

# Removal of Promoter Nucleosomes by Disassembly Rather Than Sliding In Vivo Short Article

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## Summary

Previous work demonstrated the removal of nucleosomes from the *PHO5* promoter upon transcriptional activation in yeast. Removal could occur by nucleosome disassembly or by sliding of nucleosomes away from the promoter. We have now activated the *PHO5* promoter on chromatin circles following excision from the chromosomal locus. Whereas sliding would conserve the number of nucleosomes on the circle, we found that the number was diminished, demonstrating chromatin remodeling by nucleosome disassembly.

## Introduction

Nucleosomes are removed from the *PHO5* promoter during transcriptional activation in the yeast *Saccharomyces cerevisiae* (Boeger et al., 2003; Reinke and Horz, 2003). Removal may result either from disassembly (complete displacement of the histone octamer from the DNA) or from sliding (movement in *cis*, whereby the histone octamer is repositioned without losing contact with the DNA, generating a nucleosome in a different position on the same DNA molecule). Here, we describe a general method for distinguishing between these possible mechanisms and its application to *PHO5*.

Activation of the IFN- $\beta$  promoter in HeLa cells has been shown to involve the sliding of a nucleosome away from the TATA box (Lomvardas and Thanos, 2001). Conversely, repression of *POT1* and *REC104* in yeast appears to result from sliding of nucleosomes from upstream or downstream positions toward the promoters, induced by the chromatin remodeling factor Isw2p (Fazio and Tsukiyama, 2003). Sliding was inferred from a gradual shift in the positions of nucleosomes. A similar, gradual movement of nucleosomes was previously demonstrated in vitro upon treatment of chromatin with NURF (Hamiche et al., 1999), a member of the ISWI family of chromatin remodeling complexes isolated from *Drosophila* (Tsukiyama and Wu, 1995).

ISWI complexes slide nucleosomes without apparent perturbation of their structure, since the isolated complexes do not alter the nuclease accessibility of nucleosome core particles (which have no free DNA and so have no possibility of sliding) and since they do not alter the topology of closed circular nucleosomal arrays (Aalfs et al., 2001; Langst et al., 1999). The SWI/SNF and RSC chromatin remodeling complexes also appear to cause nucleosome sliding (Jaskelioff et al., 2000; Whitehouse et al., 1999), but in contrast with ISWI complexes, they do

cause significant perturbation of nucleosome structure. The isolated complexes expose DNA in core particles and diminish the linking number of closed circular nucleosomal arrays in the presence of topoisomerase (Jaskelioff et al., 2000; Kwon et al., 1994; Lorch et al., 2001). These and other observations have been interpreted in terms of the unpeeling of DNA from the histone octamer. The DNA may loop out and rebind to the octamer, creating a bulge, whose propagation around the nucleosome results in sliding.

Does chromatin remodeling generally occur by nucleosome sliding in vivo? We have investigated this question with the use of chromatin circles excised by a site-specific recombinase from yeast chromosomes. We previously employed such circles to determine the fate of nucleosomes following transcriptional activation of the *PHO5* promoter. Circles formed from the activated promoter showed a reduction in linking number corresponding to the loss of an equivalent number of nucleosomes, and results of sedimentation and limit digestion analysis led to the same conclusion (Boeger et al., 2003). To determine whether the nucleosomes were removed by disassembly or sliding, we have now formed the chromatin circles first and activated transcription afterward. Disassembly of nucleosomes on such preformed circles would cause the same nucleosome loss as before, whereas sliding would conserve the number of nucleosomes. The results were clearly consistent with disassembly rather than sliding.

## Results

The *PHO5* gene of *S. cerevisiae* is repressed by a high concentration of inorganic phosphate in the growth medium and activated at a low concentration. Repression and activation are mediated by the Pho80p/Pho85p complex and by Pho4p, respectively. In *pho4 $\Delta$  cells, *PHO5* is therefore constitutively repressed, whereas mutations impairing Pho80p render *PHO5* constitutively active (Lenburg and O'Shea, 1996).*

We previously generated *PHO5* chromatin circles in the activated state from *pho80 $\Delta$  cells (Boeger et al., 2003; Griesenbeck et al., 2003). As activation was constitutive, it occurred prior to excision of the circles from the chromosomal locus. Since the chromosomal locus is an open domain, the removal of nucleosomes during activation could have occurred by sliding. Now we have reversed the order of events, with the excision of chromatin circles before activation. Circles were formed in wild-type cells grown in high-phosphate medium and were activated by transfer of cells to phosphate-free medium. Since a circle is a closed domain, the number of circle nucleosomes could only be diminished by disassembly and not by sliding of nucleosomes.*

Two types of *PHO5* circles were formed, one of 2250 bp containing the entire *PHO5* gene and promoter and one of 750 bp containing only the promoter. The structures of activated and repressed promoter chromatin were unaltered by excision of the circles from the chro-

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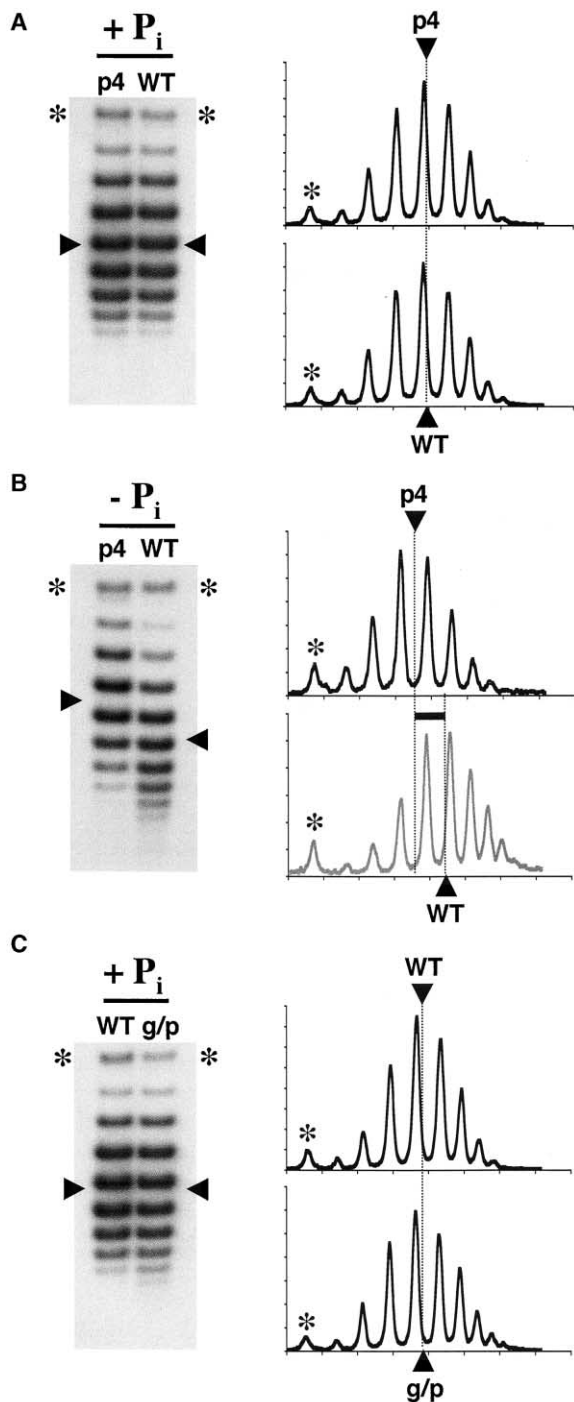


Figure 1. Topological Analysis of *PHO5* Gene Circles

Topoisomers were resolved in agarose gels containing 22  $\mu$ g chloroquine phosphate/ml. Under these conditions, all topoisomers were positively supercoiled, so those with larger linking numbers exhibited higher electrophoretic mobilities. Blot hybridization was performed with a probe spanning the entire *PHO5* gene. The autoradiography of the hybridized blot is shown on the left; the profiles of the topoisomer distributions on the right.

(A) wt and p4 refer to the topoisomer distributions of *PHO4* (yM18.17) cells and *pho4* $\Delta$  (yM38.22) cells, respectively, grown in high-phosphate medium. *PHO5* is TATA-less in strains yM18.17 and yM38.22. (B) wt and p4 refer to the topoisomer distributions of *PHO4* (yM18.17) cells and *pho4* $\Delta$  (yM38.22) cells, respectively, cultured for 8 hr in

phosphate-free medium. An arrowhead indicates the center of the distribution,  $\langle Lk \rangle$ . An asterisk (\*) marks the position of the nicked circle. (C) wt and g/p refer to the topoisomer distributions of wild-type (yM2.1) cells and *gcn5* $\Delta$ /*pho80* $\Delta$  (yM12.18) cells, respectively, grown in high-phosphate medium. A bar indicates the magnitude of  $\Delta \langle Lk \rangle$  between activated (wt) and repressed circles (p4).

mosomal locus (Boeger et al., 2003; Griesenbeck et al., 2003). Circles were produced by a site-specific recombinase, which was expressed from the *GAL1* promoter. Cells were cultured in raffinose-containing medium, and recombination was induced by the addition of galactose to the medium. About 80% of *PHO5* loci underwent recombination upon galactose induction (Griesenbeck et al., 2003). There was no further recombination during subsequent culture in phosphate-free medium (data not shown), which is important for the conclusions drawn below. Cells in which recombination did not occur probably lost the recombinase expression plasmid.

We have assessed the removal of nucleosomes by topological, limit digestion, "ChIP," and sedimentation analyses. In all cases, chromatin circles were excised from the chromosomal locus during growth in high-phosphate medium. We then compared activated chromatin circles, obtained from wild-type cells following culture in phosphate-free medium, with repressed circles, obtained from *pho4* $\Delta$  cells grown under identical conditions. In this way, we could distinguish specific effects of transcriptional activation on the *PHO5* promoter from general effects of the growth conditions on DNA and chromatin structure.

#### Topological Analysis

We could distinguish nucleosome disassembly from sliding by topological analysis, since circle topology should depend on the number of nucleosomes but not their locations. Gene circles were formed in wild-type and *pho4* $\Delta$  cells grown in high-phosphate medium. DNA was extracted either immediately after circle formation or after culturing the cells for an additional 5–20 hr in phosphate-free medium. The topoisomer distribution of the DNA extracted immediately was the same for wild-type and *pho4* $\Delta$  cells (Figure 1A). In contrast, after continued culture of both strains in phosphate-free medium, there was a significant linking difference,  $\Delta \langle Lk \rangle$ , between circles from wild-type and *pho4* $\Delta$  cells. The linking difference increased over time, reaching a plateau value of 1.3 to 1.5 after 8–13 hr of culture in phosphate-free medium (Figure 1B). These kinetics were similar to those of appearance of the *PHO5* gene product (Barbaric et al., 2003). As in the previous study, we used strains with a TATA-less *PHO5* promoter to exclude effects of transcription on circle topology. The TATA-less promoter is transcriptionally inactive but undergoes the same chromatin transition as the wild-type promoter under activating conditions. The linking difference may therefore be attributed exclusively to a change in chromatin structure (Boeger et al., 2003).

A linking difference of  $\sim 0.5$  was observed between circles from *pho4* $\Delta$  cells grown in high-phosphate and

phosphate-free medium after circle formation in high-phosphate medium. A bar indicates the magnitude of  $\Delta \langle Lk \rangle$  between activated (wt) and repressed circles (p4).

(C) wt and g/p refer to the topoisomer distributions of wild-type (yM2.1) cells and *gcn5* $\Delta$ /*pho80* $\Delta$  (yM12.18) cells, respectively, grown in high-phosphate medium. An arrowhead indicates the center of the distribution,  $\langle Lk \rangle$ . An asterisk (\*) marks the position of the nicked circle.

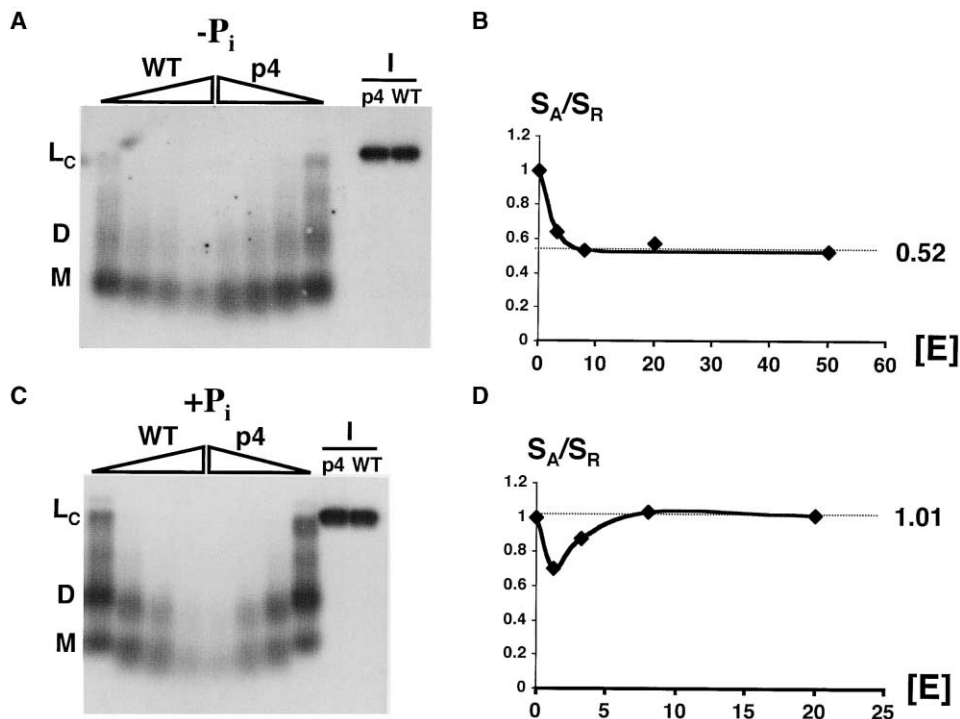


Figure 2. Limit Digestion Analysis of Promoter Circles

(A) Promoter circles (100 attomoles) from *pho4Δ* (yM39.22) cells and *PHO4* (yM3.2) cells, designated p4 and wt, respectively, and purified after 18 hr of culture in phosphate-free medium, were digested in 200  $\mu$ l with 3.2, 8, 20, and 50 U micrococcal nuclease for 5' at 37°C, or undigested circles were linearized with NcoI (I) as input control. DNA was extracted, electrophoresed in an agarose gel, blotted, and hybridized with a probe spanning the entire promoter circle. The positions of the mono- (M) and dinucleosome (D) in the gel are indicated at the left margin of the autoradiography. L<sub>c</sub> indicates the position of the linearized circle.

(B) The total hybridization signal S was measured by integrating over each lane of the blot in (A). The ratio S<sub>A</sub>/S<sub>R</sub> of hybridization signals for activated, wt, and repressed circles, p4, at equal enzyme concentration was normalized for small differences in input, see I in (A), and plotted against enzyme concentration (E).

(C) Promoter circles (100 attomoles) from *pho4Δ* (yM39.22) cells and *PHO4* (yM3.2) cells, designated p4 and wt, respectively, and purified after culture in high-phosphate medium, were digested in 200  $\mu$ l with 1.28, 3.2, 8, and 20 U micrococcal nuclease for 12.5' at 37°C or linearized with NcoI (I). DNA was extracted, electrophoresed in an agarose gel, blotted, and hybridized with a probe spanning the entire promoter circle.

(D) Limit digestion analysis was performed as described in (B).

phosphate-free media (Figures 1A and 1B). The shift may reflect a difference in the helical repeat of DNA between cells cultured in high-phosphate and phosphate-free media, inasmuch as the helical repeat is dependent on ionic conditions. The  $\langle Lk \rangle$  of circles in *pho4Δ* cells remained constant between 5 and 20 hr of culture in phosphate-free medium (data not shown), indicating that the change in intracellular conditions affecting DNA conformation occurred rapidly and further indicating that chromatin structure was constant over the time course of induction.

As a control, we sought to confirm that circle topology would not be affected by nucleosome sliding. We compared circles formed in wild-type cells in high-phosphate medium with circles formed in *gcn5Δ/pho80Δ* cells under the same conditions. Whereas nucleosomes occupy defined locations at the *PHO5* promoter in wild-type cells, they are randomly located in *gcn5Δ/pho80Δ* cells under repressing conditions (Almer and Horz, 1986; Gregory et al., 1998). There was no detectable linking difference between the circles from wild-type and *gcn5Δ/pho80Δ* cells (Figure 1C), showing that circle topology depended on the number of nucleosomes and

not on their locations. The linking difference observed between activated and repressed gene circles therefore argues against a sliding mechanism for the removal of nucleosomes from the *PHO5* promoter.

#### Limit Digestion Analysis

We used limit digestion analysis to measure the fraction  $\phi$  of unaltered nucleosomes retained on the activated promoter circle;  $\phi$  is given by the ratio of activated circle DNA after micrococcal nuclease digestion to repressed circle DNA after digestion, in the limit of high nuclease concentration (Boeger et al., 2003). In the case of nucleosome sliding,  $\phi$  is expected to be one, since the number of nucleosomes on the circle remains constant.

The size of the gene circle, advantageous for the precise measurement of linking differences, made it unsuitable for limit nuclease analysis because the number of nucleosomes affected by activation was small compared to the total number of nucleosomes on the circle. For this reason, we turned from gene circles, containing 12 nucleosomes before activation, to promoter circles, which contain only 3 nucleosomes (Griesenbeck et al., 2003). Promoter circles were formed in wild-type and

*pho4Δ* strains in high-phosphate medium and then cultured for an additional 18 hr in phosphate-free medium to fully activate the promoter. Since any promoter sequences remaining at the chromosomal locus due to incomplete excision would also be detected by limit digestion analysis, we purified the promoter circles by differential centrifugation and affinity chromatography (Griesenbeck et al., 2003). The purified circles were digested with increasing concentrations of micrococcal nuclease, followed by DNA extraction, gel electrophoresis, and blot hybridization with a probe that spans the entire promoter circle (Figure 2A). A hybridization signal was measured by integration over an entire lane of the blot; the ratio of hybridization signals for activated and repressed circles approached a limit at high nuclease concentration of  $\phi = 0.52$ , equivalent to  $3 \times \phi = 1.56$  unaltered nucleosomes retained on activated circles (Figure 2B). In contrast,  $\phi$  for circles from wild-type and *pho4Δ* cells maintained in high-phosphate medium was  $\sim 1$ , showing that the circles bore the same number of nucleosomes before activation (Figures 2C and 2D). The value of  $\phi$  less than one found for activated promoter circles is indicative of nucleosome disassembly and inconsistent with a sliding mechanism of activation.

#### ChIP Analysis

The removal of nucleosomes from the activated *PHO5* promoter was previously revealed by a loss of histones, measured by a variant of chromatin immunoprecipitation (ChIP) analysis employing circles formed after activation in strains expressing oligo-histidine-tagged histone H2B (Boeger et al., 2003). Here, we have applied the same procedure to circles formed before activation, in cells cultured in high-phosphate medium. The cells were treated with formaldehyde to crosslink histones to DNA, either before or after an additional 16 hr of culture in phosphate-free medium to activate the *PHO5* promoter. Extracts from crosslinked cells were applied to a metal-affinity resin for binding the oligo-histidine tag, and the fraction of promoter circles retained on the resin after extensive washing was measured by real-time PCR. Activated circles from wild-type cells grown in phosphate-free medium were less efficiently retained on the affinity resin than repressed circles from *pho4Δ* cells grown in phosphate-free medium and repressed circles from wild-type cells grown in high-phosphate medium (Figures 3A and 3B). On the other hand, circles from wild-type cells and *pho4Δ* cells grown in high-phosphate medium were indistinguishable by this analysis as were *LEU2* circles (concomitantly generated with *PHO5* circles) from wild-type cells and *pho4Δ* cells grown in phosphate-free medium (Figures 3A and 3C). Similar results were obtained by ChIP with the use of an antibody against the C-terminal tail of histone H3 (data not shown). These results are indicative of histone loss from the promoter circle upon activation, consistent with nucleosome disassembly but not with a sliding mechanism for promoter activation.

#### Sedimentation Analysis

We find that the loss of nucleosomes upon transcriptional activation previously documented for the *PHO5* promoter at the chromosomal locus (Boeger et al., 2003)

can also be detected by sedimentation analysis of chromatin circles. Thus, activated promoter circles isolated from *pho80Δ* cells sediment more slowly than repressed circles isolated from wild-type cells (Figure 4A). This effect could not be attributed to nucleosome sliding, because circles isolated from *pho80Δ/gcn5Δ* cells sedimented indistinguishably from repressed circles isolated from wild-type cells (Figures 4A and 4B). We have performed the same analysis on circles formed before activation. Circle formation was in high-phosphate medium and activation was by culture for 15 hr in phosphate-free medium as described above. Such activated circles sedimented more slowly than repressed circles from *pho4Δ* cells cultured in phosphate-free medium or repressed circles from wild-type cells cultured in high-phosphate medium (Figures 4A and 4C). These results are consistent with the loss of associated proteins from circles upon activation and provide a further argument against a sliding mechanism for the removal of nucleosomes from the activated promoter.

It may be noted that circles formed in wild-type cells and activated by culture in phosphate-free medium migrated slightly faster than circles formed in *pho80Δ* cells (Figures 4B and 4C). This result is consistent with topology and limit nuclease digestion analyses, indicating that circles from wild-type cells cultured in phosphate-free medium bear, on average, about half a nucleosome more than circles from *pho80Δ* cells (see Discussion). The difference is presumably due to less than complete inhibition of Pho80p/Pho85p function in phosphate-free medium.

#### Discussion

The results of each of four independent analyses of *PHO5* chromatin circles are consistent with a disassembly but not a sliding mechanism for the removal of nucleosomes from the activated *PHO5* promoter. This conclusion is strengthened by quantitative consistency of the results. Two of the methods used, topological analysis and limit digestion, provide quantitative measures of nucleosome loss. In the absence of constraints on linker DNA, the linking difference due to the loss of a nucleosome is about one (Germond et al., 1975; Keller, 1975; Shure and Vinograd, 1976; Simpson et al., 1985; White et al., 1988; Zivanovic et al., 1988). The linking difference  $\Delta\langle Lk \rangle$  between activated and repressed promoter circles is therefore related to the fraction of nucleosomes,  $\phi$ , retained on the activated promoter circle by

$$\phi = 1 - \frac{1}{N} \Delta\langle Lk \rangle,$$

where  $N$  is the number of nucleosomes on the repressed promoter circle. Based on our finding of  $\Delta\langle Lk \rangle = 1.4 \pm 0.1$  and with  $N = 3$ ,  $\phi$  is expected to be  $0.53 \pm 0.03$ , in good agreement with the value of  $\phi = 0.52$  determined by limit digestion analysis. This quantitative agreement strongly argues for the loss of 1.4 nucleosomes from activated circles rather than partial unfolding of nucleosomes. Since nucleosome loss occurred from a closed domain it must have been due to disassembly.

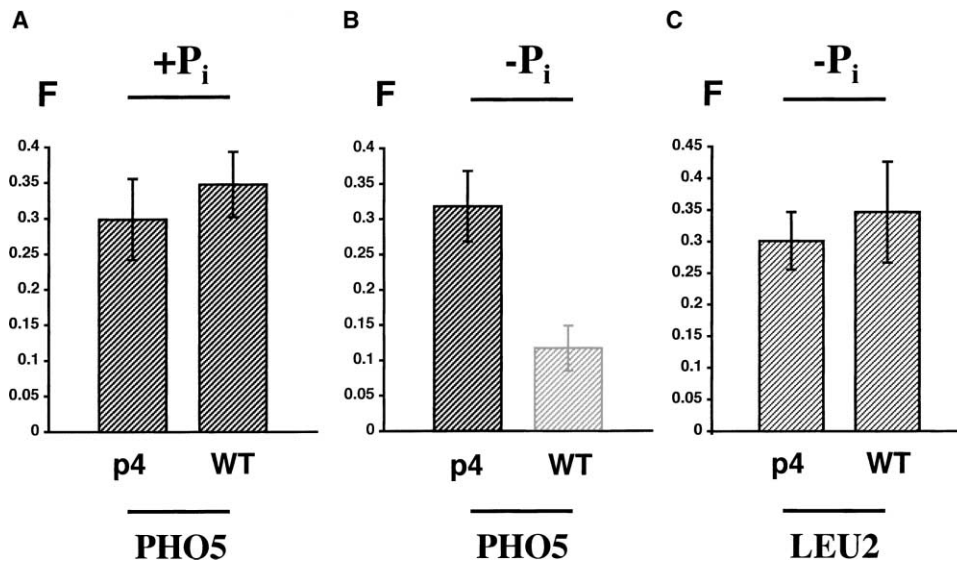


Figure 3. ChIP Analysis of Promoter Circles

Circles crosslinked in vivo were incubated with talon affinity resin for binding of the His-tagged histone H2B expressed in yM24.1 (*PHO4*) and yM41.23 (*pho4Δ*) cells. The fraction (F) of circles retained on the matrix after extensive washing was determined by real-time PCR using circle-specific primers.

(A) Retention of promoter circles from yM24.1 (wt) and yM41.23 (p4) cells crosslinked in high-phosphate medium.

(B) Retention of promoter circles from yM24.1 (wt) and yM41.23 (p4) cells crosslinked after 16 hr of culture in phosphate-free medium.

(C) Retention of *LEU2* circles from yM24.1 (wt) and yM41.23 (p4) cells crosslinked after 16 hr of culture in phosphate-free medium.

It may be asked if the small size of promoter circles altered the mechanism of the remodeling reaction, skewing it toward nucleosome disassembly rather than sliding. Such an effect of circle size seems unlikely for a number of reasons. First, the quantitative measures of the chromatin transition are consistent between promoter circles and gene circles, three times larger. Second, promoter and gene circles are formed by recombination with equal efficiencies (Griesenbeck et al., 2003), arguing against an energetic penalty upon formation of the promoter circle, as might be expected if the circle represented a strained conformation. Finally, promoter circles are rather large relative to the number of nucleosomes they bear, with only 60% of their DNA contained in core particles, compared to 80% for gene circles.

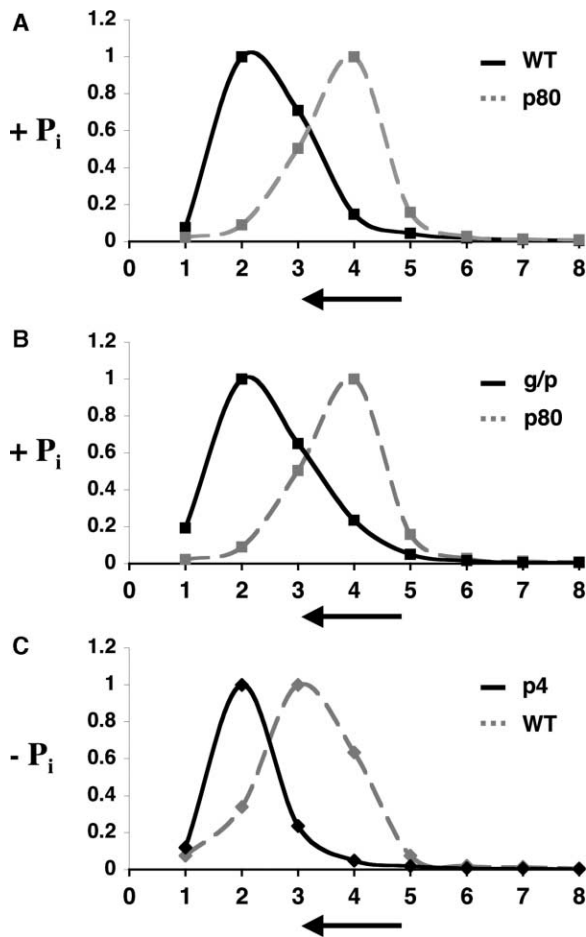
While the loss of nucleosomes from circles activated after excision is substantial, the value of about 1.4 nucleosomes observed is somewhat less than the 1.9 nucleosomes lost by the reverse procedure of activation before excision from the chromosomal locus (Boeger et al., 2003). Since the accessibility to nuclease digestion of the promoter at the chromosomal locus was identical to that on the circle following cell culture in phosphate-free medium (data not shown), the difference in the two  $\Delta\langle Lk \rangle$  values is unlikely to be due to circle formation. Rather, it is almost certainly due to the difference in the methods of transcriptional activation, a shift to phosphate-free medium in the present work, and the use of a *pho80Δ* mutant strain in the previous work. As already noted, the inactivation of the Pho80p/Pho85p complex upon shift to phosphate-free medium may be incomplete, compared with the total abrogation of Pho80p function in a deletion mutant.

Nucleosomes retained on the activated promoter par-

tially occupy positions not found in the repressed state (Boeger et al., 2003), raising the possibility of sliding during activation. Indeed, our experiments address only the ultimate fate of promoter nucleosomes and not the details of the activation process, which could involve mobilization of nucleosomes and other mechanisms. We conclude that histones must be dislodged from the DNA and not simply transferred to an adjacent location. Such nucleosome disassembly has the advantage of restricting the range of structural effects of gene activation. Rearrangements of nucleosomes over a larger chromosomal domain would be required if nucleosomes were removed from the *PHO5* promoter by sliding alone. Such long-range effects might interfere with the regulation of neighboring genes. The perturbation of chromatin structure upon activation of many inducible promoters may be less dramatic than that of the *PHO5* promoter, and in these cases, sliding of a small number of nucleosomes over short distances may suffice (Lomvardas and Thanos, 2001). In no case, however, has the possibility of nucleosome disassembly upon promoter activation been excluded, and it may occur in general.

The partial occupancy by nucleosomes at three sites of the activated *PHO5* promoter has been attributed to a steady state of disassembly and reassembly of nucleosomes (Boeger et al., 2003). It has recently been shown that nucleosome assembly occurs during interphase at several loci in *Drosophila* cells (Ahmad and Henikoff, 2002). Such replication-independent assembly of nucleosomes may be due, in part, to a disassembly/reassembly process occurring at transcriptionally active promoters.

As *PHO5* gene and promoter circles lack *ARS* elements, nucleosome loss occurs in the absence of DNA



**Figure 4. Sedimentation Analysis of Promoter Circles**  
Promoter circles enriched by differential centrifugation were sedimented in a 5%–30% maltose gradient. Circle distributions were determined by real-time PCR using circle-specific primers. Circle concentration relative to that at the peak of the distribution, determined by real-time PCR, is plotted on the ordinate against the gradient fraction number on the abscissa. Arrows indicate the direction of sedimentation.  
(A) Sedimentation distribution of circles isolated from *PHO80* (yM3.2) cells and *pho80Δ* (yM9.7) cells (wt and p80, respectively) grown in high-phosphate medium.  
(B) Sedimentation distribution of circles isolated from wild-type (yM3.2) cells and *gcn5Δ/pho80Δ* (yM14.7) cells (wt and g/p, respectively) grown in high-phosphate medium.  
(C) Sedimentation distribution of circles isolated from *pho4Δ* cells (yM39.22) and *PHO4* (yM3.2) cells (p4 and wt, respectively) cultured for 15 hr in phosphate-free medium after circle formation in high-phosphate medium.

replication (data not shown). This supports the conclusion from previous work that chromatin remodeling at the *PHO5* promoter does not require replication (Schmid et al., 1992). Evidence has been presented for a role of the INO80 complex, a close relative of the SWI/SNF complex in *PHO5* remodeling (Steger et al., 2003). We have found, however, that *PHO5* is constitutively active in *pho80Δ/ino80Δ* mutant cells and can be fully activated in *ino80Δ* cells as well as in cells lacking ARP8, a component of the INO80 complex (data not shown). Likewise, homologs of *INO80* are dispensable for *PHO5* activation,

including *SNF2* (Gaudreau et al., 1997), *ISW1* and *ISW2* (Kent et al., 2001), *FUN30*, *RDH54*, *RAD26*, *CHD1*, *SWR1*, *RAD54*, *RAD5*, *YLR247C*, *RIS1*, *YFR038W*, and *TRM82* (data not shown). Factors responsible for nucleosome loss at the *PHO5* promoter therefore remain to be identified.

#### Experimental Procedures

##### Genetic Elements

Plasmid pJSS4.1 for expression of the LexA adaptor was derived from pJSS3.1 by exchanging the *URA3* gene with the *HIS3* gene. Plasmid pB3.1 for inducible expression of R-recombinase has been described (Griesenbeck et al., 2003).

##### Yeast Strains and Media

Strains yM3.2, yM8.14, yM9.7, and yM18.17 have been described (Boeger et al., 2003). Strain yM24.1 was derived from yM3.2 by tagging histone H2B as described (Boeger et al., 2003). Strains yM39.22, yM38.22, and yM41.23 were derived from yM3.2, yM18.17, and yM24.1, respectively, by replacing the *PHO4* open reading frame with the *URA3* gene by homologous recombination. Strains yM12.18 and yM14.7 were derived from strains yM8.14 and yM9.7, respectively, by replacing a 0.92 kb *MluI*/*NdeI* fragment at the *GCN5* locus with the *URA3* gene by homologous recombination. Following circle formation, cells were transferred from SCR medium lacking leucine (or leucine and histidine) into SCD medium lacking phosphate (or histidine and phosphate).

##### Topological Analysis

Topoisomer distributions were revealed as described (Boeger et al., 2003), except that 22 μg/ml of chloroquine-phosphate (Sigma) was used in gel electrophoresis. The distributions were analyzed according to Depew and Wang (1975). Thus, <Lk> refers to the center of the Gaussian envelope function fitting the distribution.

##### Limit Digestion Analysis

Chromatin circles were IgG-affinity purified from cells transformed with plasmids pJSS4.1 and pB3.1 as described (Griesenbeck et al., 2003). Circles were digested with micrococcal nuclease followed by DNA extraction, gel electrophoresis, and blotting as described (Boeger et al., 2003; Griesenbeck et al., 2003). The blot was hybridized with a probe spanning the entire promoter circle.

##### ChIP Analysis

The retention of promoter circles on a metal-affinity matrix (talon resin) was measured as described (Boeger et al., 2003), except that circles were eluted from the affinity matrix by incubating the resin with 40 μg of proteinase K in 400 μl of proteinase K buffer (50 mM Tris [pH 8.0], 20 mM EDTA, 200 mM NaCl, 0.5% SDS) for 1 hr at 37°C followed by phenol/chloroform extraction. Input DNA was prepared from 50 μl of crosslinked circles, and 400 μl of proteinase K buffer was added for subsequent reversal of crosslinks at 65°C. For each sample of crosslinked circles, DNA was prepared from three input aliquots and three affinity precipitations. Each DNA sample was measured in triplicate by real time PCR, generating a total of 18 measurements (9 measurements for input DNA and 9 measurements for DNA retained on the column). From the 18 measurements, 9 × 9 = 81 ΔCT values could be derived, providing 81 data points for the calculation of mean value and standard deviation of the fraction of circles retained on the matrix (Boeger et al., 2003).

##### Sedimentation Analysis

After breaking cells mechanically, chromatin circles were enriched by differential centrifugation as described (Griesenbeck et al., 2003). About 100 attomoles of enriched circle were mixed with 100 μg of denatured salmon sperm DNA, dialyzed against 25 mM HEPES [pH 7.5], 220 mM NaCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 3 mM benzimidazole at 4°C, and analyzed in a 5%–30% maltose gradient as described (Boeger et al., 2003), except that the distribution of circles in the gradient was determined by real-time PCR using circle-specific primers.

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