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A nucleosome precludes binding of the transcription factor Pho4 *in vivo* to a critical target site in the *PHO5* promoter

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Activation of the *Saccharomyces cerevisiae PHO5* gene by phosphate starvation is accompanied by the disappearance of two pairs of positioned nucleosomes that flank a short hypersensitive region in the promoter. The transcription factor Pho4 is the key regulator of this transition. By *in vitro* footprinting it was previously shown that there is a low affinity site (UAS_p1) which is contained in the short hypersensitive region in the inactive promoter, and a high affinity site (UAS_p2) which is located in the adjacent nucleosome. To investigate the interplay between nucleosomes and Pho4, we have performed *in vivo* footprinting experiments with dimethylsulfate. Pho4 was found to bind to both sites in the active promoter. In contrast, it binds to neither site in the repressed promoter. Lack of binding under repressing conditions is largely due to the low affinity of Pho4 for its binding sites under these conditions. Despite the increased affinity of Pho4 for its target sites under activating conditions, binding to UAS_p2 is prevented by the presence of the nucleosome and can only occur after prior disruption of this nucleosome in a process that requires UAS_p1. Protection of the *PHO5* UAS_p2 by the nucleosome is not absolute, however, since overexpression of Pho4 can disrupt this nucleosome even when UAS_p1 is deleted. Also under these conditions, with only UAS_p2 present, all four nucleosomes at the *PHO5* promoter are disrupted, whereas no chromatin change at all is observed when both UAS elements are destroyed.

Key words: active chromatin/*in vivo* footprint/nucleosome disruption/Pho4/yeast

Introduction

There is increasing evidence that the basic subunit of chromatin, the nucleosome, is integrally involved in gene regulation. In most cases in which the influence of the chromatin structure on gene expression has been studied, the presence of nucleosomes at a promoter has been found to have a repressive effect on its activity (reviewed in Kornberg and Lorch, 1991, 1992; Felsenfeld, 1992; Svaren and Hörz, 1993; but see Schild *et al.*, 1993; McPherson *et al.*, 1993). As a consequence, mechanisms must exist to alleviate nucleosome mediated repression of inducible genes when the promoter is activated. It has indeed been found for a number of genes that high level transcription is accompanied by chromatin changes in regulatory regions (Almer *et al.*, 1986; Evans *et al.*, 1990; Reik *et al.*, 1991; Bresnick *et al.*, 1992; Axelrod *et al.*, 1993; Gross *et al.*, 1993). In several systems, it has been shown that the chromatin rearrangement is not simply the result of transcription, but rather occurs independently (Pham *et al.*, 1991; Gilbert *et al.*, 1992; Hirschhorn *et al.*, 1992; Lee and Garrard, 1992; Morgan and Whitlock, 1992; Fascher *et al.*, 1993), lending further support to the notion that it is a required step in the cascade of gene activation. Very little is known, however, about the mechanism of nucleosomal rearrangement. In model experiments, Workman and Kingston (1992) have shown *in vitro* that Gal4 can lead to the destabilization of a nucleosome containing Gal4 target sites, provided that nonspecific competitor DNA is present in the assay mixture. Similarly, alleviation of H1 mediated repression in a *Drosophila in vitro* system requires an RNase sensitive component, also suggesting the requirement for a histone acceptor (Croston *et al.*, 1992).

In our own experiments we are attempting to elucidate the mechanism of a nucleosome rearrangement at the *PHO5* promoter in *Saccharomyces cerevisiae*. In the repressed state, the *PHO5* promoter is covered by two pairs of nucleosomes which flank a short hypersensitive region (Almer and Hörz, 1986) (see Figure 1). Upon activating the gene by starving the cells of phosphate, these four nucleosomes disappear and the entire promoter becomes accessible to the transcriptional machinery (Almer *et al.*, 1986). Two positive regulatory proteins contribute to this transition, the products of the *PHO2* and the *PHO4* genes (Fascher *et al.*, 1990). However, overproduction of Pho4 can compensate for a lack of Pho2, while the reverse is not true (Fascher *et al.*, 1990), indicating that Pho4 can act as the primary trigger.

By *in vitro* footprinting, we have shown that there are two binding sites for Pho4 at the *PHO5* promoter (Vogel *et al.*, 1989), which correspond to the two UAS elements found by deletion mutagenesis (Rudolph and Hinnen, 1987). One is contained in the hypersensitive region, the

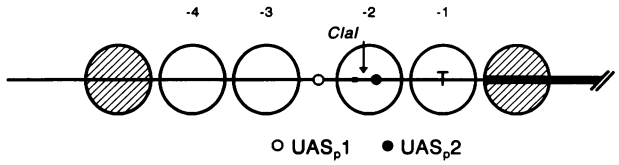

Fig. 1. Chromatin structure at the repressed *PHO5* promoter. Nucleosomes −1, −2, −3 and −4 (large open circles) are removed upon activation (Almer *et al.*, 1986). The two Pho4 binding sites, UAS_p1 (○) and UAS_p2 (●) and a Pho2 binding site (■) found by *in vitro* footprinting (Vogel *et al.*, 1989) are marked. T denotes the TATA box (Rudolph and Hinnen, 1987). The location of a *ClaI* site at −275 relative to the coding sequence (solid black bar) is shown.

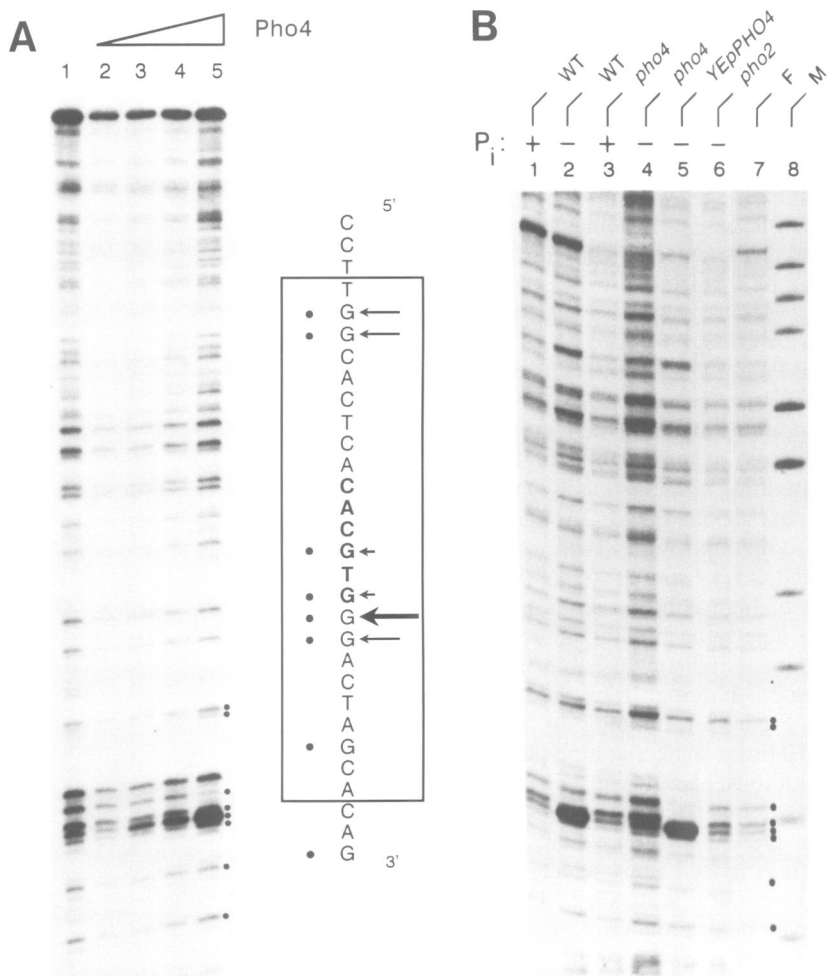

Fig. 2. DMS footprint analysis of Pho4 binding to the *PHO5* UAS_p2 *in vitro* and *in vivo*. (**A**) A *Bam*HI−*Sal*I fragment from the *PHO5* promoter was incubated with DMS in the presence of increasing amounts of recombinant Pho4 protein (lanes 2–5) or in the absence of Pho4 (lane 1) and subsequently analyzed with primer 1 as described in Materials and methods. Pho4 concentrations were increased by a factor of four in each lane. G residues within UAS_p2 are marked by dots. The Pho4 binding site, as detected by *in vitro* footprinting with DNase I (Vogel *et al.*, 1989), is boxed in the sequence. The thick arrow designates a G residue which becomes hypersensitive to DMS upon Pho4 binding, the short arrows mark guanines protected by Pho4 and the medium arrows G residues whose reactivity with DMS is unchanged by Pho4 binding. (**B**) Yeast cells were treated with DMS and analyzed with primer 1 as described in Materials and methods. The strains and growth conditions are indicated at the top (WT = YS18, *pho4* = YS22, *pho2* = YS19). YEpPHO4 means YS18 containing the Pho4 overexpression plasmid YEpPHO4, F is free DNA and M a labeled *Hpa*II digest of pBR322 that serves as a molecular weight reference. G residues within UAS_p2 are marked by dots.

other one within nucleosome −2 (see Figure 1). This raises the obvious question of how the chromatin structure of the *PHO5* promoter affects the ability of Pho4 to interact with its binding sites, and, in turn, how interaction of Pho4 leads to modification of the nucleosome structure. It was therefore important to determine the conditions under which Pho4 binds to its two target sites *in vivo*. To do so, we performed *in vivo* footprinting experiments using dimethylsulfate (DMS). At the same time, we investigated the role of the two UAS elements in the chromatin transition by deleting one or both of them in strains that overexpress Pho4.

Results

Binding of Pho4 to its binding site is detected by DMS footprinting

We have previously demonstrated interaction of Pho4 with its target sites in the *PHO5* promoter by *in vitro* footprinting with DNase I (Vogel *et al.*, 1989). In order to find out if binding of Pho4 also affects the reactivity of the DNA towards DMS, we first performed *in vitro* footprinting experiments with DMS. DMS was the most appropriate footprinting reagent for our purposes because it can permeate intact cells, obviating the need to isolate nuclei, and also because histone−DNA interactions in the nucleosome do not noticeably affect reactivity of the DNA towards DMS (Jackson and Felsenfeld, 1987). Therefore, interactions of nonhistone proteins with the DNA are selectively detected. Rather than using end-labeled DNA for our *in vitro* experiments, we used an unlabeled restriction fragment as binding template, and a linear PCR protocol which was used in the subsequent *in vivo* experiments. Figure 2A shows the results for UAS_p2 of the *PHO5* promoter in the presence of different amounts of recombinant Pho4. It can be seen that there are changes in the reactivity of G residues within the binding site: there is enhancement of one G residue (position −247) which increases continuously with increasing Pho4 concentration, and at high concentrations of Pho4 (lane 5)

there is clear protection of a different G residue three nucleotides away (position −250). That the binding of a protein can lead to both protection and enhancement of G reactivities is a common occurrence (see for example Ogata and Gilbert, 1979). We conclude from this experiment that footprinting with DMS is a suitable method for monitoring Pho4 interaction with the DNA and, furthermore, that (at least for this UAS element) the assay is quite sensitive, reflecting the extent of binding over a Pho4 concentration range of >16-fold.

Interaction of Pho4 with UAS_p2 in vivo

In order to test whether Pho4−UAS_p2 interaction could also be observed *in vivo*, we exposed to DMS cells that had been grown in conditions under which the *PHO5* promoter was either repressed or induced, i.e. high phosphate or no phosphate conditions. The genomic DNA was isolated and analyzed as described in Figure 2A. In induced cells (Figure 2B, lane 2) Pho4 appears to bind to UAS_p2 since the pattern generated is very similar to that obtained *in vitro* in the presence of high concentrations of Pho4. In contrast, there is no detectable binding in repressed cells (lane 1) as the pattern is indistinguishable from the free DNA pattern (lane 7). Judging from the *in vitro* titration experiment in Figure 2A, binding of Pho4 to UAS_p2 must be at least 10 times weaker at high phosphate than at no-phosphate conditions. In *pho4* cells, no evidence for protein binding was found, regardless of whether phosphate was absent or present in the medium (lanes 3 and 4), confirming that it is indeed Pho4 which changes the reactivity of the G residues at UAS_p2. The Pho4 dependent changes are even more pronounced when Pho4 is overproduced (lane 5). In a *pho2* strain, in which phosphate starvation cannot disrupt *PHO5* chromatin (Fascher *et al.*, 1990), there is no detectable binding of Pho4 to UAS_p2 even in the absence of phosphate (lane 6). In conclusion, these experiments show that in all cases, binding of Pho4 to UAS_p2 is observed only when this site is in a non-nucleosomal configuration (Fascher *et al.*, 1990), and binding is not observed when a nucleosome is present.

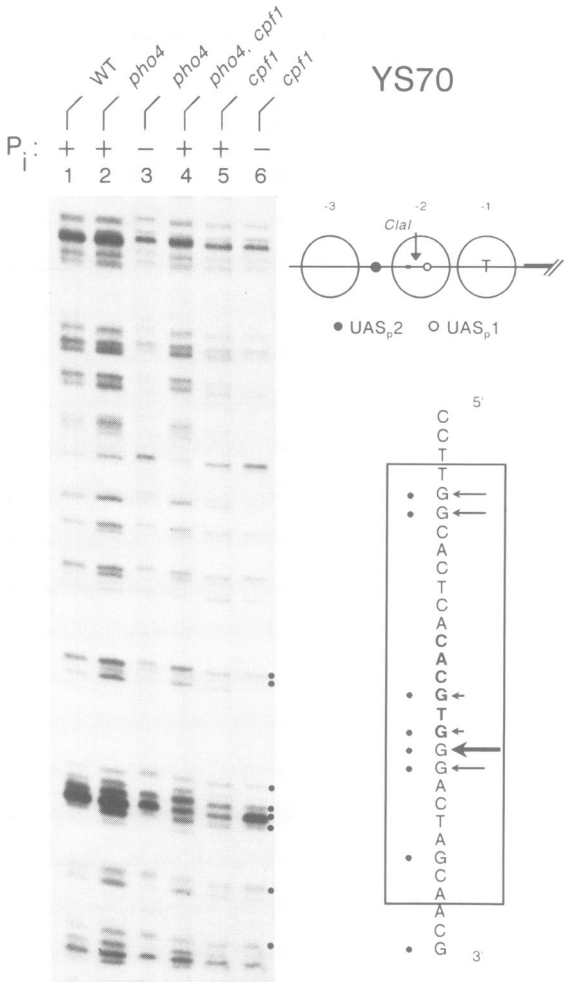

Fig. 3. DMS *in vivo* footprint analysis of the *PHO5* UAS_p2 in YS70 and derivatives thereof. Cells from YS70, in which the two UAS elements are inverted (see schematic and Materials and methods), and YS70 derivatives as indicated at the top (*pho4* = YS72; *cpf1* = YS71; *pho4*, *cpf1* = YS73) were grown under the conditions listed, treated with DMS and analyzed as in Figure 2 with primer 2, which reveals the methylation pattern of the same strand of UAS_p2 as depicted in Figure 2.

The nucleosome blocks binding of a helix−loop−helix protein to UAS_p2

In order to investigate if the nucleosome was directly responsible for the lack of interaction of Pho4 with UAS_p2, we decided to construct a new strain (YS70) by exchanging UAS_p1 and UAS_p2 at the *PHO5* promoter. When we analyzed the *PHO5* promoter in this strain by nuclease digestion we found that it adopted the same chromatin structure under repressing conditions as the wild-type strain (data not shown), i.e. positioned nucleosomes and a short hypersensitive region, now encompassing UAS_p2, however. The chromatin transition which accompanies gene activation occurred in the same way as in the wild-type promoter.

We then assayed for factor binding to UAS_p2 in the newly generated strain YS70 by DMS treatment as described above. The characteristic hypersensitivity of the G_{-247} residue (compare Figure 2), was now also present under repressing conditions, i.e. in the inactive promoter (Figure 3, lane 1). Essentially the same pattern was obtained in a *pho4* strain, however (Figure 3, lanes 2 and 3), indicating that it was not or not only Pho4 interacting with the UAS element under these conditions. This was not completely unexpected since Pho4 is a member of the helix−loop−helix family of proteins that bind cognate sequences with a common 6 bp core (Murre *et al.*, 1989). Several members of this family have also been demonstrated in yeast, e.g. Cpf1 (Bram and Kornberg, 1987; Cai and Davis, 1990; Mellor *et al.*, 1990), Fbf1 (Schuller *et al.*, 1992), Ino2 (Nikoloff *et al.*, 1992) and Ino4 (Hoshizaki *et al.*, 1990). Cpf1 had been a likely candidate for a protein interacting with Pho4 binding sites, since Cpf1 has been shown to bind to the Pho4 sites at the *PHO5* promoter *in vitro* (Fisher and Goding, 1992), and there is indication that Pho4 can compensate for certain defects caused by the loss of Cpf1 (O'Connell and Baker, 1992). The results of the double disruption of *PHO4* and *CPF1* (Figure 3, lane 4) demonstrate that binding to UAS_p2 in a *pho4* strain was indeed due to the Cpf1 protein.

In the following experiment we used a *cpf1* strain to look for Pho4 binding to UAS_p2 in YS70 because of Cpf1

interference in our *in vivo* binding assay. The results are shown in Figure 3, lanes 5 and 6. At repressing conditions, there was only little binding to UAS_p2 as judged from the reactivity of G residue −247 relative to −248, which is the most sensitive measure of Pho4 binding (see Figure 2A). Under activating conditions, however, there is strong binding of Pho4 to UAS_p2 in the *cpf1* derivative of YS70 (Figure 3, lane 6).

There are two important conclusions from these results. The nucleosome confers clear protection to UAS_p2 against Cpf1 binding as shown by comparing YS70 with our wild-type YS18 strain (compare lane 1 in Figure 2B and lane 1 in Figure 3). Secondly, the affinity of Pho4 for its binding site seems to be significantly lower at repressing (high phosphate) conditions than at activating conditions. A test of the ability of the nucleosome to protect against Pho4 binding should therefore be performed under activating conditions in order for it to be meaningful. Under those conditions, however, the nucleosome is disrupted in a process that requires the internucleosomal site UAS_p1 (Fascher *et al.*, 1993), and its potential protective effect can no longer be investigated. We therefore had to use a slightly different strategy, as described in the next section.

The nucleosome also protects UAS_p2 against binding of Pho4

In order to analyze if Pho4 was able to bind to its target site when present in a nucleosome we needed to find experimental conditions under which nucleosome −2 persisted even under activating conditions, i.e. when the affinity of Pho4 for UAS_p2 was high. Such conditions are available in a strain in which UAS_p1 is deleted (IH12). In this strain, phosphate starvation does not lead to nucleosome disruption at the *PHO5* promtoer (Fascher *et al.*, 1993). When we tested Pho4 binding to UAS_p2 in this strain, we found that there was virtually no binding at activating conditions (Figure 4, lane 2). This experiment confirms that the nucleosome prevents Pho4 from interacting with UAS_p2, and that binding of Pho4 can only occur when the nucleosome is disrupted, which in turn requires interaction of Pho4 with UAS_p1.

High Pho4 levels overcome the protection of the PHO5 UAS_p2 by the nucleosome

As shown above, in the absence of UAS_p1, Pho4 cannot directly gain access to the remaining UAS_p2 because it is present within a nucleosome. To investigate if there is an absolute requirement of UAS_p1 for the chromatin modulation, we decided to overexpress the Pho4 protein in a strain lacking UAS_p1, since increasing Pho4 levels in a wild-type strain leads to a constitutively open promoter (Figure 5A, lanes 1–4) as also shown previously (Fascher *et al.*, 1990). When we overexpressed Pho4 in IH12, the strain lacking UAS_p1, the *Cla*I site was protected at high phosphate but accessible at low phosphate conditions, indicating disruption of nucleosome −2 under inducing conditions (Figure 5A, lanes 5–8). The chromatin change was not confined to this nucleosome as shown by DNase I digestion (Figure 5B). Under conditions of phosphate starvation, the chromatin structure at the *PHO5* promoter changes with the disruption of four positioned nucleosomes in very much the same way as is typical for the wild-type promoter at normal Pho4 dosage. These

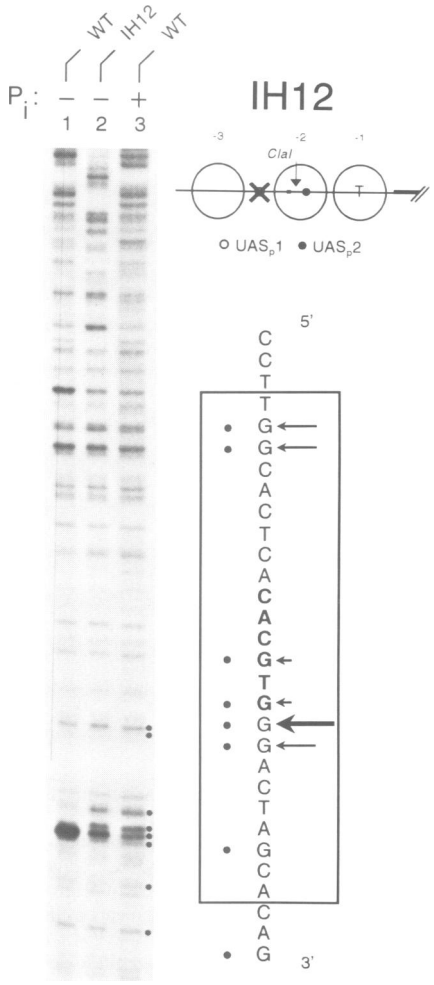

Fig. 4. DMS *in vivo* footprint analysis of the *PHO5* UAS_p2 in IH12. Wild-type (IH2) cells and IH12 cells, grown under the conditions indicated, were treated with DMS and analyzed with primer 1 as described in Materials and methods.

experiments demonstrate that the nucleosome does not provide an absolute barrier to interaction of UAS_p2 with the Pho4 protein.

The remodeling of the chromatin structure in IH12 in response to high expression levels of Pho4 requires interaction of Pho4 with UAS_p2. This is shown by analysis of IH52, a derivative of IH12 in which UAS_p2 was also mutated: no trace of opening was observed after overexpression of Pho4 under high as well as no phosphate conditions (Figure 5A, lanes 9–12).

Pho4 binds to UAS_p1 only when the promoter is active

We also examined Pho4 binding to UAS_p1. *In vitro*, there are again changes in the pattern induced by Pho4 (Figure 6, lanes 1 and 2). The differences are not as striking as for UAS_p2, mostly because Pho4 binding to this UAS element does not cause hyperreactivity of any G residue, but there is clear protection of one G within the binding site, and some protection also of a G residue close by. The fact that there is no Pho4 induced hyperreactivity of G residues as found for UAS_p2 is probably due to their particular location within the Pho4 binding site and the sequence context.

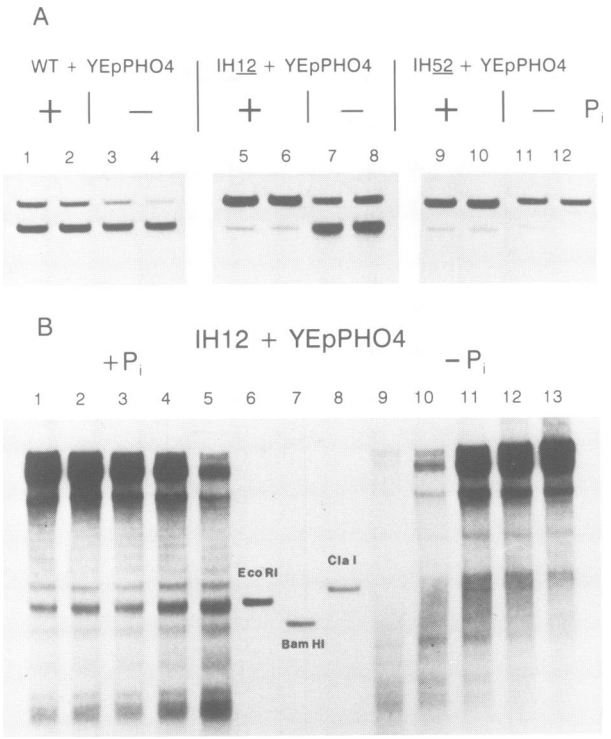

Fig. 5. Overexpression of Pho4 can compensate for the lack of UAS_p1 in the chromatin transition at the *PHO5* promoter. IH12 transformed with the plasmid YEpPHO4 was grown either in high phosphate ($+P_i$) or phosphate-free medium ($-P_i$) and analyzed by digestion with *Cla*I (A) or DNase I (B). The chromatin structure of IH2 (wild-type *PHO5* promoter) and IH52 (lacking both UAS elements at the *PHO5* promoter) transformed with the plasmid YEpPHO4 was examined by *Cla*I digestion for comparison (A). The *Cla*I site is located inside nucleosome −2 (see Figure 1). (**A**) Nuclei containing ~10 µg of DNA were digested for 60 min at 37°C in 200 µl with 50 (lanes 1, 3, 5 and 7) or 200 U of *Cla*I (lanes 2, 4, 6 and 8). In order to monitor cleavage of the *Cla*I site at position −275, DNA was isolated, cleaved with *Hae*III, analyzed in a 1% agarose gel, blotted and hybridized with probe D (Almer *et al.*, 1986). A 1.38 kb *Hae*III fragment is generated if *Cla*I does cleave and a 1.07 kb *Hae*III−*Cla*I fragment if the *Cla*I site is accessible. (**B**) Nuclei were digested for 20 min with 0.5, 1, 2, 4 or 6 U/ml DNase I (lanes 1–5 and 13–9, respectively). DNA was isolated, digested with *Apa*I, separated in a 1.5% agarose gel, blotted and hybridized with probe D. The *Apa*I site is at position −1340 and contains the upstream *Hae*III site used in panel A. Lanes 6–8 contain restriction nuclease double digests of IH12 genomic DNA with *Apa*I+*Eco*RI, *Apa*I+*Bam*HI and *Apa*I+*Cla*I, respectively, to generate marker fragments. The *Eco*RI site replaces UAS_p1 in IH12 and marks the short hypersensitive site which is characteristic of the repressed state (see Figure 1).

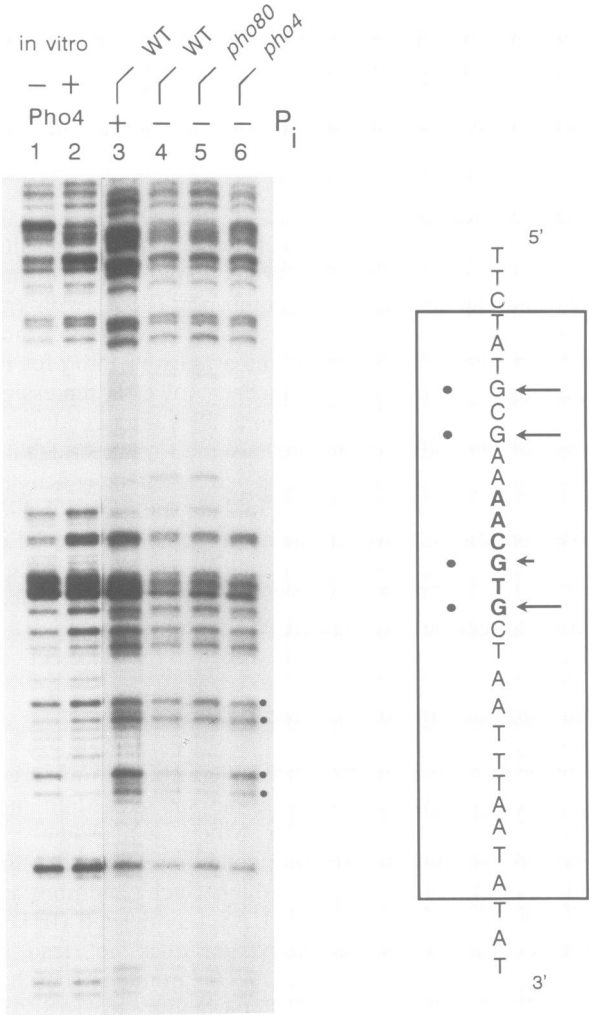

Fig. 6. DMS footprint analysis of Pho4 binding to the *PHO5* UAS_p1 *in vitro* and *in vivo*. A *Bam*HI−*Sal*I fragment from the *PHO5* promoter was incubated with DMS in the absence (lane 1) or in the presence (lane 2) of recombinant Pho4 protein (at the concentration used in lane 5 of Figure 2A) and subsequently analyzed with primer 3 as described in Materials and methods. In lanes 3–6 yeast cells grown in the presence or absence of phosphate as indicated were treated with DMS and analyzed as described in Figure 2 with primer 3 (WT = YS18; *pho80* = YS31; *pho4* = YS22). Protection of a band close to the top of the gel represents binding of Pho4 to UAS_p2. Pho4 does not confer hypersensitivity to any G residue in this strand of UAS_p2 (data not shown).

When UAS_p1 is assayed *in vivo*, the same protection is observed in the pattern from induced cells (Figure 6, lanes 4 and 5) as *in vitro*. This is again due to Pho4 since there is no protection in a *pho4* strain analyzed under the same conditions (Figure 6, lane 6). Binding of Pho4 to UAS_p1 in wild-type cells is not observed, however, for the repressed promoter (lane 3) since the pattern is indistinguishable from the free DNA pattern (lane 1). Because of the lower sensitivity of our DMS assay with UAS_p1, we cannot estimate how much weaker the binding is at high than at low phosphate conditions. Nevertheless, this finding is in agreement with results described above which show that the binding affinity of Pho4 for its target site is regulated by the phosphate level.

Discussion

Occupancy of Pho4 binding sites in vivo

Using DMS footprinting, we have shown that binding of Pho4 to either UAS element in the *PHO5* promoter is detected only when the promoter is activated. One possible scenario had been that Pho4 would be constitutively bound to UAS_p1 in the hypersensitive site, but this is clearly not the case. We also know that binding of Pho4 is not responsible for creating the short nucleosome-free region in the repressed promoter, because the repressed chromatin structure is identical in a *pho4* strain (Fascher *et al.*, 1990).

The DNA binding affinity of Pho4 is substantially increased by phosphate starvation. Binding to UAS_p1 is only observed when the cells are grown in medium lacking phosphate. Similarly, there is only a small amount of detectable binding to UAS_p2, which has a higher affinity

for Pho4 *in vitro* (Vogel *et al.*, 1989), when this site was moved into the nucleosome-free region. Recent work by Kaffman *et al.* (1994) has shown that Pho4 is phosphorylated under repressed conditions by a cyclin–CDK complex composed of Pho80 and Pho85. It seems likely, therefore, that the binding affinity of Pho4 is regulated by phosphorylation.

Our evidence shows that even when the affinity of Pho4 is increased by phosphate starvation, it can bind to UAS_p2 only when this site is in a nucleosome-free region. When it is present in its normal location within nucleosome −2, interaction with Pho4 is prevented by the nucleosome. This conclusion is based on experiments in which a promoter derivative lacking UAS_p1 was used to prevent disruption of nucleosome −2, since we have previously shown that the disruption requires the presence of UAS_p1 (Fascher *et al.*, 1993). Lack of binding of Pho4 to UAS_p2 in a promoter lacking UAS_p1 gives further support to the concept that a vital function of UAS_p1 is to trigger disruption of the adjacent nucleosome.

Nucleosomal inhibition of Pho4 binding is also demonstrated in the accompanying paper (Svaren *et al.*, 1994). Pho4 derivatives which lack an activation domain can bind to UAS_p1 but not UAS_p2 in the native *PHO5* promoter. When UAS_p2 is present in the hypersensitive site, however, the truncated derivatives can then bind to UAS_p2 (Svaren *et al.*, 1994).

Cpf1 and the phosphatase regulon

UAS_p2 can potentially interact not only with Pho4 but also with Cpf1, another member of the basic helix–loop–helix family of proteins. We have, however, never observed any binding of Cpf1 to UAS_p1. Cpf1 has a number of interesting properties. It is relatively abundant, binds to centromeres and contributes to the mitotic stability of chromosomes (Bram and Kornberg, 1987; Cai and Davis, 1990; Mellor *et al.*, 1990). At the same time, Cpf1 binding sites are found in many promoters, and the Cpf1 protein must be required for methionine biosynthesis since *cpf1* strains are auxotrophic for methionine. Under normal conditions there appears to be no functional redundancy between Pho4 and Cpf1. However, overexpression of Pho4 or disruption of the *PHO80* gene suppresses methionine auxotrophy of a *cpf1* strain (O'Connell and Baker, 1992). The reverse is not the case: Cpf1 overexpression does not suppress the phenotype of a *pho4* mutant (O'Connell and Baker, 1992). This may be due to the fact that Cpf1 does not seem to be a conventional transcriptional regulator since lexA–Cpf1 fusion proteins do not induce a reporter gene with a lexA binding site (Thomas *et al.*, 1992).

An important corollary of these findings is that nucleosome −2 protects UAS_p2 from inappropriate interaction with Cpf1. Pho4 and Cpf1 have quite similar binding specificities (Fisher and Goding, 1992). Our work shows that a nucleosome can help determine which protein of a homologous family binds to a certain element. We have also observed Cpf1 binding to a constitutively nucleosome-free Pho4 binding site in the native *PHO8* promoter. The activity of the *PHO8* promoter is somewhat higher in a *cpf1* strain, and we have evidence that Cpf1 can interfere with the activity of Pho4 at the *PHO8* promoter (S.Barbaric, J.Svaren and W.Hörz, manuscript in preparation).

Cooperativity of nucleosome disruption

The presence of two target sites for Pho4 in the *PHO5* promoter suggests the possibility that they interact cooperatively to activate transcription. Consistent with this possibility, deletion of either UAS results in <10% of wild-type activity (Rudolph and Hinnen, 1987). However, activation by Pho4 is not cooperative when two binding sites are placed upstream of the *CYC1* promoter; activation in this context is proportional to the number of binding sites (Sengstag and Hinnen, 1988). Furthermore, UAS_p1 alone can activate transcription to almost 50% of wild-type if nucleosome −2 is entirely deleted (Straka and Hörz, 1991). Therefore, the two UAS elements may be required not to cooperatively interact with the basal transcription apparatus, but rather to overcome nucleosomal repression of the *PHO5* promoter.

Overexpression of Pho4 derivatives that lack the ability to disrupt nucleosomes leads to occupancy of UAS_p1 but not UAS_p2 (Svaren *et al.*, 1994). This result demonstrates that UAS_p1 is accessible to a transcription factor in the inactive chromatin configuration by virtue of its presence in the short hypersensitive site. Therefore, an increase in the affinity of Pho4 for its targets upon shifting cells to phosphate-free medium would lead to binding of Pho4 to UAS_p1. Multiple mechanisms could be involved in this first step: dissociation of Pho80 (Okada and Toh-e, 1992), dephosphorylation of Pho4 (Kaffman *et al.*, 1994) or interaction with Pho2 (Sengstag and Hinnen, 1988; Brazas and Stillman, 1993). Establishment of binding at UAS_p1 would trigger chromatin disruption, thereby allowing Pho4 to bind to the second site.

Interaction between transcription factors and nucleosomes

Pho4 bound at UAS_p1 probably contacts and destabilizes adjacent nucleosomes, because nucleosomes lacking internal Pho4 sites can be disrupted by binding of Pho4 to a nearby site (Almer *et al.*, 1986; Straka and Hörz, 1991). An alternative, but not incompatible, possibility is that Pho4 can interact with UAS_p2 even while it is incorporated in nucleosome −2. Although nucleosome −2 inhibits binding of Pho4 to UAS_p2, this protection is not absolute because overexpressing Pho4 in a strain that lacks UAS_p1 disrupts the nucleosome structure at the *PHO5* promoter. Interaction between Pho4 and the nucleosomal site must have therefore occurred transiently. Interestingly, even with UAS_p1 deleted the chromatin transition still involves all four nucleosomes, which implies a concerted structural transition affecting a chromatin microdomain rather than individual disruption of each nucleosome by factor attack. It should be kept in mind, however, that although we have shown that nucleosome disruption by Pho4 can occur in the absence of DNA replication (Schmid *et al.*, 1992), the possibility exists that replication may be required for Pho4 to attack a site within a nucleosome under these special circumstances (deletion of UAS_p1 and overexpression of Pho4).

In conclusion, these results have demonstrated that the transition from inactive to active chromatin at the *PHO5* promoter is a finely tuned process that rests on the balance between histone–DNA interactions on the one hand and the binding efficiency of a transcription factor to its binding sites on the other. The latter depends on the

number and affinity of the binding sites, concentration of the transcription factor, and a process that senses the phosphate concentration in the medium and regulates the binding strength of the factor. In the accompanying paper (Svaren *et al.*, 1994) we address the actual mechanism by which Pho4 modulates/interferes with histone–DNA interactions in the nucleosome.

Materials and methods

Yeast strains and media

YS18 (*MAT*α, *his3*-11, *his3*-15, *leu2*-3, *leu2*-112, *ura3*Δ5, can^R) has been described (Sengstag and Hinnen, 1987). YS19 (*pho2*) and YS22 (*pho4*) are both null mutations derived from YS18 by eviction of the respective gene (Sengstag and Hinnen, 1988) and were kindly provided by A.Hinnen. YS31 (*pho80*) was derived from YS18 by disrupting the *PHO80* gene. YS70 is identical to YS18 except that the two UAS elements at the *PHO5* promoter are inverted. The wild-type sequence from position −373 to −341 of the *PHO5* promoter was replaced by the following sequence: CCT**TGGCACTCACACGTGGGACTAG**C-AACGCGT and from position −265 to −237 by GCTTAT**TAAATTA-GCACGTTTTCGCATA**GAACTGAATTC [bases protected by Pho4 in DNase I footprints (Vogel *et al.*, 1989) are in bold]. YS71 (*cpf1*), YS72 (*pho4*) and YS73 (*cpf1*, *pho4*) were constructed from YS70 by eliminating the *PHO4* gene as described (Sengstag and Hinnen, 1988) and/or the *CPF1* gene with the disruption plasmid pMF33 (Mellor *et al.*, 1990) kindly provided by M.Funk. IH12 (formerly called IH2Δ12; Fascher *et al.*, 1993) is a derivative of IH2 (*MAT***a**, *trp1*, *his4*-519, *leu2*-3, *leu2*-112, *ura3*-251, *ura3*-328, *ura3*-373, *ade2*; Rudolph and Hinnen, 1987) with a deletion in the *PHO5* promoter that removes UAS_p1. IH52 was derived from IH12 by mutating the central hexanucleotide of the *PHO5* UAS_p2 from CACGTG to AAGCTT. In some experiments, the strains contained a Pho4 expression plasmid (YEpPho4) (Fascher *et al.*, 1990).

Strains without plasmids were either grown in YPDA (2% peptone, 1% yeast extract, 2% glucose, 100 mg/l adenine), i.e. under conditions of *PHO5* repression, or in phosphate-free medium (Almer *et al.*, 1986) to induce *PHO5*. Strains containing a plasmid were grown under repressing conditions in 0.67% yeast nitrogen base without amino acids (Difco), supplemented with 2% glucose, the necessary amino acids, uracil and adenine, or in phosphate-free medium as described for strains without plasmids.

Yeast transformation

Yeast transformation was by the lithium acetate procedure of Ito *et al.* (1983) and transformants were identified by Southern blotting using appropriate DNA probes.

Isolation of yeast nuclei, nuclease digestion, gel electrophoresis, hybridization and DNA probes

All methods used were described previously (Almer *et al.*, 1986) or are explained in the figure legends. Biodyne B nylon membranes (Pall, Dreieich, Germany) were used for Southern transfer. Probes were labeled by the random primer method (Feinberg and Vogelstein, 1983).

In vivo DMS footprinting

Yeast cells were treated with DMS as described by Giniger *et al.* (1985) with the following modifications. The indicated yeast strains were grown in a 500 ml culture to 2–4 × 10^7 cells/ml. The cells were centrifuged, resuspended in 6 ml of medium and divided into four aliquots of 1.5 ml. Two microliters of DMS were added to each tube and the cells were incubated at room temperature for 9 min (for inactive cells) or 20 min (for active cells). Incubation was terminated by the addition of 40 ml of cold TEN buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 40 mM NaCl).

Yeast DNA was isolated, treated with RNase in 0.6 ml TE for 1 h at 37°C and precipitated by the addition of 24 µl of 5 M NaCl and 0.4 ml isopropanol. The DNA was then resuspended in 0.4 ml, digested with *Eco*RV to reduce its viscosity, precipitated, resuspended in 100 µl of piperidine (diluted 1:10 in water) and then placed in a 90°C water bath for 30 min. The DNA was precipitated with ethanol, lyophilized extensively to remove all traces of piperidine, and redissolved in 200 µl TE for a 500 ml culture of 2 × 10^7 cells/ml.

Primer extension using Taq polymerase

A gel-purified primer was labeled with T4 polynucleotide kinase and then purified from unincorporated label by a 1 ml column of G-50 Sephadex. The following primers were used: 5′-GCCAAGTAAGGTG-ACC (primer 1), 5′-GCCGTATTCAATTAACTC (primer 2) and 5′-GACGTCGTCTATAAAC (primer 3). Approximately 5 µg of DNA (or 3 ng of plasmid DNA that had been treated with DMS *in vitro* for a free DNA control) were mixed with 150 000 c.p.m. of the radiolabeled primer in 50 µl of a buffer containing 10 mM Tris–HCl, pH 8.3, 10 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml gelatin and 0.25 mM of each dNTP. Five units of *Taq* polymerase (Boehringer) were added, and the samples were incubated at 95°C for 2 min followed by 30 cycles of 95°C for 1 min, 45°C for 1 min and 70°C for 2 min.

After completion of the primer extension, 6.6 µl of 1% SDS, 100 mM EDTA, 1 mg/ml proteinase K was added, and the samples were digested for 30 min at 45°C. The DNA was precipitated with the addition of 4 µl of 3 M NaCl and 150 µl of ethanol. Each pellet was washed with 70% ethanol, redissolved in a denaturing gel loading buffer, boiled for 3 min and then loaded on an 8% polyacrylamide sequencing gel.

DMS treatment in vitro

Three nanograms of a *Bam*HI–*Sal*I fragment from the *PHO5* promoter was placed in 3.5 µl of 10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 2 mM DTT, 0.1 mM EDTA, 3.5 mM $MgCl_2$, 10 µg/ml poly(dI–dC) for 10 min at room temperature. Then, 2.1 µl of 1.25% DMS in 10 mM $MgCl_2$, 50 mM sodium cacodylate pH 8.0, 1 mM EDTA was added and left for 3 min at room temperature. The reaction was terminated by the addition of 1.3 µl of 1.5 M sodium acetate, pH 7.0, 1 M β-mercaptoethanol, and then precipitated with ethanol. Where indicated, Pho4 protein, partially purified from the cell lysate of transformed *Escherichia coli* (Vogel *et al.*, 1989) obtained from K.Vogel, was added to a concentration of up to 15 µg/ml in the initial binding reaction. The DMS treated DNA was treated with piperidine as described above.

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