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Chromatin and Cancer: Causes and Consequences

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Abstract In this review, we discuss recent evidence implicating chromatin structure in the etiology of cancer. In particular, we present evidence indicating that inappropriate regulation of chromatin structure inhibits normal cell differentiation pathways and stimulates uncontrolled cell proliferation, with the outcome being oncogenesis. Such inappropriate chromatin structures arise as a consequence of (i) chromosomal rearrangements that fuse gene-specific activators with global co-regulators, drastically altering activator function; (ii) hypermethylation of tumor suppressor gene promoters, resulting in their inactivation; or (iii) mistargeted nuclear compartmentalization of growth-control genes and their regulators, resulting in the up- or down-regulation of such genes. How does chromatin silence genes? Recent results from model in vivo systems argues that chromatin can repress transcription at two levels: (i) by sterically interfering with the binding of transcription factors to the promoter, thereby blocking initiation; and (ii) at a step subsequent to the binding of activators and recruitment of the preinitiation complex. J. Cell. Biochem. Suppl. 35:61–68, 2000. Published 2001 Wiley-Liss, Inc.[†]

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CHROMATIN AND GENE REGULATION

It is now clear that a large number of sequence-specific transcription factors regulate gene expression through their ability to remodel chromatin structure. While some, like human CTF-1, may work by directly binding to the nucleosomal histones [Alevizopoulos et al., 1995], others mediate remodeling indirectly, through recruitment of multisubunit enzymatic complexes. Three types of enzymatic activity can modify chromatin structure. ATP-dependent chromatin-remodeling enzymes, epitomized by the highly conserved SWI/SNF complex, promote changes in chromatin structure by loosening contacts between the N-terminal tails of the core histones and the DNA, resulting in increased nucleosomal mobility [reviewed in Kingston and Narlikar, 1999]. A second class of remodeling enzymes consists of the nuclear histone acetyltransferases (HATs) that covalently modify specific lysine residues within the N-terminal tails of the four core histones. Acetylation of histone tails can disrupt the higher order folding of the chromatin fiber as well as loosen histone–DNA interactions within individual nucleosome cores [Luger et al., 1997]. Moreover, by analogy with phosphotyrosine–SH2 domain interactions, acetylation may mark histones for interaction with bromodomain proteins, which bind with high affinity to acetyl lysines [Strahl and Allis, 2000]. Acetylation, similar to phosphorylation, is a dynamic process. Underscoring this point, a large number of enzymes that deacetylate histones (histone deacetylase complexes [HDACs]), some existing in association with ATP-dependent remodeling enzymes [Zhang et al., 1999], have also been identified. Inappropriate recruitment of any one of these chromatin-modifying activities to growth-control genes can result in cancer.

One of the best examples of this is acute promyelocytic leukemia (APL), which is associated with at least two different chromosomal arrangements that drastically modify the chromatin-remodeling capability of the retinoic acid nuclear receptor (RAR). RAR is a sequence-specific DNA binding factor that regulates

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genes important for myeloid and epithelial cell differentiation; it heterodimerizes with RXR (retinoid X receptor), binds to target promoters, and actively represses transcription [reviewed in Redner et al., 1999]. Repression of target genes is mediated through the ligand binding region of RAR, which interacts with two co-repressors, N-CoR and SMRT, that recruit an intermediary protein, Sin3A. Sin3A in turn recruits the effector molecule, HDAC1. These sequential interactions result in local deacetylation of histones and formation of a repressive p 300, chromatin structure. Upon binding retinoic acid, RAR undergoes a conformational change leading to the dissociation of the co-repressor complex. This RAR intermediate now recruits p 300, a large co-activator complex containing multiple HATs (see below), resulting in acetylation of adjacent nucleosomes.

The most frequent translocation associated with APL, t(15;17), results in the fusion of RARα to PML (promyelotic leukemia), a protein that functions as a scaffold in the formation of PML bodies (see below). Fusion of RARα to PML blocks the ligand-induced conformational change in RARα, resulting in the constitutive repression of genes required for myeloblast differentiation. Treatment of PML-RARα myeloid cell lines with pharmacological levels of retinoic acid (RA) or with trichostatin A (TSA), an HDAC inhibitor, induces their differentiation, consistent with a critical role for hypoacetylated, repressive chromatin in leukemogenesis [He et al., 1998].

Another common genetic lesion in APL is the t(11;17)(q23;q21) translocation. This results in fusion of RARα to the promyelocytic leukemia zinc finger (PLZF) protein, required for proper patterning of the limb and axial skeleton [Barna et al., 2000]. PLZF is a co-repressor protein that independently binds HDACs. Thus, APL caused by the PLZF-RARα fusion is resistant to RA treatment. Some success in the treatment of this type of APL has been obtained by the use of both HDAC inhibitors and RA, again implicating a chromatin-based etiology [He et al., 1998] [reviewed in Melnick and Licht, 1999; Redner et al., 1999].

ALM1-ETO is the most common oncoprotein found in acute myeloid leukemia (AML). It results from a t(8;21) reciprocal translocation, joining the N-terminal DNA-binding domain of the AML-1 sequence-specific activator to a putative repressor, ETO (Eight Twenty One). While the physiological role of ETO is unknown, it is comprised of four highly conserved domains. The N-terminal domain is homologous to *Drosophila* $TAF_{II}110$ and may play a role in ETO nuclear body formation; the adjacent domain is responsible for homodimerization; a third conserved domain, located in the C-terminal half, is of unknown function; and the C-terminal zinc finger domain confers repression through interaction with N-CoR and SMRT. Thus, the AML1-ETO fusion protein may exert its oncogenic effect through the inappropriate recruitment of HDACs to the promoters of genes regulated by AML1, leading to a repressive chromatin structure that blocks transcriptional activation [reviewed in Redner et al., 1999].

The mixed lineage leukemia (MLL) gene is the human homologue of *Drosophila trithorax* (*trx*), which is required for homeotic gene expression. In certain types of leukemia, MLL is found fused to either CBP or p300, which are abundant and multifunctional co-activators possessing intrinsic HAT activity [Giles et al., 1998]. The CBP/p300 acetyltransferase interacts with a large number of nuclear proteins, including gene-specific activators, TFIIB, and other co-activators. In so doing, it serves as a transcriptional integrator for a variety of extracellular stimuli in coordinating cell growth, differentiation, and even apoptosis. Thus, in contrast to the foregoing examples, MLL-CBP or MLL-p300 may be leukemogenic through inappropriate gene activation, possibly of genes not normally regulated by these proteins.

In addition to the fusion of global co-regulators to gene-specific activators, other types of misregulated chromatin remodeling activity have been implicated in oncogenesis. For example, mutations in the *BRCA1* tumor suppressor gene predispose women to familial breast cancer. *BRCA1* plays important roles in DNA repair as well as in homologous recombination. It also acts as a transcriptional co-activator and requires the chromatin remodeling activity of the human SWI/SNF complex. Loss of exon 11 is a frequent *BRCA1* mutation; it leads to loss of interaction with Brg1, the catalytic subunit of a major hSWI/SNF complex [Bochar et al., 2000]. Mutations in a second hSWI/SNF subunit, SNF5, have also been identified in certain malignant pediatric tumors. These and other observations suggest an important role for hSWI/SNF in regulating cell differentiation

and proliferation. How might this happen? hSWI/SNF is a critical co-activator of many transcription factors. At nuclear receptor and *BRCA1*-regulated genes, SWI/SNF recruitment may act to increase nucleosomal fluidity, thereby facilitating the subsequent binding of transcription factors to the chromatin template. An activated state could be secured by the subsequent acetylation of the nucleosomal histones by CBP/p300, P/CAF, or other HATs [Kingston and Narlikar, 1999]. Alternatively, the fluid chromatin could be locked into place by the binding of other gene-specific activators or components of the preinitiation complex (PIC), TATA-binding protein (TBP) in particular. In other instances, SWI/SNF may facilitate the formation of a repressive chromatin structure. Association of a second hSWI/SNF complex, hBrm, with the retinoblastoma (Rb) tumor suppressor protein acts to decrease the activation of E2F1, an activator of genes involved in cell-cycle progression, thereby promoting G1 arrest [Trouche et al., 1997]. A repressed state might be achieved by subsequent recruitment of histone deacetylase activity or the binding of sequence-specific repressors.

Evidence for temporal ordering in the binding of chromatin remodeling and modification complexes at gene promoters has been obtained for mitotically activated genes in the yeast, *Saccharomyces cerevisiae* [Krebs et al., 2000]. In the case of the particularly well-studied *HO* gene, transient binding by a far-upstream activator (Swi5) is followed by the recruitment of SWI/SNF, which in turn recruits an abundant histone acetylase complex (SAGA [Spt-Ada-Gcn5 acetyltransferase]); together these complexes effect acetylation of promoter chromatin, facilitating the binding of a second activator, SBF [Cosma et al., 1999]. The latter presumably recruits TBP and other components of the PIC, leading to transcriptional activation.

In summary, inappropriate activity of any one of the three chromatin modifying complexes can result in misregulation of genes involved in cell growth and differentiation, ultimately leading to oncogenesis.

DNA METHYLATION, CHROMATIN, AND GENE REGULATION

Methylated cytosine residues (m^5Cyt), occurring within the palindromic sequence CpG, mark locations of transcriptionally silent DNA within the mammalian genome. Islands rich in CpG dinucleotides are often found in promoter regions of "housekeeping" genes that are actively transcribed; these ~1 kb islands are typically not methylated. In contrast, CpG islands associated with tissue-specific genes are subject to methylation during embryogenesis and development. Methylation of the CpG sites within the promoters of these genes can effectively, and heritably, lock the genes in an inactive state. In interesting con rast, methylation of CpG sites downstream of the transcription start site does not block transcription, implying that methylation acts locally and at the level of initiation, not elongation, in mammalian cells [Jones and Laird, 1999].

Inappropriate DNA methylation of tumor suppressor gene promoters contributes directly to progression of some cancers. For example, mutations within the *BRCA1* structural gene are not typically found in sporadic breast cancers, in contrast to familial breast cancers (see above). Nonetheless, cell lines derived from such tumors are often heavily methylated in the gene's promoter region, correlating with an absence of *BRCA1* expression [Rice and Futscher, 2000]. Similarly, abnormal methylation of CpG sites in the promoter of $p16^{INK4A}$ can result in its inactivation. $p16^{INK4A}$ encodes a cyclin-dependent kinase inhibitor that acts upstream of Rb, maintaining Rb in an active state. $p16^{INK4A}$ can be inactivated by deletion, point mutation, and DNA methylation. In certain colon cancers, $> 90\%$ of $p16^{INK4A}$ inactivation is through *de novo* promoter methylation [Herman et al., 1995]. Similarly, inactivation of *hMLH1*, a gene encoding a DNA mismatch repair component, occurs ~70% of the time through promoter hypermethylation in sporadic colorectal carcinomas [Herman et al., 1998]. Inappropriate methylation of other tumor-suppressor genes, including Rb and Von-Hippel Lindau, has also been linked to their functional inactivation. Thus, inactivation of genes critical to growth control can occur without changes in DNA sequence, leading to malignancy.

How does methylation block transcription initiation? One possibility is that factor binding is inhibited by the presence of the 5-methyl group, which can sterically interfere with major groove-binding proteins. However, this appears not to be a generally applicable mechanism since (i) promoters bearing multiple m^5Cyt

residues are transcribed as efficiently as those lacking m^5Cyt when chromatin is absent; and (ii) binding of certain abundant activators that have CpG in their DNA recognition elements, Sp1 and CTF-1 in particular, is not affected by m^5Cyt (although the binding of others, including NF-κB, c-Myc, E2F, and AP-2, is) [Eden and Cedar, 1994]. Instead, DNA methylation appears to principally exert its repressive effects through alteration of local chromatin structure. This is brought about by the binding of two abundant nuclear proteins, MeCP2 and MBD3, to methylated DNA [reviewed in Bird and Wolffe, 1999]. MeCP2 contains an m^5Cyt-binding domain (MBD) and a transcriptional repressor domain (TRD). The TRD directly interacts with the co-repressor Sin3A, which in turn interacts with HDACs 1 and 2, leading to targeted deacetylation of promoter-associated nucleosomes [Jones et al., 1998; Nan et al., 1998]. MBD3 is associated with the Mi2/NuRD complex, an ATP-dependent remodeling complex with histone deacetylase activity [Zhang et al., 1999]. Recruitment of these histone-modifying complexes to regions of methylated DNA by MeCP2 and MBD3 explains why methylated DNA is often associated with hypoacetylated histones. Addition of TSA is not sufficient to fully relieve repression associated with DNA methylation. However, when TSA is used in conjunction with pretreatment with 5-deoxy-azacytidine, a demethylating agent, such repression can be overridden [Cameron et al., 1999]. This implies that recruitment of histone deacetylase activity is not the sole mechanism by which DNA methylation elicits repression.

An interesting case in which *loss* of DNA methylation is thought to underlie oncogenesis is the ICF (immunodeficiency/centromeric region instability/facial anomalies) syndrome, characterized by hypomethylation as well as decondensation of centromeres. Two-thirds of cells in lymphoblastoid cell lines derived from ICF patients show chromosomal fusions between the centromeric regions of chromosomes 1 and 16, resulting in multi-radial arms [Tuck-Muller et al., 2000]. Methylation may be critical to ensure packaging of centromeric sequences into repressive chromatin structures, thereby preventing homologous recombination. Thus, in ICF patients, loss of methylation results in loss of repressive chromatin structure and consequent genome instability.

NUCLEAR SUBLOCALIZATION, CHROMATIN, AND GENE SILENCING

While the nucleolus, site of ribosomal RNA synthesis, is the best-understood subnuclear structure, it is not the only one. Many other, functionally distinct subcompartments exist within the nucleus. For example, several co-repressors implicated in leukemogenesis, PML, PLZF, ETO, and Ikaros, are normally associated with HDACs and show subnuclear localization. Interestingly, in certain cases, the nuclear structures defined by these proteins are disrupted in cancers. For example, fusion of PML with RARα results in loss of PML bodies and formation of microspeckles. PML bodies, present in all mammalian cells, contain a rich assortment of proteins including sequence-specific activators, co-activators, co-repressors, as well as the heterochromatin proteins 1α and 1β (HP1α and HP1β [reviewed in Seeler and Dejean, 1999]. Treatment of APL cell lines with retinoic acid results in the reappearance of the PML bodies, concomitant with their differentiation [Koken et al., 1994]. In the case of AML1-ETO, the fusion protein is targeted to nuclear structures defined by ETO, which are distinct from those containing AML1 [Odaka et al., 2000]; such mistargeting may underlie the phenotype associated with this gene fusion.

Ikaros, a sequence-specific transcriptional repressor required for proper lymphoid development, localizes to pericentromeric heterochromatin. This targeting is achieved through the protein's zinc finger DNA binding domain, and is due to the presence of high-affinity binding sites within centromeric repeats [Cobb et al., 2000]. Genes silenced by Ikaros also co-localize to pericentromeric heterochromatin, suggesting that Ikaros may recruit target genes to this inactive nuclear fraction. This co-localization pattern is lost in leukemia cells that contain mutated forms of Ikaros [Sun et al., 1999].

The presence of ETO bodies raises the question of whether chromatin remodeling enzymes are principally (or exclusively) found in concentrated foci in the nucleus. If so, how can they be recruited to individual promoters? Or, in analogy to Ikaros, might individual promoters be recruited to these bodies? While these questions remain unresolved, a potential insight into the function of PML, PLZF, and ETO bodies is provided by the recent identification of MAD (matrix associated deacetylase) bodies. These

are similar in size and number to PML bodies, but are clearly distinct from other subnuclear structures. The MAD bodies contain class I and class II deacetylase complexes as well as N-CoR and SMRT co-repressors. Inhibition of deacetylase activity using TSA and sodium butyrate results in the loss of MAD bodies [Downes et al., 2000]. Thus, subnuclear compartmentalization occurs only under conditions where the chromatin remodeling enzymes are catalytically active. This could mean that such particles represent the actual sites of chromatin-mediated repression or activation.

THE YEAST *HSP82* HEAT SHOCK GENE: TWO DISTINCT MECHANISMS FOR CHROMATIN-MEDIATED REPRESSION

The foregoing examples suggest a strong correlation of misregulated chromatin remodeling with oncogenesis, and raise questions as to mechanism. It is difficult to perform such analysis in mammalian cell culture systems due to their genetic intractability. The use of simpler, more tractable organisms such as the yeast *S. cerevisiae* can allow a more exacting molecular dissection of the mechanisms involved in chromatin regulation of gene expression. In the final section of this review, we describe a model system that our laboratory has used to address the mechanism(s) by which chromatin regulates promoter activity. The model employs the yeast *HSP82* heat shock gene, a dynamically regulated, stress-responsive gene. Under both non-inducing and acutely inducing conditions, the promoter region of *HSP82* is assembled into a nuclease-hypersensitive, nucleosome-disrupted structure [Szent-Gyorgyi et al., 1987]. Nonetheless, despite the absence of canonical nucleosomes, the default state of the promoter is a stable dinucleosome. This is based on two lines of evidence. First, reconstitution of promoter DNA with core histones results in the formation of two translationally positioned nucleosomes: Nuc -1, centered over the core promoter and Nuc -2, centered over the upstream regulatory elements [A.M. Erkine and D.S. Gross, unpublished observations]. And second, when the gene-specific activator, heat shock factor

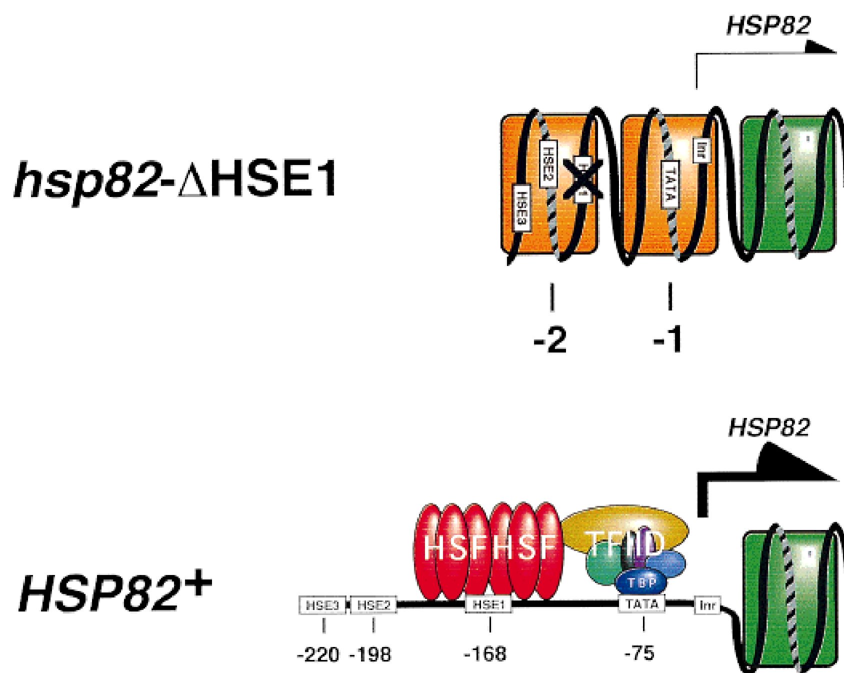

Fig. 1. Euchromatin-mediated repression in *S. cerevisiae*. A pair of precisely positioned nucleosomes occupy the repressed *hsp82*-ΔHSE1 promoter, impairing the binding of cognate transcription factors to their regulatory sites. Nuc -1 assembles core promoter DNA, obviating binding of TBP, and Nuc -2 assembles the upstream promoter sequence, impairing binding of HSF. This allele is transcribed at less than 1% the frequency of the wild-type gene (*HSP82*$^{+}$), symbolized by the thin transcription arrow. The actual stoichiometry of HSF trimers bound to the *HSP82*$^{+}$ promoter is unknown; the non-induced state is depicted. In the induced state, all three heat shock elements are occupied and twice as much HSF is bound to the DNA [Erkine et al., 1999; Sekinger and Gross, 2001].

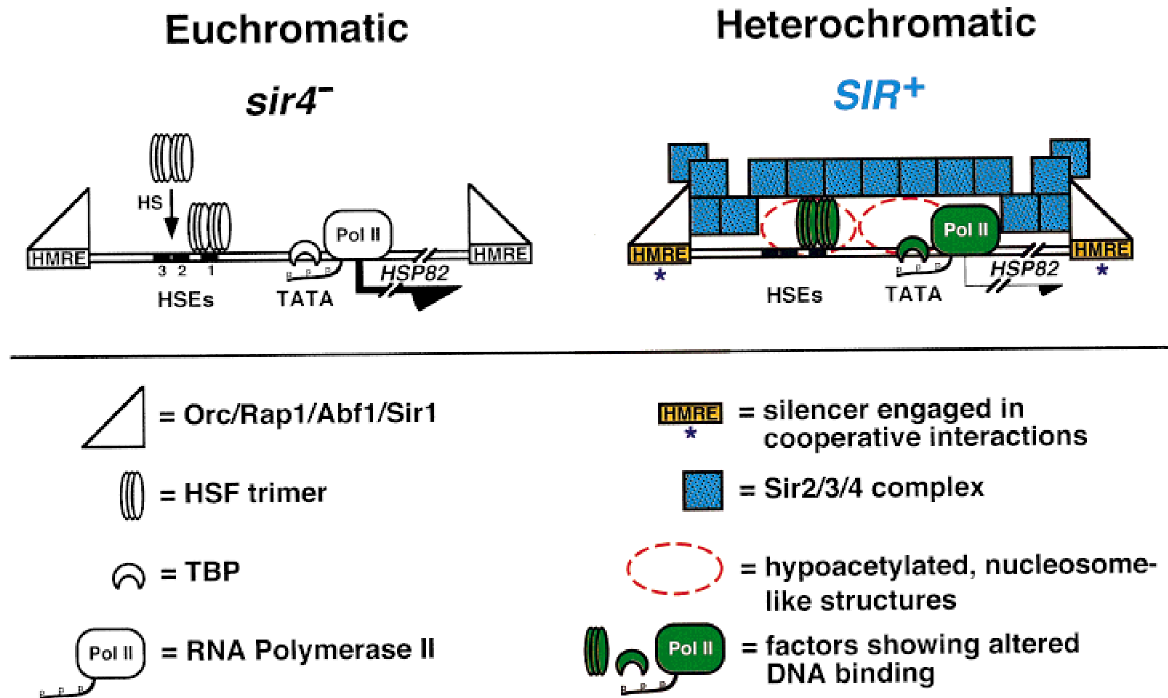

Fig. 2. Heterochromatin-mediated repression in *S. cerevisiae*. Schematically illustrated are three key transcription factors (HSF, TBP, and Pol IIo) present at the *HMRE/HSP82* locus in *sir4*⁻ and *SIR*⁺ strains. In euchromatin (*sir4*⁻), these proteins remain productively bound and *trans*-activate the linked gene (robust transcription symbolized by thick arrow). In the heterochromatic state (*SIR*⁺), hyperrepression is established by linear propagation of Sir proteins, which are associated with hypoacetylated nucleosomes (dashed ovals). This structure alters the binding properties of these regulatory factors (but does not displace them) and strongly represses expression of the gene (thin arrow). It is plausible that these sequence-specific DNA binding proteins gain access to their target sites following passage of the replication fork, prior to chromatin maturation [Venturi et al., 2000]. Thus, positive and negative regulators of transcription co-exist within heterochromatin.

(HSF), is prevented from binding in vivo, as is the case in a promoter mutant lacking the high-affinity site for HSF, the nuclease hypersensitive region is replaced by virtually the identical structure [Gross et al., 1993] (Fig. 1, *hsp82*-ΔHSE1). Importantly, while the two remaining HSF binding sites and TATA box are unmutated, they remain unoccupied and *hsp82* transcription is repressed >100-fold. Thus, the pair of nucleosomes that assemble the *hsp82*-ΔHSE1 promoter repress expression by sterically impairing the binding of the cognate transcription factors. Conclusive evidence for this comes from the observation that enforced overexpression of HSF can lead to its stable binding to the two low-affinity heat shock elements [Venturi et al., 2000]. As this binding occurs exclusively within the S-G2 window of the cell cycle—a time when chromatin is disassembled and then reassembled—it suggests that HSF competes with histones in occupying the *hsp82* promoter. *hsp82*-ΔHSE1 may thus serve as a model system to understand how chromatin-mediated repression takes place at euchromatic promoters containing positioned, stable nucleosomes.

An entirely different mechanism of repression is mediated by the yeast *SIR* silencing genes. These genes encode proteins—Sir2, Sir3, and Sir4—that form a nucleosome-binding complex that is recruited to specific chromosomal regions and thereby transcriptionally silences such regions through the formation of a specialized, heterochromatic-like structure. Interestingly, the Sir2-Sir3-Sir4 complex is primarily localized into subnuclear foci [reviewed in Cockell and Gasser, 1999]; in this regard it bears an intriguing resemblance to the nuclear bodies described above. Locus-specific repression is thought to occur through recruitment of the Sir complex by sequence-specific DNA binding proteins, RAP1, ABF1, and ORC, that bind telomeres and mating-type silencers [reviewed in Loo and Rine, 1995]. Once recruited, the Sir protein complex propagates along nucleosomes by binding the hypoacety-

lated N-terminal tails of histones H3 and H4 [reviewed in Grunstein, 1998]. Our laboratory has addressed how *SIR* repression is achieved by targeting *HMRE* mating-type silencer elements 5′ and 3′ of the chromosomal *HSP82* gene. Efficient *SIR*-dependent silencing of *HSP82* is accompanied by a dramatic reconfiguring of its promoter chromatin structure, as the constitutive DNase I hypersensitive region is replaced by two novel, nucleosome-like structures. Interestingly, DNA footprints mapping to the UAS and TATA regions persist, yet are clearly altered from the wild-type state [Sekinger and Gross, 1999]. Indeed, essentially normal levels of HSF and two components of the general transcriptional machinery, TBP and Pol II, remain associated with *HMRE*/*HSP82*, despite 100-fold reduction in transcription and assembly of the promoter DNA into a complex containing *SIR* silencing proteins and hypoacetylated histones [Sekinger and Gross, 2001] (see Fig. 2). Importantly, both recruitment- and elongation-competent forms of RNA polymerase, Pol IIa and Pol IIo (hypo- and hyperphosphorylated), respectively, are present at the hyperrepressed promoter. Co-occupancy of activators with silencing proteins is not unique to the heat shock promoter: the naturally silenced *HMR*a1 promoter, similarly assembled into a repressive chromatin structure, is also occupied by TBP, Pol IIa, and Pol IIo [Sekinger and Gross, 2001]. Thus, *SIR*-generated heterochromatin represses transcription principally at a step subsequent to both activator binding and PIC formation, probably at either promoter clearance or early elongation. We propose that like *SIR*-silenced *HSP82*, chromatin-mediated repression in mammalian cells will, at least in certain cases, act at a post-initiation step. Indeed, elongational pausing is a common regulatory mechanism at mammalian euchromatic genes, including *c-myc*, *c-fos*, and *hsp70* [Brown et al., 1996]. That DNA methylation or Ikaros-mediated silencing might work principally at this level would not be surprising, given their similarities to the *SIR*-silencing mechanism outlined above.

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