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Common Themes in Mechanisms of Gene Silencing

Review

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Summary

The assembly of DNA into regions of inaccessible chromatin, called silent chromatin, is involved in the regulation of gene expression and maintenance of chromosome stability in eukaryotes. Recent studies on Sir2-containing silencing complexes in budding yeast and HP1- and Swi6-containing silencing complexes in metazoans and fission yeast suggest a common mechanism for the assembly of these domains, which involves the physical coupling of histone modifying enzymes to histone binding proteins.

Introduction

Cell differentiation requires precise control mechanisms that allow the expression of only the appropriate subset of genes in each cell type. Evidence from a number of experimental systems suggests that eukaryotic cells maintain or "remember" their gene expression programs, specified during embryonic development, through heritable modifications in the structure of their chromosomes.

Gene silencing is a primary mechanism that contributes to maintenance of committed gene expression patterns. Silencing involves the inactivation of chromosome domains that contain key regulatory genes by packaging them into a specialized chromatin structure that is inaccessible to DNA binding proteins (Grewal, 2000; Paro, 1993). Silencing proteins assemble such inaccessible DNA domains by acting directly on nucleosome structure. The nucleosome is the basic unit of folding in eukaryotic chromosomes and is composed of 147 base pairs of DNA wrapped about two turns around an octamer of the four core histones (Luger et al., 1997; reviewed in Kornberg and Lorch, 1999). The folding of genes into nucleosomes and higher order chromatin structures is a repressive event. It has recently become clear that this folding is the focal point for many of the processes that regulate gene expression and chromosome dynamics. For example, a large number of chromatin remodeling complexes have been identified that use the energy of ATP hydrolysis in order to perturb histone-DNA interactions and facilitate the binding of regulatory proteins and the transcription machinery to DNA (reviewed in Kingston and Narlikar, 1999; Peterson and Workman, 2000; Struhl, 1996; Tamkun, 1995). Silencing mechanisms act in the opposite direction by what appears to involve the masking of nucleosomes from the liberating activity of the remodeling factors (reviewed in Francis and Kingston, 2001).

Studies in distantly related organisms, including mammals, flies, and yeast, are beginning to shine light on the molecular mechanisms that assemble silenced chromosome domains. A remarkable outcome of these studies is that despite the divergence of many of the proteins that are involved in gene silencing in various systems, the overall molecular mechanisms appear to be very similar across the phylogenetic spectrum. Specifically, silencing by two apparently unrelated groups of proteins in yeasts and metazoans involves the direct covalent modification of the histones by silencing proteins. Moreover, in both instances, enzymes that carry out these modifications are physically associated with histone binding proteins in silencing complexes, and these complexes require specific modifications of histone tails by their associated enzymes in order to bind to histones and spread along the chromatin fiber. In this review, I discuss recent studies of two types of eukaryotic gene silencing complexes that have given rise to the above common principles. These are the Sir2-containing silencing complexes that have so far only been studied in budding yeast, and the HP1 and Swi6 complexes that mediate silencing in metazoans and fission yeast, respectively.

What Is Gene Silencing?

In defining gene silencing, it may be useful to state how it is distinguished from promoter-specific gene repression. An important characteristic of silencing is that it acts in a regional rather than promoter- or sequence-specific manner to generate large domains of DNA that are inaccessible to DNA binding proteins (see Rine, 1999). For example, in yeast, different RNA polymerase II promoters, as well as RNA polymerase III promoters, inserted within silent chromatin domains are repressed (Huang et al., 1997; Schnell and Rine, 1986). Consistent with the idea that silencing entails the assembly of a generally repressive chromatin domain, silent DNA regions are also less accessible to the cellular recombination machinery and to exogenous enzymatic probes, such as the *dam* methyltransferase and restriction endonucleases (Gottschling, 1992; Loo and Rine, 1994; Singh and Klar, 1992; Wallrath and Elgin, 1995). A second important property of silent chromatin domains is their persistence through mitotic and meiotic cell divisions such that a particular chromatin structure (DNA and its associated proteins) is replicated during the process of chromosome duplication (reviewed in Grewal, 2000). This mode of inheritance, commonly referred to as epigenetic inheritance, is believed to underlie cellular memory mechanisms that maintain cell identity and stable patterns of gene expression in eukaryotes.

Despite the generally inaccessible properties of silenced chromatin, discussed above, these domains must somehow be replicated and repaired, suggesting the existence of mechanisms that regulate access to silent chromatin. In fact, silent chromatin domains appear to be accessible to certain classes of DNA binding proteins or enzymes. For example, recognition sites for the FLP and Cre recombinases located within yeast si-

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lent chromatin domains are accessible to these enzymes when each is expressed at high levels (Cheng et al., 1998; Holmes and Broach, 1996), and transcriptional activators can gain access to yeast silent chromatin within a restricted window during the cell cycle (Aparicio and Gottschling, 1994). In *Drosophila*, the T7 bacteriophage RNA polymerase can access its promoter even when it is located within a silent DNA region (McCall and Bender, 1996). Moreover, it has recently been shown that in budding yeast silencing proteins and the general transcription machinery can cooccupy DNA at some promoters (Sekinger and Gross, 2001). Together, these findings suggest that silent chromatin is much more dynamic than previously thought. Nonetheless, the idea that silent chromatin is less accessible to DNA binding proteins is supported by a convincing body of evidence and is not necessarily inconsistent with the above data.

Gene Silencing and Heterochromatin

Heterochromatin was first identified cytologically in insect and plant cells by Emil Heitz in the 1920s based on its distinct behavior during the cell cycle (cited in Eissenberg and Elgin, 2000). Whereas most chromosome regions undergo cycles of condensation and decondensation as cells enter and exit mitosis, heterochromatin maintains a condensed appearance throughout the cell cycle. The revelation that the heterochromatic state is associated with gene inactivity came from subsequent studies in *Drosophila*, which showed that inactivation of genes translocated to the vicinity of heterochromatin correlates with the spreading of heterochromatin over these genes (reviewed in Weiler and Wakimoto, 1995). Gene silencing shares the central properties of general inaccessibility and epigenetic inheritance with heterochromatin. Therefore, although silent chromatin domains, unlike heterochromatin, are not always cytologically distinguished, they are often referred to as heterochromatic.

In fission yeast and metazoans, heterochromatin is generally associated with centromeric and telomeric chromosome regions (Allshire et al., 1994; Spofford, 1976). Recent studies in *Drosophila* and fission yeast have established a role for heterochromatin in centromere function (Hsieh and Fire, 2000; Karpen and Allshire, 1997; Kellum and Alberts, 1995). For example, mutations in gene products that interfere with the formation of heterochromatin result in defects in chromosome segregation presumably because some aspect of centromere and/or kinetochore function is perturbed (Allshire et al., 1995; Ekwall et al., 1999; Hari et al., 2001; Kellum and Alberts, 1995). The precise function of heterochromatin at centromeres and telomeres is poorly understood and is likely to involve an important structural role that is independent of transcriptional silencing.

The first molecular connection between developmental gene silencing and heterochromatin came from the identification of two *Drosophila* gene products, Heterochromatin Protein 1 (HP1) and Polycomb (Pc) (James and Elgin, 1986; Paro and Hogness, 1991). HP1 and Pc appear to take part in unrelated cellular processes involving the formation of centromeric heterochromatin and the stable inactivation of homeotic selector genes during development, respectively. However, the two proteins share a region of sequence similarity at their amino termini named the chromo domain. This similarity gave rise to the conjecture that similar molecular mechanisms may be involved in the formation of heterochromatin and the stable inactivation of genes during development. A number of subsequent studies support the basic notion that there is a mechanistic connection between the Polycomb group and heterochromatin (reviewed in Eissenberg and Elgin, 2000).

Finally, gene silencing and heterochromatin are often associated with repetitive DNA sequences and may be involved in stabilizing such sequences. Most repetitive DNA elements are somehow recognized and inactivated by either transcriptional or posttranscriptional mechanisms (reviewed in Henikoff, 1998; Hsieh and Fire, 2000). Transcriptional inactivation of repetitive DNA is likely to involve silencing mechanisms. For example, in fission yeast and metazoans, centromeric and telomeric heterochromatin is composed of various repetitive DNA sequences (Blackburn, 1984; Murphy and Karpen, 1995). In *Drosophila*, modifiers of heterochromatic gene silencing and the Polycomb group genes both appear to be involved in inactivation of repeated transgenes, supporting the existence of a silencing-based mechanism that regulates repetitive DNA (Dorer and Henikoff, 1994; Pal-Bhadra et al., 1999). Moreover, in budding yeast, silencing-based mechanisms inhibit hyperrecombination within the highly repetitive ribosomal RNA gene array (rDNA), and, in both budding and fission yeasts, transcription of RNA polymerase II genes inserted within the rDNA is silenced (Bryk et al., 1997; Fritze et al., 1997; Smith and Boeke, 1997; Thon and Verhein-Hansen, 2000). The underlying mechanisms for these diverse functions of hetreochromatin involve the assembly of chromatin domains that inhibit access to DNA binding proteins. In at least two types of silencing complexes, discussed below, these mechanisms involve the physical coupling of histone modifying enzymes to histone binding proteins.

SIR-Mediated Gene Silencing in Budding Yeast

One of the best-studied examples of eukaryotic gene silencing occurs in the budding yeast *Saccharomyces cerevisiae*. Gene silencing in yeast was first discovered at the silent mating type or homothallic (*HM*) loci and later at telomeric DNA regions (Gottschling et al., 1990; Klar et al., 1981; Nasmyth et al., 1981). Silencing at the *HM* loci is essential for maintenance of haploid cell identity (Herskowitz et al., 1992). Reporter genes inserted near yeast telomeres become silent in a stochastic fashion, giving rise to a mixed population of cells where the reporter gene is either "on" or "off" and these on and off states are stably maintained for many generations (Gottschling et al., 1990). Such stable variegated states in yeast are similar to classical position effects on gene expression observed in *Drosophila*, where, for example, DNA rearrangements that bring the *white* gene near heterochromatin result in its variegated expression in genetically identical cells (Weiler and Wakimoto, 1995). In yeast, silencing also appears to contribute to maintenance of telomeric repeats (Palladino et al., 1993). Therefore, as is the case in metazoans, silencing in yeast regulates cell identity and is also associated with land-

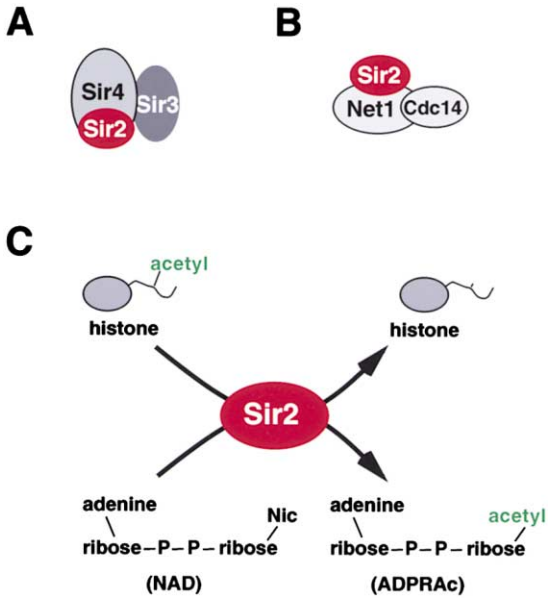

Figure 1. The Yeast Silencing Protein Sir2 Is a Component of Two Distinct Silencing Complexes and Is an Enzyme that Couples Histone Deacetylation, NAD Hydrolysis, and Synthesis of O-acetyl-ADP-ribose

The SIR complex containing Sir2, Sir3, and Sir4 mediates mating type and telomeric silencing (A). The RENT complex containing Sir2, Net1, and Cdc14 mediates rDNA silencing (B). The overall Sir2 reaction mechanism is shown in (C). NAD is represented as adenine-ribose-P-P-ribose-Nic; P and Nic denote phosphate and nicotinamide, respectively. O-acetyl-ADP-ribose (ADPRAc) is represented as adenine-ribose-P-P-ribose-acetyl. See text for references.

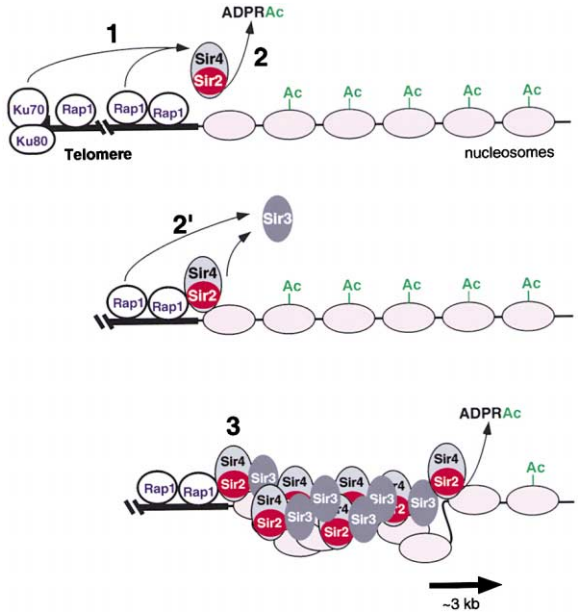

Figure 2. Model for Step-Wise Assembly of Silent Chromatin in Budding Yeast

Telomere binding proteins, the yKu70/yKu80 heterodimer and Rap1, recruit the Sir2/Sir4 complex to DNA (step 1). Following deacetylation of histone tails by Sir2 (step 2), the Sir3 protein is recruited via interactions involving Rap1, Sir4, and histone tails and binds to nucleosomes (shown as purple ovals) by interacting with the deacetylated histone tails (step 2′). Multimerization of Sir3 and Sir4 then results in additional rounds of modification and binding, and spreading of the complex along nucleosomes (step 3). Ac, acetyl group on amino-terminal lysines of histones; ADPRAc, O-acetyl-ADP-ribose. See text for details and references.

mark chromosome structures. However, unlike fission yeast and metazoans, centromeres in budding yeast do not appear to be associated with silent chromatin.

The regulatory DNA sites and many of the proteins involved in the nucleation and assembly of silent chromatin at the *HM* loci and telomeres have been identified and provide a foundation for understanding a mechanism of gene silencing. Silencing at these loci is mediated by a multiprotein nucleosome binding complex called the SIR complex (Figure 1A) (Aparicio et al., 1991; Hecht et al., 1995; Klar et al., 1979; Moazed et al., 1997; Rine and Herskowitz, 1987; Strahl-Bolsinger et al., 1997). This complex contains the Sir2, Sir3, and Sir4 proteins and is recruited to DNA by interactions with proteins that bind to chromosome ends or to specific regulatory sites called silencers. For example, within the yeast telomeric repeats, the Rap1 protein binds to multiple sites and together with chromosome end binding proteins yKu70 and yKu80 recruits the SIR complex via interactions with the Sir3 and Sir4 proteins (Figure 2) (Boulton and Jackson, 1998; Kyrion et al., 1993; Laroche et al., 1998; Moretti et al., 1994). Interestingly, the SIR complex appears to be assembled in a step-wise fashion (Moazed et al., 1997). The Sir2 and Sir4 proteins form a tightly associated complex both in vitro and in vivo (Moazed et al., 1997; Strahl-Bolsinger et al., 1997). The interaction of this complex with Sir3 appears to be a regulated step since an efficient Sir3-Sir4 interaction can only be detected either with truncations of the Sir4 protein that remove its amino terminus or under conditions that preserve SIR-nucleosome interactions (Moazed et al., 1997; Moretti et al., 1994; Strahl-Bolsinger et al., 1997). An intriguing possibility is that interactions that regulate the assembly of the SIR complex also control its nucleosome binding and silencing activity.

Pioneering studies by Grunstein and colleagues provided the first convincing evidence for the roles of histones and chromatin structure in silencing. The amino-terminal tails of histones H3 and H4 are universally conserved in eukaryotes but surprisingly are not required for growth in yeast. However, these tails play an essential role in silencing (Kayne et al., 1988). Mutational analysis suggests that the deacetylated state of specific lysine residues within the H3 and H4 tails is crucial for efficient silencing (Braunstein et al., 1996; Johnson et al., 1990). Additional evidence suggests that these histones directly interact with the Sir proteins. First, certain *sir3* alleles could suppress the silencing defect of H4 tail mutations and, second, Sir3 and Sir4 could bind to the amino termini of H3 and H4 in vitro (Hecht et al., 1995; Johnson et al., 1990). Based on these results, Grunstein and colleagues have proposed a model for the assembly of silent chromatin that involves the recruitment of the SIR complex to DNA and its subsequent spreading along the chromosome fiber through interactions involving the binding of Sir3 and Sir4 to deacetylated histone tails. Consistent with this model, chromatin immunoprecipita-

tion experiments show that the Sir proteins are associated with DNA throughout silent chromatin domains and that histones within these domains are hypoacetylated (Braunstein et al., 1993; Hecht et al., 1996).

Hypoacetylation of histones is a conserved hallmark of silent chromatin. However, until recently, it was not known whether histones in silent chromatin domains are actively deacetylated (by enzymes associated with silencing complexes) or whether deacetylation is a passive byproduct of other assembly events. Recent discoveries about the function of a conserved yeast silencing protein, Sir2, provide a direct link between the silencing machinery and histone deacetylation, and suggest that deacetylation is physically coupled to other activities that are essential for assembly of silent chromatin. Sir2 and its homologs are unusual NAD-dependent protein deacetylases (Imai et al., 2000; Landry et al., 2000; Smith et al., 2000). Sir2 can efficiently deacetylate histones in vitro, and although a direct demonstration is still lacking, the studies discussed above strongly suggest that Sir2 also acts on histones in vivo. Interestingly, unlike the Rpd3 class of histone deacetylases, which catalyze conventional amide hydrolysis reactions, Sir2 couples deacetylation to hydrolysis of NAD and transfers the acetyl group from its substrate to an NAD cleavage product to form O-acetyl-ADP-ribose (ADPRAc) (Figure 1C) (Tanner et al., 2000; Tanny and Moazed, 2001). While the enzymatic activity of Sir2 is absolutely required for silencing in vivo (Imai et al., 2000; Tanny et al., 1999), the possible significance of the unusual coupling that involves NAD hydrolysis and generates ADPRAc is presently unclear (reviewed in Moazed, 2001). ADPRAc has been speculated to act as an effector or signaling molecule for a step in assembly of silent chromatin (Tanner et al., 2000; Tanny and Moazed, 2001).

The coupling of deacetylation or other enzymatic activities to the assembly of the SIR complex on chromatin has profound implications for the mechanisms of nucleation, spreading, and possibly inheritance of silent chromatin domains. A Sir2 protein that is enzymatically inactive assembles into silencing complexes with wild-type efficiency, but its association with silent chromatin domains is impaired (Tanny et al., 1999). Thus, modification of histones or other proteins by Sir2 is required for the efficient binding of the SIR complex to chromatin. This finding suggests a step-wise model for the assembly of silencing proteins along the chromatin fiber (Figure 2). In this model, the recruitment of the Sir2/Sir4 complex to DNA is followed by deacetylation of the tails of histones H3 and H4 by Sir2. This step allows the binding of the Sir3 and Sir4 proteins to the deacetylated histone tails and the recruitment of a new Sir2/Sir4 complex. Repetitions of this modification binding cycle would then result in the spreading or polymerization of the SIR complex outward from the nucleation site (Figure 2). This coupling of histone binding activities to specific histone modifications appears to be a common theme shared by silencing complexes in fission yeast and metazoans, which will be discussed later.

Sir2 and Silencing in the Ribosomal RNA Gene Array

In most eukaryotes, the ribosomal RNA genes, which encode the 35S precursor RNA and the 5S RNA, exist as tandem arrays of approximately 100–200 copies at one or more chromosomal locations and are sequestered into a nuclear subdomain called the nucleolus (reviewed in Carmo-Fonseca et al., 2000; Shaw and Jordan, 1995). While most repetitive DNA in eukaryotes is subject to inactivation that in some instances involves transcriptional silencing, the rRNA gene array (rDNA) is often cited as an exception to the general phenomenon of repeat-induced silencing because these repeats are very highly transcribed (for example, see Hsieh and Fire, 2000). However, as mentioned previously, in both budding and fission yeasts, rDNA chromatin structure is regulated by silencing (Bryk et al., 1997; Fritze et al., 1997; Smith and Boeke, 1997; Thon and Verhein-Hansen, 2000). Although the general mechanism is poorly understood, rDNA silencing in budding yeast requires the Sir2 protein and its enzymatic activity, suggesting that deacetylation and a mechanism that is related to SIR-mediated silencing are involved in regulation of rDNA structure (Bryk et al., 1997; Fritze et al., 1997; Imai et al., 2000; Smith and Boeke, 1997; Tanny et al., 1999). At least two possible models can be proposed to explain the paradoxical association of rDNA silencing with a highly transcribed part of the genome. The first model is that rDNA is packaged into a type of silent chromatin that limits access to the RNA polymerase II transcription machinery and recombination enzymes but is somehow accessible to RNA polymerase I. In an alternative model, since only about half of rRNA genes are transcribed at any given time, rDNA silencing would only act on the subset of repeats that are not actively transcribed, in an indiscriminate manner similar to other examples of silencing.

Apart from its role in stabilizing a repetitive DNA region that is essential for growth, rDNA silencing has been shown to be a determinant of replicative life span in yeast. Increased recombination rates at rDNA, as would result from loss of silencing in *sir2* mutant cells, result in excision of rDNA repeat units as extrachromosomal circles (Sinclair and Guarente, 1997). Retention and exponential amplification of these circles in mother yeast cells has been proposed to result in eventual senescence (Sinclair and Guarente, 1997). It has recently been shown that a *C. elegans* homolog of Sir2 is involved in regulation of life span, suggesting that this role of Sir2-like proteins is conserved in eukaryotes. (Tissenbaum and Guarente, 2001). It remains to be determined whether Sir2 affects life span in multicellular eukaryotes by mechanisms that are similar to its silencing functions in yeast.

In budding yeast, rDNA silencing requires a distinct multiprotein complex that contains the Sir2, Net1, and Cdc14 proteins but not the Sir3 and Sir4 proteins (Figure 1B). Net1, the core subunit of this complex, localizes Sir2 to rDNA and is required for rDNA silencing (Straight et al., 1999). In addition to its role in rDNA silencing, Net1 (also known as Cfi1) is required for the regulation of exit from mitosis and regulates the activity of the Cdc14 protein phosphatase by sequestering it in the nucleolus until telophase (Shou et al., 1999; Visintin et al., 1999). Chromatin immunoprecipitation experiments show that both Net1 and Sir2 are associated with DNA fragments throughout the length of rDNA repeats (Gotta et al., 1997; Straight et al., 1999), suggesting that the Net1/Sir2 complex can spread along chromatin. How-

Table 1. Conservation of Silencing Proteins in Budding Yeast, Fission Yeast, and Metazoans

Budding Yeast (*S. cerevisiae*)	Fission Yeast (*S. pombe*)	Fruit Fly (*D. melanogaster*)	Human (*H. sapiens*)
Sir2	Spb16d10.07cp[a]	Sir2[a]	SIRT1[a]
Sir3	—	—	—
Sir4	—	—	—
Net1	—	—	—
Hda1[b]	Clr3	GC1770	HDAC5
Set1, Set2[c]	Clr4	Su(var)3-9	SUV39H1
Rpd3[b]	Clr6	Rpd3	HDAC1
—	Swi6	HP1	HP1α, β, and γ
—	Rik1	—	—

Silencing proteins in each organism are shown in vertical columns, and homologs, when present, are shown in horizontal rows.
[a] A role in gene silencing for these proteins has not been demonstrated. See Brachmann et al. (1995) and Frye (1999) for alignments of Sir2-like proteins.
[b] Although present in *S. cerevisiae*, these NAD-independent histone deacetylases appear to be required for promoter-specific gene repression. In fact, Rpd3, either directly or indirectly, antagonizes mating type, telomeric, and rDNA silencing in *S. cerevisiae* (Rundlett et al., 1996).
[c] The *S. cerevisiae* Set2 protein shares the SET domain and the flanking cysteine-rich regions with the Clr4 and SUV39H1 proteins (Rea et al., 2000) (Saccharomyces Genome Database). The Set1 protein contains a SET domain but lacks the flanking cysteine-rich region and is required for telomeric silencing (Nislow et al., 1997).

ever, the mechanism of this spreading appears to be fundamentally different from that of the SIR complex at the *HM* loci and telomeres. For example, the association of Net1 with rDNA fragments does not require Sir2, and enzymatically inactive Sir2 localizes to rDNA with nearly wild-type efficiency (Straight et al., 1999; Tanny et al., 1999). Thus, a step-wise assembly mechanism is either absent or is regulated in a Sir2-independent manner at rDNA. It also remains to be determined whether Net1 or an unknown component of the complex contains a histone binding activity that may promote the spreading of Net1 throughout rDNA.

HP1- and Swi6-Based Silencing Mechanisms

As mentioned earlier, metazoan chromosomes contain large stretches of silent chromatin at their centromeric and telomeric DNA regions. HP1 is a structural component of heterochromatin that was first identified in *Drosophila* and is conserved in organisms ranging from fission yeast to human (Eissenberg and Elgin, 2000; James and Elgin, 1986) (Table 1). In support of its role in the formation of heterochromatin, the gene encoding HP1, *Su(var)2-5*, is a dosage-dependent regulator of heterochromatic silencing (Eissenberg et al., 1990). HP1 and its homologs all share a chromodomain and a related domain, called the chromo shadow domain (Eissenberg and Elgin, 2000). In *Drosophila*, the chromo domain of HP1 is required for its localization to centromeric heterochromatin (Platero et al., 1995). In keeping with their structural conservation, HP1 homologs in distantly related organisms have similar silencing functions. For example, as is the case in *Drosophila*, the fission yeast homolog of HP1, Swi6, is a structural component of silent chromatin domains and is required for centromeric, mating type, and telomeric silencing (reviewed in Grewal, 2000). *swi6* mutants are also defective in centromere function (Ekwall et al., 1995; Klar and Bonaduce, 1991; Lorentz et al., 1994), supporting a role for heterochromatin in chromosome segregation, first suggested by studies of HP1 mutant embryos in *Drosophila* (Kellum and Alberts, 1995).

Biochemical studies suggest that the role of HP1 in silencing involves its interaction with histones. Here again, assembly of silent chromatin appears to be regulated by the physical coupling of a histone binding protein with a histone modifying enzyme (reviewed in Jenuwein, 2001). One of the mammalian HP1 homologs, HP1β (also called M31), is in a stable complex with another conserved protein, named SUV39H1 (Aagaard et al., 1999). Like HP1, the *Drosophila* and fission yeast homologs of this protein, Su(var)3-9 and Clr4, respectively, are required for heterochromatic gene silencing (Ekwall and Ruusala, 1994; Tschiersch et al., 1994). Recently, SUV39H1 and Clr4 were shown to possess intrinsic methyltransferase activity with specificity for lysine 9 at the amino terminus of histone H3 (Rea et al., 2000). Methylation of this lysine residue is conserved in many eukaryotes (Strahl et al., 1999), and the physiological significance of this modification has been demonstrated by a series of experiments in fission yeast and metazoan systems. First, methylation of H3-lysine 9 is specific for a reporter gene inserted within the silent DNA region spanning the *mat2* and *mat3* loci in fission yeast and requires a functional Clr4 protein (Nakayama et al., 2001). Second, in fission yeast, the assembly of silent chromatin domains and methylation of H3-lysine 9 require the function of Clr3 and Clr6 proteins, which are histone deacetylases of the Rpd3 and Hda1 classes (Nakayama et al., 2001). Deacetylation of the H3 tail at lysine 9 and other positions is proposed to precede methylation of lysine 9. Finally, methylation of H3-lysine 9 has been shown to create a binding site for the HP1 and Swi6 proteins, which bind to H3-lysine 9 through their conserved chromodomains (Bannister et al., 2001; Lachner et al., 2001). Both HP1 in metazoans and Swi6 in *S. pombe* can multimerize through interactions that involve their chromo shadow domains (Cowieson et al., 2000; Smothers and Henikoff, 2000). Thus, modification of a histone tail is followed by its association with a specific histone binding protein, which can then multimerize and spread along the chromatin fiber (Figure 3).

Interestingly, the Polycomb protein can also bind to histone tails, suggesting that the assembly mechanisms discussed for the HP1/SUV39H1 may also apply to Polycomb-mediated silencing (Breiling et al., 1999; Lachner et al., 2001). However, while requiring the chromodo-

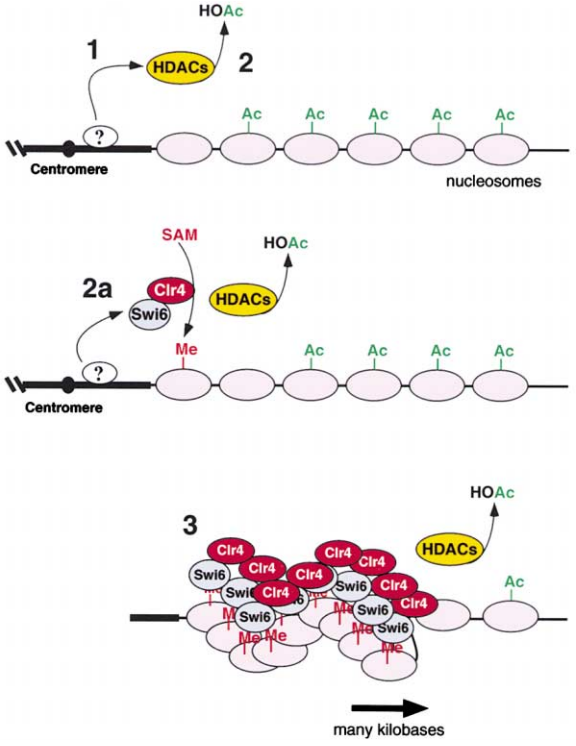

Figure 3. Model for Step-Wise Assembly of Silent Chromatin Domains in Fission Yeast

Following recruitment to DNA by protein(s) that have not yet been identified (step 1), histone deacetylases (HDACs, Clr3, and Clr6) deacetylate histone tails (step 2). The H3-specific methyltransferase, Clr4, then methylates lysine 9 of H3 and creates a binding site for the Swi6 protein (step 2a). Self-association of the Swi6 protein and subsequent rounds of modification and binding result in the spreading of the complex along nucleosomal DNA for several kilobases (step 3). Model adapted from Nakayama et al. (2001). Similar models have been proposed for mammalian HP1/SUV39H1 assembly (Bannister et al., 2001; Lachner et al., 2001). SAM, S-adenosyl-methionine; Me, methyl group on lysine 9 of histone H3; HOAc, acetate. See text for additional references.

main, the binding of polycomb to histones and nucleosomes displays specificity distinct from that of HP1 and is not sensitive to the methylation state of the H3 tail (Breiling et al., 1999; Lachner et al., 2001). Moreover, PRC1, a purified Pc complex, is associated with only substoichiometric amounts of the Rpd3 histone deacetylase and does not contain a Su(var)3-9-like methyltransferase subunit (Saurin et al., 2001). Further, in vitro, PRC1 can inhibit chromatin remodeling of nucleosome arrays that are assembled with tailless histones, suggesting that interactions involving other structural features of the nucleosome are likely to contribute to PRC1-dependent silencing (Shao et al., 1999). On the other hand, another Pc group complex, the E(z)Esc complex, contains the Rpd3 histone deacetylase as well as a SET domain protein, E(z), which is likely to have methyltransferase activity (Tie et al., 2001; also see Rea et al., 2000). Since silencing of homeotic genes in *Drosophila* requires components of both the E(z)Esc and PRC1 complexes, it remains possible that a step-wise mechanism involving both histone deacetylation and methylation is also involved in assembly of Polycomb group repressors on chromatin.

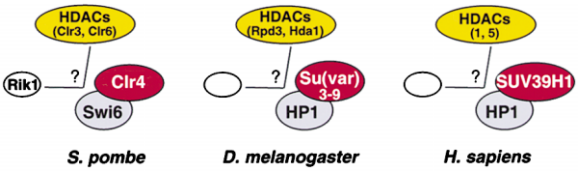

Figure 4. The Swi6/HP1 Silencing Complex Is Conserved in the Fission Yeast, *S. pombe*, and Metazoans

The human HP1β protein has been shown to be associated with the methyltransferase protein SUV39H1. By analogy, Swi6 and the *Drosophila* HP1 are represented in association with SUV39H11 homologs Clr4 and Su(var)3-9; these interactions are strongly supported by genetic and colocalization experiments, but physical evidence for the association of Swi6 with Clr4 or HP1 with Su(var)3-9 is lacking. In *S. pombe*, HDACs and the zinc finger protein Rik1 are required for the association of Swi6 with silent chromatin, but it is unknown whether Rik1 and HDACs are physically associated with Swi6 or Clr4. See text for references.

Common Pathways for Assembly of Silent Chromatin Domains

With the exception of histones, proteins involved in SIR-mediated silencing in budding yeast and in HP1/Swi6-mediated silencing in fission yeast and metazoans share no obvious similarity at the primary sequence level. Despite this lack of sequence conservation, the overall pathway of assembly of silent chromatin by these complexes appears to be strikingly similar (Figures 2 and 3). In both cases, enzymes that modify histone tails are physically coupled to proteins that specifically recognize and bind to the histone tails marked by these enzymes. Therefore, a similar step-wise assembly pathway can be envisioned for each situation. In the case of the SIR complex, the NAD-dependent histone deacetylase, Sir2, is tightly associated with the Sir4 protein. Following recruitment of the Sir2/Sir4 complex to DNA, Sir2 deacetylates specific histone tail lysines and may create a binding site for the Sir4 and Sir3 proteins. Sir3 is recruited to chromatin via interactions that appear to involve both the histone tails and Sir4. Homo- and heteromultimerization of the Sir3 and Sir4 proteins would then allow subsequent rounds of binding and modification to take place resulting in the spreading of the SIR complex along the chromatin fiber (Figure 2).

In the case of HP1 and Swi6 silencing complexes, histone methyltransferases (SUV39H1 or Clr4) are physically associated with histone binding proteins, HP1 and Swi6 (Figures 3 and 4). Here, efficient binding requires that histone tails are first deacetylated by Rpd3 and Hda1 class deacetylases and then methylated at the H3-lysine 9 by Clr4 (or SUV39H1 in mammals). As with the Sir3 and Sir4 proteins, HP1 proteins are able to multimerize (Cowell and Austin, 1997; Nielsen et al., 2001a). Thus, binding and modification cycles can be reiterated to promote the spreading of HP1/Swi6 silencing complexes along the chromatin fiber (Figure 3). It is important to note that HP1 and Polycomb can also bind to nucleosomes from which the histone tails have been removed by trypsin digestion, and HP1 can associate

via its chromodomain with the core domain of histone H3 (Breiling et al., 1999; Nielsen et al., 2001a; Zhao et al., 2000). It is therefore possible that interactions that do not involve the H3 amino terminus or specific histone modifications also contribute to the stable association of HP1 silencing complexes with chromatin.

The Histone Code Hypothesis and Epigenetic Inheritance

The histone code hypothesis and related proposals state that posttranslational modifications of histone tails such as acetylation, methylation, and phosphorylation serve to create specific binding sites for chromatin regulatory proteins (Braunstein et al., 1993; Strahl and Allis, 2000; Thompson et al., 1994; Turner, 2000). The studies discussed above provide support for these proposals and suggest that histone modifications are indeed involved in the assembly of silent chromatin domains. Recent studies by Grewal and coworkers provide further support for these ideas by showing that an extensive heterochromatic domain spanning the *mat2* and *mat3* silent mating type loci in *S. pombe* is highly enriched for histone H3 methylated at lysine 9, and that, in contrast, H3 methylated at lysine 4 is specific to the surrounding nonsilenced region (Noma et al., 2001). On the other hand, HP1 and SUV39H1 also participate in methylation of H3 lysine 9 and promoter-specific gene repression directed by the retinoblastoma (Rb) protein, indicating that in human cells H3-lysine 9 methylation is not specific for heterochromatin (Nielsen et al., 2001b).

An important question now is whether particular combinations of histone modifications are sufficient to mark chromatin states for epigenetic inheritance, for example, in a manner analogous to the way that DNA methylation patterns can be stably inherited based on recognition of hemimethylated DNA after DNA replication. A direct prediction of such a hypothesis is that particular combinations of histone modifications should be able to recruit silencing complexes to chromatin even in the absence of the observed physical coupling of modifying enzymes, such as Sir2, Clr4, or SUV39H1, to histone binding proteins, such as Sir3-Sir4, Swi6, or HP1, respectively. The acetylation state of histone tails appears to be very dynamic in vivo, and although methylated histones are thought to turn over more slowly, to date the kinetics of acetylation and methylation have only been measured using bulk histones and turn over rates for specific modifications remain to be determined (Annunziato et al., 1995; Waterborg, 2001). Epigenetic inheritance is likely to require a close association between enzymes that modify histone tails and proteins that recognize these modifications and remain associated with histones throughout the cell cycle. But additional experiments are required to resolve this issue.

Concluding Remarks

The studies outlined above strongly suggest that despite the divergence of molecular components, mechanisms of heterochromatic gene silencing in budding yeast, fission yeast, *Drosophila*, and mammals are similar. Conservation of Sir2-like proteins in fission yeast and multicellular eukaryotes raises the possibility that SIR-based repression mechanisms work side by side with HP1/Swi6-based silencing in these systems (see Table 1). It will be interesting to learn whether the SIR- and HP1/Swi6-based silencing mechanisms play complementary or redundant roles in gene inactivation. Moreover, several budding yeast proteins contain potential methyltransferase domains, similar to the SET domain in SUV39H1 and Clr4 proteins (Table 1), and their possible roles in silencing remain to be determined. In all of these cases, there is a great need for in vitro systems that reconstitute silencing activity. Such in vitro systems are essential for understanding the step-wise assembly of silencing proteins on chromatin templates and for determining whether histone modifications or other events mark silent chromatin domains for epigenetic inheritance.

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