Instituto Juan March
de Estudios e Investigaciones

138 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Regulation of Eukaryotic Genes in their Natural Chromatin Context

Organized by

K. S. Zaret and M. Beato

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Introduction
M. Beato and K. S. Zaret

Long strands of eukaryotic DNA are packaged into the nucleus of the cell by DNA wrapping around octamers of histone proteins, creating nucleosome core particles, and by further packaging of the nucleosome cores into chromatin. The organization of eukaryotic DNA in chromatin influences all processes requiring access to genetic information, including gene transcription, DNA replication, repair, and recombination. These processes are critical for normal cellular physiology and are frequently perturbed in disease states. The significance of chromatin structure for the regulation of gene expression is underscored by the discovery that numerous transcription factor co-regulators, as well as proteins that control DNA metabolism, modify the packaging of DNA in chromatin. Many of these co-regulators, and protein complexes thereof, modify chromatin packaging either by covalently altering the histone proteins or by ATP-dependent, nucleosome remodeling. Furthermore, proteins that modulate DNA-dependent functions are often sensitive to histone modification states. Relevant histone modifications include phosphorylation, acetylation, methylation, and ubiquitylation, each of which are associated with stable states of repression or activation, or the transition from one state to another. The biochemistry of these transcriptional co-regulatory complexes has been studied intensively, but their biological significance in the regulation of individual genes is poorly understood. This workshop focused on the biological context and cellular signficance of chromatin in gene regulation and other DNA-dependent functions.

To date, most studies in this field have focused on the potential role of transcriptional regulatory complexes in artificial contexts, rather than with physiological, regulatory pathways and native DNA sequence contexts. This distinction in approach is understandable, because assessing the biochemical parameters of regulatory proteins in highly controlled reactions is critical to define the proteins' mechanisms of action. On the other hand, evidence is emerging that in some cases, the artificial DNA sequence arrangements and templates studied *in vitro* are inaccurate models of the *in vivo* situation. For example, nucleosomes can be positioned precisely on certain artificial DNA sequences *in vitro*, but the same DNA sequences can exclude a nucleosome when introduced into native chromatin in the cell. Results such as these emphasize the need to understand how DNA sequences function in a native chromatin context. In addition, some parameters of native chromatin context, such as nuclear localization, cannot yet be reconstituted *in vitro*, yet are emerging as a significant influence on gene activity.

Recent technical developments, such as chromatin immunoprecipitation (ChIP) and high resolution *in vivo* genomic analysis, have opened new possibilities for addressing the role of chromatin structure and its modifications in the regulation of genes in their native sequence context in the cell. Results are beginning to accumulate showing that transcription factors and coregulator complexes may strongly depend on the precise organization of the target DNA sequences in nucleosomes and in higher order chromatin structures. In some systems, the packaging of DNA in cellular chromatin determines the nature of the interaction of regulatory proteins with their cognate *cis* regulatory elements and, thus, the outcome of the transcriptional response. Thus, detailed studies that accurately determine native chromatin structures in cells provide a framework for *in vitro* reconstitution of more native-like structures, for subsequent mechanistic analysis.

The aim of the proposed workshop is different from those of previous chromatin meetings. Our intention was to compare the results obtained by groups working with various natural genes in their native chromatin environment, in order to get a more realistic picture of the actual mechanism used by regulatory protein complexes to fulfill their physiological function. To this end, we included genetic and biochemical talks by 25 leading scientists, combined in 5 sessions covering different levels of complexity. The biological systems were from *S. cerevisiae, Drosophila, Tetrahymena*, the mouse and human, and mammalian viruses. Both *in vivo* and *in vitro* systems were described, with the latter based on chromatin structures observed *in vivo*. There was excellent discussion at the meeting, which helped to shed new light on the crucial aspects of chromatin function in physiologically relevant systems.

Miguel Beato and Ken S. Zaret

Session 1: Disruption of native chromatin during activation
Chair: Susan M. Gasser

Correlating in vivo promoter occupancy with cofactor requirement for chromatin remodeling at the yeast *PHO5* promoter

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Under conditions of phosphate starvation the *PHO5* promoter of *S. cerevisiae* undergoes a striking chromatin transition, whereby a chromatin microdomain consisting of four positioned nucleosomes is completely disrupted (Svaren and Hörz, 1997). How this remodeling event is achieved is not yet understood. It is assumed that Pho4, the main transcriptional activator of *PHO5*, recruits transcriptional coactivators to the promoter including ATP-dependent remodeling machines as well as histone acetyltransferases. Although Gcn5 is not required for full activation of *PHO5* the rate of *PHO5* activation is greatly decreased in its absence (Barbaric et al., 2001). This delay in transcriptional activation is specifically due to slow chromatin remodeling of the *PHO5* promoter, whereas the transmission of the phosphate starvation signal to the *PHO5* promoter progresses at a normal rate. Interestingly, other components of SAGA are alo required for *PHO5* activation, but their absence generates a very different effect. We have asked the question, which cofactors are recruited to the *PHO5* promoter upon activation and what the precise sequence of events during recruitment is. Chromatin immunoprecipitation (ChIP) analysis employing tagged versions of the Pho4, Ada2 and Snf2 proteins was used to determine the kinetics with which these factors bind to the promoter. Data on how the recruitment of one coactivator affects the recruitment of others is presented. The contribution and significance of SAGA and of histone acetylation for chromatin remodeling at the *PHO5* promoter is discussed.

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The *in vivo* dynamics of promoter topography during transcriptional regulation in *Saccharomyces cerevisiae*: the ADH2 and RPGs systems

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The involvement of chromatin structure and organization in transcriptional regulatory pathways has become evident. One unsolved question concerns the molecular mechanisms of chromatin remodeling during *in vivo* promoter activation. By using high resolution *in vivo* analysis we have explored the modifications occurring on two different promoters during the steps encompassed between derepression-activation and RNA elongation.

Alcohol dehydrogenase2. When the glucose content of the medium is lowered, yeast cells modify the positions of specific nucleosomes. In the ADH2 promoter, the nucleosomes -1 and +1 (respectively: the TATA and RIS nucleosomes) change the distribution of their positions by few nucleotides in the direction of transcription. Such repositioning does not occur in the absence of the ADH2 transcriptional activator Adr1 or in the presence of its DNA binding domain alone, and occurs even when the catalytic activity of the RNA polymerase II is impaired, suggesting that the Adr1 activation domain mediates the recruitment of some factors to correctly preset the relevant sequences for subsequent transcription steps. A detailed and exhaustive analysis of the effects of acetylations/deacetylations on the relevant positions of H2 and H4 on the whole activation process, and on the timing of recruitment of several components of the regulatory machinery (including TBP) is presented.

Ribosomal Proteins Genes. We have analyzed in detail the structure of RAPI-UAS_{RPG} complexes in *S. cerevisiae* cells using multi-hit $KMnO_4$, UV and micrococcal nuclease high-resolution footprinting. Three copies of the Rapl protein are simultaneously bound to the promoter in exponentially growing cells, as shown by $KMnO_4$ multi-hit footprinting analysis, causing extended and diagnostic changes in the DNA structure of the region containing the UAS_{RPG}. Aminoacid starvation does not cause loss of Rap1p from the complex; however *in vivo* UV-footprinting reveals the occurrence of structural modifications of the complex. Moreover, low-resolution micrococcal nuclease digestion shows that the chromatin of the entire region is devoid of positioned nucleosomes but is susceptible to changes in accessibility to the nuclease upon aminoacid starvation. The implications of these results (as well as their extension to other RPGs promoters) for the mechanism of Raplp action are discussed.

These studies were performed in collaboration with M. Grunstein (UCLA), D. Shore (Geneva) and E. Young (Seattle) and their groups. Recent publications on these matters to be found in JBC (march 02), EMBO J (march 02), JMB (in press), Biochemistry (in press).

Chromatin-specific regulation of transcription by recombinant Wnt and Notch enhancer complexes *in vitro*

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A common feature of the Wnt/Wg and Notch developmental signaling pathways is the ligand-dependent mobilization of dedicated co-activators that target specific DNA-binding enhancer factors. To further assess the role of these co-activators in the assembly and activation of enhancer complexes on chromatin, we have initiated studies to reconstitute Wnt and Notch enhancer activity *in vitro* with purified recombinant proteins. To date we have shown that β-catenin, a transcriptional co-activator of LEF-1 in Wnt/Wg-responsive enhancers, enhances transcription strongly in a chromatin-specific manner *in vitro*. In addition, β-catenin can bind co-operatively with LEF-1 to chromatin, but not nonchromatin, templates. We further found that different naturally-occuring isoforms of LEF-1 vary significantly in their intrinsic ability to bind nucleosomal templates in the absence of β-catenin, and that the amino terminus of LEF-1 represses binding to chromatin, but not nonchromatin, templates. Transcriptional activation *in vitro* requires both the armadillo repeats as well as the C terminus of β-catenin, whereas the phosphorylated N terminus appears to be inhibitory to transcription *in vitro*. A fragment spanning the C terminus (CT) and ARM repeats 11 and 12 (CT-ARM), but not the CT alone, functions as a dominant negative inhibitor of LEF-1-β-cat activity *in vitro* and can block ATP-dependent binding of the complex to chromatin. LEF-1-β-cat transactivation *in vitro* was also repressed by recombinant ICAT (inhibitor of β -catenin and Tcf-4), a physiological inhibitor of Wnt/Wg signaling that interacts with ARM repeats 11 and 12. Neither ICAT nor the CT-ARM inhibitory fragment disrupted the LEF-1-β-cat complex after it was bound stably to chromatin. Studies are underway to assess the chromatin remodeling complex utilized by LEF-1-β-cat to access the nucleosomal template and to identify the factors that interact with the CT-ARM region of β-catenin.

Signaling through the Notch pathway activates the proteolytic release of the Notch intracellular domain (ICD), which functions as a dedicated transcriptional co-activator of CSL enhancer-binding proteins. In contrast with our findings with the minimal Wnt/Wg complex, the recombinant Notch ICD:CBF1 enhancer complex is transcriptionally inactive on chromatin templates *in vitro*. Instead, we find that activation of transcription *in vitro* requires an additional co-activator, Mastermind (MAM), which has been shown to assemble stoichiometrically into the Notch enhancer complex. We find that MAM provides two activation domains necessary for Notch signaling in *Xenopus* embryos, only one of which (TAD1) contributes to the initiation of transcription in the minimal system used here. We find that the TAD1 region of MAM binds CBP/p300 and promotes nucleosome acetylation at Notch enhancers *in vitro*. Importantly, co-expression with MAM and CBF1 promotes the modification and proteolysis of the Notch ICD *in vivo*. We find that enhanced phosphorylation and turnover of the ICD requires the TAD2 region of MAM, which is a

glutamine-rich activation domain essential for Notch signaling *in vivo*. Thus MAM may function as a timer to couple transcription activation with turnover of the Notch enhancer complex.

Further studies with the Notch and Wnt/Wg transcription systems may reveal important differences in the strategies used by these developmental signaling systems, and provide an approach to understand the roles of additional co-activators that are assembled into each regulatory complexes.

Double bromodomain proteins, Brd2 and Brd4 interact with acetylated chromatin and regulate cell growth

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Bromodomain is a conserved α-helical motif present in a number of nuclear proteins that interact with and modify chromatin. Brd2 (formerly Ring3) and Brd4 (formerly MCAP) are mammalian members of the BET family of bromodomain proteins. They possess two bromodomains and another motif, the ET domain. Despite the extensive conservation from yeast to humans, the function and mode of action of the BET family proteins are largely unknown. We found that both Brd2 and Brd4 associate with non-centromeric regions of chromosomes during mitosis, and are required for mitosis (1). Brd4 is induced in response to growth stimuli and thus expressed in proliferating cells. Supporting multiple roles in cell growth, cells over-expressing or under-expressing Brd4 are defective in cell cycle progression from G1 to S. Recently we have shown that Brd4 interacts with a component of the DNA replication machinery, the RFC in NIH-3T3 and HeLa cells and that this interaction likely accounts for the regulation of G1 to S progression (2). In an effort to gain further understanding of the role of the BET family proteins, we examined the interaction of Brd2 and Brd4 with chromatin in living mouse or human cells. By FLIP analyses performed with transfected GFP-Brd4 in TSA treated cells, we show that Brd4 has a greater affinity for highly acetylated chromatin than less acetylated counterparts. Analysis with a series of Brd4 deletions and mutations showed that the interaction with acetylated chromatin is dependent on a cooperative action of the two bromodomains. By flow cytometry-based FRET analysis, we observed that Brd2 also interacts with acetylated histones in living cells and affects transcription. These results will be discussed in the context of the function of the BET family proteins conserved throughout eukaryotes.

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Silenced chromatin is permissive to activator binding and PIC recruitment

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Chromatin is thought to repress transcription by limiting access of the DNA to transcription factors. Using a yeast heat shock gene (HSP82) flanked by mating-type silencers as a model system, we have found that repressive, SIR-generated heterochromatin is permissive to the constitutive binding of an activator, HSF, and two components of the preinitiation complex (PIC), TBP and Pol II. Both recruitment-competent [IIa] and elongation-competent [IIo] isoforms of Pol II are detected at normal levels, despite transcription being reduced 100-fold. These factors cohabitate the promoter with Sir silencing proteins and deacetylated nucleosomal histones. The heterochromatic HMRa1 promoter is also occupied by TBP, Pol IIa, and Pol IIo. The abundance of Sir proteins recruited to the HSP82 promoter linearly correlates with the efficiency of silencing, indicating that Sir2p, Sir3p, and Sir4p act in a stoichiometric, not catalytic, fashion. In marked contrast to silencing mediated by heterochromatin, inactivation of euchromatic HSP82 conferred by deletion of the high-affinity HSF site is characterized by greatly diminished interaction of the same three positive regulators – HSF, TBP, and Pol II – with DNA. Thus, *S. cerevisiae* heterochromatin uses a novel mechanism to silence gene expression, whereby positive regulators gain access to promoter DNA yet RNA pol II, although recruited, is elongationally paused. Interestingly, following heat shock, transcription of heterochromatic HSP82 is increased >400-fold. Current experiments are aimed at identifying the molecular determinants of both heterochromatin-mediated Pol II pausing and activator-induced Pol II release.

Session 2: Utilization of chromatin structure during activation
Chair: Vincenzo Pirrotta

Synergisms on the MMTV promoter in chromatin

Miguel Beato

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Steroid hormones influence a plethora of cellular functions, which differ depending on the nature of the target cell and on the constellation of other signals impinging on a particular cell at a given time. To achieve the necessary coordination with other signalling pathways for various hormones and factors in the highly connected intracellular space, steroid hormones use a variety of mechanisms. Until very recently attention has been mainly focussed on the transcriptional effects of steroid hormones. These effects are mediated by intracellular hormone receptors, which participate in multiple interactions with DNA, other sequence-specific transcription factors, transcriptional co-regulators, and the general transcriptional machinery (Beato et al., 1995). In the last years a great effort has been devoted to understanding the nature of the transcriptional co-regulators and how they mediate the interaction of the hormone receptors with chromatin remodelling complexes and the transcriptional apparatus (Freedman, 1999). Considerable progress has been achieved leading to the recognition of covalent and conformational chromatin changes as key steps in transcriptional regulation by steroid hormone receptors and other transcription factors.

The packaging of the thousand of millions of base pairs of the eukaryotic genome in chromatin serves the compaction of the long DNA double chain within the confines of the cell nucleus. However, it is becoming progressively clear that the topological constrains imposed on the double helix by its folding around nucleosomes and in higher order chromatin structure have also important consequences for the various processes involving manipulations of the genetic information, such as DNA replication, recombination, repair and transcription. Not only does the packaging in chromatin represent an obstacle for factor access to DNA sequence information, but in certain cases the specific three-dimensional organization of DNA in chromatin prevents or facilitates interactions, which would be different on free DNA. Present research in the field aims at understanding the nature of the structural information encompassed in the highly specific folding of the DNA double helix in chromatin.

We approach these issues using the regulation of the MMTV promoter by progesterone as a model. Hormonal induction of the this promoter is mediated by a regulatory unit including, among other *cis* elements, five binding sites for steroid hormone receptors, so-called HREs, upstream of a binding site for the transcription factor NF1. Induction requires the integrity of these *cis*-acting elements, but the corresponding factors do not bind cooperatively to promoter DNA and do not synergize in cell-free transcription with DNA templates. The promoter is organized on a phased nucleosome, which exposes only 2 of the 5 HREs for binding of the hormone receptors and preclude binding of NF1. Hormone treatment results in rapid simultaneous occupancy of all 5 HREs and the NF1 site on the surface of a nucleosome-like particle. The functional synergism between hormone receptors and NF1 depends on positioned nucleosomes (Chávez and Beato, 1997), but does not require the proline-rich transactivation functions of NF1 (Di Croce et al., 1999), which is important in a different promoter context. Receptors bound to the accessible HREs recruit the NURF complex, which in the presence of ATP remodels the MMTV chromatin and facilitates NF1 binding (Di Croce et al., 1999). Binding of NF1 stabilizes an open nucleosome conformation

and favours full receptor binding (Prado et al., 2002). Thus positioned nucleosomes account for constitutive repression and participate in hormonal induction by mediating the reciprocal synergism between receptors and NF1, in a process involving ATP-dependent nucleosome remodelling. Furthermore, these results confirm that two transcription factors can synergize in very different ways depending on the chromatin organization of promoter.

Another interesting aspect of chromatin structure relates to the influence of histone H1 on the organization and transcription of MMTV chromatin. *In vitro*, linker histones bind asymmetrically to the distal end of the promoter nucleosome and protects additional 26-29 nucleotides of linker DNA (G. Vicent, M.J. Meliá and M.B., submitted). Binding of histone H1 stabilize the nucleosome, but progesterone receptor (PR) binds to H1 containing nucleosomes with higher affinity than to the octamer core particle. Moreover, MMTV minichromosomes containing histone H1 are transcriptional repressed but are transcribed more efficiently in the presence of PR and NF1 (R. Koop, L. Di Croce and M.B., unpublished). Thus the extent of hormonal induction increases in chromatin containing histone H1, suggesting a precise organization of the relevant promoter sequences in chromatin optimises the interaction of PR with the exposed HREs and improves the hormonal response of the promoter.

In addition to their direct transcriptional effects, steroid hormones have been found to influence the activity of many other signalling pathways by so-called non-genomic mechanisms. These effects are mediated by interactions at the membrane or cytoplasmic level and offer a possibility for integration of the steroid hormone signals at the entry site of many other physiological signals acting via membrane receptors. In breast cancer cells estrogens stimulate cell proliferation and this effect can be blocked by inhibitors of the MAP kinase signalling pathway. This pathway is transiently activated by estrogens through an interaction of the classical estrogen receptor (ERα) with the SH2 domain of c-Src (Migliaccio et al., 1996). Activation of c-Src is followed by transient activation of Ras, Raf and Erk1/2.

Progestins also stimulate proliferation of breast cancer cells in culture via a rapid and transient activation of the Src-Ras-Erk cascade (Migliaccio et al., 1998). However, activation by progestins requires expression of both progesterone receptor (PR) and ERα and is inhibited not only by antiprogestins but also by antiestrogens (Migliaccio et al., 1998). PR is precipitated with antibodies to ERα and vice versa, demonstrating the existence of a intracellular complex between the two hormone receptors (Migliaccio et al., 1998). However, antibodies to PR do not precipitate c-Src, suggesting that the activation of the Src/Ras/Erk cascade by progestins is indirect and mediated by an interaction of PR with the unliganded ERα, which itself activated c-Src. We find that two domains of the N-terminal half of PR (ERID-I and –II) interact with ERα directly and independently, and are both required for the activation by progestins of the Src-Erk cascade in cells equipped with PR and ERα. PR also contains a proline-rich region, which interacts directly with c-Src *in vitro*. However, this region can be deleted without influencing the strong activation of the pathway seen in the presence of ERα. Moreover, ERID-I and -II are not relevant for the transcriptional activation of a progesterone reporter gene.

These findings open the way for a dissociation of the progesterone signalling by crosstalk with the MAP kinase pathways and by transcriptional mechanism, which will allow the identification of the gene networks regulated by each of these different. The possible

connexion between the rapid activation of kinase cascades and the direct gene regulation via HREs remains an important issue to be addressed in the future.

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Steroid receptor mediated histone modifications and chromatin remodeling *in vivo*

Trevor K. Archer

With the use of histone proteins eukaryotes are able to compact DNA into chromatin and institute novel mechanisms with which to regulate gene expression, mechanisms that may not be available to prokaryotes. The mouse mammary tumor virus (MMTV) promoter has been used extensively as a model to explore the processes that activate transcription and remodel chromatin. Initial experiments demonstrated that glucocorticoids acting through the glucocorticoid receptor (GR), a ligand-activated transcription factor, could reversibly alter MMTV chromatin structure (Zaret and Yamamoto, 1984). Analysis of Chromatin||Protein interactions in comparison to DNA||Protein interactions revealed the formation of the pre-initiation complex at the promoter in response to glucocorticoid mediated chromatin remodeling (Archer et al., 1992). However, for the promoters assembled as chromatin prolonged exposure to hormone resulted in the eviction of the pre-initiation complex and the cessation of transcription (Lee and Archer, 1994).

Subsequent analysis of the cycle of PIC formation and eviction from the promoter revealed that this remodeling of chromatin is transient such that upon prolonged exposure to hormone the promoter becomes refractory to glucocorticoids (Lee and Archer, 1998). Further experiments demonstrated that this refractory state requires the continual presence of hormone and can be reversed by its removal. As these changes were specific for chromatin we focused on reversible modifications of histones as a potential mechanism to explain our observations. Our experiments showed that the promoter was inactivated via a mechanism whereby histone H1 was dephosphorylated in response to glucocorticoids. Removal of glucocorticoids resulted in the rephosphorylation of histone H1 and the reacquisition of transcriptional competence by the promoter. Finally, we demonstrated that H1 on the MMTV promoter is dephosphorylated when the promoter is unresponsive to glucocorticoids. These observations provided strong evidence that phosphorylated H1 was intimately linked with the GR-mediated disruption of MMTV chromatin *in vivo*.

To extend our analyses, we explored the mechanistic link between GR mediated histone H1 dephosphorylation and silencing of the MMTV-promoter by describing the putative kinase responsible for H1 phosphorylation (Bhattacharjee et al., 2001). Both *in vitro* kinase assays and *in vivo* protein expression studies suggested that in hormone treated cells the ability of cdk2 to phosphorylate histone H1 was decreased. To address the role of cdk2 and histone H1 dephosphorylation in the silencing of the MMTV-promoter, we used potent cdk2 inhibitors, Roscovitine and CVT-313 to generate an MMTV promoter which is associated predominantly with the dephosphorylated form of histone H1. Both compounds blocked phosphorylation of histone H1 and under these conditions the GR was unable to remodel chromatin, recruit transcription factors to the promoter or stimulate MMTV mRNA accumulation.

Histone H1 is not a unique species in that most mammals have at least 6 isoforms for which independent genes have been identified and designated as ,H1.0, H1.1, H1.2, H1.3, H1.4 and H1.5 (Albig et al., 1991)

Given this diversity we were intrigued to investigate if the specific effects with respect to GR activated transcription might indicate that specific isoforms were subject to dephosphorylation (Banks et al., 2001). Using electrospray mass spectrometry, we demonstrated that prolonged dexamethasone treatment differentially effects a subset of the six somatic H1 isoforms in mouse cells. H1 isoforms H1.0, H1.1, and H1.2 are non-responsive to hormone where as prolonged dexamethasone treatment effectively dephosphorylated the H1.3, H1.4, and H1.5 isoforms. The protein kinase inhibitor staurosporine, shown to dephosphorylate histone H1 and down-regulate MMTV in cultured cells, appears only to completely dephosphorylate the H1.3 isoform. Finally, these results suggest a model where cdk2 directed histone H1.3 phosphorylation is an essential component of GR-mediated chromatin remodeling and activation of the MMTV promoter *in vivo*.

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The dynamics of nuclear receptor interactions with gene targets

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Activation of transcription in eucaryotic systems is associated in current models with the formation of a stable preinitiation complex. We previously reported the direct observation of steroid receptor binding to a tandem array of the mouse mammary tumor virus (MMTV) promoter in living cells. Through the use of photobleaching techniques, we unexpectedly found that the glucocorticoid receptor (GR) exchanges rapidly with regulatory elements in the continued presence of ligand (1). Using fluorescence recovery after photobleaching (FRAP) we have now examined the dynamic behavior of several green fluorescence protein (GFP) tagged transcription factors at the MMTV tandem array. The glucocorticoid receptor interacting protein 1 (GRIP1) exhibits a half maximal time for fluorescent recovery of 5 sec., the same rapid exchange as observed for GR. In contrast, the large subunit of RNA polymerase II (RPB1) shows a very slow exchange, requiring thirteen minutes for complete fluorescence recovery.

We have also reconstituted the GR-dependent nucleoprotein transition with chromatin assembled on MMTV DNA. The remodeling event is ATP-dependent, and requires either a nuclear extract from HeLa cells or purified human Swi/Snf (2). Through the use of a direct interaction assay (magnetic bead Apull-down@), we demonstrate recruitment of human Swi/Snf to MMTV chromatin by GR (3). We find that GR is actively displaced from the chromatin template during the remodeling process. Displacement requires the presence of ATP, can be reversed by the addition of apyrase, and is specific to chromatin templates. The disengagement reaction can be induced with purified human Swi/Snf, but the reaction occurs less efficiently, and is further stimulated by the inclusion of nuclear extract. Binding of the secondary transcription factor, NF-1, to the promoter is facilitated by GR-induced chromatin remodeling. Both the in vitro and in vivo results are consistent with a dynamic model (Ahit and run@) in which GR first binds to chromatin after ligand activation, recruits a remodeling activity, and is simultaneously lost from the template.

These findings suggest a new model for nuclear receptor function (4) in which the receptors reside on response elements in chromatin only for brief periods, then return to the template for successive binding events. We further observed that the template undergoes a cascade of modification events, which are triggered by the initial receptor binding event. We suggest that the receptor may recruit different cofactors during successive "return-to-template" events, and thus participate in biochemically distinct processes during each binding cycle. This view represents a major departure from classic endocrine models, which envisage the receptor as statically bound to responsive promoters in the continued presence of hormone.

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Opening chromatin in early development

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Our laboratory investigates the first transcription factors to bind a gene in development, focusing on how such factors gain access to their target sequences in compacted chromatin and elicit local chromatin opening. We are also interested in the basic mechanisms by which chromatin is compacted into higher-order structures.

We previously showed that essential HNF3 and GATA-4 binding sites are occupied on a transcriptional enhancer of the mouse albumin gene in the endoderm, prior to the binding of additional factors that activate the gene in liver development. Controlled loss of GATA and HNF3 site occupancy correlates with loss of the potential to activate albumin transcription, and ectopic expression of GATA and HNF3 in non-endoderm cells endows the potential to activate albumin. To understand the functions of such initial chromatin binding factors, prior to gene activation, we created artificial nucleosome arrays that span the albumin enhancer. The arrays were compacted with linker histone H1 to generate a closed chromatin configuration, where the DNA became resistant to diverse enzymatic probes. By various assays, we found that purified HNF3 and GATA-4 proteins can bind to their sites in highly compacted chromatin and create a local domain of accessibility. Chromatin opening occurs in the absence of ATP-dependent nucleosome remodeling enzymes. Other DNA binding factors, including GAL4, C/EBP, and NF1, lack this chromatin opening activity. Using a sequential chromatin immunoprecipitation approach, we discovered that in liver nuclei, HNF3 is bound to nucleosomes at the albumin enhancer. Taken together, these studies show that DNA binding factors that initiate the assembly of regulatory complexes in chromatin are sufficient to open local chromatin structure and remain stably bound to nucleosomes during gene activation.

The chromatin opening properties of HNF3 are dependent upon a C-terminal domain that binds certain core histone proteins, independently of HNF3's DNA binding domain. Current investigations are centered on identifying the histone amino acids targeted by HNF3. We wish to determine whether those amino acids are critical for inter-nucleosome interactions that promote higher-order chromatin structure, and whether the displacement of those interactions, by HNF3's C-terminal domain, are critical for chromatin opening.

Mitotic recombination and DNA replication during the *Schizosaccharomyces pombe* cell cycle

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Genetic recombination between sister chromatids plays an important role in maintaining genome integrity during DNA replication. We have studied the relationship between DNA replication and recombination *in vivo* during the mitotic cell cycle in *Schizosaccharomyces pombe* by two-dimensional gel electrophoresis and targeting of the *ura4*$^+$ gene marker at various genomic loci. Our results indicate that Holliday junctions and the rate of marker integration significantly increase during the S phase at regions containing DNA replication origins. On the other hand, mutations in several genes involved in recombinational repair negatively affect DNA replication and cause the accumulation of abnormal intermediates. Chromatin analysis across the ars1 replication origin, located upstream from the *hus5*$^+$ gene, revealed a pattern of positioned nucleosomes that represents the chromatin framework where transcription, replication, initiation and genetic recombination take place. These results point to a close relationship between these three processes *in vivo*.

Session 3: Histones and DNA modifications in gene regulation
Chair: Katherine A. Jones

Translating the histone code: A tale of tails

C. David Allis

Over the past decade, research into the genomes of many diverse organisms has produced an enormous quantity of information on the chromosomal location. sequence, and structure of thousands of genes. The human genome is estimated to contain 30,000 –40,000 unique genes; the DNA sequence and chromosomal location of all these genes are becoming widely known. A central challenge facing the biomedical community is how to derive medically -valuable knowledge about the function of these genes from the now-available DNA sequence data.

Though every gene exists within every cell in the human body, only a small percentage of genes are activated in any given cell. To manage this genetic information efficiently, nature has evolved a sophisticated system that facilitates access to specific genes. This system relies on a DNA-histone protein complex called chromatin to efficiently package the genetic information that exists within each cell. This packaging system making certain genes more readily accessible to transcription factors and other machinery that must engage our genetic template. It is only now, after the description of oncogenes in the 1980's, tumor suppressor genes in the 1990's, and the recent sequencing of the human genome, that attention will focus more squarely on the molecular engines which drive the mutation, loss, and aberrant regulation of genes resulting in cancer. Chromatin modifications, and the regulation of the enzymes responsible for adding or subtracting them, are poised to take center stage in the study of cancer in the current post-genomic or epigenomic era. Moreover, the implications of chromatin and its modification are just beginning to be appreciated in clinical oncology. The identification of altered DNA methylation and histone acetylase activity in a range of human cancers, coupled with the use of HDAC inhibitors in the treatment of leukemia and epithelial malignancies (ie lung cancer) make a compelling argument. It is clear that the regulatory signals provided by chromatin modifications will revolutionize our view of cancer as new models of 'epigenetic carcinogenesis are advanced. We favor the view that there exists an epigenetic indexing system for our genome, or a 'histone code', that represents a fundamental regulatory mechanism that acts outside of the DNA itself. We predict that this 'code' impacts on most, if not all, chromatin-templated processes with far-reaching consequences for cell fate decisions and for normal and pathological development [see Strahl and Allis, 2000; Cheung et al., 2000; Jenuwein and Allis, 2001).

The importance of chromatin-based epigenetic mechanisms of gene regulation is implied by the fact that the human genome contains much fewer genes than originally expected. In the words of others:

"The modest number of human genes means that we must look elsewhere for the mechanisms that generate the complexities inherent in human development and the sophisticated signaling systems that maintain homeostasis. There are a large number of ways in which the functions of individual genes and gene products are regulated. The degree of "openness" of chromatin structure and hence transcriptional activity is regulated by protein complexes that involve histone and DNA enzymatic modifications."

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X-inactivation; an increasingly complex code of silence

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Most genes on one of the two X chromosomes in female mammals are inactivated at the blastocyst stage of development, a process of dosage compensation that equalizes levels of X-linked gene products between XX females and XY males [1]. X inactivation provides a valuable paradigm of developmentally regulated, long-term silencing. Progression to the inactive state requires completion of a series of steps described as *counting* (inactivation is triggered only in cells with more than one X), *choice* (one X remains active, while all additional Xs are inactivated), *initiation* of the silencing process and *spreading* across the chromosome, and *stabilisation* or *maintenance* (once in place, X inactivation is almost irreversible). The inactive X (Xi) shares many properties with constitutive heterochromatin. It is late replicating, methylated at CpG islands, shows a general underacetylation of core histones and is enriched in H3 di-methylated at lysine 9 and depleted in H3 di-methylated at lysine 4. Xi also has properties that make its chromatin unique. For example, it is coated with the RNA product of *Xist*, a gene that is expressed only from Xi, and appears to be enriched in the histone variant macroH2A.

To define the roles played by different chromatin modifications in the various stages of the X inactivation process, we have used mouse embryonic stem cells (ES cells) as a model system. ES cells are derived from the inner cell mass of the pre-implantation blastocyst and female ES cells have two active X chromosomes. The X inactivation process is initiated when the cells differentiate *in vitro*. In previous experiments we have studied the timing of events associated with X inactivation [2]. Down-regulation of X-linked gene expression is detected within 1-2 days, as is coating with *Xist* RNA and late replication. Global deacetylation is first detected only at day 4, whereas methylation of CpG islands is not seen until much later, 2-3 weeks from the onset of differentiation. Deacetylation of all four core histones is initiated at the same time and proceeds in parallel. It seems that CpG methylation and global histone deacetylation are involved in *stabilisati* of the inactive state.

However, local changes in histone acetylation may be involved at earlier stages (*initiation* or *choice*). A region of about 100Kb upstream of *Xist* is hyperacetylated in undifferentiated female ES cells and deacetylated by day 2 of differentiation [3]. This same region has been shown to be highly enriched in H3 di-methylated at lysine 9 [4] and, by deletion analysis, found to be required for X inactivation [5]. Our recent results suggest that HDAC-mediated deacetylation of this region may be essential for the normal progression of X inactivation.

Histone acetylation may also play a role in *counting*. By Chromatin ImmunoPrecipitation (ChIP), we have shown that all four core histones on X-linked genes in female ES cells are 2-3 fold more acetylated than those on X-linked genes in male cells or on autosomal genes. Thus, acetylation provides an epigenetic mark that distinguishes X-linked genes in XX and XY cells. This hyperacetylation has been detected in all XX ES lines so far

studied, with the exception of D102, a line with a 65Kb deletion downstream of *Xist* and whose behaviour suggests a deficiency in the counting process [6]. Hyperacetylation of endogenous X-linked genes is not induced in XY ES cells carrying additional copies of the X inactivation centre (*Xic*) as YACs, even though X inactivation can be initiated (usually on the transgene) in these transgenic cells [7]. It remains to be determined whether the hyperacetylation we detect is present on both female X chromosomes, or just one. Experiments with mutant ES lines are in progress to resolve this.

Xi shows distinctive patterns of H3 methylation, staining weakly with antibodies against H3 di-methylated at lysine 4 and relatively strongly with antibodies to H3 di-methylated at lysine 9 [4, 8, 9]. These H3 lysines can be mono-, di- and tri-methylated *in vivo* [10]. We find that antisera raised against di-methylated peptides show little or no cross-reaction with the equivalent tri-methylated peptides, and *vice versa*, an observation that should be taken into account when interpreting results obtained with such antisera. Our recent results with novel antisera to di- and tri-methylated H3 demonstrate the functional significance of methylation levels in X inactivation, and throw new light on the relationship between histone methylation and acetylation.

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Independent dynamic regulation of histone H3 phosphorylation and acetylation during immediate-early gene induction

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Induction of the Immediate-Early (IE) genes c-*fos* and c-*jun* by diverse agents is associated with the phosphorylation and acetylation (phosphoacetylation) of histone H3 and acetylation of histone H4. We have mapped the extent and complexity of nucleosomal modifications at specific positions along these genes. For comparable nucleosomes at similar positions along the gene, a large population becomes highly acetylated on histone H3 and H4, whereas phosphoacetylation was found on a much smaller population of the same nucleosomes. Inhibiting histone H3 phosphorylation with kinase inhibitors did not measurably alter the enhanced acetylation of these nucleosomes. Furthermore, whereas H3 phosphorylation is a MAP kinase-mediated inducible event, we found acetylation to be continuously turning over by the targeted action of HATs and HDACs, even in quiescent cells in the absence of any stimulation or gene transcription. Our data contradict published models in the literature whereby phosphoacetylation of histone H3 is proposed to arise because phosphorylation at serine 10 directs subsequent acetylation at lysine 14, arguing instead for independent dynamic turnover of acetyl and phosphate groups at these genes, the two modifications coinciding on only a small fraction of nucleosomes. These studies indicate that phosphorylation and acetylation are independently and dynamically regulated and provide insights into the complexity of multiple histone modification during Immediate-Early gene induction.

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Function and mechanisms of imprinting and epigenetic reprogramming

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We are interested in the regulation of imprinted genes in the mammalian genome, and in their role in development and disease. The Igf2 gene contains three differentially methylated regions (DMRs), two of which are methylated on the active paternal copy. This has given rise to the proposal that these DMRs contain silencers which can be regulated by DNA methylation. Both DMRs have been deleted by knockouts and shown to contain silencer or activator sequences that are important for regulation of imprinting or expression. In vitro transfection assays confirmed the presence of methylation sensitive silencer or activator sequences in these regions.

Igf2 is an important regulator of fetal growth and is highly expressed both in the fetus and in the placenta. In order to address its role specifically in placental function, we have examined a placenta specific knockout of Igf2. This results in intrauterine growth restriction of both the placenta and the fetus. Specific assays for placental transfer functions revealed altered transfer in mutant placentae, thus providing an explanation for the fetal growth restriction.

Differential methylation in imprinted genes needs to be maintained in somatic cells of offspring. This is not trivial since the paternal genome undergoes genome wide demethylation in the zygote, and the maternal genome demethylation during cleavage division in the preimplantation embryo. This is followed by de novo methylation around implantation. The biological purpose of this epigenetic reprogramming of the genome is not known. We have asked whether highly methylated somatic nuclei become reprogrammed during cloning. We found that in most cloned embryos aspects of reprogramming were aberrant, suggesting that reprogramming is important for the totipotency of the nucleus, particularly during cloning. Aberrant reprogramming may contribute to the low efficiency of cloning.

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DNA-repair in chromatin and transcribed genes

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In eukaryotic cells, the regulation of all DNA-dependent processes is intimately coupled to structural and dynamic properties of packaging DNA in nucleosomes and higher order chromatin structures. We study repair of UV-induced DNA-lesions by photolyase and nucleotide excision repair (NER) in yeast *S.cerevisiae*. Since UV-lesions (cyclobutane-pyrimidine dimers (CPDs) and pyrimidine-(6-4)-pyrimidone photoproducts (6-4PPs)) are generated at dipyrimidines and pyrimidine clusters, the repair approach allows to investigate DNA-accessibility everywhere in the genome. Photolyase is an elegant molecular tool, since it appears to act as an individual protein, its activity is light dependent and therefore can be precisely regulated in living cells. Photolyase is very efficient and rapidly repairs DNA-lesions in open regions (promoters, origins of replication; less than 15 minutes), while nucleosome repair requires about 2 hours. NER is slower than photoreactivation by photolyase. NER is a complex pathway which might require nucleosome remodelling activities to access DNA in nucleosome substrates and generate the space for the repair reactions. Transcription of DNA by RNA-polymerases affects chromatin structure and DNA-repair. RNA-polymerases are blocked at DNA-lesions in the transcribed strand and promote NER (transcription coupled repair by RNA-polymerase II) or inhibit photolyase. The following results will be presented: (i) A comparative analysis of chromatin structure and DNA-repair by photolyase in the *URA3* gene at high resolution. The results provide insight in dynamic properties of nucleosomes in vivo. (ii) An analysis of DNA-repair in yeast heterochromatin. (iii) DNA-repair in the nucleolus. We found that photolyase as well as NER have unrestricted access to nucleolar chromatin. The active genes are more rapidly repaired than the silenced genes and appear to maintain an open chromatin structure after damage formation and during repair.

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HMGB1 has a role in chromatin remodelling and regulation of transcription

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HMGB1 is an abundant chromatin protein endowed with "architectural" activity: though it has no sequence specificity, it is recruited by several DNA-binding proteins to bend the DNA. It establishes protein-protein contacts with a variety of interactors (HOX and OCT proteins, steroid receptors, p53, TBP, TFIIA, RAG1 and some viral proteins), and facilitates their binding to DNA target sites.

Hmgb1-/- mice are born, but die within 24 hours. Embryonic fibroblasts (MEFs) from Hmgb1-/- mice have impaired GR responses: dexamethasone-driven transcription is reduced as compared to +/+ cells, and is restored by Hmgb1 transfection.

In vitro, Glucocorticoid Response Elements (GREs) are not bound by GR alone (and bandshifts do not occur), whereas GR binds to GREs in the presence of HMGB1.

We aim to understand in molecular terms how HMGB1 helps control the transcription of a GR-responsive gene within its normal chromosomal location.

We looked for dexamethasone-regulated genes in Hmgb1-/- and +/+ MEFs by mRNA micro-array analysis. Among several found, we selected the 24p3 gene, that codes for a secreted stress protein of the lipocalin superfamily. By RT-PCR we verified that 24p3 is tightly dex-regulated, and its activation is impaired in Hmgb1-/- MEFs. Nucleosomes are translationally positioned on the 24p3 promoter and relocate over the GREs in response to dexamethasone. We showed in vitro that HMGB1 kick-starts nucleosome sliding by chromatin remodelling machines: we propose that GR-recruited HMGB1 initiates the nucleosome repositioning required for the transcriptional activation of 24p3, and serves as a linchpin between transcription factors and remodelling machines.

Genomic targets of the c-Myc oncoprotein

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The c-Myc protein is induced by mitogens, binds a specific DNA element (CACGTG or E-box) at chromosomal target loci and contributes an essential function in gene activation. This function consists, at least in part, in promoting localized histone acetylation in chromatin 1-3. In spite of this essential role in mitogen-induced transcription, experimental activation of Myc in quiescent cells fails to induce most of its target genes 1. This restricts the validity of screening strategies based primarily on mRNA expression for the identification of Myc targets. To circumvent this limitation, we have developed a high-throughput screen for chromosomal loci that are physically bound by Myc *in vivo*. First, bioinformatic tools were used to find promoter-associated E-boxes in human genomic DNA. In a second step, we used quantitative chromatin immunoprecipitation 1 to assess the binding of Myc to hundreds of selected E-boxes in live human cells. Myc bound approximately 30% of the promoter sequences analyzed, but only a negligible fraction of randomly selected E-boxes. By extrapolation, we predict that several thousand E-box-containing promoters are targeted by Myc in human cells. Our progress in characterizing the regulation of these genomic targets will be presented.

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Session 4: Long distance chromatin regulation and remodelling
Chair: Peter B. Becker

Chromatin boundaries as barriers against position effects

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Insulators are DNA elements that can possess two defining properties: An insulator can block the action of an external promoter on an enhancer when it lies between them. It can also serve as a barrier against the encroachment of adjacent condensed chromatin. When such an insulator is placed on both sides of a transgene, the barrier function provides protection against position effects, in which expression levels depend on the site of integration.

We have been studying the insulator element located at the 5' end of the chicken beta-globin locus, which possesses both of these defining properties. We have shown that enhancer blocking arises from a single sequence element in the insulator that binds the factor CTCF. We have found however that the barrier function does not depend at all on CTCF, but rather on the binding of other proteins to additional sites within the 250 bp 'core' insulator element. In order to understand the function of this insulator in its natural setting, we have examined the patterns of histone modification over the entire chicken beta-globin locus as well as upstream elements.

Patterns of histone methylation and acetylation are consistent with a role of the insulator in protecting against the propagation of a condensed chromatin structure that lies adjacent to the beta-globin locus. These results have now been compared with the patterns of histone modification and DNA methylation over insulated and uninsulated reporter genes in stably transformed cell lines. The data support a model in which insulators maintain histone acetylation over the protected regions, and in so doing directly prevent gene inactivation and indirectly prevent DNA methylation at critical sites in the transgene, which would otherwise serve as further inactivation signals.

Globin gene activation: A stochastic process

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Models for stochastic gene activation have been invoked to explain mono-allelic expression patterns of genes involved in immune and cellular responses, dosage compensation and cell commitment, but have also been proposed for gene activation in general.

We have tested the generality of a stochastic model at the allelic level using the alpha and beta globin genes, as they play no role in any cellular selection processes. Allelic transcription patterns and mRNA levels in single cells showed an imbalance of alpha versus beta globin expression in a significant proportion of cells and different probabilities in the activation of alpha and beta globin genes. Interestingly once a cell chooses a particular pattern of expression, that pattern becomes fixed in the cell.

These data provide strong evidence for a stochastic basis of gene activation in general, which has broad implications for a variety of processes from the evolution of different layers of gene control to phenotypic differences between genetically identical cells and organisms.

Targeting of the histone acetyltransferase MOF to the male X chromosome for dosage compensation in flies

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Drosophila and humans resemble each other with respect to their heteromorphic X chromosomes: male and female cells are characterized by XY and XX genotypes, respectively. Female cells thus have a double dose of X-linked genes. However, it seems important that cells of both sexes express X-linked genes to an equivalent degree. 'Dosage compensation', thus involves the adjustment of expression of X chromosomal genes at the level of transcription. In human female cells this is achieved through permanent inactivation of one X chromosome as facultative heterochromatin. By contrast, flies transcribe genes from both female X chromosomes but increase transcription from the single male X chromosome roughly two fold. Despite of the obvious differences, dosage compensation in both species relies on common principles. In either case tuning of gene expression involves epigenetic mechanisms such as the formation of stable chromatin structures with particular histone modification patterns and the coating the X chromosome by non-coding RNA transcribed from the X chromosome.

The hyperactive X chromosome in flies is characterized by acetylation of histone H4 at lysine 16. The histone acetyltransferase responsible for this epigenetic mark, MOF, is targeted to the X chromosome as part of a dosage compensation complex, including non-coding roX RNA and the male-specific lethal proteins MSL-1, MSL-2, MSL-3 and MLE as well as the histone H3 kinase JIL-1. Our laboratory is interested in the principles that target the dosage compensation machinery to the X chromosome, in defining the role of non-coding RNA in complex assembly and function and in elucidating the structural consequences of histone H4 acetylation. We previously found that recombinant MOF was able to specifically acetylate histone H4 at lysine 16 and that this modification suffices to substantially de-repress transcription of chromatin templates in vitro and in vivo (1). MOF is a member of the MYST family of histone acetyltransferases, characterized by chromodomain signatures and a rare C_2HC zinc finger motif. This zinc finger is involved in substrate recognition: if impaired, histone acetylation will not occur despite of an intact HAT domain (2).

Three of the known subunits of the dosage compensation complex, MOF, MLE and MSL-3 are able to interact with RNA and require the presence of RNA, most likely roX RNA for stable interaction with chromatin. We recently showed that MOF binds roX RNA in vivo through its chromo-domain signature (3).

Targeting the X-chromosome also involves X chromosomal DNA sequences. Pioneering work of the Kuroda, Lucchesi and Baker labs have led to the formulation of a model whereby the dosage compensation complex initially assembles on some 35 X chromosomal 'entry sites' from which it 'spreads' over the reminder of the X chromosome. In close collaboration with the Kuroda lab we recently identified one such entry site (4).

Current efforts of the laboratory are directed at (i) characterizing other entry sites in order to define the consensus properties of targeting elements; (ii) characterizing the protein-DNA interactions that define X chromosomal targeting; (iii) understanding the principle which assures the 'coating' of the X chromosome; and (iv) elucidating the role of non-coding RNA in complex assembly and function by comprehensive structure-function analysis. Progress along these lines of research will be presented.

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Analysis of the contribution of GAGA-dsap18 interaction to the functional regulation of the bithorax complex of *Drosophila*

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We have recently reported that GAGA interacts with dSAP18, a polypeptide that, in mammals, was found associated with the Sin3-HDAC co-repressor. GAGA-dSAP18 interaction likely plays a role on the regulation of homeotic gene expression. In polytene chromosomes, dSAP18 and GAGA co-localise at the region of the bithorax complex (BX-C) where GAGA is known to be bound to some silenced PREs. In addition, we observe a genetic interaction between Trl and a deficiency that uncovers dSAP18 . Flies heterozygous for the GAGA mutation Trl67 and hemizygous for Df(3R)sbd26 show a homeotic transformation of segment A6 into A5. Through the mobilisation of a P-element insertion in the 5'UTR of dSAP18 (line EP(3)3462) we have generated a number of dSAP18 mutant alleles. Several of them are homozygous lethal but not in front of Df(3R)sbd26. In good agreement with our previous results, the newly generated dSAP18 alleles show the homeotic transformation described above when transheterozygous for EP(3)3462 or Df(3R)sbd26. A similar homeotic transformation is observed with Rpd3 mutant alleles. These results suggest that, through recruitment of the Sin3-HDAC complex, GAGA might participate in the regulation of homeotic gene expression but, contrary to what would be anticipated for such an interaction, it contributes to the relief of repression at the iab-6 element. GAGA-dSAP18 interaction might also participate in the functional regulation of the Fab-7 element since they co-localise at the Fab-7 element in transgenic lines carrying ectopic copies of the Fab-7 element. At present, we are dissecting the *cis*-regulatory elements that in the Fab-7 element are responsible for this co-localisation.

Mechanistic differences between different ATP-dependent chromatin remodelers

Hua-Ying Fan, Geeta Narlikar, Xi He and Robert E. Kingston

ATP-dependent chromatin remodelers regulate chromatin structure and, thus, play an indispensable role in all nuclear processes involving DNA. Three classes of ATP-dependent remodelers have been identified, and the number of unclassified, homologous proteins with similar activities is increasing. A fundamental question concerns how different chromatin remodelers participate in different biological processes.

Several models have been proposed to explain the biochemical activities of ATP-dependent chromatin remodelers. Members of the three classes of ATP-dependent remodelers, or these remodeler-containing complexes, all can cause changes in nucleosome translational positions. Therefore, 'sliding' of the histone octamer along the DNA has been proposed to be a common mechanism for ATP-dependent chromatin remodelers. Nonetheless, sliding may not be an optimal model to explain chromatin remodeling at densely packed nucleosomes *in vivo*.

We have compared and contrasted the biochemical activities of BRG1 and SNF2h in protocols that examine positions of nucleosomes and rates of remodeling. These proteins represent the motor proteins of two major families of human ATP-dependent remodeling complexes, the SWI/SNF family and the ISWI-based family. We have found that these two proteins generate distinct remodeled products. The rates of remodeling and products generated by SNF2h are consistent with sliding being its primary mechanism; in contrast, the behavior of BRG1 is inconsistent with sliding being an obligatory aspect of its mechanism. Instead, it appears that BRG1 can make DNA accessible on the surface of the histone octamer. Despite these differences, both proteins generate multiple products that are continuously inter-converted suggesting that interconversion of products might be a universal property of ATP-dependent remodelers. These studies imply that these two remodelers use distinct mechanisms for creating accessible DNA, one that works efficiently for changing nucleosome position, and another that can create accessibility without changing nucleosome position.

Session 5: Regulation of heterochromatin
Chair: Gary Felsenfeld

Dynamics of functional interphase chromatin

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The higher-order organization of the eukaryotic nucleus is proposed both to reflect specialized aspects of nuclear function, and to influence the efficiency and timing of nuclear events. We have examined the organization of chromatin in the interphase nucleus of budding yeast using real-time fluorescence imaging of GFP tagged chromosomal domains. Four distinct types of chromosomal sites were tested: one early replicating and one late replicating internal region of the chromosome, a centromere and multiple different telomeres. By analysing the "micromovement" by rapid time-lapse microscopy, we find that internal regions on chromosome arms are highly dynamic in G1, in contrast to telomeres and centromeres. Two partially redundant mechanisms anchor telomeres to the nuclear envelope : one involving yKu and one that relies on the repressed chromatin state induced by Sir proteins. The rapid movement of internal regions is suppressed when ATP is depleted and can be modulated by mutation of *mcm7*, a component of a hexameric helicase. Changes in chromatin dynamics during the response to DNA damage, transcriptional activation and DNA replication will be discussed.

Chromatin organization by histone lysine methylation

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Diverse post-translational modifications of histone N-termini represent an important epigenetic mechanism for the organization of chromatin structure and the regulation of gene activity. Within the last two years great progress has been made in understanding the functional implications of histone methylation, in particular through the characterization of histone methyltransferases (HMTases) that direct the site-specific methylation of lysine positions in the histone H3 N-terminus. Histone lysine methylation has been linked with pericentric heterochromatin formation, X inactivation, Polycomb-group (Pc-G) dependent repression and epigenetic gene regulation at euchromatic positions. Moreover, studies on the stability of histone lysine methylation also revealed the surprising involvement of an RNA component in the higher-order structuring of pericentric heterochromatin. Together, these regulatory roles strongly establish histone lysine methylation as a central epigenetic modification for the organization of eukaryotic chromatin with far-reaching implications for proliferation, cell-type differentiation, overall development, gene expression, genome stability and cancer.

Epigenetic control of gene silencing and maintenance of cell identity

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In development and during transition from totipotency to differentiated state, epigenetic control of gene silencing guarantees heritable repression of genes not required in specific cell types. Chromatin dynamics is thought to play a major role in silencing memory function. In *Drosophila*, the proteins of the Polycomb group (PcG) act as multi-protein complexes that maintain cell identity by "freezing" transcription states of developmentally regulated genes. Despite the considerable progress in the identification and characterization of PcG protein complexes both in flies and in mammals, the mechanisms by which they work *in vivo* and in particular their role in promoter silencing remain elusive.

PcG proteins bind to defined DNA-elements (PREs) that convey epigenetic inheritance throughout development. The role of PREs in chromatin regulation is still unclear. Growing evidence suggest that a simple model based on a constitutive heterochromatin/transcriptionally inhert/inaccessible state of chromatin may not apply to PcG and other repressed loci. As the ultimate goal of epigenetic silencing factors is to block RNA synthesis at core promoters of their target genes, promoter structure is likely contain a key site for transcriptional memory mechanisms.

In early *Drosophila* embryogenesis, inactive state of homeotic gene promoters is determined by segmentation transcription factors that interact with basal trascription machinery. At the same time when segmentation genes define the expression state of homeotic genes, PC (and TRX) proteins bind not only not to PREs but also to core promoters. Thus, we wondered whether the presence of PcG at repressed promoters would imply a direct targeting of RNA Polymerase and basal transcription factors by PcG. By chromatin immunoprecipitation approach (X-ChIP) we found that TATA-binding protein (TBP), the initiation factors TFIIB and TFIIF, differentially phosphorilated RNA Polymerase II, histone deacetylase (RPD3) hypo-acetylated and methylated histones H3 are constitutive components of PcG repressed promoters. Furthermore, we and others found that PcG proteins interact *in vitro* with GTFs. We used RNA interference to *knock down* PcG in SL2 cells. Strikingly, we observed a full derepression of all PcG controlled promoters independent from cell type specific activators. Non-PcG targets were not affected. We propose that epigenetic silenced state of developmentally regulated genes is achieved by constitutive block operated by PcG onto a paused/non-elongating polymerase. An important corollary to these findings is that repressed genes, and in particular developmentally regulated genes retain, in principle the ability to be reactivated even in the absence of specific factors.

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Polycomb silencing and promoter interactions

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Polycomb silencing complexes are assembled at Polycomb Response Elements (PREs) of homeotic genes in the very early embryo but they do not prevent the initiation of transcription dictated by appropriate enhancer elements. Silencing sets in only during gastrulation at promoters that are not transcriptionally active at that time. This may be explained by the fact that an essential component, the PSC protein is not recruited at the PRE but at the promoter, by interaction with TFIID. After blastoderm, a rearrangement takes place, probably involving looping of the PRE complex to contact the promoter. Using a reporter transgene construct, we find that a hsp26 promoter repressed by the PRE retains TFIID and RNA polymerase but is unable to open the strands to form an initiation complex. These results suggest that, rather than preventing access of transcription factors to chromatin, PcG silencing complexes act primarily at the promoter complex.

Recruitment of PcG complexes at the PRE occurs in the pre-blastoderm embryo by multiple DNA binding components, two of which GAGA factor and PHO protein. These factors, together with other unidentified DNA binding components together recruit a complex including PC, PH, ESC and EZ and RPD3 proteins. Targeted acetylation interferes with the formation of the silencing complex but not with the silencing mechanism itself, once the complex has formed. Nevertheless, a short burst of histone acetylation at blastoderm prevents the stable maintenance of the repressed state in later development. We suppose that histone acetylation interferes with the maintenance of histone methylation. The ESC/EZ complex contains in fact a histone methyltransferase activity. Work is in progress to determine how the chromatin methylated by the PcG complex differs from that methylated by heterochromatin proteins or by a Trithorax complex.

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POSTERS

The DNA chaperone function of HMGB1 facilitates ACF-dependent nucleosome sliding

T. Bonaldi, G. Längst, R. Stronher, P. B. Becker and M.E. Bianchi

The significant stability of the nucleosome structure is counteracted by the fact that nucleosomes are moved around all the time when most of nuclear processes take place, for example transcription, replication, repair.

At least two different mechanisms affect nucleosome sliding: covalent modification of histones themselves and mobilization by ATP-dependent machines (1) (2).

The mechanism by which remodelling machines relocate the DNA over the histone octamer still remains elusive: the two most popular scenarios assume that not all the weak histone-DNA interactions are broken simultaneously, but only a small number at any given time. The "twist diffusion model" argues that a small set of histone-DNA interactions is disrupted when the topology of DNA is altered, for example by varying the DNA twist (3).

The "bulge model" suggests that local loops of DNA are detached from the histone octamer surface, starting at the sites where the DNA enters its path around the particle, and are displaced progressively along the length of nucleosome. Such looping could be initiated by thermal energy or by specific condition of high ionic strength, and remodelling machines are postulated to catalyze these events.

Längst and co-workers tested the validity of the "twist diffusion" model by introducing randomly positioned nicks in the nucleosomal DNA substrate: the nicks, although leading to relaxation of any topological stress, did not prevent sliding. This argues against involvement of DNA twist in nucleosome mobilization and – on the contrary - supports the bulging model (4). We reasoned that the rate-limiting step in this process must be the generation and then the propagation of the bulge. So, a protein that can recognize and/or generate local distortions of DNA might facilitate the formation and the propagation of the bulge, and increase the efficiency of nucleosome sliding. HMGB1 is the archetype of proteins that bend and distort DNA, and it is a good candidate to help "lubricate" nucleosome sliding.

In this work we show that the addition of HMGB1 in a well-defined nucleosome sliding system (5) operated by recombinant ACF enhances nucleosome mobility. We also verify that HMGB1 facilitates ACF binding to the nucleosome without being stable part of the complex. The highly dynamic binding of HMGB1 to the nucleosome appears to be a major feature; as a matter of fact, an HMGB1 tailless derivative, which interacts with chromatin in a less dynamic way, strongly inhibits the ACF-mediated sliding. Our data suggest that HMGB1 is able to increase chromatin "fluidity" by generating strategic DNA bends, or "bulges" which are profitably used by ACF to induce nucleosome sliding. Conversely, the ability of HMGB1 to facilitate nucleosome sliding provides further support for the bulge mechanism.

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E-cadherin repression by Snail family factors during tumour progression involves the recruitment of HDACs

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E-cadherin is the main molecule implicated in maintenance of cell to cell contacts and epithelial tissue architecture (1). However, during some process taking place during development and tumour progression cell-cell contacts must be restructured and reorganised. These changes are tightly controlled during development, but became unrestrained during tumour progression, and the lost of the epithelial characteristics leads to the acquisition of invasive and metastatic potential, frequently associated to the acquisition of a mesenchymal phenotype, known as Epithelial-Mesenchymal Transition (EMT)(2).

Several mechanisms have been implicated in the downregulation of E-cadherin expression during EMT, including genetic, epigenetic and transcriptional changes (3). The Snail family of zinc finger transcription factors is one of the candidates for E-cadherin control during development and tumour progression. Indeed, mouse Snail transcription factor has been described as a direct repressor of E cadherin expression in epithelial cell lines (4, 5). However, very little is still known about the mechanisms underlying Snail-mediated E-cadherin repression. In this context, we are investigating the potential involvement of chromatin acetylation. Our results suggest that mSnail repress E-cadherin promoter in a Histone Deacetylase (HDAC) dependent manner. Snail-mediated repression of E-cadherin promoter in epithelial cells is liberated by treatment with Trichostatin A (a HDAC inhibitor) and it is increased by cotransfection of Snail with HDAC-1. Similar results have been obtained in the analysis of mouse Slug, another member of this family highly related to Snail. These findings are in agreement with the analysis of E-cadherin promoter activity in E-cadherin deficient carcinoma cells, with endogenous expression of Snail and Slug, in which deacetylation appears to be one of the mechanisms implicated in E-cadherin repression.

The results obtained so far strongly suggest that repression of E-cadherin by Snail family transcription factors involves the recruitment of HDACs and the subsequent modification of chromatin acetylation status. At present, chromatin immunoprecipitation assays (ChIPs) are being carried out with different cell lines to further characterise the in vivo involvement of HDACs in E-cadherin repression induced by Snail factors.

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In vivo approaches to study the relationship between chromatin structure and transcription elongation in yeast

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A high number of eukaryotic genes have been described to be regulated at the transcription elongation level. Most of them has been studied *in vitro*, using nuclear extracts and biochemical techniques. Several general transcription factors that operate at the elongation level have been identified in this way, and some of them influence elongation by modulating chromatin structure. The presence of close homologs of those factors in *Saccharomyces cerevisiae* makes this organism a tentative model to study transcription elongation.

¿How can chromatin be efficiently transcribed *in vivo*, in spite of being highly packaged? We are trying to give a genetic answer to this question. After *in vitro* mutagenesis of the genes encoding histones H3 and H4, we have isolated a collection of histone mutants conferring sensitivity to 6-azauracil, a drug that impairs transcription elongation by reducing UTP and GTP intracellular pools. We hope that the detailed characterization of these mutants will contribute to uncover the different elements that contribute to transcription elongation through chromatin.

In addition we are developing *in vivo* approaches to study transcription elongation in yeast. In order to measure the efficiency of transcription elongation *in vivo*, we have designed new systems containing reporter genes that are specially sensitive to transcription elongation defects. These new reporters enable to quantify transcription elongation through any given gene and, in combination with *in vivo* run-on assays, provide an estimation of the contribution of chromatin structure to the transcription elongation process.

Regulation of V(D)J recombination in a chromosomal context

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V(D)J recombination is a developmentally regulated process, which is responsible for the generation of antigen receptor diversity at the immune system. Lineage- and developmental regulation of V(D)J recombination is controlled at the level of substrate accessibility to the recombinase machinery. Chromatin structure at antigen receptor loci is modified in a lineage- and developmental stage-dependent manner to control substrate accessibility to the recombinase machinery. It has been demonstrated that enhancers led to developmental-specific long-range chromatin structural changes that allow RAG proteins to access to the RSSs within a particular genomic region at these loci. Interestingly, recent studies have established a very tight correlation between enhancer-dependent developmental regulation of histone H3 hyperacetylation and V(D)J recombination at antigen receptor loci suggesting that histone acetylation status defines the nature of a recombinationally competent state.

The T-cell receptor (TCR) alpha/delta locus is a very interesting model to study developmental regulation of V(D)J recombination because the two genes that it contains have different programs during thymocyte maturation. The TCR delta gene is rearranged and expressed at CD4-CD8-CD25+CD44- (DN-III) thymocytes, whereas the TCR alfa gene is not rearranged and expressed until CD4+CD8+ (DP) thymocytes. Furthermore, TCR delta gene transcription is downregulated in the transition form DN-III to DP thymocytes. The particular organization of this locus, with the TCR delta gene nested between the Valpha and Jalpha gene segments, determines that the entire TCR delta gene is deleted upon TCR alpha gene rearrangement. Hence, studies on the control of developmental V(D)J recombination at these genes are important to understand the mechanisms that regulate the generation of alphabeta and gammadelta T lymphocytes. To elucidate the molecular bases for developmental regulated transcription and V(D)J recombination at the TCR alpha/delta locus, in vivo occupancy and function of the TCR delta enhancer (Edelta), the TCR alpha enhancer (Ealpha), and the T early alpha (TEA) promoter in developing thymocytes was studied. These experiments indicated that Edelta and Ealpha function as developmentally-stage specific enhancers in thymocytes differentiating along the alphabeta pathway, with Edelta "on" and Ealpha "off" at DN-III thymocytes, and Edelta "off" and Ealpha "on" at DP thymocytes. Developmental downregulation of Edelta is associated with a loss of Edelta occupancy. Interestingly, Ealpha and TEA promoter are occupied as early as DN stage III, even though these elements cannot collaborate to drive germline transcription and Valpha to Jalpha rearrangement at this stage. The data indicated that this is a consequence of two distinct events: (1) removal of the TCR delta gene from the influence of Ealpha as a result of chromosomal excision by Valpha to Jalpha rearrangement, and (2) inactivation of Edelta due to loss of transcription factor occupancy. To elucidate the molecular mechanisms for the regulation of gene expression at the TCR genes during T-cell development by enhancers is important to identify the multiprotein complexes that are assembled on them during cell development, as well as to study of nuclear localization of antigen receptor genes during T-cell development.

Suppression of basal HIV transcription by integration into centromeric heterochromatin: a new molecular mechanism for HIV latency

Albert Jordan and Eric Verdin

We have previously reported that the site of integration site of the HIV-1 provirus into the cell genome plays an important role on its transcriptional activity. Based on these observations, we predicted that a small fraction of integration sites might exhibit such low basal activity that no Tat messenger RNA would accumulate. Such situation would keep the provirus locked in the early phase of transcription and would result in a functional state of latency. To test this model, we constructed an HIV-based retroviral vector containing the Tat and GFP open reading frames separated by an internal ribosome entry site. We infected Jurkat cells with virus particles containing this vector and cloned infected cells. Most infected cell lines contained actively transcribed vectors as predicted by our previous observations. However, we identified a single clone (out of 300) exhibiting a true state of transcriptional latency. No GFP was measured under basal conditions. Following treatment with phorbol esters, a marked increased in viral transcription was noted. Sequencing of the integration site demonstrated that the proviral genome has integrated in a centromeric heterochromatin region. Using a PCR-based assay, we have obtained further evidence that integration into centromeric regions is a general mechanism leading to latent infection in vitro. Similar results have been obtained using a full-length HIV vector. Furthermore, we are currently comparing the remodeling state and histone modifications code of Nuc-1, a nucleosome positioned immediately downstream of the transcription start in the HIV promoter, when integrated into euchromatin or heterochromatin.

Comparing CHRAC and ISWI: Sequence directed nucleosome mobility and binding on the surface of a nucleosome

Gernot Längst and Peter B. Becker

We recently showed that the ATPase ISWI, the catalytic core of the chromatin accessibility complex (CHRAC), and CHRAC induce movement of intact histone octamers to neighbouring DNA segments without facilitating their dispacement to competing DNA in trans. In the nucleosome mobility assay, nucleosomes are assembled on short DNA fragments and positioned at the center or the end of the DNA. Using a DNA fragment originating from the mouse ribosomal promoter, we can show that ISWI moves the centrally positioned nucleosome to only one end of the DNA fragment. This is a sequence specific property of the ribosomal DNA because other DNA fragments have been identified moving centrally positioned nucleosomes to different border positions, or even laterally positionend nucleosomes to the center of the DNA fragment. In agreement with this sequence directed nucleosome mobility of the rDNA fragment we observed orientation dependent binding of CHRAC and ISWI to the surface of the nucleosome. ISWI and CHRAC interact with the border of the nucleosome, where the DNA enters and leaves the histone octamer. ISWI binds only to one side of the nucleosome whereas CHRAC interacts with both sides of the nucleosome. We also analyzed the mechanistics of nucleosome mobility and propose a model whereby DNA bending rather than twisting induces nucleosome mobility.

A role for the Snf1p kinase in coordinating recruitment of Snf-Swi and acetylation of histones H3 and H4 for SUC2 derepression

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Multiple chromatin-modifying activities are involved in the control of gene expression in eukaryotes. Upon derepression of the glucose-repressible SUC2 gene, both the ATP-dependent Snf-Swi complex and the histone acetyltransferases (HATs), Gcn5p and Esa1p, were rapidly recruited to the SUC2 promoter. Snf-Swi bound continuously to the derepressed promoter. The increased acetylation of histones H3 and H4, shown to be solely dependent on Gcn5p and Esa1p, respectively, was most marked immediately following derepression. Snf-Swi could be recruited to the SUC2 promoter without the Gcn5p and Esa1p activities. Conversely, acetylation of SUC2 histones could occur in the absence of Snf-Swi nucleosome-remodeling activity. The independence of Snf-Swi recruitment and histone acetylation suggests that Snf-Swi and the HATs contribute to SUC2 transcription through parallel pathways. Importantly, the Snf1p kinase activity, which is essential for SUC2 gene derepression, was required for Snf-Swi recruitment, H3 acetylation, and to a lesser extent, H4 acetylation. This suggests that Snf1p plays a key role in coordinating the apparently independent Snf-Swi nucleosome-remodeling and histone acetylation activities in SUC2 derepression.

Chromosomal integration of retinoic acid response elements prevents cooperative transcriptional activation by RAR and RXR

Bruno Lefebvre , Céline Brand , Philippe Lefebvre, and Keiko Ozato

All-*trans* retinoic acid receptors (RAR) and 9-*cis* retinoic acid receptors (RXR) are nuclear receptors known to activate cooperatively transcription from retinoid-regulated promoters. By comparing the transactivating properties of RAR and RXR in P19 cells using either plasmid or chromosomal reporter genes containing the mRARb2 gene promoter, we found contrasting patterns of transcriptional regulation in each setting. Cooperativity between RXR and RAR occurred at all times with transiently introduced promoters, but was restricted to a very early stage (<3h) for chromosomal promoters. This time-dependent loss of cooperativity (LOC) was specific for chromosomal templates containing two copies of a retinoid responsive element (RARE) and was not influenced by the spacing between the two RAREs. This LOC suggested a delayed acquisition of RAR full transcriptional competence since (i) cooperativity was maintained at RAR ligand sub-saturating concentrations, (ii) overexpression of SRC1 led to LOC and even to strong repression of chromosomal templates activity and (iii) LOC was observed when additional *cis*-acting response elements were activated. Surprisingly, histone deacetylase inhibitors counteracted this LOC by repressing partially RAR-mediated activation of chromosomal promoters. LOC was not correlated to local histone hyperacetylation, nor to alteration of the constitutive RNA polymerase II (RNAP) loading at the promoter region. Unexpectedly, RNAP binding to transcribed regions was correlated to the RAR activation state, as well as to acetylation levels of histone H3 and H4, suggesting that RAR acts, at the mRARb promoter, by triggering the switch from a RNA elongation-incompetent RNAP form towards a RNA elongation-competent RNAP.

Mechanisms of transcriptional activation of the CCAAT-binding NF-Y

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The CCAAT-box is a widespread promoter element that is activated by NF-Y a trimer composed of the histone fold NF-YB-NF-YC and of NF-YA. NF-Y efficiently binds nucleosomal DNA. We find that acetylated NF-YB and NF-YA, but not NF-YC, are present in mammalian cells and that inhibition of HDAC activity in vivo decreases CCAAT-binding. NF-YA and NF-YB are acetylated in vitro by p300 and mutational analysis identified residues NF-YA within the HAP2 conserved domain, and in NF-YB within histone fold lysines involved in DNA recognition; indeed, acetylation of NF-Y in vitro leads to inhibition of DNA-binding. Binding of non-acetylated NF-Y to an MHC class II Ea promoter nucleosome recruits p300, largely increasing its HAT activity on hisones and NF-Y. This effect depends upon an intact CCAAT and the NF-Y capacity to associate nucleosomes. Indeed, acetylation of p300 and NF-Y increases their reciprocal affinity. Thus when recruited on a nucleosome, p300 HAT activity targets histones as well as a transcription factor required for recruitment.

In another set of experiments, we studied in detail the mechanisms of NF-Y-mediated p300 recruitment on the Cyclin B2 promoter, containing triple CCAAT boxes that are bound by NF-Y in ChIP assays in vivo. Binding of NF-Y to the three sites is normally not cooperative in EMSAs, but addition of p300 leads to strong cooperative effects. Recruitment of p300 is mediated by the NF-Y Q-rich activation domains in NF-YA and NF-YC. The precise arrangements of the three sites are also crucial for functional p300 interactions.

Heterochromatin Protein 1 homologue in *C. elegans* acts in germline and vulval development

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Proteins of the highly conserved heterochromatin protein 1 (HP1) family have been found to function in the dynamic organization of nuclear architecture and in gene regulation throughout the eukaryotic kingdom (Eissenberg and Elgin, 2000) (Jones et al., 2000). In addition to being key players in heterochromatin-mediated gene silencing, HP1 proteins may also contribute to the transcriptional repression of euchromatic genes via the recruitment to specific promoters (Nielsen et al., 2001). To investigate the role played by these different activities in specific developmental pathways, we identified HP1 homologues in the genome of *C. elegans* and used RNA-mediated interference (RNAi) to study their function. We have shown that one of the homologues, HPL-2, is required for the formation of a functional germline and for the development of the vulva by acting in an Rb-related pathway. We suggest that by acting as repressors of gene expression, HP1 proteins may fulfill specific functions in both somatic and germline differentiation processes throughout development.

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Impairment of chromatin assembly increases genomic instability

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Homologous DNA recombination plays an essential role in DNA repair and genomic stability. Homologous recombination can lead to loss of heterozygosity and genetic rearrangements that have been observed to be the cause of several different genetic diseases, including cancer. Chromatin organization plays critical roles in the regulation of transcription and replication, and specific chromatin remodeling machineries have been reported to be active regulators of such processes. In recombination, like in any other DNA metabolic process, different machineries have to deal with nucleosomes. This fact makes chromatin an essential factor to be considered during the recombination process. To underscore the role of chromatin in mitotic recombination we have designed a genetic assay to vary the levels of histone H4, thereby modulating the nucleosome density. Under these experimental conditions, we have shown that a depletion of histone H4 in yeast cells leads to 1) an increase in the frequency of mitotic recombination between direct and inverted repeats and 2) a defect in DNA repair of double strand break (DSB)-induced lesions. Importantly, overexpression of histone H4 does not affect those events.

To get insight in the molecular mechanism underlying the recombination events stimulated by partial depletion of histone H4 we have analyzed their connection with other DNA associated processes. In contrast to other related recombination events, partial depletion-stimulated recombination is not dependent on transcription through the recombination system. Impairment of chromatin assembly by deletion of either the chromatin assembly factor ASF1 or the amino terminal tail of histone H4 leads to increments in mitotic recombination and defects in repair of DSB-induced damage. Besides, depletion of histone H4 and deletion of ASF1 generate identical chromatin modifications as determined by MNase I accessibility. Our results provide evidence connecting proper chromatin organization and regulation of mitotic recombination in yeast cells.

Genetic interactions suggest a role for Htz1 protein in transcription elongation

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The variant histone H2A.Z, encoded by HTZ1 in *S. cerevisiae*, regulates gene transcription and is partially redundant with chromatin remodeling (SNF/SWI) and modifying (SAGA) complexes. We have carried out a synthetic lethal screen to identify other proteins that function in concert with Htz1 to regulate essential functions in *S. cerevisiae*. One of the genes identified in the screen encodes RPB2, the second largest subunit of RNA pol II. The rpb2-2 mutation is dominant in the absence of Htz1 function, as if the template associated mutant polymerase arrests and blocks transcription by wild type pol II. This suggested that Htz1 might have a role in elongation, either releasing the paused polymerase or improving its processivity. In support of this hypothesis, we found that htz1D cells are sensitive to 6-azauracil (6-AU), a drug that leads to depletion of the RNA precursors UTP and GTP. To begin to investigate the role of Htz1 in transcription elongation we analyzed the genetic interactions between HTZ1 and genes encoding known transcription elongation factors including DST1, SPT4, SPT5, SPT6, SPT16 and ELP3. The dst1D htz1D, spt4D htz1D and elp3D htz1D double mutants are all impaired for growth and both, spt4D htz1D and dst1D/htz1D cells are hypersensitive to 6-AU. We also identified two mutations in SPT5 that are synthetic lethal with htz1D as well as mutations in SPT6 and SPT16 that suppress 6-AU sensitivity of htz1D. These data are consistent with a role of Htz1 in transcription elongation.

Hormonal regulation of chromatin structure by the Forkhead Transcription Factor TTF-2

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The goal of this study is to understand the role of chromatin structure in the hormonal regulation of a tissue specific promoter.

Thyroperoxidase, TPO, is an essential enzyme in thyroid hormone synthesis. The minimal promoter necessary and sufficient to confer tissue specificity and hormonal responsiveness has been identified (1) and contains binding sites for three tissue-specific transcription factors, TTF-1, TTF-2, Pax-8 and for the ubiquitous transcription factor NF-1 (2). The forkhead factor TTF-2 is the main mediator of thyrotropin/cAMP and insulin/IGF-1 regulation of TPO gene expression (3).

Here we analyzed TPO promoter regulation in cells under two different hormonal conditions where the gene is or not expressed.

By *in vivo* footprinting analysis we demonstrate a hormonal regulation for the binding of all the transcription factors to the TPO promoter. Strikingly, only TTF-2 expression correlates with TPO gene activation (3), indicating that this factor is limiting for TPO expression. Studies done with HNF-3 demostrate a chromatin remodeling role for forkhead factors (4). These facts together with the observation that HNF-3 can regulate TPO activation *in vitro* in the same manner as TTF-2 (5), led us to investigate the possibility for hormonal regulation of the TPO promoter chromatin structure. MNase and DNase I sensitivity assays revealed the presence of nucleosomal particles over the active TPO promoter *in vivo*. In the absence of hormonal stimuli, the DNase I hypersensitivity pattern is altered, indicating changes in the chromatin conformation. Future studies will involve more detailed analysis of the hormonal regulation of chromatin structure by forkhead factors.

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Control of CBP co-activating activity by arginine methylation

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The histone acetyl transferases CBP (*CREB Binding Protein*) and the related p300 protein function as key transcriptional co-activators in multiple pathways. In the case of transcriptional activation by nuclear receptors, CBP and p300 are recruited by co-activators of the p160 family, which GRIP-1 belongs to. Recently, GRIP-1 has also been shown to activate transcription through the recruitment of the arginine methyl transferase CARM1.

Here, we report that CBP and p300 are specifically methylated by CARM1 on conserved arginine residues *in vitro*. We also show that CBP is methylated by CARM1 *in vivo*. Finally, we provide functional evidence that arginine residues which are methylated by CARM1 play a critical role for transcriptional activation by GRIP-1. Thus, our data indicate for the first time that arginine methylation is an important mechanism for modulating co-activator transcriptional activity.

Heterochromatin formation and silencing in *Saccharomyces cerevisiae*

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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 vicinity1-4. 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. Thus, silencing at natural subtelomeric regions is expected to depend on the nature of the specific subtelomeric genes and on their location1,2,5-10.

The right telomeric region of *Saccharomyces cerevisiae* chromosome VI silence the expression of the first subtelomeric ORF located after the TAS (YFR057w), through a process that requires the structural integrity of heterochromatin10. Several proteins are known to be involved in heterochromatin formation, including RAP1, SIR2, SIR3, SIR4 and histones H3/ H4. We have found that at the end of the right telomeric region of chromosome VI, SIR4 is independently recruited by RAP1, while the recruitment of SIR2 and SIR3 are completely dependent on the presence of SIR4 protein. In contrast, further along telomeric heterochromatin, all the SIR proteins are required for SIR spreading and silencing of YFR057w. These data demonstrate that SIR4 helps initiate the sequential association of other SIR proteins at RAP1 sites allowing the subsequent spreading of the silent state and is a key factor that determines heterochromatin formation and function.

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Down-regulation of the nucleosome assembly pathway by the *S. cerevisiae* Rad53 kinase

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Several lines of evidence from our laboratory have recently converged into a model where the *S. cerevisiae* Rad53 protein kinase plays a pivotal role to down-regulate the nucleosome pathway in response to DNA damage and histone overexpression. First, rad53D and catalytically impaired rad53 kinase mutant cells are extremely sensitive to histone over-expression. This effect is due to the fact that rad53 mutant cells fail to degrade over-expressed histones and, as a result, accumulate large amounts of free histones in the nucleus. Second, following various types of DNA damage or inhibition of DNA replication, newly synthesised histones and chromatin assembly factors re-localise from the nucleus to the cytoplasm. Interestingly, this re-localisation is also Rad53 kinase-dependent. Third, we find that histone mRNAs disappear in a Rad53 kinase-dependent manner when S-phase cells are treated with the alkylating agent MMS, which is known to slow down DNA replication. We propose that down-regulation of the nucleosome assembly pathway is an intrinsic component of the DNA damage response, which prevents cytotoxic effects due to histone overexpression when cell cycle delays due to DNA damage occur either in late G1 or throughout S-phase. Our progress towards identifying targets of the Rad53 kinase in the nucleosome assembly pathway will also be presented.

Asymmetric binding of histone H1 stabilizes MMTV nucleosomes and the interaction of progesterone receptor with the exposed HRE

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Packaging of Mouse Mammary Tumor Virus (MMTV) promoter sequences in nucleosomes modulates access of DNA binding proteins and influences the interaction among DNA bound transcription factors. Here we analyze the binding of histone H1 to MMTV mononucleosomes assembled with recombinant histones and study its influence on nucleosome structure and stability as well as on progesterone receptor (PR) binding to the hormone responsive elements (HREs). The MMTV nucleosomes can be separated into three main populations, two of which exhibited precise translational positioning. Histone H1 bound preferentially to the 5´distal nucleosomal DNA protecting additional 27-28 nt from digestion by micrococcal nuclease. Binding of histone H1 was unaffected by prior crosslinking of protein and DNA in nucleosomes with formaldehyde, suggesting that the apposition of histone H1 did not require dissociation of the nucleosome structure. Neither the translational nor the rotational nucleosome positioning was altered by histone H1 binding, but the nucleosomes were stabilized as judged by the kinetics of nuclease cleavage. Unexpectedly, binding of recombinant PR to the exposed distal HRE in nucleosomes was enhanced in the presence of histone H1. This enhanced PR affinity may contribute to the positive effect of histone H1 on the hormonal activation of MMTV reporter genes.

Conservation of the function of the Polycomb group protein Ring1 in mice and flies

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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. 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, an ortholog of the *D. melanogaster Polycomb* gene. We showed recently that *Ring1A* is a new member of the PcG of genes as deduced from the phenotypes of mice with loss and gain of *Ring1A* function. A singularity of the *Ring1* genes, however, is that in contrast to other vertebrate PcG genes, no known mutations of *Drosophila* PcG genes encoding Ring1 homologs have been identified yet.

We found that the product of *Dring,* the *Drosophila* ortholog of mammalian *Ring1* genes, encodes a chromosomal protein that binds to >100 specific sites, most of which bind also the PcG proteins Polycomb, Polyhomeotic, Polycomblike and Posterior sex combs. This is in agreement with the finding that Dring is among the components of the Polycomb repressing complex 1 isolated from *Drosophila* embryos.

Based on the localization of the *Dring* gene in polytene chromosomes we searched for deficiencies that may show a Polycomb phenotype throughout the 97C-98B interval. In such an interval localizes also the Polycomb mutation *Sce*, which is not molecularly characterized. We found a deficiency which, in combination with *Sce1*, was lethal. In turn, that deficiency also interacted with *Polycomb* (*Pc3* allele). The phenotype of both the offspring of *Sce1* germ line mutant females and of males with the deficiency was identical to that of *Sce1* mutants lacking the maternal component. Such a phenotype is characterized by the homeotic transformation of all the segments to the identity of that the 8th abdominal segment.

We then attempted to rescue the Sce1 phenotype (maternal and zygotic) using a UAS-DRing construct. The generalized and early expression of *Dring* complemented totally the Sce mutation. In addition, a more limited *DRing* expression such as that driven by a *paired*-GAL4 line resulted in partial rescue, restricted to the paired expressing segments. A similar phenotypic rescue was also observed in transgenic flies expressing the murine Ring1A protein. From these results we conclude that there is a functional conservation of Ring1 proteins in mice and flies.

TGFβ-dependent regulation of cyclin A expression

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Cytokines of the TGFβ family regulate biological processes ranging from cellular proliferation to differentiation or development. Signaling by TGFβ from cell membrane to nucleus can be mediated by SMAD proteins which act both as signaling proteins and transcription factors. In particular, Smad2 and Smad3 are serine phosphorylated in a TGFβ-dependent fashion, associate with Smad4 and translocate to the nucleus where they bind target gene promoters. We and others have previously shown that cyclinA2 transcription is inhibited in response to TGFβ, via a CRE site on the cyclinA promoter.

We have been studying the involvement of SMAD proteins and the role of both the CRE site and the inverted CCAAT box, located 25bp downstream of the CRE site, for that repression. By transient transfections, we found that both the SMADs and the transcription factor NF-Y are involved in the TGFβ dependent repression of cyclinA2 expression and we could correlate this role with an increased DNA binding activity in gel shifts. Using chromatin imunoprecipitation coupled to quantitative PCR, we were able to show an enhancement of both NF-Y and SMAD binding to the endogenous cyclin A promoter over the two hour time course of TGFβ treatment. Altogether, these data suggest that SMADs can mediate TGFβ-dependent signaling to the cyclin A promoter and that both the CRE and the inverted NF-Y sites are targets for this signaling.

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