On the induced MMTV promoter, the DNA binding domain of NF1 is required for the detection of an open chromatin conformation as a DNase I hypersensitive region. We hypothesize that triggering the shift to an open chromatin conformation by activated GR is the rate limiting step in promoter activation, and that NF1 binding is required for the stabilization of this chromatin transition.

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Dimerization of the largest subunit of Chromatin Assembly Factor 1: importance *in vitro* and during *Xenopus* early development

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To date the *in vivo* importance of chromatin assembly factors during development in vertebrates is unknown. Chromatin Assembly Factor 1 (CAF-1) represents the best biochemically characterized factor promoting chromatin assembly during DNA replication or repair in human cell free systems. Here, we identify a *Xenopus* homolog of the largest subunit of CAF-1 (p150). Novel dimerization properties are found conserved in both *Xenopus* and human p150. A region of 36 amino acids required for p150 dimerization was identified. Deletion of this domain abolishes the ability of p150 to promote chromatin assembly *in vitro*. A dominant negative interference based on these dimerization properties occurs both *in vitro* and *in vivo*. In the embryo, nuclear organization was severely affected and cell-cycle progression was impaired during the rapid early cleaving stages of *Xenopus* development. We propose that the rapid proliferation at early developmental stages necessitates the unique properties of an assembly factor which can ensure a tight coupling between DNA replication or repair and chromatin assembly.

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Heterochromatin protein 1 interacts with BRG1 and BRM, the SNF2 homologs of the mammalian SWI/SNF complexes

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Heterochomatin protein 1 (HP1) is an evolutively conserved heterochromatin associated protein. *Drosophila* HP1 seems to be involved in both transcriptional silencing of locus close to the heterochromatin and transcriptional activation of heterochromatic genes. Mammals HP1-like proteins also affect transcription of endogenous or transiently transfected promoter. However, the mechanism by which HP1-like proteins affect transcription is unknown. We show that HP1a interacts with BRM (SNF2a) and BRG1 (SNF2b), the two SNF2/SWI2 homologues present in the mammalian SWI/SNF complexes. Pull down experiments showed the existence of two HP1a interaction domains in both polypeptides BRM and BRG1. The interaction is dependent on the HP1a chromodomain. Furthermore, GFP-tagged BRG1 variants can be recruited, by cotransfected HP1a, to pericentromeric heterochromatic regions. Interestingly , BRM is able to activate the RSV promoter in the presence of HP1a, but not when HP1a is absent. Our results suggest that HP1a may function in transcriptional activation by recruiting the SWI/SNF complexes.

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Drosophila polycomb group proteins are associated with dTAFII proteins in polycomb repressive complex 1

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In *Drosophila* the expression of the homeotic and other genes is controlled by the opposing actions of the Polycomb and trithorax groups of proteins. We have previously purified Polycomb Repressive Complex 1 (PRC1) containing a number of Polycomb group proteins from *Drosophila* embryos and shown it to antagonise chromatin remodeling by the SWI/SNF complex. The recent completion of the *Drosophila* genome sequencing projec has allowed us to identify the protein composition of the PRC1 complex using mass spectrometric based techniques. We show that Polycomb group proteins and TATA binding protein associated factors (dTAFIIs) constitute a large part of the *Drosophila* PRC1 complex. Additional components include proteins previously linked to the Polycomb group, proteins originally found in other chromatin modifying complexes, and the trithorax group DNA-binding protein, Zeste. This analysis establishes a connection between proteins required for stable maintenance of transcriptionally repressed states and proteins that associate with the general transcriptional machinery.

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Transcriptional repression by the retinoblastoma protein through the recruitment of a histone methyl transferase

Didier Trouche

The E2F transcription factor controls the cell cycle-dependent expression of many S phase-specific genes. Transcriptional repression of these genes in G0 and at the beginning of G1 by the Rb protein is crucial for the proper control of cell proliferation. Rb has been proposed to function through the recruitment of histone deacetylases. In agreement with that, histones on many E2F-regulated promoters evolve during G1 from a hypoacetylated to a hyperacetylated state. We show here that Rb also interacts with a histone methyl transferase, which specifically methylates K9 of histone H3. This strict specificity together with co-immunoprecipitation experiments from transfected cells, indicate that this histone methyl transferase is likely to be the recently described heterochromatin-associated protein Suv39H1. We also found that Suv39H1 and Rb co-operate to repress E2F activity and that Suv39H1 could be recruited to E2F1 through its interaction with Rb. Taken together, these data indicate that Suv39H1 is involved in transcriptional repression by Rb, and suggest an unexpected link between E2F regulation and heterochromatin. Finally, our results show that transcriptional repression by Suv39H1 involves histone deacetylases, suggesting a model in which histone methyl transferases and deacetylases function interdependently to repress E2F activity.

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Silencing at yeast subtelomeric regions: who is in?

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In *Saccharomyces cerevisiae,* the heterochromatin at telomeres spreads into subtelomeric regions and can silence the expression of reporter genes when they are transplanted near to their vicinity[1-4]. This transcriptional repression is conditioned by the strength of the promoters that are silenced, by the presence or absence of subtelomeric TAS (Telomere Associated Sequences) and by the distance to the telomeres[1,2,5,6]. Thus, silencing at natural subtelomeric regions is expected to depend on the nature of the specific subtelomeric genes and on their location. Hence the importance of identifying natural systems undergoing telomeric silencing. Such systems should be suitable models for the characterization of this kind of silencing and should help to understand its biological significance. Previous reports have described the existence of several transcriptional units that undergo telomeric silencing in its natural context[7-9]. Here, I report the silencing of genes that belong to the two major subtelomeric gene families from *S. cerevisiae*. These results are discussed.

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Axial skeleton deffects and Hox gene expression alterations reveal genetic interactions between the murine Ring1A and M33 Polycomb genes

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The products of the Polycomb group (PcG) of genes act as transcriptional repressors involved in the maintenance of homeotic gene expression patterns during development (1). Initially identified in *D. melanogaster*, both molecular and genetic evidence show that the PcG system is conserved throghout evolution (1). In our study of the mammalian PcG system we cloned two genes which encode the Ring1A and Ring1B proteins, by means of their interaction with M33 (2), an ortholog of the D. melanogaster Polycomb gene. We showed recently that Ring1A is a new member of the PcG of genes, although no mutant has been identified in *D. melanogaster*, yet. Mice lacking or overexpressing Ring1A showed homeotic transformations and other alterations of the axial skeleton (3). Transformation of vertebral identities in Ring1A-deficient mice, however, are anterior instead of posterior which are the usual transformations seen in other PcG mutant mice (4-7). In addition, the penentrance and expressivity of phenotypes in Ring1A +/- mice indicate a singular dependency on Ring1A gene dosage. Previous evidence in both *D. melanogaster* and mice shows genetic interactions between PcG products (8), which is consistent with their action as macromolecular complexes. Because of the unexpected phenotypes of Ring1A-deficient mice, we wished to study mice doubly deficient for the Ring1A and M33 genes. Here we report the analysis of axial skeleton of these mice, which shows stronger M33-related phenotypes, whereas Ring1A-specific alterations are almost lost. Analysis of Ring1A -/-;M33+/- and Ring1A +/- ;M33 +/- mice showed the dosage dependence of such interaction. However, these skeletal alterations were not accompanied by shifts in the mesodermal expression domain of Hox genes, at least in 11.5 dpc embryos. Instead, we observed anteriorization of the rostral domains of expression of some Hox genes in the neuroectoderm. Taken together, these results provide evidence that genetic interactions between PcG genes, such as Ring1A and M33, is more complex th.

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Identification of novel subunits of Elongator, a histone acetyltransferase complex involved in transcript elongation and a component of elongating RNA polymerase II

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Elongator is a histone acetyltransferase complex which occurs either free or associated with the elongating form of RNA polymerase II. Previously identified components of Elongator are p150/Elp1, the WD40 repeat protein p90/Elp2, and the catalytic subunit p60/Elp3.

To analyse the composition of Elongator in detail, we have purified the complex using epitope-tagged Elp1 and found that Elongator is a complex composed of six subunits. In addition to the three known subunits, we identified three novel components, p51/Elp4, p35/Elp5, and p30/Elp6. Elongator is composed of two sub-complexes: one consisting of the three largest and the second of the three newly identified subunits. Elp4, –5 and –6 are encoded by non-essential genes. Disruption of the gene encoding Elp4 results in a phenotype strongly resembling elp1, elp2 and/or elp3 mutant strains and include temperature and salt sensitivity and slow adaptation to changed growth conditions. So far, no additional defects were found in elp1 elp4 double mutants suggesting that the six subunit form of Elongator is a functional entity in vivo. We identified homologs of Elp4 in a variety of higher eukaryotes, suggesting that Elongator is structurally conserved from yeast to human. The purification of Elongator allowed us to characterise its biochemical properties. We find that Elongator is a histone acetyltransferase with a strong preference for histone H3 and -to a lesser extent- H4, while no acetylation of H2A and H2B was detected. Finally, we demonstrate that Elongator can bind to both naked and nucleosomal DNA.

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