Nucleosomes Unfold Completely at a Transcriptionally Active Promoter

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Summary

It has long been known that promoter DNA is converted to a nuclease-sensitive state upon transcriptional activation. Recent findings have raised the possibility that this conversion reflects only a partial unfolding or other perturbation of nucleosomal structure, rather than the loss of nucleosomes. We report topological, sedimentation, nuclease digestion, and ChIP analyses, which demonstrate the complete unfolding of nucleosomes at the transcriptionally active PHOS promoter of the yeast Saccharomyces cerevisiae. Although nucleosome loss occurs at all promoter sites, it is not complete at any of them, suggesting the existence of an equilibrium between the removal of nucleosomes and their reformation.

Introduction

The packaging of promoter DNA in nucleosomes inhibits transcription in vitro (Imbalzano et al., 1994; Knezetic and Luse, 1986; Lorč et al., 1987; Taylor et al., 1991) and in vivo (Han and Grunstein, 1988). This inhibition is relieved by a change in nucleosome structure or internucleosomal associations or both, as shown by the conversion of promoter DNA from a nuclease-resistant to a nuclease-accessible (“hypersensitive”) state upon transcriptional activation. It was once thought that nucleosomes were entirely lost from transcriptionally active promoters. More recent studies, however, have raised the possibility that nucleosomal DNA is rendered accessible in vivo without a complete unfolding of the nucleosome (Aalfs and Kingston, 2000).

Three previous studies demonstrated an absence of nucleosomes from nucleosome-hypersensitive promoter regions. The limitation of these studies was a lack of correlation between transcriptional activation and nucleosome loss. First, Drosophila heat shock promoter DNA could not be crosslinked with histones either before or after heat shock, suggesting that the promoters were nucleosome free in both active and repressed states (Karpov et al., 1994). Second, restriction endonuclease digestion of the transcriptionally active chicken β-globin promoter released a 115 bp fragment with the electrophoretic mobility of naked DNA, but the mobility of this fragment prior to activation was not assessed (McGhee et al., 1981). Finally, electron microscopy revealed a nucleosome-free gap of ~400 bp over the promoter of 15%–25% of viral minichromosomes in SV40-infected cells, but the relationship of the gap to transcriptional activity could not be determined (Jakobovits et al., 1980; Saragosti et al., 1980).

Two lines of evidence have pointed to the retention of histones on transcriptionally active promoters, raising the possibility that nucleosomes are retained as well. First, chromatin immunoprecipitation experiments demonstrated the occurrence of hyperacetylated histones on active promoters (Kuo and Allis, 1998; Struhl, 1998). This appeared to be compatible with accessibility to nuclease digestion, since nucleosomes reconstituted in vitro with highly acetylated histones exhibit increased accessibility (Anderson et al., 2001; Lee et al., 1993; Bettesse-Dadée et al., 1996). Second, chromatin-remodeling complexes have been isolated from yeast and metazoan cells that expose nucleosomal DNA to nuclease attack without displacement of histones (Cairns et al., 1996; Cote et al., 1994; Imbalzano et al., 1994; Narlikar et al., 2002). These remodeling complexes can leave nucleosomes in a stably altered state (Lorch et al., 2001; Schnitzler et al., 1998), retaining a degree of nuclease accessibility (Cote et al., 1998; Lorch et al., 1998; Schnitzler et al., 1998), which has been attributed to a conformational change of the nucleosomal core (Schnitzler et al., 1998).

Here we investigate the extent to which nucleosomes are unfolded following transcriptional activation of the PHOS promoter of yeast. Classical studies of Hörz and coworkers have made PHOS a paradigm for understanding the relationship of chromatin structure to transcription (Svaren and Hörz, 1997). The PHOS gene encodes a secreted acidic phosphatase, whose expression is activated in response to a lack of inorganic phosphate in the growth medium. A region of the promoter protected by four nucleosomes in the repressed state becomes accessible to nuclease attack following activation. This chromatin transition exposes crucial promoter elements: a binding site (UASp2) for the Pho4p transcriptional activator protein, as well as the TATA box and transcription start site. The functional significance of the chromatin transition is further established by molecular genetic analysis. Depletion of histone H4 in vivo results in UAS-independent activation of PHOS in high phosphate media (Han and Grunstein, 1988; Han et al., 1988). Deletion of the activation domain of Pho4p abolishes the chromatin transition and eliminates binding of the truncated activator to UASp2 (Svaren et al., 1994; Venter et al., 1994).

Possibilities that have been considered to account for the chromatin transition at the PHOS promoter include the removal of nucleosomes, the selective loss of histone H2A/H2B dimers, and histone hyperacetylation (Almer et al., 1986; Fascher et al., 1993). Furthermore, since increased nuclease accessibilities exclusively result from altered internucleosomal associations without any accompanying change in nucleosome structure.

We have investigated the basis of the chromatin transition by direct analysis of the promoter region, in both repressed and activated states, following excision with
endonucleases in vitro or with the use of a site-specific recombinase in vivo (Ansari and Gartenberg, 1999). The results disclose the fate of nucleosomes and provide a starting point for future studies of the mechanism.

Results

Topological Analysis of Promoter Chromatin
A loss of nucleosomes and unraveling of the associated DNA produces a change in topology that is reflected in the topoisomer distribution of the DNA in circular form. We could release the PHO5 region from yeast chromatin in circular form in vivo by the approach of Gartenberg and colleagues (Ansari and Gartenberg, 1999), which employs the R recombinase of Zygosaccharomyces rouxii. This enzyme brings together two distant RS elements, which we introduced by homologous recombination at the PHO5 locus in the linker between nucleosomes –4 and –3 (Figure 1) and at the end of the open reading frame. Recombination was controlled in these modified strains by expression of the R recombinase transgene from the inducible GAL1 promoter. Addition of galactose to the growth media of the modified formly accessible to DNase I cutting. The same pattern was recapitulated in a nucleosome (Figure 1). The pattern was the same as that obtained from wild-type cells, except that the introduction of an RS element upstream of nucleosome –3 created a DNase I-hypersensitive site at that position, indicating that the RS element was not incorporated in a nucleosome (Figure 1).

In pho80Δ strains, the DNase I digestion pattern was altogether different, regardless of the phosphate concentration in the growth medium (Figure 1). The region encompassing nucleosomes –3 to –1 was almost uniformly accessible to DNase I cutting. The same pattern was found for wild-type cells grown in low phosphate medium (Almer et al., 1986). The results of DNase I digestion under both activating and repressing conditions recapitulate the previous findings of Hörz and coworkers (Almer et al., 1986; Gregory et al., 1998).

The nearly uniform accessibility to DNase I digestion seen under activating conditions could result from a lack of nucleosome positioning, rather than a change in nucleosome structure or nucleosome loss. A lack of positioning would render sequences within nucleosomes more accessible and linker sequences less accessible to nucleosome digestion. On the contrary, both nucleosomal and linker sequences became more accessible to restriction endonuclease digestion upon transcriptional activation. About 7% of ClaI sites in nucleosome –2 were accessible under repressing conditions, whereas ~80% of ClaI sites were readily cleaved under activating conditions (Figure 2A). A BstEII site in the linker between nucleosomes –1 and –2, accessible to digestion under repressing conditions, became even more accessible to DNA digestion (Figure 2A).
under activating conditions (Figure 2A). These results of restriction endonuclease digestion, like those of DNase I digestion, recapitulate the previous findings of Horz and coworkers (Almer et al., 1986; Gregory et al., 1998), and show that insertion of RS elements and activation by means of the pho80Δ mutation do not perturb the nucleosomal organization or chromatin transition at the PHO5 promoter.

We also performed restriction endonuclease digestion to determine whether excision of PHOS chromatin circles by the R recombinase and subsequent manipulation of the circles in vitro had any effect on the PHOS chromatin structure. We isolated the PHOS circles by differential centrifugation, followed by affinity chromatography with a GST-LexA adaptor molecule that was bound to protein A-coupled agarose beads using an anti-GST antibody. Circles were eluted from the column with a site-specific protease to cleave the adaptor protein. The results of restriction endonuclease digestion of the isolated PHOS circles were very similar to those obtained for PHOS at the chromosomal locus (compare Figures 2A and 2B). With the exception of nucleosome –3, which appeared to be shifted toward nucleosome –2, PHOS promoter nucleosomes were in the same positions on repressed circles (isolated from PHO80 strains) as at the chromosomal locus. All restriction sites tested showed increased accessibility on activated chromatin circles (isolated from pho80Δ strains), again similar to activated chromosomal locus (Figure 2). We conclude that circle formation and isolation conserve important structural features of activated and repressed promoter chromatin.

Having established the conservation of chromatin structure on circles, we could employ the circles for topological analysis of the chromatin transition at the PHOS5 promoter. Following induction of circle formation in cultures of PHO80 and pho80Δ strains, DNA was extracted and topoisomerons were resolved by gel electrophoresis. We found a linking difference between activated (A) and repressed (R) circles of ∆LkA,R = 1 ± 0.2 (Figure 3A). In the absence of other factors, the number of nucleosomes lost from a circular DNA molecule is identical to the observed linking difference ∆Lk, assuming the circles are at relaxation equilibrium (Germond et al., 1975; Keller, 1975; Prunell, 1998; Shure and Vino-grad, 1976; Simpson et al., 1985; Zivanovic et al., 1988).

When activated and repressed gene circles are compared, however, there is another factor to be considered: the effect of transcription on circle topology. To eliminate the effect of transcription, we replaced the TATA box of the PHOS promoter with an unrelated sequence. No PHOS5 mRNA could be detected by blot hybridization in TATA-less pho80Δ cells (data not shown). The chromatin transition at the PHOS5 promoter in pho80Δ strains was indistinguishable between TATA box wild-type and TATA box mutant strains (data not shown), as expected from previous studies (Fascher et al., 1993). The linking difference ∆LkA,R measured in TATA box mutants may therefore be attributed entirely to the chromatin transition. The result was ∆LkA,R = 1.85 ± 0.05 (Figure 3B and 3C), consistent with an average loss of ~1.85 nucleosomes, or conversely the retention of ~1.15 of the three promoter nucleosomes.

To test the assumption that activated and repressed circles were at relaxation equilibrium in vivo, we brought the isolated circles to relaxation equilibrium in vitro by treatment with topoisomerase. The linking difference ∆LkA,R was unaffected by this treatment, showing that the assumption was valid (Figure 3C).

Sedimentation Analysis of Promoter Chromatin
A change in topology could arise, wholly or in part, from a change in the twist or writhe of DNA in the linker
regions between nucleosomes, rather than from an effect on the nucleosomes themselves. The observed $\Delta Lk^{\text{in vivo}}$ could also reflect a partial disruption of all promoter nucleosomes rather than the complete loss of a few. To determine the fates of individual promoter nucleosomes upon gene activation, we turned to sedimentation analysis, which is capable of distinguishing a nucleosome from naked DNA and also from various altered forms, such as particles that have lost histone H2A/H2B dimers, or the stably altered nucleosomes produced by chromatin-remodeling complexes, which migrate as dimers (Lorch et al., 1998; Schnitzler et al., 1998).

Micrococcal nuclease was used to cleave linker DNA between nucleosomes, releasing nucleosome monomers and higher multimers from the PHO5 promoter region of isolated nuclei, as shown by sedimentation analysis (Figure 4). Nucleosomes –1, –2, and –3 (N-1, N-2, and N-3) were recovered from PHO80 nuclei with comparable efficiencies. Yields of N-2 and N-3 from pho80Δ nuclei were much diminished, compared with the level of nucleosomes from a control region, the inactive LYS2 gene. The yield of N-1 was less affected by transcriptional activation. Evidently, N-1 persists to a considerable extent, whereas N-2 and N-3 are lost upon activation or undergo an alteration in structure that renders them susceptible to micrococcal nuclease attack. Notably, core particles recovered from the activated promoter are indistinguishable from their counterparts.
on the repressed promoter with respect to DNA length and sedimentation behavior.

Naked DNA resulting from loss of N-2 and N-3, or altered forms of these nucleosomes susceptible to micrococcal nuclease digestion, could not be detected because they would be destroyed by digestion. We therefore turned to the use of restriction endonucleases. A 220 bp DNA fragment spanning N-2 could be released by digestion with BstEII and HaeIII (Figure 2). As a control, we incubated naked N-2 DNA with isolated nuclei to be sure that the release of the naked fragment from nuclei would not be masked by its interaction with nuclear proteins. The N-2 region released from PHO80 nuclei sedimented at the same rate as nucleosomes produced by micrococcal nuclease digestion (Figures 5A and 5A'). By contrast, the N-2 region released from pho80Δ nuclei cosedimented with naked DNA (Figures 5A and 5A').

The N-3 region could be cleaved with BamHI and HaeIII to yield a 140 bp fragment. Because the size was less than the 147 bp length of core nucleosomal DNA, there was very little release of N-3 nucleosomes from PHO80 nuclei, as judged from sedimentation analysis (data not shown). On the other hand, the N-3 fragment was efficiently released from pho80Δ nuclei, cosedimenting with nuclei sedimenting with naked control fragment (Figure 5B). Evidently N-3, like N-2, is largely lost upon transcriptional activation.

The N-1 region could be excised by BstEII and HaeIII as a 216 bp fragment. When released from PHO80 nuclei, this fragment sedimented as a nucleosome (Figures 5C and 5C'). When released from pho80Δ nuclei, the same fragment sedimented differently, unlike a nucleosome, but also unlike naked control DNA (Figures 5C and 5C'). This distinctive sedimentation behavior was not due to interaction of transcription factors with the TATA box in the N-1 region, since a control experiment performed with the pho80Δ strain in which the TATA box was replaced by an unrelated sequence (see above) gave the same results (data not shown).

We sought to increase the resolution of the analysis by excising a smaller piece of N-1 DNA from pho80Δ nuclei. We took advantage of results of partial micrococcal nuclease digestion and indirect end label analysis, showing that N-1 frequently adopts a position on activated promoters about 30 bp downstream of its position on repressed promoters (Figure 6A). (This analysis was performed on isolated PHO5 chromatin circles to avoid crosshybridization with DNA of PHO5 homologs.) The shift in position of N-1 upon activation exposed the TATA box but not the transcription start site, and rendered an artificial ClaI site introduced in the TATA box mutant highly accessible to digestion, while a Sall site introduced about 20 bp downstream of N-1 became more protected (Figure 6B). Cleavage of isolated pho80Δ TATA mutant nuclei with ClaI and Haell released a 146 bp N-1 fragment, which was resolved by sedimentation into a mixture of nucleosomes and naked DNA (Figure SD).

In some pho80Δ TATA mutant nuclei, the ClaI site in the N-1 region was protected by N-1 (Figure 6B), and cleavage at the ClaI site in the N-2 region, exposed by the loss of N-2, released a 316 bp fragment in ClaI/ Haell digests. This larger fragment cosedimented with a nucleosome and was clearly distinguishable from naked control DNA of the same length (Figure 5E). Superposition of the sedimentation profiles of the 146 and 316 bp fragments yielded a bimodal distribution with peaks at the positions of nucleosomes and naked DNA (Figure 5F). This distribution corresponded to a mixture of 60% nucleosomes and 40% naked DNA.

Sedimentation analysis thus demonstrates a change in the structure of promoter nucleosomes upon PHO5 activation, ruling out the possibility that the altered state of activated promoter chromatin exclusively results from a change in internucleosomal associations. The sedimentation analysis further provides evidence for only two states of activated promoter DNA, naked or nucleosomal. It may be asked whether the appearance of naked DNA is an artifact of manipulation in vitro. Nucleosomes that were partially unfolded in vivo might have become fully unfolded in vitro. Alternatively, loosely bound, perhaps extensively modified histones might have been lost during manipulation in vitro. Indeed, we treated samples with excess naked DNA prior to sedimentation to minimize nonspecific protein-DNA interaction, and it might be imagined that loosely bound histones were removed at this stage. We could control against nucleosome unfolding in vitro by topological analysis. Such unfolding would selectively alter the topology of the activated circles, increasing the linking difference between activated and repressed circles, ∆Lk₄₆. As already mentioned, however, treatment of isolated chromatin circles with topoisomerase did not alter ∆Lk₄₆. We extended this analysis by treating isolated chromatin circles with competitor DNA at 37°C, followed by relaxation with topoisomerase. After DNA treatment the majority of the N-2 region released from activated circles migrated as naked DNA in a gel electrophoresis (data not shown). However, the linking difference between activated and repressed circles remained constant (Figure 3C), demonstrating that exposure to naked DNA did not selectively affect activated promoter chromatin.

As a control against the possible loss of loosely bound histones, we treated activated cells with formaldehyde and repeated the sedimentation analysis of the N-3 region. (We chose this region to avoid any confusion that might arise from the crosslinking of transcription factors to the DNA.) Despite the crosslinking, the N-3 fragment released from pho80Δ nuclei still cosedimented with the naked control fragment (data not shown). We extended the crosslinking analysis by chromatin immunoprecipitation (ChIP) with the use of strains expressing His-tagged histone H2B. Circles crosslinked to tagged H2B were retained on a TALON column. PHO5 circles, as well as circles containing the LEU2 selection marker from the recombinase expression plasmid (see Experimental Procedures), were detected by real-time quantitative PCR (Figure 8A). The use of circles, produced by specific excision from the genomic locus, affords a possible advantage over conventional ChIP analysis, which relies on random fragmentation of DNA by sonication. The LEU2 circles provided an internal control for the consistency of the analysis and lack of change at a locus unlinked from PHO5. Expression of the tagged histone, confirmed by immunoblot analysis (data not shown), did not interfere with the chromatin transition at the PHO5 promoter, as shown by the change in accessibility to cutting by ClaI (Figure 8B). The results of quantitative PCR analysis showed a significant reduction in
Figure 5. Fragmentation of PHO5 Chromatin by Restriction Endonucleases and Sedimentation Analysis

The procedure was as in Figure 4. The DNA concentration relative to that at the peak of the distribution, determined from the radioactivity in the blot, is plotted for each gradient fraction on the ordinate. The numbers of the fractions are indicated on the abscissa. The distributions derived from PHO80 and pho80Δ nuclei are blue and green, respectively. Reference distributions are brown. The reference distribution shown in (B), (D), and (F), labeled C (for core particle), was obtained by reconstitution of a core particle on a 140 bp DNA fragment in vitro. The two peaks in these distributions identify the positions of core particles and naked DNA (asterisk) in the gradients.

(A) Distribution of the N-2 region released as a 220 bp fragment from PHO80 (N-2R) and pho80Δ (N-2A) nuclei by BstEII/HaeII digestion. (A') The distribution of the N-2 region released by MNase digestion from PHO80 nuclei (CN-2) and the distribution of the naked 220 bp N-2 DNA fragment (D-2220) are shown for reference.

(B) Distribution of the N-3 region released as a 140 bp fragment from pho80Δ (N-3A) nuclei by BamHI/HaeII digestion. The distribution of the N-3 region released from PHO80 nuclei (CN-3) by MNase digestion is shown for reference.

(C) Distribution of the N-1 region released as a 216 bp fragment from PHO80 (N-1R) and pho80Δ (N-1A) nuclei by BstEII/HaeIII digestion. (C') The distribution of the N-1 region released by MNase digestion from PHO80 nuclei (CN-1) and the distribution of the naked 216 bp N-1 DNA fragment (D-1216) are shown for reference.

(D) Distribution of the N-1 region released as a 146 bp fragment from pho80Δ (N-1A) nuclei by ClaI/HaeIII digestion.

(E) Distribution of the N-1 region released as a 316 bp fragment from pho80Δ nuclei (N-1A) by ClaI/HaeIII digestion. The distribution of the N-1 region released by MNase nuclei (C N-1) and the distribution of the naked 316 bp N-1 DNA fragment (D-1316) are shown for reference.

(F) Distribution of N-1A results from the superposition of distributions N-1A (146 bp, [D]) and N-1A (316 bp, [E]).

Neither sedimentation nor ChIP analysis provides any evidence for the selective loss of H2A/H2B dimers, frequently suggested as basis for the exposure of nucleosomal DNA upon transcriptional activation (Hayes and Wolffe, 1992; Tse et al., 1998). The resolution of our analysis would have been sufficient to distinguish H3/H4 tetramer particles from nucleosomes and from naked histone octamers.

During the amount of crosslinkable histone on the activated promoter (Figure 8A). A similar result was obtained with the use of antibodies against the C-terminal tail of histone H3, retained on a protein A column (data not shown). These results suggest that histones are lost from the activated promoter in vivo and not during subsequent treatment and handling in vitro.
Nuclease Digestion

where $[E]$ is the nuclease concentration, and $k$ is a constant.

Limit digestion by micrococcal nuclease, converting the occupancy by nucleosomes of particular DNA sequences. To this end, nuclei isolated from PHO80 and pho80Δ cells were digested with increasing concentrations of micrococcal nuclease, followed by DNA extraction, gel electrophoresis, and blot hybridization. The strengths of the hybridization signals obtained with a probe from the PHO5 open reading frame were closely comparable for the wild-type and mutant cells, showing that the numbers of nuclei analyzed and general accessibility to digestion were about the same (Figure 7A). In contrast, hybridization with a probe spanning the region from N-1 to N-3 revealed more rapid digestion of promoter DNA in the nuclei from pho80Δ cells (Figure 7A).

The ratio of the hybridization signals for the mutant (transcriptionally activated, A) and wild-type (repressed, R) cells, $S_A/S_R$, approached a limit value of 0.36 at high nuclease concentrations (Figure 7C). This limit value corresponds to the ratio of nucleosomes present on the promoter under activating and repressing conditions, as shown by the following line of argument.

We assume that every promoter sequence exists in a state of high nuclease accessibility (denoted D) or a state of low nuclease accessibility (denoted C, for core particle). The concentration of a promoter DNA, $[P]$, is then

\[ [P] = [C] + [D]. \]  

(1)

It can be shown that core particle DNA is digested by MNase according to a first order rate law under our experimental conditions (data not shown). Thus,

\[ \frac{d[C]}{dt} = -k[E][C], \]  

(2)

where $[E]$ is the nuclease concentration, and $k$ is a constant $>0$. The solution of (2) is

\[ [C](t) = [C_0] \exp \{-k[E]t\}, \]  

(3)

where $[C_0]$ is the original concentration at $[E] = 0$ or $t = 0$. From Equation 1, Equation 3 and the fact that linker DNA is digested much faster than core particle DNA follows that, at constant $t$, $[P]_A/[P]_R$ approaches $[C_0]_A/[C_0]_R$ for large $[E]$, where $A$ and $R$ denote the activated and repressed promoter states. Finally, since $[P]$ is proportional to the hybridization signal $S$, it follows that $S_A/S_R$ approaches $[C_0]_A/[C_0]_R$ for large $[E]$. So although $k > 0$ and core particles are also degraded by MNase, it is possible to derive the starting concentration of nucleosomes on the promoter sequence, $[C_0]$, from the nuclease digestion kinetics. The observed asymptotic behavior of $S_A/S_R$ approaching a limit $= 0$ (Figure 7C) is indicative of equal rate constants $k$ for core particles on the activated and repressed promoter sequences, further supporting the conclusion that the nucleosomes possess identical structures on these sequences.

We conclude that, on average, $\sim 0.36 \times 3 = 1.1$ nucleosomes are associated with the PHO5 promoter, or conversely $\sim 1.9$ nucleosomes are lost from the promoter, upon transcriptional activation. We extended the analysis to individual promoter nucleosomes, with the use of isolated PHO5 chromatin circles to exclude the possibility of crosshybridization between nucleosome probes and the DNA of PHO5 homologs (Figure 7B). The ratio.
Figure 7. Kinetics of Micrococcal Nuclease Digestion

(A) Nuclei isolated from PHO80 (yM2.1) or pho80Δ (yM8.14) cells, designated R and A, respectively, were digested in 200 μl with 1, 5, 10, or 20 U/ml micrococcal nuclease for 20 min. DNA was extracted, electrophoresed in an agarose gel, blotted, and hybridized with a probe spanning the open reading frame (ORF) or the domain of nucleosomes N-1 to N-3 (Pro).

(B) PHO5 circles (150 attomol of DNA) purified from PHO80 (yM2.1) or pho80Δ (yM8.14) cells, designated R and A, respectively, were digested in 200 μl with 0.5, 1.5, and 4.5 U of micrococcal nuclease for 5 min at 37°C. DNA was extracted, electrophoresed in an agarose gel, blotted, and hybridized with probes for the N-1, N-2, and N-3 regions.

(C) The total hybridization signal S was measured by integrating the profile of radioactivity in each lane of (A) and (B). The ratio of values S for activated, A, and repressed, R, states is plotted against the nuclease concentration [E] (in arbitrary units).

Discussion

Classical studies have shown the conversion of promoter chromatin to a nuclease-accessible state upon transcriptional activation. Recent work on histone acetylation and chromatin-remodeling complexes has raised the question whether this conversion reflects a stable alteration of nucleosome structure rather than the complete loss of nucleosomes. We present evidence from topological, sedimentation, ChIP, and quantitative nuclease digestion analyses for the complete unfolding of nucleosomes at the PHO5 promoter of yeast.

Sedimentation analysis demonstrates a structural change of three promoter nucleosomes upon PHO5 activation (Figure 5). Topological analysis shows that this structural change is accompanied by a change in linking number of PHO5 gene circles of +1.85 ± 0.05 (Figures 3B and 3C). The linking change is preserved upon treatment of isolated circles with topoisomerase, demonstrating that topoisomer distributions are in relaxation equilibrium in vivo (Figure 3C). The linking change thus provides a measure of the structural alteration accompanying transcriptional activation at the PHO5 locus. The linking change may result exclusively from the change in nucleosome structure or it may arise, wholly or in part, from changes in the twist or writhe of linker DNA.

We first consider the possibility that the linking difference is due entirely to the change in nucleosome structure. In this case, the linking difference could reflect the complete unfolding of 1.85 promoter nucleosomes, implying the retention of 1.15 unaltered nucleosomes on the activated promoter, or it could arise from a partial unfolding of all three promoter nucleosomes. The results of quantitative nuclease digestion are in close agreement with the expectation for the complete unfolding of nucleosomes, demonstrating the retention of 1.1 unaltered nucleosomes at the activated promoter (Figure 7). Nucleosomes retained on the activated promoter are equivalent to their counterparts on the repressed pro-
Nucleosome Removal upon Promoter Activation

Figure 8. Association of Histones with the PHO5 Promoter, Assessed by ChIP Analysis with His-Tagged Histone H2B
(A) Chromatin circles from repressed (yM40.1) or activated (yM25.1) cells expressing His-tagged H2B, designated R and A, respectively, were crosslinked and processed as for ChIP analysis except with the use of a TALON affinity matrix for binding the tagged histone H2B. The fraction of input DNA retained on the affinity matrix was measured by real-time quantitative PCR and is graphed for PHO5 promoter circles and for an unlinked LEU2 control circle. The heights of the bars correspond to the mean values calculated from at least nine ChIP analyses for each strain. Error bars indicate the sample standard deviation. Untagged strains expressed only wild-type histone H2B.
(B) Accessibility of the ClaI site in repressed, R, and activated, A, PHO5 promoter chromatin from H2B-tagged strains was compared as in Figure 2. The arrowhead marks the uncut promoter.

The results of sedimentation and electrophoretic analysis, showing the conversion of nucleosomal into naked DNA, further support this conclusion (Figure 5 and data not shown). In the case of N-1, the sedimentation results show the loss of 0.4 nucleosomes (Figure 5F), identical with the value obtained for this position from limit nuclease digestion (Figure 7B). The amount of naked promoter DNA fragment revealed by sedimentation therefore accounts completely for the increase in nuclease accessibility. This result argues against the possibility that the naked promoter DNA fragment represents only a small fraction of the total and does not reflect the behavior of the majority at that nucleosomal position.

As mentioned above, the observed linking difference could arise, wholly or in part, from a change in twist or writhe of linker DNA. In this case, the near perfect quantitative agreement between topological and nuclease digestion data would have occurred by chance, which seems very unlikely. The quantitative agreement between the two analyses suggests instead that changes in linker DNA conformation do not contribute to the observed topological difference between activated and repressed circles. Furthermore, if nucleosomes at the activated promoter unfold only partially in vivo, it must be assumed that they disintegrated in vitro during sedimentation analysis. However, the topological results were unaffected by the exposure of chromatin circles to conditions under which naked DNA is released from the activated promoter, showing that no further unfolding of nucleosomes occurred upon manipulation of the circles in vitro (Figure 3C). Finally, crosslinking in vivo failed to retain histones of unfolded nucleosomes, as demonstrated by sedimentation and ChIP analyses (Figure 8 and data not shown). Once more, the experimental evidence argues against the possibility of a partially unfolded nucleosome at the activated PHO5 promoter. Although nucleosome loss occurs at all sites (Figure 5), it is not complete at any of them (Figure 4), suggesting the existence of an equilibrium between the removal of nucleosomes and their reformation. The position of this equilibrium differs markedly for the different sites. The rates of removal and reformation may depend on the DNA sequence or on the properties of the chromatin-remodeling activities involved. Transcription factor binding might also compete with nucleosome formation at some sites.

The combination of removal and reformation of nucleosomes can reconcile apparently conflicting results of previous studies regarding the nature of the transcriptionally activated state. Removal of nucleosomes accounts for nuclease accessibility. Reformation of nucleosomes explains the retention of histones revealed by chromatin immunoprecipitation.

The coincidence of transcriptional activation with the loss of nucleosomes from promoter elements suggests that a nucleosome-free state is a prerequisite for the function of these elements. The removal of nucleosome −2 from UASp2 might be a requirement for the binding of basic helix-loop-helix factors like Pho4p. This requirement may not be general, as some classes of DNA binding transcriptional activator proteins may be capable of interacting with their recognition sites in the presence of a nucleosome. The observed loss of nucleosome −1, on the other hand, probably reflects a universal requirement for the removal of nucleosomes from core promoter elements prior to transcription. The occurrence of nucleosomes, nonetheless, at the core promoter in 60% of pho80Δ cells suggests that the promoter remains tightly controlled even under activating conditions, frequently assuming a repressed state. A fully repressed state, with all three nucleosomes in their positions on the PHO5 promoter is very unlikely in pho80Δ cells: if the three nucleosomes occupy their positions independently, the probability for all positions occupied simultaneously is only 0.6 \times 0.3 \times 0.18 = 0.032.

While our findings indicate the removal of nucleosomes from a promoter upon transcriptional activation, they shed no light on the mechanism of removal. Possibilities range
from complete disassembly to sliding histone octamers. In the case of PHO5, sliding over distances of at least 300 bp and concomitant rearrangement of nucleosomes over a larger chromosomal domain would be required.

The removal of nucleosomes represents a definitive endpoint of chromatin remodeling. Covalent modifications of histones, such as acetylation, associated with transcriptional activation of many promoters (Struhl, 1998), or the alteration of core particle structure, as observed with chromatin-remodeling complexes in vitro, may pertain to intermediate steps of chromatin remodeling in vivo.

Experimental Procedures

Genetic Elements

Yeast DNA was cloned using Pfu polymerase (Stratagene). The integrity of cloned PHOS DNA was confirmed by DNA sequencing. Sequences were flanked with RS elements using pABX22 (kindly provided by M. Gartenberg). The lexA cluster is a concatenator of three copies of the lexA operator from the CoEl gene of E. coli. pB2 was derived from pHS451-RecR (kindly provided by M. Gartenberg) by flanking the LEU2 gene with RS elements. A bacterial GST-LexA expression plasmid was generated by inserting a PCR fragment encompassing the lexA open reading frame of E. coli into the EcoRI site of pGEX-6P-1 (Amersham Pharmacia).

Yeast Strains and Media

All yeast strains in this study were derived from strain YS18 (Sengstag und Hinnen, 1987), kindly provided by W. Hörz. Cells were transformed using the lithium acetate method.

RS elements were inserted into the BamHI ~440 bp upstream of the PHOS TATA box and together with the lexA operator cluster into the PstI site ~90 bp downstream of the PHOS stop codon by homologous recombination (strains yM2.1, yM8.14, yM18.17, yM19.2). To generate strains yM3.2 and yM9.7, RS elements were inserted into the same BamHI site as described above and between the Dral and Sall sites at the beginning of the PHOS open reading frame and between the Dral and Sall sites 30 bp downstream from the start codon. This introduces a Sall site 30 bp downstream from the start codon. To generate pgo803 strains yM7.8, yM8.14, yM9.7, and yM19.2, an ~330 bp EcoRV/ClaI fragment was deleted from PHO80 and replaced by a HIS3 cassette using homologous recombination. Strains yM18.17 and yM19.2 were derived from yM2.1 and yM8.14, respectively, by replacing the 24 bp PHOS core promoter sequence AAATGAAACGTATATAAGCGCTGA with TCATCGATCCCCGGG (data not shown), demonstrating that topoisomers are positively supercoiled at 15 μg/ml. Topoisomer distributions were analyzed as suggested by Depew and Wang (1975). Thus, ∆Lk refers to the center of the Gaussian envelope function fitting the topoisomer distribution. Linking differences, ∆Lk, given in the text are mean values of four independent experiments ± maximum deviation.

Topoisomerase Treatment of Isolated Chromatin Circles

About 100 attomole of affinity-purified chromatin circles was treated with 4 U of wheat germ topoisomerase I (Promega) for 1 hr at 30°C in 100 μl of buffer R (see above) containing 0.1 mg/ml insulin.

Sedimentation Analysis

Nuclei (~40 μg of DNA) were digested with either 200 U of each restriction enzyme or with 4 U of MNase in a volume of 400 μl at 37°C for 1 hr or 20 min, respectively. The reaction was terminated by adding 47.5 μl of 1 M Tris (pH 7.5), 20 μl of 0.5 M EDTA, and 100 μg of denatured salmon sperm DNA. Following incubation for an additional 30 min at 37°C to allow adsorption of nonspecific DNA binding proteins to the salmon sperm DNA, the nuclei were chilled on ice and centrifuged at 4000 × g for 5 min at 4°C. The supernatant was applied to a 12 ml 5%–30% maltose gradient containing 25 mM HEPES, 0.1 mg/ml BSA, 1 mM sodium hydrogen sulfite, 1 μg/ml protease inhibitor cocktail (Boehringer), 1 mM EDTA, and centrifuged at 35,000 rpm in a SW41 rotor (Beckman) for 19 hr at 4°C. Gradient fractions were phenol/chloroform extracted and dialyzed against TE (pH 8.0). DNA was precipitated with ethanol, resuspended in TE (pH 8.0), electrophoresed in a 2% agarose gel, blotted, and hybridized with the probes indicated.

Nucleosome core particles were reconstituted as described (Lorch et al., 1987). The sedimentation behavior of naked DNA under the conditions used was assessed by mixing DNA fragments (5 fmol) with nuclei (~40 μg DNA), addition of Tris, EDTA, and salmon sperm DNA, as described above, and incubation for 1 hr at 37°C. Treatment of naked DNA fragments in this way did not alter their peak positions in maltose gradients (data not shown). Ssax values were taken from the literature (Noll and Noll, 1989; Simon et al., 1978).

Biot Hybridization and Quantitation of Nucleic Acids

Nucleic acids were transferred from agarose gels to nylon membranes (Schleicher & Schuell) in 20 × SSC as described (Sambrook et al., 1989). DNA probes for hybridization were generated by random
prime labeling using [(α-32P)dATP (Amersham). Quantitative analysis was performed with a PhosphorImager (Molecular Dynamics) and ImageQuant software.

ChIP Analysis
Yeast strains yM40.1, yM25.1, yM32.2, and yM9.7 transformed with recombinase promoter circles and the recombinase expression plasmid were grown to a density of ~2 × 10^7 cells/ml in 2× SCR lacking leucine. Adding galactose to 2% induced chromatin circle excision. Recombinase induction produces two circles in these strains: the PHO5 promoter circles and LEU2 circles derived from the recombinase expression plasmid.

After recombination, circles were crosslinked in vivo by adding formaldehyde (Fuka) to a final concentration of 1% and incubating for 15 min at room temperature. To quench the formaldehyde reaction, glycine was added to a final concentration of 125 mM and the incubation was extended for 5 min. Extracts were prepared as described above for endonuclease analysis with the exception that cells were washed twice in sorbitol before and after spheroplasting. glycine was added to a final concentration of 125 mM and incubating for 15 min at room temperature. To quench the formaldehyde reaction, glycine was added to a final concentration of 125 mM and the incubation was extended for 5 min. Extracts were prepared as described above for endonuclease analysis with the exception that cells were washed twice in sorbitol before and after spheroplasting. Instead of isolating nuclei from ficoll-containing buffer, spheroplasts were lysed in 400 µl buffer L (1% SDS, 10 mM EDTA, 50 mM Tris [pH 8.0], protease inhibitors as above) then diluted with 3.6 ml buffer D (1% Triton X-100, 1 mM EDTA, 167 mM NaCl, 20 mM Tris [pH 8.0], protease inhibitors as above) to yield buffer LD. Immediately before use in ChIP experiments, free EDTA was complexed by the addition of a 5-fold excess of Mg2+.

For each immunoprecipitation (IP), aliquots of extract were added to an equal volume of 2× IR buffer (50 mM Tris [pH 8.0], 20 mM EDTA) and reserved for subsequent measurement of the input concentration of circles. Separately, aliquots (350 µl) of extract were added to 50 µl of TALON resin (Clontech) equilibrated in buffer EQ (0.1% SDS, 23 mM Tris [pH 8.0], 0.9% Triton X-100, 150 mM NaCl) and rotated slowly for 30 min at room temperature. The resin was recovered by brief centrifugation and washed with buffer EQ twice for 10 min and three times for 5 min. Bound circles were eluted with 350 µl of 2× IR buffer. After mixing and pelleting the resin by centrifugation, 300 µl of eluate was taken for subsequent analysis. Both input and eluate were treated with Proteinase K (Sigma), and crosslinks were reversed by incubation overnight at 65°C. All samples were extracted with phenol/chloroform, and circle DNA was precipitated with cold ethanol. DNA pellets were resuspended in 30 µl of 10 mM Tris (pH 8.8). Circles were linearized with Cial (New England Biolabs) to produce a unique DNA sequence that could be distinguished from contaminating genomic DNA by PCR.

The relative concentrations of circle DNA in the input and eluate were determined by real-time quantitative PCR using an Opticon instrument (MJ Research) and circle-specific primers in a reaction inoculated with 2 µl of linearized DNA. Reaction efficiency 2 was determined to be 1.8 by replicate analyses of dilution series prepared from purified circle DNA (linearized, data not shown). Base-line-subtracted datasets of fluorescent intensities were plotted against cycle number on a log scale, and a fluorescence threshold was chosen that intersected the fluorescence traces in their linear range. For each sample the cycle at which this threshold was attained was recorded (CT value). Ratios in DNA concentrations between input and eluate were calculated from the difference in their CT values (ΔCT). The fraction (F) of input circles retained by the resin was calculated as: F = αΔCT, where α is a scaling factor to account for the fold difference between the amount of extract reserved as input aliquots and the amount of extract exposed to the resin.

For each tagged strain, at least three replicate experiments were performed to measure F. In each replicate experiment, three IPs were performed in parallel (at least nine IPs per strain). For each IP input and eluate, samples were measured in triplicate (18 PCR reactions per IP or at least 162 measurements per strain for each circle). PHOS promoter circles and the LEU2 control circles were measured in separate PCR reactions run in parallel with identical enzyme batches (Dynamo Mix, MJ Research). Pair-wise ΔCT calculations were performed to generate a sample distribution of values for F. We report the mean and standard deviation of this sample distribution for the PHOS and control circles for each strain.

Occasional outlying CT and F values were rejected when they deviated by more than two standard deviations from their respective means. Descriptive statistics were recalculated after outliers were rejected. Untagged control strains were yM32.2 and yM9.7.

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References


