

## Molecular basis of transcriptional silencing in budding yeast

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**Abstract:** Transcriptional silencing is a phenomenon in which the transcription of genes by RNA polymerase II or III is repressed, dependent on the chromosomal location of a gene. Transcriptional silencing normally occurs in highly condensed heterochromatin regions of the genome, suggesting that heterochromatin might repress transcription by restricting the ability of sequence-specific gene activator proteins to access their DNA target sites. However, recent studies show that heterochromatin structure is inherently dynamic, and that sequence-specific regulatory proteins are able to bind to their target sites in heterochromatin. The molecular basis of transcriptional silencing is plainly more complicated than simple steric exclusion. New ideas and experiments are needed.

*Key words:* transcriptional silencing, heterochromatin, accessibility.

**Résumé :** L'atténuation transcriptionnelle (« silencing ») est un phénomène dans lequel la transcription d'un gène par l'ARN polymérase II ou III est réprimée selon la localisation chromosomique du gène. L'atténuation transcriptionnelle se produit habituellement dans des régions d'hétérochromatine très condensée du génome, ce qui suggère que l'hétérochromatine pourrait réprimer la transcription en limitant l'accès des protéines activatrices spécifiques d'une séquence à leurs sites cibles dans l'ADN. Cependant, des études récentes montrent que la structure de l'hétérochromatine est dynamique et que les protéines régulatrices spécifiques d'une séquence peuvent se lier à leurs sites cibles dans l'hétérochromatine. Le mécanisme moléculaire de l'atténuation transcriptionnelle est nettement plus complexe qu'une simple exclusion stérique. De nouvelles idées et expériences sont nécessaires.

*Mots clés :* atténuation transcriptionnelle, hétérochromatine, accessibilité.

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### Introduction

Chromosomes comprise two cytologically distinct types of regions: relatively uncondensed euchromatin, and highly condensed heterochromatin. Heterochromatin affects many aspects of the function of genes that are contained within it or nearby, including replication timing, recombination frequency, and transcription level. This phenomenon is known as a position effect. In *Saccharomyces cerevisiae*, budding yeast, heterochromatin is not visible cytologically, yet position effects have been identified in three locations: telomeres (Gottschling et al. 1990), the rDNA locus (Fritze et al. 1997; Smith and Boeke 1997), and the silent mating-type loci, *HML* and *HMR* (Schnell and Rine 1986). When a gene transcribed by RNA polymerase II (Pol II) or RNA polymerase

III (Pol III) is placed at these locations, its transcription is repressed. This phenomenon is known as transcriptional silencing. Transcriptional silencing depends on the location of the gene, but not the sequence of the gene. It involves a specialized chromatin structure, called silenced chromatin.

Several proteins that are important for silencing have been identified. Four silencing information regulators (Sir proteins), Sir1p, Sir2p, Sir3p, and Sir4p, are needed for transcriptional silencing at *HM* (Haber and George 1979; Klar et al. 1979; Rine et al. 1979; Rine and Herskowitz 1987), whereas only Sir2p, Sir3p, and Sir4p are required for silencing at telomeres (Aparicio et al. 1991), and only Sir2p is required for silencing of rDNA (Fritze et al. 1997). Sir2p is a NAD<sup>+</sup>-dependent histone deacetylase (Imai et al. 2000), and silencing requires the histone deacetylase activity of this enzyme. No enzymatic activity of other Sir proteins has been found; they are believed to function only as structural proteins. Mutations in the histone H3 and H4 N-terminal tail domains affect silencing (Kayne et al. 1988; Thompson et al. 1994). The N-terminal tails of H3 and H4 are hypoacetylated in silenced chromatin, and Sir2p-dependent hypoacetylation of specific lysine residues in the H3 and H4 tails is important for efficient silencing. Other proteins — Abf1p, Ard1p, Cdc7p, Cdc14p, Nat1p, Net1p, Orc1p, Rap1p, Sum1p, and Top1p — are also involved

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in transcriptional silencing, although less is understood about their roles (Loo and Rine 1995; Huang 2002).

*Cis*-acting elements, known as silencers, that regulate silencing at *HM* loci have been identified. Two silencers, referred to as E and I, flank both *HMR* and *HML* (Abraham et al. 1984; Feldman et al. 1984). The well-studied *HMR*-E silencer comprises binding sites for the origin replication complex, Rap1p, and Abf1p (Brand et al. 1987). Telomeres contain many Rap1p binding sites (Strahl-Bolsinger et al. 1997). The silencers that regulate rDNA silencing remain unknown.

### How is silenced chromatin assembled?

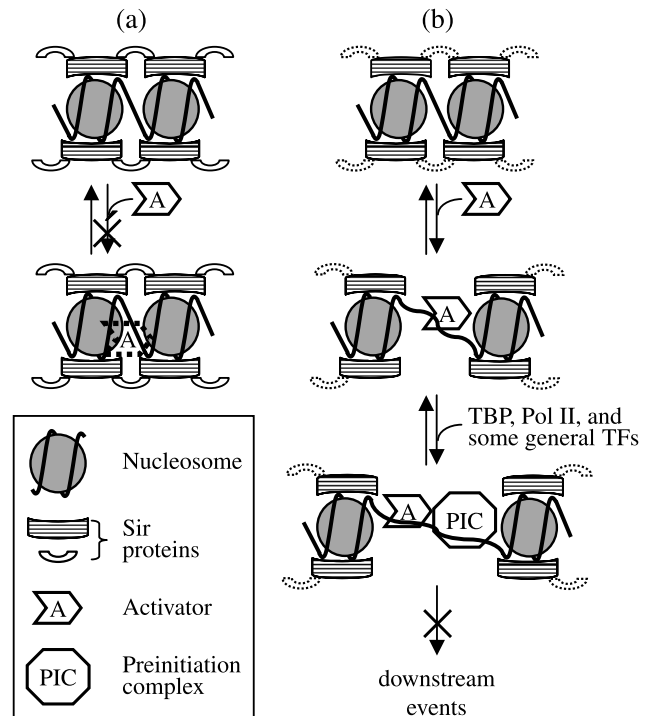
At *HM* loci, Rap1p binds to *HML*-E, *HML*-I, and *HMR*-E, where it cooperates with the origin of replication complex and (or) Abf1p to initiate silencing. Rap1p also binds to its recognition sites in internal tracts of  $C_{1-3}A/TG_{1-3}$  DNA at telomeres. The recruitment of a Sir protein complex to silenced chromatin is mediated by the interactions between Rap1p and Sir4p (Luo et al. 2002). Once targeted to *HM* silencers or telomeres, Sir2p deacetylates histone tails. Additional Sir complexes bind to the deacetylated nucleosomes. Repetition of this process leads to Sir proteins spreading along chromatin to form a silenced chromatin structure (Rusche et al. 2002). How far Sir proteins can propagate depends on the dosage of Sir proteins (Suka et al. 2002) and on the presence of DNA "boundary" elements that block further spreading (Bi and Broach 2001). In contrast to silencing at *HM* and telomeres, silencing of rDNA requires a protein complex termed RENT, which contains Sir2p, Net1p, and Cdc14p, and is localized in the nucleolus (Straight et al. 1999). Net1p is responsible for recruiting Sir2p to rDNA repeats, but how Net1p is recruited to rDNA is unknown.

Early studies showed that the establishment of transcriptional silencing at *HMR* and *HML* requires that cells pass between the G1 and M phases of the cell cycle, the period during which DNA is replicated. This finding hinted that DNA replication may be required for the establishment of silencing (Miller and Nasmyth 1984). However, a recent study tested this hypothesis directly and found that establishment of silencing is independent of DNA replication (Li et al. 2001). It remains unknown what event between the early S phase and the M phase of the cell cycle is involved in the establishment of silencing.

### How does silenced chromatin affect transcription?

The mechanism by which silenced chromatin represses transcription is not understood. There are two prevailing models. A model based on steric hindrance (Fig. 1a) supposes that silent chromatin represses transcription by preventing site-specific gene activator proteins from binding to their DNA target sites. An alternative model, based on downstream transcriptional inhibition (Fig. 1b), supposes that activators may have unhindered access to their target sites in heterochromatin, and that transcription is instead blocked at a step that is downstream from preinitiation-complex recruitment. Both models have experimental support; moreover, they are not necessarily mutually exclusive.

**Fig. 1.** Two models for transcriptional silencing. (a) Steric hindrance model. DNA in silenced chromatin is inaccessible to site-specific gene activator proteins. (b) Downstream inhibition model. Activators bind to their targets in heterochromatin; transcription is blocked in a step downstream from preinitiation-complex recruitment. The Sir proteins are shown in dotted-outline to suggest that the silenced chromatin may be dynamic in some way that allows activator proteins to bind.



Many early studies support the steric hindrance model. The HO endonuclease, which initiates mating-type interconversion, cleaves a specific sequence at either *MATa* or *MATα*, but not the same sequences at the silenced *HMLα* or *HMRa* loci (Strathern et al. 1982), suggesting that the silenced loci are inaccessible to HO endonuclease. Similarly, DNA sequences near telomeres are more refractory to the *Escherichia coli* Dam DNA methyltransferase than are those same or other DNA sequences located in euchromatin (Gottschling 1992), and pyrimidine dimers at the active *MATα* locus are removed preferentially to those at the inactive *HMLα* locus (Terleth et al. 1989); these findings suggest that access to DNA in silenced chromatin is restricted from Dam methylase and from the proteins required for DNA repair, respectively. Finally, related results are obtained in isolated nuclei, where *HMR* DNA of wild-type strains is less accessible to digestion by many restriction endonucleases and by the HO endonuclease, than is *HMR* DNA of *sir<sup>-</sup>* strains (Loo and Rine 1994).

Other studies, however, argue against the steric hindrance model. In all of the experiments described above, except those using the HO endonuclease *in vivo*, the silenced chromatin is still partially accessible to the DNA-modifying enzymes: accessibility of the silenced chromatin is quantitatively reduced but not eliminated, yet transcription of the silenced chromatin is completely blocked. Moreover, with *Drosophila* tissue culture cells, restriction endonuclease

accessibility assays on isolated nuclei show that the silencing-like phenomena of gene inactivation by Polycomb protein, and position effect variegation, are not associated with any significant change in the accessibility of the inactivated chromatin (Schlossherr et al. 1994). Some biological processes requiring protein–DNA interaction, such as homologous recombination (Holmes and Broach 1996), retrotransposon integration (Zou et al. 1996), and site-specific recombination (Cheng et al. 1998), do occur at silenced chromatin in vivo. Taken together, these observations suggest that silenced chromatin is more dynamic and accessible than is assumed by the steric hindrance model. This reasoning suggests that other mechanism(s) may function with, or instead of, reduced DNA accessibility, to completely repress transcription.

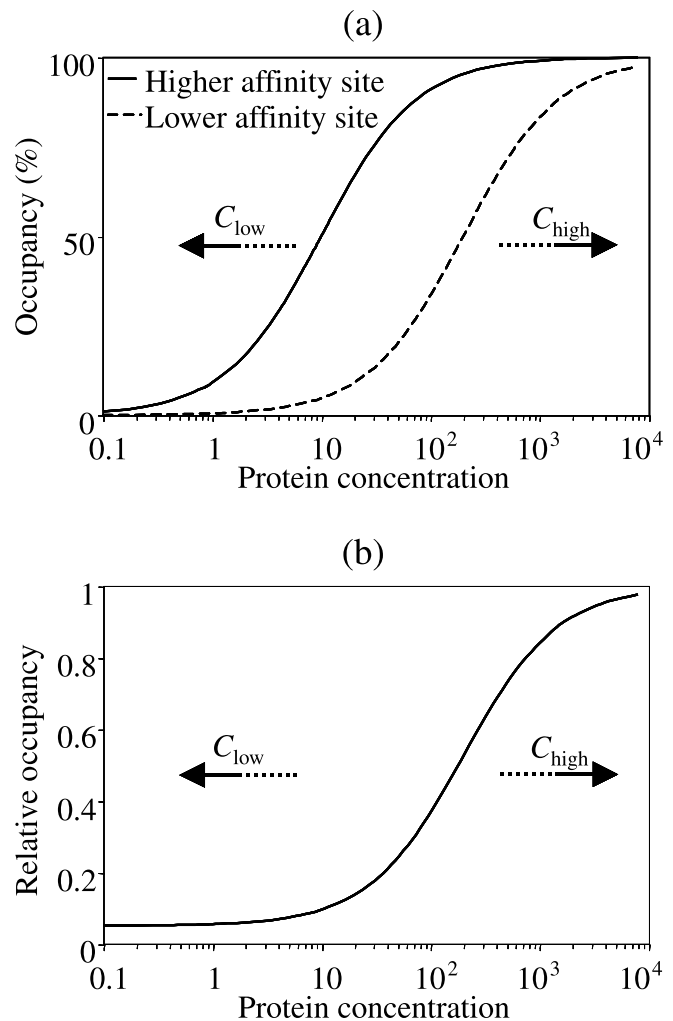
Several other studies simultaneously argue against the steric hindrance model and support the downstream inhibition model (Fig. 1*b*). The promoter region of *HML*  $\alpha 1$  and  $\alpha 2$  is nucleosome free, and sequences between the two TATA boxes are actually more sensitive to micrococcal nuclease at (silenced) *HML* than at (active) *MAT* $\alpha$  (Weiss and Simpson 1998); the *HMR*  $\alpha 1$  promoter is actually occupied by the TATA-box binding protein (TBP) and Pol II, even when the gene is silenced; and the promoter of a *HSP82* gene silenced by *HMR*-E silencers is permissive to the constitutive binding of heat shock transcription factor (HSF), TBP, and Pol II (Sekinger and Gross 1999, 2001). These discoveries suggest that transcription of silenced chromatin is blocked downstream from preinitiation-complex recruitment.

As mentioned above, these two distinct models for mechanisms of transcriptional silencing are not necessarily exclusive. Although regulated DNA accessibility appears not, on its own, to provide an all-or-nothing response, it may nevertheless contribute to the silencing phenomenon. First, high relative accessibility at a euchromatic site, compared with a heterochromatic location of the same DNA sequence, may translate to high relative affinity at the euchromatic site. The accessibility assays may probe the high-concentration region of the binding curve, where differences in resulting occupancy are much smaller than in the low-concentration region (Fig. 2). If the binding of general transcriptional factors to DNA in vivo occurs within the low-concentration region, as seems likely, then much more Pol II may be assembled at a euchromatic promoter (high accessibility, thus, high effective affinity) than at a silenced promoter (low accessibility, low effective affinity).

Second, regulated accessibility at heterochromatic loci may also contribute to transcriptional silencing through large cooperative effects. If each component of the Pol II transcription and regulatory machinery binds at heterochromatic sites with slightly less affinity (a slight cost in free energy) relative to their binding at euchromatic sites, the total cost in free energy and occupancy can be enormous, because Pol II contains many DNA-interacting proteins. In accordance with this view, the two models shown in Fig. 1 are not completely distinct.

In this context, studies that have established that increased concentration of a cellular activator can overcome heterochromatic silencing (Aparicio and Gottschling 1994; Lundgren et al. 2000) can be interpreted with either of the two competing mechanisms shown in Fig. 1. One interpretation (Fig. 1*a*) is that silenced chromatin regions quantitatively

**Fig. 2.** Binding curves for a protein at two DNA sites having different affinities. (a) Absolute occupancy of a protein at two DNA sites having different effective affinities is plotted against protein concentration. The high- and low-affinity sites may correspond to identical DNA sequences placed in euchromatic and heterochromatic regions, respectively. The solid line shows occupancy at the higher-affinity site, the dashed line shows occupancy at the lower-affinity site. (b) Ratio of protein occupancy at the lower-affinity site to that at the higher-affinity site. This parameter represents the ability of a protein to discriminate between the two sites and preferentially occupy the one having higher effective affinity. In the low-concentration region, the ratio of occupancy at the higher-affinity site to that at the lower-affinity site approaches the ratio of dissociation constants (20 fold, in this example), whereas in the high-concentration region, the ratio of occupancies approaches 1.



hinder (but do not fully prevent) accessibility, thus quantitatively reducing the activator's occupancy at the silenced locus, compared with the activator's occupancy at chromatin regions that are not silenced. In this view, in accord with Fig. 2, the effect of the increased activator concentration is to increase occupancy at the silenced locus to a value closer to that obtained at nonsilenced loci by the wild-type concentration of activator. However, an alternative interpretation is that the silenced chromatin hinders (but again does not com-

pletely prevent) the ability of bound activator to recruit the rest of the Pol II transcription machinery (Fig. 1*b*). In accordance with this view, the effect of the increased activator concentration is to compensate for poor recruitment of the Pol II transcription machinery by driving the occupancy of bound activator to greater levels than occurs normally at nonsilenced loci, thereby compensating for a slightly decreased affinity of other cooperatively interacting protein partners.

Third, it is also possible that large inherent consequences of regulated accessibility are masked in studies such as those described above, which are carried out on populations of unsynchronized cells. For example, consider a population of cells that comprises two subpopulations that differ from each other only at the epigenetic state of heterochromatic gene. In one subpopulation, a heterochromatic gene is derepressed and accessible, while in the other subpopulation, the same heterochromatic gene is silenced and inaccessible. A population average will reveal accessibility at the silenced gene, even though the subpopulation that is actually silenced at that time does not allow accessibility. Alternatively, heterochromatic DNA might be inaccessible throughout most of the cell cycle, yet accessible when cells are at a certain cell cycle stage, for example, during DNA replication. Again, a population average of the unsynchronized cells will reveal accessibility at a silenced locus, even though there may be no accessibility at that same locus throughout the rest of the cell cycle. For these reasons, it is possible that existing studies overestimate the extent to which the silenced chromatin remains accessible to DNA-binding proteins.

Although regulated accessibility may prove to be more important than studies to date suggest, transcription can also be regulated at many other steps, including general transcription factor recruitment, the formation of the open polymerase complex, promoter escape, and Pol II elongation. Histone acetylation might be a prerequisite for the recruitment of some transcription factors, and the hypoacetylation of silenced chromatin might prevent transcription factor recruitment. Consistent with this view, acetylation of histone H4 K8 mediates the recruitment of the SWI/SNF complex, and acetylation of K9 and K14 in histone H3 is important for the recruitment of TFIID during activation of the IFN- $\beta$  gene (Agalioti et al. 2002). Moreover, Sir2p might deacetylate not only histones, but also transcription factors, and the deacetylation of a transcription factor might reduce its activity. In support of this model, mammalian Sir2p homologs regulate p53 function via deacetylation (Luo et al. 2001; Vaziri et al. 2001). Sir proteins might also interact directly with the transcription machinery to block transcription. Although there is no direct evidence that Sir proteins interact with any component of transcription machinery, two *Drosophila* proteins, HP1 and Polycomb repressive complex 1 (PRC1), both of which are involved in gene silencing, do interact directly with TATA-binding protein-associated factors (TAFs) (Saurin et al. 2001; Vassallo and Tanese 2002). Finally, silenced chromatin might be more rigid than euchromatin, so that the conformation changes required for open complex formation, promoter escape, and Pol II elongation might be restricted in silenced chromatin. Deacetylation of histone tails increase the positive charge, likely strengthening the interactions between histones and DNA and potentially suppressing certain chromatin conformational changes. Beyond that, the

binding of Sir proteins to nucleosomes might also contribute to a more rigid structure of silenced chromatin.

rDNA silencing differs from silencing at *HM* and telomeres, not only because rDNA silencing requires different transacting factors, but also because rDNA silencing occurs at a location that is actively transcribed by RNA polymerase I (Pol I). There are ~150 copies of the rRNA genes in the yeast genome. Not all copies are actively transcribed by Pol I in normal growth conditions. Remarkably, it has been shown that rDNA silencing requires the presence of active Pol I transcription machinery at the silenced gene (Buck et al. 2002; Cioci et al. 2003). This finding rules out the possibility that rDNA silencing takes place only in the subset of rRNA repeats that are not actively transcribed by Pol I. Moreover, this finding means that silenced rDNA repeats are *not* inaccessible to site-specific DNA-binding proteins, because the silenced rDNA is accessible to the Pol I transcription machine. The DNA accessibility at rDNA loci is only slightly enhanced by *sir2* or *top1* deletion, when probed with  $\lambda$  exonuclease and micrococcal nuclease, and only minor differences exist in the rDNA accessibility to the restriction enzyme EcoR V among WT, *sir2*, *top1*, and Sir2p overexpressing strains (Cioci et al. 2002). Nevertheless, despite the many apparent differences between silencing of rDNA and that of *HM* and telomeres, silencing at all three types of loci may still use the same basic molecular mechanisms to repress transcription.

## Future directions

The available data show that regulated DNA accessibility, on its own, cannot account for transcriptional silencing, and that sequence-specific binding proteins bind to DNA sites in silenced chromatin. Transcriptional silencing likely represents a combination of many distinct mechanisms, each providing a quantitatively modest effect. When the effect of each mechanism is evaluated individually, no all-or-nothing response can be detected. However, through a combination of multiple regulated steps, transcription can be tightly repressed. It will be important to determine quantitatively at which mechanistic step(s) transcriptional silencing represses Pol II or Pol III transcription. To understand the role of DNA accessibility in transcriptional silencing more clearly, studies with synchronized cells and experiments that discriminate epigenetic states are required.

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