Molecular Cell, Vol. 15, 69–81, July 2, 2004, Copyright ©2004 by Cell Press

Precise Nucleosome Positioning and the TATA Box Dictate Requirements for the Histone H4 Tail and the Bromodomain Factor Bdf1

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Summary

Acetylation of histone tails plays a key role in chromatin dynamics and is associated with the potential for gene expression. We show here that a 2–3 bp mispositioning of the nucleosome covering the TATA box at *PHO5* induces a dependency on the acetylatable lysine residues of the histone H4 N-terminal region and on the TFIID-associated bromodomain factor Bdf1. This dependency arises either through fusion of the *PHO5* promoter to a *lacZ* reporter or by mutation of the TATA box in the natural gene. The results suggest that promoters in which the TATA box is either absent or poorly accessible on the surface of a nucleosome may compensate by using Bdf1 bromodomains and acetylated H4 tails to anchor TFIID to the promoter during the initial stages of transcription activation. We propose that nucleosome positioning at the nucleotide level provides a subtle, but highly effective, mechanism for gene regulation.

Introduction

Chromatin remodeling is an intimate part of both transcriptional activation and repression, and considerable evidence has accumulated to suggest that the N-terminal histone tails may play a key role. Histone tail acetylation correlates with regions of chromatin with the potential for gene expression (Braunstein et al., 1993, 1996; Grunstein, 1997; Kuo et al., 1996; Struhl, 1998; Wang et al., 1998), and patterns of histone acetylation appear to define chromosomal domains and be inherited during mitosis (Ekwall et al., 1997; Jeppesen and Turner, 1993; Monson et al., 1997). Consistent with acetylation playing a key role in gene expression, chromatin immunoprecipitated from the promoters of transcriptionally active genes appears to be hyperacetylated (Braunstein et al., 1996; Cosma et al., 1999), and HATs are known to function in large protein complexes as transcription cofactors in many organisms (Grant et al., 1997; Ikeda et al., 1999; Allard et al., 1999). Conversely, histone deacetylases have also been implicated in transcription repression (Kuo and Allis, 1998; Rundlett et al., 1996). In addition, the histone tails have been implicated in nucleosome recognition by a variety of chromatin interacting complexes. For example, bromodomains, found in Swi2/Snf2 or Gcn5, appear to recognize specifically acetylated lysine residues within histone tails and can anchor their respective complexes to acetylated promoter nucleosomes (Jacobson et al., 2000; Ornaghi et al., 1999; Owen et al., 2000; Dhalluin et al., 1999; Hassan et al., 2002; Hudson et al., 2000). These observations have led to the conclusion that histone tails play a key role in regulation at most, if not all, genes and that one role of transcription activators is to target acetylation complexes to promoters (Berger, 1999). However, precisely why some promoters appear to require different chromatin remodeling or modifying activities to others remains a largely unresolved question.

Perhaps the best example of specific chromatin remodeling accompanying transcription activation is provided by the *S. cerevisiae PHO5* gene that encodes a repressible acid phosphatase (APase) (Almer and Hörz, 1986; Almer et al., 1986). In high phosphate, *PHO5* is repressed and the promoter is associated with an array of positioned nucleosomes with a single DNase1 hypersensitive site termed HS2 located between nucleosomes −2 and −3. The position of HS2 correlates with a low affinity binding site for the transcription activator Pho4 (Venter et al., 1994; Vogel et al., 1989), but under repressed conditions, Pho4 (Venter et al., 1994) and TFIID (Sekinger and Gross, 2001) are absent from the DNA. On switching to low phosphate, transcription activation is accompanied by remodeling and apparent loss of precisely four nucleosomes from the promoter (Almer et al., 1986; Boeger et al., 2003; Reinke and Hörz, 2003), facilitating access of Pho4 to HS2 in association with the homeodomain protein Pho2 (Barbaric et al., 1996, 1998), and occupation of a second Pho4 binding site at UAS_{P2} (Venter et al., 1994). Chromatin remodeling at the *PHO5* promoter is independent of both DNA replication and transcription activation (Fascher et al., 1993; Schmid et al., 1992), but is nevertheless absolutely dependent on the Pho4 activation domain (Svaren et al., 1994, McAndrew et al., 1998). In addition it has recently been demonstrated that activation of the *PHO5* promoter requires the Spt3 and Spt7 components of the SAGA complex (Barbaric et al., 2003), but is largely independent of Gcn5 (Gregory et al., 1998), although the kinetic of *PHO5* activation is delayed in the absence of Gcn5 (Barbaric et al., 2001). The delayed kinetic of activation in a *gcn5* background is consistent with the observation that the Gcn5 and Esa1 HATs appear to be responsible for the acetylation of histones H3 and H4, respectively, seen at the *PHO5* promoter under repressed conditions (Vo-

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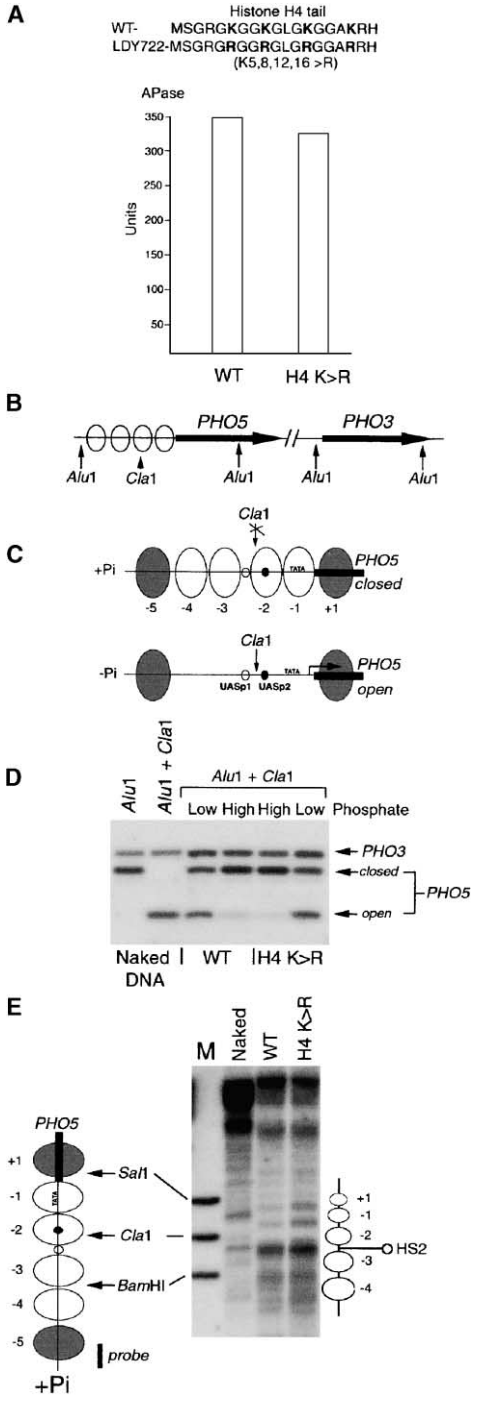


Figure 1. *PHO5* Promoter Activity and Chromatin Opening Is Largely Independent of Four Acetylatable Lysine Residues in the Histone H4 N-Terminal Tails

(A) Comparison of acid phosphatase activity between a wild-type strain (PKY44a) and a strain (LDY722) in which histone H4 lysines 5, 8, 12, and 16 have been mutated to arginine and are therefore nonacetylatable under no-phosphate conditions.

(B) Schematic showing relative locations of *PHO5* and the highly related *PHO3* genes.

(C) Schematic showing relative positions of nucleosomes over the *PHO5* promoter under repressed and induced conditions and the location of the Cla1 site.

(D) Cla1 accessibility assay used as a measure of chromatin opening at *PHO5* in a wild-type (PKY44a) strain and a strain in which the acetylatable lysine residues in the histone H4 N-terminal tail have been mutated to arginine (LDY722). Note that the probe used hybridizes to the *PHO3* gene.

(E) Micrococcal nuclease assay across the *PHO5* promoter in a wild-type (PKY44a) and H4 tail mutant strain (LDY722). The relative locations of the nucleosomes and probe are indicated.

gelauer et al., 2000). In addition, the acetylatable lysine residues in the histone H4 N-terminal tail are essential for activation of a *PHO5-lacZ* reporter (Durrin et al., 1991). In contrast, we show here that transcription of the natural *PHO5* gene is largely independent of the acetylatable lysine residues in either the histone H3 or histone H4 N-terminal tails. Moreover, our results suggest that a requirement for the H4 tail and the bromodomain factor Bdf1 is dictated by the affinity of the promoter for TFIID which is in turn determined either by the precise rotational position of the DNA relative to the surface of the core histones within a nucleosome containing the TATA box or by the presence or absence of a recognizable TATA element.

Results

Acetylation of the Histone H4 Tail Is Dispensable for Transcription Activation and Chromatin Remodeling at the *PHO5* Promoter

Under repressed conditions, both the histone H3 and histone H4 N-terminal tails are hyperacetylated at the *PHO5* promoter compared to the coding region by the Gcn5 and Esa1 HATs, respectively (Vogelauer et al., 2000). Activation of the *PHO5* promoter on switching to low-phosphate conditions exhibits a delayed kinetic in a strain lacking Gcn5 (Gregory et al., 1998; Barbaric et al., 2001), while a *PHO5-lacZ* reporter is absolutely dependent on the acetylatable lysine residues in the histone H4 tail (Durrin et al., 1991). Taken together, these data suggest that histone acetylation is important for activation of *PHO5* in low phosphate. Surprisingly, however, under fully inducing (no-phosphate) conditions, a strain expressing a version of histone H4 in which lysines 5, 8, 12, and 16 have been mutated to arginine (H4 K>R) (Durrin et al., 1991) reproducibly produced at least 90% of the acid phosphatase activity seen in the wild-type strain (Figure 1A).

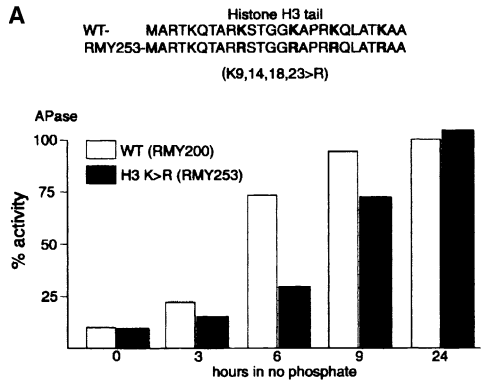
Consistent with the wild-type levels of acid phosphatase expressed in the H4 mutant strain, both chromatin remodeling and nucleosome positioning at the *PHO5* promoter were unaffected by the inability to acetylate lysines 5, 8, 12, and 16 of the histone H4 N-terminal tail (Figures 1B–1E). Under repressing, high-phosphate conditions, the *PHO5* promoter is covered by an array of four positioned nucleosomes. Switching to low phosphate induces Pho4-dependent remodeling of the chromatin covering the *PHO5* promoter, leading to a 600 bp region of the promoter becoming hypersensitive to nuclease digestion. To assay chromatin remodeling at the *PHO5* promoter, wild-type or histone H4 mutant strains were permeabilized (Kent et al., 1993; Kent and Mellor, 1995), and the accessibility of a Cla1 site located within positioned nucleosome −2 was measured. The ability of this enzyme to gain access to its recognition

sequence within the *PHO5* promoter is a reliable and quantitative assay which has been used previously to provide an accurate reflection of whether the chromatin across the entire *PHO5* promoter is in an open or closed conformation (for examples, see Almer et al., 1986; Gregory et al., 1998; McAndrew et al., 1998; Svaren et al., 1994). A map of *PHO5* and the adjacent *PHO3* gene and the relative positions of the restriction sites used in the assay is shown in Figure 1B, and a more detailed map of the *PHO5* promoter and the relative locations of the four positioned nucleosomes as well as the Cla1 site used for the chromatin opening assays is shown in Figure 1C. The results obtained from the Cla1 accessibility assays using the wild-type and H4 mutant strain backgrounds under either repressing or fully inducing conditions are presented in Figure 1D. In the wild-type strain, increased Cla1 accessibility is observed under inducing (low-phosphate) conditions, reflecting the chromatin remodeling which accompanies the derepression of the *PHO5* promoter. In the H4 mutant strain, the Cla1 accessibility is indistinguishable from that observed using the wild-type strain. Thus, the chromatin opening and transcription activation characteristic of the derepression of the *PHO5* promoter under low-phosphate conditions are not only independent of Gcn5, but may be entirely independent of histone H4 tail acetylation by any HAT.

In addition to the presence of highly positioned nucleosomes, the *PHO5* promoter under repressing, high-phosphate conditions is characterized by a nuclease hypersensitive (HS2) site located between nucleosomes -2 and -3 at UAS_{P1}, a Pho4 binding site. Although the ability of Pho4 to activate transcription and remodel chromatin at the *PHO5* promoter was unaffected by the presence of a nonacetylatable form of histone H4, it was nevertheless possible that the H4 mutation would affect the relative positioning of the nucleosomes under repressing conditions. However, the micrococcal nuclease digestion pattern obtained under high-phosphate conditions was identical when comparing a wild-type or H4 K>R mutant strain (Figure 1E), with the ladder of bands characteristic of highly positioned nucleosomes and the nuclease hypersensitive site (HS2) flanked by positioned nucleosomes -2 and -3 being readily apparent. Thus, ability to acetylate the H4 N-terminal tail lysines appears to be dispensable for positioning the nucleosomes across the *PHO5* UAS in high-phosphate as well as for activation of *PHO5* transcription and chromatin remodeling.

Results obtained previously by Northern blotting of *PHO5* mRNA (Mann and Grunstein, 1992) indicate that mutation of lysines 9, 14, 18, and 23 to arginine within the H3 tail fails to affect the final levels of *PHO5* expression attained after induction in low-phosphate medium. However, the kinetic of induction is somewhat slower (Figure 2A), consistent with the slower kinetic of activation of *PHO5* observed in a *gcn5* strain (Barbaric et al., 2001) as well as the basal levels of Gcn5-dependent H3 acetylation observed at *PHO5* under repressed conditions (Vogelauer et al. 2000).

Despite the absence of a significant requirement for the lysine residues or either the histone H3 or H4 N-terminal tails, it has been reported that specific mutations affecting lysine residues in the H3 and H4 tails can in



B

Strain	H3	H4		Low Pi	High Pi
WZY63 (WT)	WT	WT	*GCN5*	940	30
WZY76	WT	WT	*gcn5*	860	20
WZY160	K9R	K5,12R	*GCN5*	1070	35
WZY161	K9R	K5,12R	*gcn5*	1120	10
WZY141	K9Q	K5,12Q	*GCN5*	1020	40
WZY143	K9Q	K5,12Q	*gcn5*	1010	40
WZY94	K14R	K8,16R	*GCN5*	1250	30
WZY93	K14Q	K8,16Q	*GCN5*	1080	30
WZY156	K14Q	K8,16Q	*gcn5*	820	5

Figure 2. *PHO5* Promoter Activity Is Largely Independent of Acetylatable Lysine Residues in Both the Histone H3 and H4 N-Terminal Tails

(A) Comparison of acid phosphatase activity between a wild-type strain (RMY200) and a strain (RMY253) in which histone H3 lysines 9, 14, 18, and 23 have been mutated to arginine. Activity is presented relative to that observed after 9 hr (460 units) in no-phosphate medium in the wild-type strain.

(B) Comparison of acid phosphatase activity in strains containing the indicated mutations in histone H3 and H4 tails in the presence or absence of Gcn5 after 24 hr in no-phosphate medium.

combination, but not alone, have a profound effect on cell growth, viability, and regulation of transcription. In general, the effects of combinations of H3 and H4 tail lysine substitution mutations are significantly enhanced by the absence of the Gcn5 HAT (Zhang et al., 1998). To discount the possibility of a combinatorial effect of H3 and H4 tail mutations on the regulation of *PHO5*, we examined the expression of *PHO5* in a series of strains in which specific combinations of H3 and H4 tail residues cannot be acetylated. The results, summarized in Figure 2B, indicate that irrespective of which combination of H3 or H4 tail mutants was used, no effects were observed on the final induced levels of *PHO5* achieved in low phosphate either in the presence or absence of Gcn5. Thus, in contrast to many promoters, the subset of acetylatable lysine residues in the H3 or H4 N-terminal tails targeted by these mutations is not required to achieve high levels of *PHO5* expression on induction in low phosphate. We note, however, that our interpretation of the absence of a requirement for the acetylation of the H4-tail lysines is limited by the specific mutant tails assayed and that some role for specific residues may be revealed using additional mutations in the H4 tail.

The Histone H4 Tail Plays a Critical Role in Regulation of a *PHO5-lacZ* Reporter

Although our results indicate that expression of *PHO5* is independent of the acetylatable lysines 5, 8, 12, and

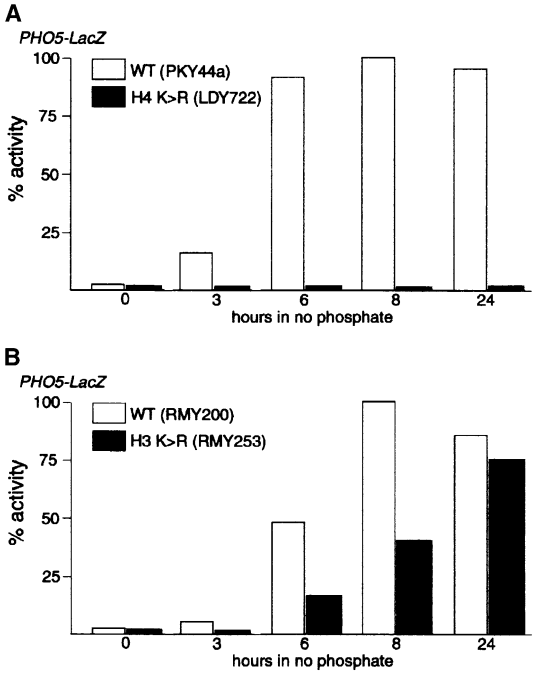


Figure 3. Requirements for the Activity of a *PHO5-lacZ* Reporter

(A) Derepression of the *PHO5-lacZ* reporter in the indicated wild-type and H4 mutant strains over time. Activity is presented relative to that observed after 8 hr in no-phosphate medium.

(B) Derepression of the *PHO5-lacZ* reporter in the indicated wild-type and H3 mutant strains over time. Activity is presented relative to that observed after 8 hr in no-phosphate medium.

16 in the amino-terminal tail of histone H4, it has been reported previously that the *PHO5* promoter is absolutely dependent on these residues when fused to a *lacZ* reporter gene and is also impaired in a strain lacking amino acids 4–28 of the H4 tail (Durrin et al., 1991). In agreement with the previous study, β-galactosidase activity arising from a *PHO5-lacZ* reporter was abolished in the H4 K>R mutant strain (Figure 3A). By contrast, in the H3 mutant background (Figure 3B), the activity of the *PHO5-lacZ* reporter reached 80% of wild-type levels after 24 hr in low phosphate, but was somewhat delayed compared to the wild-type strain. Thus, like the chromosomal gene, acetylation of the H3 tail is apparently largely dispensable for activation of *PHO5-lacZ,* but derepression of the *PHO5-lacZ* reporter is critically dependent on the H4 tail lysines, and the remaining wild-type histone tails are unable to compensate. Furthermore, the requirement for the histone H4 tail lysine residues was not a result of the episomal nature of the *PHO5-LacZ* reporter, as the expression of a plasmid-based *PHO5* gene behaved like the chromosomal gene in the H4 K>R strain (data not shown).

We suspected that understanding the origins of the differential requirements for the H4 tail between *PHO5* and the *PHO5-lacZ* reporter would reveal a fundamental insight into the role played by the H4 tail in regulation of gene expression. We ruled out the possibility that the transcription factors Pho2 and Pho4 were limiting in the H4 K>R mutant. Western blotting (Figure 4A) revealed similar levels of Pho2 protein in both the wild-type and H4 mutant strains, and similar results were obtained for Pho4 (Figure 4B). Moreover, 2-fold overexpression of Pho4 from a plasmid where Pho4 is expressed from its own promoter leads to some degree of constitutive activation of the *PHO5-lacZ* promoter in the wild-type background in high phosphate and around 2-fold elevated expression in low phosphate, but little activity above background in the strain bearing the K>R mutant version of histone H4 irrespective of the level of Pho4 expressed (Figure 4B). Thus, the defect in activation of the *PHO5-lacZ* reporter in the H4 K>R strain cannot be overcome even in the presence of excess activator.

One possible explanation for the differences observed between the *lacZ* reporter and the *PHO5* gene was that chromatin opening at the promoter fused to *lacZ* was uncoupled from transcription activation. In other words, Pho4-dependent remodeling of the *PHO5* promoter fused to *lacZ* could occur, but the mutated version of the histone H4 tail could not support transcription. To investigate this possibility, wild-type or histone H4 K>R mutant strains were permeabilized (Kent et al., 1993; Kent and Mellor, 1995), and the accessibility of the Cla1 site located within positioned nucleosome −2 was measured. As expected, the Cla1 site was not accessible in the chromatin in either the wild-type or mutant strains in high phosphate, indicating that this site in the promoter is protected by nucleosomes (data not shown). However, in low phosphate, the Cla1 site is accessible in the wild-type strain but not in the H4 mutant background (Figure 4C), indicating that the acetylatable lysines of the H4 tail are required for chromatin opening of the *PHO5* promoter when fused to *lacZ*.

Although the defect in activation of the *PHO5-lacZ* reporter in the H4 K>R strain was in chromatin opening, micrococcal nuclease digestion revealed that the *PHO5* promoter in both the natural context and when fused to *lacZ* is covered with a similar array of positioned nucleosomes with a digestion pattern clearly distinct from that observed using naked DNA (Figure 4D). Thus, the nuclease hypersensitive site corresponding the UAS_{P1} located between nucleosomes −2 and −3 is present, and nucleosomes −3 and −4 appear to occupy very similar positions. However, close examination reveals a number of subtle differences. In particular, there is a small shift in the cleavage between nucleosomes −1 and −2 in *PHO5-lacZ*, the nuclease sensitive sites defining the boundaries of nucleosome −1 are less well defined, and the nuclease protection defining nucleosome +1 is less evident in the *PHO5-lacZ* chromatin. The results are consistent with the nucleosomes toward the downstream side of the promoter being marginally less well positioned than in the natural context and may reflect a propagation of imprecise nucleosome positioning from the reporter gene into the promoter.

The *lacZ* Reporter Induces a 2–3 bp Shift in the −1 Nucleosome at *PHO5*

If nucleosome positioning within the *lacZ* coding sequences induces a subtle mispositioning of nucleosomes at the downstream side of the *PHO5* promoter, we predicted that extending the *PHO5* sequences from +81 might insulate the promoter from the effects of *lacZ*. We tested this by fusing *lacZ* at position +267,

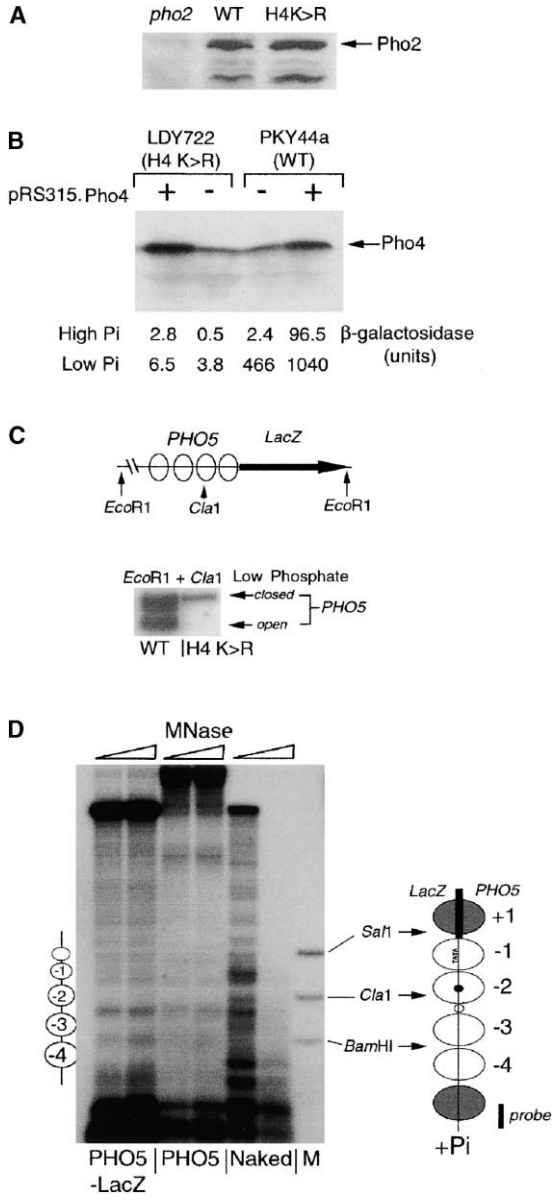


Figure 4. The Histone H4 Tail Is Required for Chromatin Opening of the *PHO5-lacZ* Reporter

(A) The failure to derepress the *PHO5-lacZ* reporter in the H4 mutant strain is independent of Pho2 levels. A Western blot using anti-Pho2 antibody and yeast extract derived from the indicated wild-type (PKY44a) is presented. H4 K>R (LDY722) and *pho2* delete (Y702) strains.

(B) The failure to derepress the *PHO5-lacZ* reporter in the H4 mutant strain is independent of Pho4 levels. A Western blot using anti-Pho4 antibody and yeast extract derived from the indicated strains is presented. Samples in which Pho4 levels have been increased by using a low copy number Pho4-expression vector (pRS315.PHO4) are indicated. The corresponding activity of the *PHO5-lacZ* reporter in these strains grown under high- or low-phosphate conditions is also shown.

(C) Cla1 accessibility assay using the *PHO5-lacZ* reporter in the wild-type (PKY44a) and H4 K>R mutant (LDY722) strains grown in low-phosphate medium. The expected locations of the nucleosomes (open circles) positioned over the *PHO5* promoter in a wild-type strain are shown relative to the EcoR1 and Cla1 sites in the *PHO5-lacZ* reporter. The blot was probed with a ClaI (+846)-EcoRV (+1134) fragment from the *lacZ* coding region.

in frame with the N-terminal coding sequences of *PHO5*. The results obtained (Figure 5A) revealed that while the *PHO5* promoter extending to +81 was dependent on the H4 tail lysine residues, the inclusion of additional *PHO5* sequences to +267 restored the ability of the promoter to function in the H4 tail K>R mutant background.

Since the *PHO5* promoter extending to +267 was independent of the H4 tail, we next compared the MNase cleavage pattern across the promoters extending to either +81 or +267 when fused to *lacZ*. The results (Figure 5B) again indicated that while the two promoters exhibited a highly similar pattern of nucleosome positioning, subtle differences between the two promoters were apparent; most notably, the cleavage between the −1 and −2 nucleosomes was marginally broader in the +267 context than in the promoter extending to +81. Again, these data suggest that the precise positioning of the −1 nucleosome might be a key factor in determining the dependence on the H4 tail.

To explore in greater detail the basis of this difference, we undertook a high-resolution analysis of the MNase cleavage pattern on each strand of the DNA at the −1 nucleosome by oligonucleotide-mediated labeling and selection of the MNase cleavage products. The results of this assay (Figure 5C) demonstrate that the single-strand MNase cleavage pattern within nucleosome −1 is clearly shifted by around 2–3 bp when comparing the +81 to the +267 versions of the *PHO5* promoter-*lacZ* reporters irrespective of which strand was examined, confirming the impression gained from the low-resolution MNase assays shown in Figure 5B.

To verify that the 2–3 bp mispositioning of the −1 nucleosome was indeed responsible for the H4 tail-dependency of the *PHO5*(+81)-*lacZ* reporter, we tested a promoter in which we introduced a 2 bp deletion in the region of the promoter that would be protected by nucleosome −1 (Figure 5D) and assayed its expression in a wild-type strain as well as in the H4 K>R mutant strain. Strikingly, and in contrast to its parent *PHO5*(+81) *lacZ* reporter, the 2 bp deletion mutant was independent of the acetylatable lysines of the H4 N-terminal tail (Figure 5E) and was induced to a level similar to that observed using the promoter extending to +267. The result is consistent with a 2 bp alteration in the positioning of nucleosome −1 inducing an absolute dependency on the H4 tail.

Mispositioning of the −1 Nucleosome Induces a Dependency on Bdf1

From the MNase digestion patterns obtained, it would appear that the MNase cleavage pattern across the −1

(D) Micrococcal nuclease (MNase) analysis by indirect end-labeling of the *PHO5-lacZ* reporter and *PHO5* gene in a wild-type strain (Y711), in which the endogenous *PHO5* promoter is deleted, grown under high-phosphate conditions. The nucleosomal cleavage pattern with MNase (300, 600 U/ml) on the chromosomal *PHO5* promoter or *PHO5-lacZ* was probed with an Apa1-Alw41 fragment derived from the 5′ end of the *PHO5*. Cleavage of naked DNA is shown as a control. The locations of the nucleosomes (open or closed circles) at the promoter are indicated relative to the BamH1, Cla1, and Sal1 markers generated by double digestion with Apa1.

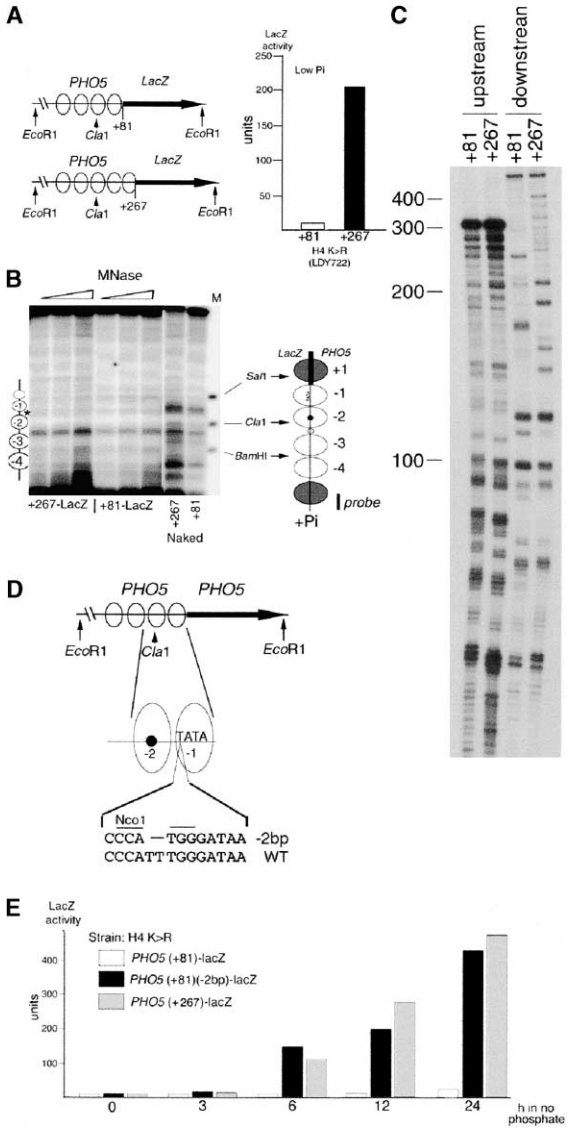


Figure 5. Dependency of the *PHO5*(+81)*-lacZ* Reporter on the Histone H4 Tail Is Caused by a 2–3 bp Displacement of Nucleosome −1

(A) Schematic showing the *PHO5*(+81)*-lacZ* reporter with the *PHO5* sequences extending to +81 or +267 and their relative activity in the H4 tail K>R mutant strain LDY722 in low phosphate.

(B) Micrococcal nuclease digestion of the +81 and +267 *lacZ* reporters.

(C) High-resolution mapping of micrococcal nuclease cleavage sites within nucleosome −1. The position of the cleavage products is located relative to the BspHI (−50 to +1527; downstream) or HaeIII (−96 to −486; upstream) sites located within nucleosome −1 (TATA −100) that were used to define the 3′ ends of the labeled MNase1 products.

(D) Schematic showing the location of the 2 bp deletion in the *PHO5* promoter.

(E) *LacZ* activity of the indicated reporters in the H4 K>R mutant strain LDY722.

nucleosome is different by around 2–3 bp when comparing the +81 and +267 *PHO5-lacZ* reporters. Since the −1 nucleosome contains the TATA box, one consequence of this small change in DNA positioning might be that the TATA box itself would be rotated relative to the nucleosome core, rendering it poorly accessible to TFIID and consequently revealing a dependency on the acetylatable lysines of the H4 tail that would not normally be required with an accessible TATA element. One explanation for these data is that an interaction between the acetylated H4 tail and a specific bromodomain factor would compensate for a poorly accessible TATA element by stabilizing TFIID at the promoter. A preliminary screen of candidate bromodomain factors revealed little or no dependency of the *PHO5-lacZ* reporter on either the Spt7 or Swi2/Snf2 bromodomains (Figures 6A and 6B), despite that fact that Spt7 itself is required for induction of *PHO5* (Barbaric et al., 2003). We therefore examined the potential role of Bdf1, a TFIID-associated factor that contains two bromodomains with the ability to bind acetylated histone H4 tails (Ladurner et al., 2003; Matangkasombut et al., 2000; Matangkasombut and Buratowski, 2003). Deletion of the *BDF1* gene, which is synthetically lethal either with mutations in the H4 tail or deletion of the *ESA1* gene (Matangkasombut and Buratowski, 2003), resulted in a delayed kinetic of activation of the natural *PHO5* gene (Figure 6C), similar to that seen on deletion of the H4 tail (Barbaric et al., 2001). Chromatin immunoprecipitation assays (Figure 6D) revealed that neither TBP nor Bdf1 could be detected at the *PHO5* promoter in high-phosphate, repressed conditions, but that both factors were recruited with a similar kinetic, consistent with Bdf1 being recruited as part of the TFIID complex. Although the final level of activation of *PHO5* was similar in a wild-type and a *bdf1* mutant strain, the *PHO5-lacZ* (+81) reporter was inactive in the *bdf1* strain (Figure 6E), consistent with its requirement for the acetylatable lysine residues of the H4 tail. By contrast, the H4 tail-independent +267 and (+81)-2bp versions of the *PHO5-lacZ* reporter were Bdf1 independent. Thus, there is a striking correlation between promoter-dependency on the H4 tail and a requirement for Bdf1.

To confirm that it was the capacity of Bdf1 to recognize the acetylated histone H4 tail that was important for its ability to support activation of the PHO5(+81)-lacZ reporter, we used vectors expressing wild-type Bdf1 or Bdf1 point mutants that exhibit a differential ability to bind the acetylated histone H4 tail or complement the growth defect of a *bdf1,bdf2* strain. These mutants and their properties have been described previously (Matangkasombut and Buratowski, 2003). The results (Figure 6F) indicate that the inability of a *bdf1* strain to induce the *PHO5*(+81)*-lacZ* reporter was complemented by expression of both wild-type Bdf1 as well as the P176A/P343A mutant that has no phenotype and has no defect in binding acetylated H4. In contrast, the Y187A,Y354A mutant which cannot support viability in a *bdf1/bdf2* background and exhibits no preference for acetylated H4 tails complements poorly, while the P194T/M195A/P361T/M362A mutant that exhibits a *ts* phenotype in *bdf1/bdf2* cells and moderate histone binding complements to an intermediate extent. Thus, the ability of Bdf1 to support activation of a *PHO5*(+81)*-lacZ* reporter correlates with its ability to recognize the acetylated H4 tail.

A TATA-less *PHO5* Promoter Is H4-Tail and Bdf1 Dependent

To mimic the presumed low affinity of the TATA box in the *lacZ* reporter in the context of the natural *PHO5* gene,

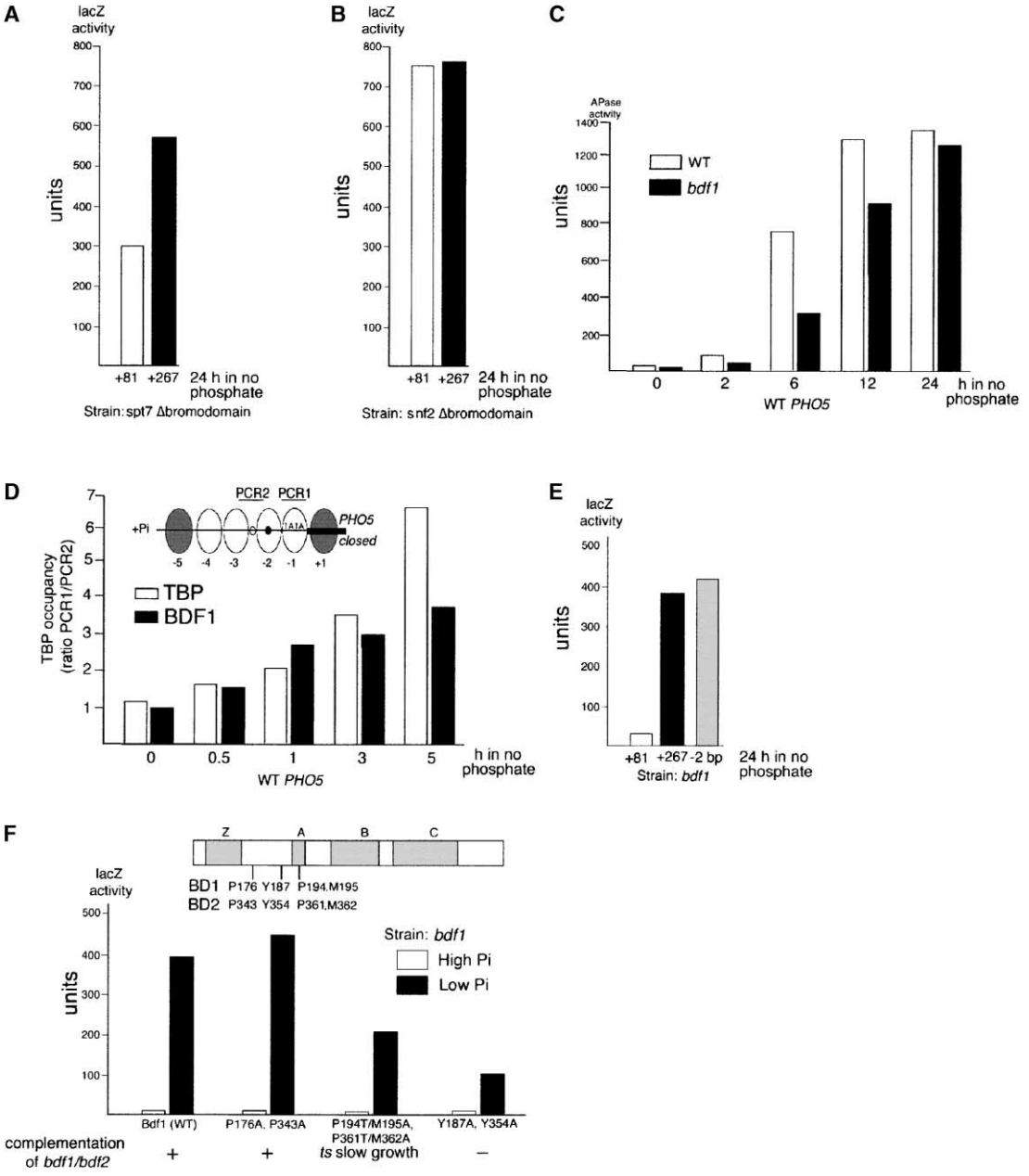


Figure 6. The *PHO5-lacZ* Reporter Is Dependent on the Bdf1 Bromodomain Factor

(A and B) Activity of the *PHO5 lacZ* reporter in strains expressing a bromodomain deletion mutant of either (A) Spt7 (FY1009) or (B) Swi2/Snf2 (BLY663).

(C) Activity of an episomal *PHO5* gene determined after induction over time in a wild-type (YSB104) or *bdf1* mutant (YSB496) strain in which the endogenous *PHO5* and *PHO3* genes have been deleted.

(D) Chromatin immunoprecipitation of TBP and HA-tagged Bdf1 in a wild-type strain using anti-TBP or anti-HA antibodies at the indicated times after induction of *PHO5* in low phosphate. The PCR products were generated using real-time PCR, and the TBP and Bdf1 occupancy at *PHO5* is expressed as the ratio of PCR product generated using primers spanning the TATA box (PCR1) to that obtained using primers spanning UAS_{P2} (PCR2).

(E) Activity of the indicated *PHO5-lacZ* reporters in a *bdf1* mutant strain (YSB496ΔP5/3).

(F) Complementation of *PHO5*(+81)-*lacZ* promoter activity in a *bdf1* strain (YSB778) using a plasmid to express wild-type Bdf1 or the indicated point mutants in the Bdf1 bromodomains BD1 and BD2. The mutants used have been characterized previously (Matangkasombut and Buratowski, 2003).

we mutated the TATA box from TATATAA to GAGATCT (Figure 7A). In a wild-type strain, the TATA-less mutant promoter exhibited a delayed kinetic of activation (Figure 7B), but after 12 hr in low-phosphate medium attained between 40% and 50% the activity of the wild-type promoter. This is consistent with the observation that in a wild-type strain, a *PHO5* promoter bearing a deletion encompassing the TATA box is competent for chromatin opening, although transcription activation in this case fails to occur (Fascher et al., 1993). By contrast,

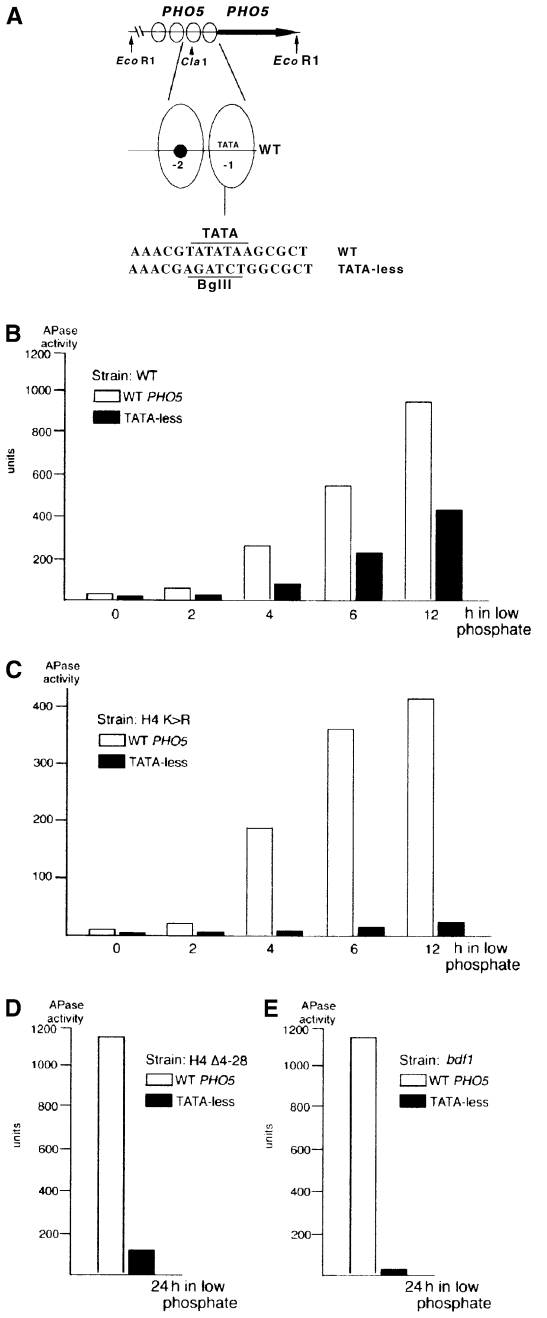


Figure 7. A TATA-less *PHO5* Promoter Is Dependent on the Histone H4 Tail and Bdf1

(A) Schematic showing the wild-type and TATA-less *PHO5* promoters.

(B–E) Acid phosphatase activity of an episomal wild-type and TATA-less *PHO5* gene in (B) a wild-type strain (YSB104ΔP5/3), (C) H4 K>R stain (LDY722ΔP5/3), (D) H4 Δ4-28 strain (PKY813ΔP5/3), or (E) the *bdf1* strain (YSB496ΔP5/3) in which the endogenous *PHO5* and *PHO3* genes are deleted. Note that both promoters were assayed in all isogenic wild-type strains with similar results.

in the H4 K>R strain, the TATA-less promoter mutant was inactive (Figure 7C). Moreover, not only were the acetylatable lysine residues critical for activation, but the integrity of the H4 tail was also required since a strain lacking amino acids 4–28 of the H4 tail also failed to activate efficiently the TATA-less *PHO5* promoter (Figure 7D). Critically, and in contrast to the wild-type *PHO5* promoter which only exhibits a delayed kinetic in the absence of Bdf1 (Figure 6C) or in a strain lacking an H4 tail (Barbaric et al., 2001), the TATA-less *PHO5* promoter was also entirely dependent on Bdf1 (Figure 7E).

Discussion

By comparing the requirements for transcription activation of a *PHO5-lacZ* reporter with that of the natural *PHO5* gene, we provide evidence that a key role for the bromodomain factor Bdf1 and the histone H4 tail is to facilitate transcription activation on a promoter where either the TATA box is absent or where it is poorly accessible within a nucleosome. Moreover, our results indicate that nucleotide level alterations in nucleosome positioning have a major effect on the requirements for transcription activation and provide a subtle but highly effective mechanism for regulating gene expression.

Precise Nucleosome Positioning and Transcription Activation

In high-phosphate conditions, neither TFIID (Sekinger and Gross, 2001) nor the transcription activator Pho4 (Venter et al., 1994) is present at the repressed *PHO5* promoter that is characterized by an array of four precisely positioned nucleosomes (Almer et al., 1986), although the promoter exhibits a degree of basal acetylation mediated by the Gcn5 and Esa1 HATs (Reinke and Hörz, 2003; Vogelauer et al., 2000). However, mutation of the acetylatable lysine residues of either the H3 or H4 N-terminal tails fails to affect the steady-state levels of *PHO5* expression achieved on switching to low-phosphate conditions, although in both cases the rate of *PHO5* derepression is somewhat reduced. Thus, at *PHO5*, unlike the *GAL* genes for example (Durrin et al., 1991), activation of transcription is largely independent of the acetylatable lysines of the H3 and H4 N-terminal tails. In contrast, a specific and absolute dependency on the acetylatable lysines in the H4 N-terminal tail can be induced by fusing the *PHO5* promoter to *lacZ*. The most likely explanation for this observation is that in the wild-type promoter, the TATA element is precisely positioned relative to the nucleosome core such that it is readily accessible by TFIID, but that in the *lacZ* fusion, the observed 2–3 bp shift in the positioning of the −1 nucleosome leads to rotation of the TATA box on the nucleosome surface, rendering it poorly accessible. The notion that the precise rotational phasing of the TATA element relative to the nucleosome core plays a key role in vivo is supported by the observation that in vitro, TBP binding to a nucleosome in the presence of the SWI/SNF complex is possible in one specific rotational position of the TATA box but not if the TATA sequence is displaced by 3 bp in either direction (Imbalzano et al., 1994). The key role of the TATA box in dictating whether a promoter is H4 tail dependent or independent was further underlined by the observation that mutation of the *PHO5* TATA element from TATATAA to AGATCTG also led to an absolute dependency of the promoter on the acetylatable lysines of the histone H4 tail.

The Role of Bdf1 at *PHO5*

The fact that deletion of residues 4–28 of the H4 tail also severely impaired activation of the *PHO5*(+81)-*lacZ* reporter (Durrin et al., 1991) as well as the TATA-less *PHO5* promoter suggested that the acetylated H4 tail is recognized by a key factor participating in the activation process. Previous work has established that acetylated histone tails can be recognized by bromodomain factors (Jacobson et al., 2000; Ornaghi et al., 1999; Owen et al., 2000; Dhalluin et al., 1999; Hassan et al., 2002; Hudson et al., 2000). Given that the integrity of the TATA box and the precise position of nucleosome −1 were key factors in determining the requirement for the H4 tail, our attention focused on Bdf1, a factor associated with TFIID that binds the acetylated histone H4 tail and associates with chromatin in a bromodomain-dependent fashion (Ladurner et al., 2003; Matangkasombut et al., 2000; Matangkasombut and Buratowski, 2003). Although Bdf1 clearly has roles independent of its association with TFIID, for example in the maintenance of euchromatin and impeding the spreading of heterochromain by competing for H4 tail binding with Sir2 (Ladurner et al., 2003), it clearly is important in gene-specific regulation, being required for activation of *GAL10* but not *CUP1* (Matangkasombut et al., 2000) for example. As first discussed by Matangkasombut and Buratowski (2003), it appears that genes most dependent on Bdf1, such as *HTA2*, *TRP3*, and the ribosomal protein genes, are those that are TAF dependent (Kuras and Struhl, 1999; Li et al., 2000), a conclusion supported by recent in-depth analysis of TFIID- and Bdf1-dependent genes (Huisinga and Pugh, 2004). Although it has been suggested that Bdf1 could bind to acetylated histone tails at promoters prior to recruitment of TFIID (Huisinga and Pugh, 2004), at the natural *PHO5* promoter Bdf1 is apparently absent under repressed conditions but is recruited with a similar kinetic as TBP on activation, suggesting that it arrives together with TFIID. In a wild-type strain, *Bdf1* is necessary for the correct kinetic of activation but not for the final steady state-levels of *PHO5* expression. In contrast the *PHO5*(+81)-*lacZ* reporter is absolutely dependent on Bdf1, and specifically on the Bdf1 bromodomains, but is independent of both the Spt7 and Swi2/Snf2 bromodomains. Thus, the dependency on Bdf1 does not reflect a more general requirement for bromodomain-histone tail interactions but is rather specific. The *PHO5* TATA-less promoter is similarly absolutely dependent on Bdf1. Our current view is that at the natural *PHO5* promoter an interaction between the Bdf1 bromodomains and the acetylated lysine residues on the H4 tail facilitates the initial steps in the activation process, but is not critical, with the −1 nucleosome being positioned precisely so that the TATA box is at its most accessible. In contrast, if the TATA box is either poorly accessible or absent, the interaction of TFIID-associated Bdf1 with the H4 tail becomes a prerequisite for loading or maintenance of TFIID on the promoter. This interpretation is supported by the observation that in vivo H4 tails appear to contribute positively to TFIID-dependent transcription (Huisinga and Pugh, 2004), while in vitro studies using purified SWI/SNF and SAGA have also implicated bromodomains as modules designed to anchor multiprotein complexes to acetylated nucleosomes at a promoter, with the Swi2/Snf2 and Gcn5 bromodomains being required to retain these complexes at a promoter in the absence of a transcription activator (Hassan et al., 2002).

The Role of Small Nucleosome Movements in Transcription Regulation

The results presented here have a number of general implications for our understanding of transcription regulation in a chromatin context. First and most obvious, the fact that fusion of the *PHO5* promoter to *lacZ* induces an absolute dependency on the histone H4 tail and Bdf1 that is not apparent at the natural promoter means that care must be taken in interpreting results obtained using reporter genes. That reporter genes induce dependencies not apparent at the natural promoter has been described before (for examples, see Nasmyth et al., 1987; Stillman et al., 1994; Tabtiang and Herskowitz, 1999), but no explanation has previously been forthcoming. It is now apparent that sequences downstream from a promoter can influence nucleosome positioning at the promoter and in particular over the TATA box.

Second, as little as 2 bp movement in nucleosome positioning can alter the requirements for chromatin remodeling in vivo. Thus, in principal, very small nucleosome movements induced by chromatin remodeling machines in vivo may render a promoter inactive even in the presence of an activator unless specific histone tails can be acetylated and recognized by specific bromodomains. Such small nucleosome movements would therefore represent a subtle and highly effective means of regulating gene expression. In this respect we note that chromatin remodeling activities such as the ISW1 and ISW2 complexes (Tsukiyama et al., 1999) are required to correctly position nucleosomes immediately downstream from many promoters and play a key role in controlling transcription elongation (Morillon et al., 2003). However, although their affects on downstream nucleosomes are readily detectable, nucleosome-nucleosome contacts similar to those we believe account for the effects of the *lacZ* reporter on the *PHO5* −1 nucleosome may mean that there are subtle and previously undetected affects of the ISWI complexes on nucleosomes over the TATA boxes of many genes, resulting in specific requirements for transcription activation. We therefore expect that small nucleosome movements will play a key role in the regulation of many promoters.

Third, although our analysis is restricted to *PHO5*, we anticipate that bromodomain-histone tail interactions will act to stabilize various components of the transcription machinery to many promoters where transcription factor binding sites are low affinity or not optimally positioned relative to nucleosomes. For example, the Bdf1-dependency of the *GAL10* promoter (Matangkasombut et al., 2000) is mirrored in a dependency on the H4 tail (Durrin et al., 1991), while the ribosomal protein genes are dependent on both Bdf1 (Matangkasombut et al., 2000), Esa1 (Reid et al., 2000), and the H4 tail (our unpublished data). However, while we might expect that a promoter lacking a recognizable TATA element will be dependent on Bdf1, we can make no predictions regarding promoters containing a TATA box, since any specific requirement for Bdf1 or the histone H4 tail will be dictated by the promoter architecture and the precise posi-

Table 1. Yeast Strains

Strain	Comments	Genotype	Source
WZY63	Wild-type	*MATa, ura3-52, lys2-801, ade2-101, trp1Δ63, his3Δ200, leu2Δ1, hht1-hhf, 1::pWZ405-F2F9-LEU2, hht2-hhf2::pWZ403-F4F10-HIS3, [pWZ414-F13]*	Zhang et al., 1998
WZY76	gcn5	*MATa, ura3-52, lys2-801, ade2-101, trp1Δ63, his3Δ200, leu2Δ1, gcn5Δ::ura3, hht1-hhf, 1::pWZ405-F2F9-LEU2, hht2-hhf2::pWZ403-F4F10-HIS3, [pWZ414-F13]*	Zhang et al., 1998
WZY160	GCN5, H3 K9R, H4 K5,12R	*MATa, ura3-52, lys2-801, ade2-101, trp1Δ63, his3Δ200, leu2Δ1, hht1-hhf, 1::pWZ405-F2F9-LEU2, hht2-hhf2::pWZ403-F4F10-HIS3, [pWZ414-F55]*	Zhang et al., 1998
WZY161	gcn5, H3 K9R, H4 K5,12R	*MATa, ura3-52, lys2-801, ade2-101, trp1Δ63, his3Δ200, leu2Δ1, gcn5Δ::ura3, hht1-hhf, 1::pWZ405-F2F9-LEU2, hht2-hhf2::pWZ403-F4F10-HIS3, [pWZ414-F55]*	Zhang et al., 1998
WZY141	GCN5, H3 K9Q, H4 K5,12Q	*MATa, ura3-52, lys2-801, ade2-101, trp1Δ63, his3Δ200, leu2Δ1, hht1-hhf, 1::pWZ405-F2F9-LEU2, hht2-hhf2::pWZ403-F4F10-HIS3, [pWZ414-F54]*	Zhang et al., 1998
WZY143	gcn5, H3 K9R, H4 K5,12R	*MATa, ura3-52, lys2-801, ade2-101, trp1Δ63, his3Δ200, leu2Δ1, gcn5Δ::ura3, hht1-hhf, 1::pWZ405-F2F9-LEU2, hht2-hhf2::pWZ403-F4F10-HIS3, [pWZ414-F54]*	Zhang et al., 1998
WZY94	GCN5, H3 K14R, H4 K8,16R	*MATa, ura3-52, lys2-801, ade2-101, trp1Δ63, his3Δ200, leu2Δ1, hht1-hhf, 1::pWZ405-F2F9-LEU2, hht2-hhf2::pWZ403-F4F10-HIS3, [pWZ414-F50]*	Zhang et al., 1998
WZY93	GCN5, H3 K14Q, H4 K8,16Q	*MATa, ura3-52, lys2-801, ade2-101, trp1Δ63, his3Δ200, leu2Δ1, hht1-hhf, 1::pWZ405-F2F9-LEU2, hht2-hhf2::pWZ403-F4F10-HIS3, [pWZ414-F48]*	Zhang et al., 1998
WZY156	gcn5, H3 K14Q, H4 K8,16Q	*MATa, ura3-52, lys2-801, ade2-101, trp1Δ63, his3Δ200, leu2Δ1, gcn5Δ::ura3, hht1-hhf, 1::pWZ405-F2F9-LEU2, hht2-hhf2::pWZ403-F4F10-HIS3, [pWZ414-F48]*	Zhang et al., 1998
RMY200	Wild-type	*MATa, ade2-101, his3Δ200, lys2-801, trp1Δ901, ura3-52 hht1,hhf1::LEU2, hht2, hhf2::HIS3 [CEN4 ARS1 TRP1 HHF2 + HHT2]*	Mann and Grunstein, 1992
RMY253	H3	*MATa, ade2-101, his3Δ200, lys2-801, trp1Δ901, ura3-52, hht1,hhf1::LEU2, hht2,hhf2::HIS3 [CEN4 ARS1 TRP1 HHF2 + pRM253 hht2 R9, 14, 18, 23]*	Mann and Grunstein, 1992
PKY44a	Wild-type	*MATa, ade2-101, arg4-1, his3Δ200, leu2-3-112, lys 2-801, Δtrp1 901, ura 3-52, thr-, tyr- Δhhf1::HIS3, Δhhf2::LEU2 [pPK25 URA3 CEN3 ARS1 HHF2]*	Kayne et al., 1988
Y711	ΔPHO5 promoter	*MATa, ade2-101, arg4-1, his3Δ200, leu2-3-112, lys 2-801, Δtrp1 901, ura 3-52,Δpho5 promoter::TRP1, thr-, tyr- Δhhf1::HIS3, Δhhf2::LEU2 [pPK25 URA3 CEN3 ARS1 HHF2]*	This study
PKY44aΔP5/3	*pho5,pho3*	*MATa, ade2-101, arg4-1, his3Δ200, leu2-3-112, lys 2-801, Δtrp1 901, ura 3-52, thr-, tyr- Δhhf1::HIS3, Δpho5,pho3:TRP1, Δhhf2::LEU2 [pPK25 ura3 CEN3 ARS1 HHF2]*	This study
LDY722	H4 K>R	*MATa, ade2-101, arg4-1, his3Δ200, leu2-3-112, lys 2-801, trp1Δ 901, ura3-52, thr-, tyr- Δhhf1::HIS3, Δhhf2::LEU2 [pPK25 URA3 CEN3 ARS1 hhf2 R 5,8,12,16](isogenic to PKY44a)*	Durrin et al., 1991
LDY722ΔP5/3	H4 K>R, *pho5,pho3*	*MATa, ade2-101, arg4-1, his3Δ200, leu2-3-112, lys 2-801, trp1Δ 901, ura3-52, thr-, tyr- Δhhf1::HIS3, Δpho5,pho3:TRP1, Δhhf2::LEU2 [pPK25 ura3 CEN3 ARS1 hhf2 R 5,8,12,16](isogenic to PKY44a)*	This study
PKY813	H4Δ4-28	*MATa, ade2-101, arg4-1, his3-201, leu2-3, leu2-112, lys2-801, trp1-901, ura3-52, thr- tyr- hhf(HIS3) hhf2 (LEU2) (isogenic to PKY44a)*	Kayne et al., 1988
PKY813ΔP5/3	H4Δ4-28, *pho5,pho3*	*MATa, ade2-101, arg4-1, his3-201, leu2-3, leu2-112, lys2-801, trp1-901, ura3-52, Δpho5,pho3:TRP1, thr-tyr- hhf(HIS3) hhf2 (LEU2) (isogenic to PKY44a)*	This study
YSB104	Wild-type	*MATa, ura3-52, leu2-3,112, his3Δ200*	Matangkasombut et al., 2000
YSB104ΔP5/3	*pho5,pho3*	*MATa, ura3-52, leu2-3,112, his3Δ200, Δpho5,pho3::HIS3*	This study
YSB496	*bdf1*	*MATa, ura3-52, leu2-3,112, his3Δ200,bdf1::LEU2*	Matangkasombut et al., 2000
YSB496ΔP5/3	*bdf1, pho5,pho3*	*MATa, ura3-52, leu2-3,112, his3Δ200,bdf1::LEU2, Δpho5,pho3::HIS3*	This study
YSB778	*bdf1*	*MATalpha, leu2Δ1, ura3-52, trp1Δ63, bdf1Δ::G418*	Matangkasombut and Buratowski, 2003
FY1009	spt7Δbromodomain	*MATa , spt7-502,his4-917δ, leu2Δ1, ura3-52*	Sterner et al., 1999
BLY663	*snf2*Δbromodomain	*Matα, snf2-bromodomainΔ, lys2-801, his3-Δ200, ura3-52*	Brehon Laurent
Y702	*Pho2*	*Matα, ade2-1, trp1-1,can1-100, leu2-3,112, his3-11,15, ura3, PHO2:HIS3*	Hirst et al., 1994

tioning of the TATA element relative to the nucleosome core.

Experimental Procedures

Yeast Strains

Yeast strains are shown in Table 1.

Functional Assays and Chromatin Analysis

Cells were grown and assayed for acid phosphatase activity produced by the *PHO5* gene as described (Svaren et al., 1994). β-galactosidase activity was assayed as described (Hirst et al., 1994). The values for the β-galactosidase and acid phosphatase assays are presented as an average of three independent experiments, each performed in duplicate. The standard deviations calculated for these data were no more than ±10%. Cla1 accessibility was assayed in permeabilized yeast (Kent et al., 1993; Kent and Mellor, 1995). MNase and DNase1 digestion of chromatin was done in permeabilized whole cells as described (Kent et al., 1993; Kent and Mellor, 1995).

High-Resolution MNase Mapping

This method was based on the procedure of Teng et al. (1997), modified in the following way. Permeabilized cells were treated with 5 U/ml of MNase for 4 min at 37°C. Naked DNA controls digested with MNase were prepared exactly as described in Kent and Mellor (1995). Around 30 μg of DNA was isolated for each MNase concentration and digested for 16 hr at 55°C with 50 U of either BspH1 or HaeIII to generate a unique end for mapping MNase cleavage sites either upstream (HaeIII −96 to −486) or downstream (BspHI −50 to +1527) of the restriction enzyme cleavage sites. The single strands to be mapped were isolated by hybridization to a biotinylated 30-mer specific to *PHO5* sequences, abutting either the BspH1 or HaeIII restriction sites within nucleosome −1 and isolated using streptavidin magnetic beads (Dynabeads; Dynal). The 3′ end was labeled by adding six adenines, using the template thymidine bases incorporated into the oligonucleotide, by incubation with ^{32}P-dATP using Sequenase and the products separated on a 6% sequencing gel. A sequence ladder for the same strand of DNA was used as a marker and was generated by PCR using modified oligonucleotides and ^{35}S-labeled nucleotides as described by Teng et al. (1997).

Plasmids

The *PHO5-lacZ* construct contained a 3 kb *PHO5* EcoRI/SalI promoter fragment cloned into pR426 (Sikorski and Hieter, 1989) and fused in-frame to a SalI fragment containing the *lacZ* gene from p502. The CEN/ARS-based Pho4 expression vector, pRS315.Pho4, was constructed by cloning a BamHI fragment containing the *PHO4* ORF into the BglII site of pRS315 into which the *PHO4* promoter as an EcoRI/BglII fragment and the *PGK* terminator as a BglII/SalI fragment had been cloned. pRS426-*PHO5* was created by cloning a 3.63 kb StuI-NciI (end-filled) fragment into the SmaI site of pRS426. The same fragment was cloned into the SmaI site of pRS316 to create a low copy number *CEN*-based version of *PHO5*. Deletion and point mutations in the *PHO5* promoter were made using the the Stratagene QuikChange Mutagenesis Kit.

Anti-Pho4 Antibody and Western Blotting

Pho4 amino acids 108–245 were expressed in *E. coli* as an in-frame fusion with GST. After purification on glutathione beads, the 100 μg GST-Pho4 fusion protein was used to inject rabbits four times at intervals of one month. Anti-GST antibodies were removed from the resulting antiserum by incubation with an excess GST protein bound to glutathione-sepharose beads for 2 hr at 4°C. The resulting supernatant was used to probe Western blots for Pho4 expression. Samples for Western blotting were prepared by harvesting yeast grown under appropriate conditions by centrifugation, resuspending the pellet in SDS lysis buffer, and boiling for 5 min before analysis by SDS-PAGE. Following electrophoresis, proteins were transferred to nitrocellulose membrane, blocked in a buffer containing 5% nonfat milk, and probed with anti-Pho4 antibody for 1 hr. After washing, blots were incubated with an anti-rabbit horseradish peroxidase-conjugated secondary antibody and washed extensively, and bands were revealed using ECL (Amersham) according to manufacturer's instructions.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation of TBP or HA-tagged Bdf1 at the *PHO5* promoter was performed essentially as described previously (Moreau et al., 2003) using real-time PCR and primers spanning either the TATA box (5′-GGGTAAACATCTTTGAATTGTCGAA and 5′-AAGCCATACTAACCTCGACTTAGCA) or UAS_{P2} (5′-ATCCGTGAT GACGATGATTTGG and 5′-CACTGACAGTCTGCAAGGTGATG).

Acknowledgments

We would like to thank Mike Grunstein, Shelley Berger, Mary Ann Osley, Brehon Laurent, Sharon Roth, Fred Winston, and Steve Buratowski for gifts of strains and plasmids. In addition, we especially appreciate the contribution of Wolfram Hörz to the interpretation of the MNase digestion pattern using the *PHO5-lacZ* reporter and Dave Stillman for providing the anti-Pho2 antibody. This work was funded by grants from The Wellcome Trust and BBSRC to J.M. and Marie Curie Cancer Care to C.G., J.L.M., and J.G. P.P. acknowledges the support of a BBSRC studentship. C.M.C. was funded by a European Union Marie Curie Fellowship, and J.L.M. was also funded by L'Association pour la Recherche Contra la Cancer.

Received: October 22, 2003
Revised: April 2, 2004
Accepted: April 28, 2004
Published: July 1, 2004

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