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Activation of the weakly regulated *PHO8* promoter in *S.cerevisiae*: chromatin transition and binding sites for the positive regulatory protein PHO4

Slobodan Barbarić[+], Klaus-D.Fascher[§] and Wolfram Hörz*
Institut für Physiologische Chemie, Universität München, Schillerstraße 44, 8000 München 2, FRG

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ABSTRACT

***PHO8* encodes an alkaline phosphatase in *Saccharomyces cerevisiae* whose transcription is regulated by the phosphate concentration in the medium. This occurs through the action of several positive and negative regulatory proteins, also involved in the regulation of other members of the phosphatase gene family. A central role is played by *PHO4*, the gene encoding a DNA binding regulatory protein. Digestion experiments with DNaseI, micrococcal nuclease and 20 different restriction nucleases show that under conditions of *PHO8* repression, there is a highly ordered chromatin structure at the promoter consisting of three hypersensitive regions, approximately 820 to 690, 540 to 510, and 230 to 160 bp upstream of the initiation codon. These hypersensitive sites are surrounded by DNA organized in nucleosomes. Gel shift analysis and *in vitro* footprinting revealed the presence of two PHO4 binding sites at the *PHO8* promoter: a low affinity site at −728 and a high affinity site at −532. Each one is located within a hypersensitive site. Upon derepression of *PHO8*, the chromatin structure changes significantly: The two upstream hypersensitive sites containing the PHO4 binding sites merge, resulting in a long region of hypersensitivity. This transition is *PHO4* dependent. However, not all of the promoter becomes nucleosome free. Instead, as a novel feature, regions of intermediate accessibility are generated upstream and downstream of the third hypersensitive site, the latter region encompassing the TATA-box. The available data fit best into a concept that these regions are organized in unstable or partly unfolded nucleosomes.**

INTRODUCTION

Much progress has been made over the past years in elucidating the mechanism of gene regulation by transcriptional enhancer elements (for reviews see refs. 1,2). There is universal agreement that these enhancers, which are often located at some distance from the RNA initiation site, bind regulatory proteins which can then act as gene activators and ultimately increase the frequency of initiation by RNA polymerase at proximal promoter elements. In these models it is usually not taken into account that the natural substrate of these processes is not free DNA but instead chromatin.

We have been interested for some time in the role that the chromatin structure plays in gene expression and have chosen yeast as an experimental system to address this question. The gene we have focussed our main attention on is the *PHO5* gene, the structural gene for a strongly regulated acid phosphatase in *S. cerevisiae* (3). We have been able to show that the chromatin structure at the *PHO5* promoter undergoes a massive transition upon induction of the gene (4,5). In high phosphate media, i.e. conditions under which the gene is repressed, there is a short hypersensitive region located about 370 bp upstream of the gene. This hypersensitive region which contains a major upstream activation site (UAS) as determined by promoter mutagenesis (6) is flanked by specifically positioned nucleosomes. Upon induction of the gene by starving the cells for phosphate, two nucleosomes upstream and two nucleosomes downstream of this hypersensitive site are selectively removed. Two positive regulatory proteins, PHO2 and PHO4 are required for *PHO5* activation (7), and we have shown that they are equally indispensable for the chromatin transition (8). PHO2 is a pleiotropic activator which is not only involved in the regulation of members of the phosphatase gene family but also other genes like *HIS4* (9) and *TRP4* (10), while PHO4 appears to be specific to the phosphatase gene family (7). At high phosphate conditions *PHO4* activity is counteracted by action of the negative regulator *PHO80*, and the protein products of the two genes have been suggested to interact directly (11).

We have now extended our investigations to the *PHO8* gene which encodes a weakly regulated alkaline phosphatase in *S.cerevisiae* (12,13). The *PHO8* gene is also regulated by the phosphate concentration in the medium and requires the same regulatory genes as *PHO5* except for the *PHO2* gene, which was proposed not to be involved in *PHO8* regulation (12). Our

* To whom correspondence should be addressed

Present addresses: [+]Laboratory of Biochemistry, Faculty of Biotechnology, Pierottijeva 6, 41000 Zagreb, Yugoslavia and [§]Regierung von Oberfranken, Ludwigstraße 20, 8580 Bayreuth, FRG

approach has been to analyze the role of these proteins in a possible modulation of the chromatin structure at the *PHO8* promoter.

Putative PHO4 binding sites at the *PHO5* promoter have been proposed on the basis of DNA sequence comparisons (6), and two of those, called UAS_p1 and UAS_p2, have been shown to actually bind PHO4 *in vitro* (7). We were interested in further defining the binding specificity of PHO4 and wanted to include the *PHO8* promoter into our analyses, with the ultimate hope of providing a scheme for the mechanism of derepression of the phosphatase gene family by the phosphate starvation signal, including both, the effects of regulatory nonhistone proteins and histone-DNA interactions.

MATERIALS AND METHODS

Yeast strains and growth conditions

YS18 (α, his3-11, his3-15, leu2-3, leu2-112, ura3-Δ5, canR). YS19 (pho2), YS22 (pho4), YS31 (pho80), YS33 (pho4,pho80) are all null mutants derived from YS18 by disruption of the respective gene. YS19 and YS22 were kindly provided by A.Hinnen. Construction of YS18 with the YEpPHO4 plasmid was previously described (8). Strains without plasmids were either grown in YPDA (2% peptone, 1% yeast extract, 2% glucose, 100 mg/l adenine), i.e. under conditions of *PHO8* repression, or in phosphate free medium (5) to induce *PHO8*. Strains containing plasmids were grown under repressed conditions in 0.67% yeast nitrogen base without amino acids (Difco), supplemented with 2% glucose, the necessary amino acids, uracil and adenine or in no phosphate medium as described for strains without plasmids.

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

All methods used were described previously (4,5). Isolated DNA fragments labeled by the random primer method (14) were used as probes.

Gel retardation assays, DNaseI footprinting

PHO4 protein, partially purified from the cell lysate of transformed E.coli (7) obtained from K.Vogel was used in gel retardation and DNaseI footprinting assays, which were performed as described before (7).

RESULTS

The pattern of hypersensitive sites at the *PHO8* promoter changes upon *PHO8* induction

We were interested in determining the organization of the chromatin structure at the *PHO8* promoter and possible changes upon derepression. Since transcription of this gene is controlled by the phosphate concentration in the medium we prepared nuclei from cells that had been grown in high phosphate as well as no phosphate media. The nuclei were digested with DNase I and the cutting pattern at the *PHO8* promoter visualized by indirect endlabeling. The results are shown in Fig.1. It can be seen that in the inactive state, there are several hypersensitive sites at the promoter marked with asterisks which we refer to as HS1 (−820 to −690), HS2 (−540 to −510) and HS3 (−230 to −160), with the coordinates giving the approximate extents of hypersensitivity. Upon derepression of *PHO8*, a distinct reorganization occurs: HS1 and HS2 merge to give one long hypersensitive region which extends slightly beyond HS2 in the downstream direction. Hypersensitivity around HS3 also undergoes some changes such that preferential cleavage occurs more upstream (see Fig.1).

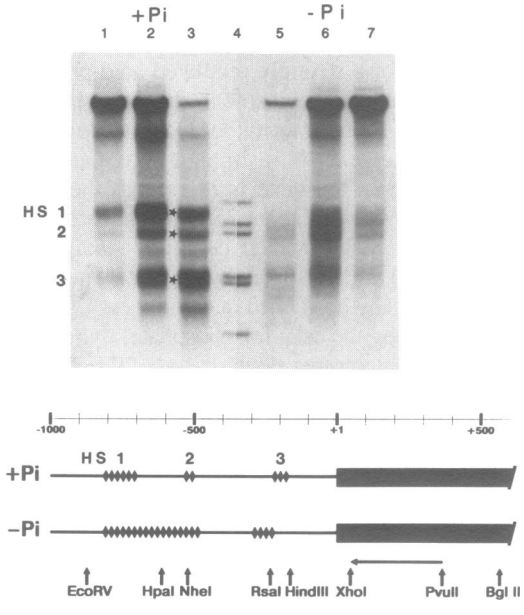

Figure 1. Hypersensitive sites at the *PHO8* promoter. Nuclei isolated from cells grown in either high phosphate (+Pi) or in no phosphate medium (−Pi) were digested for 20 min with 0.5, 1.5 and 3.5 U/ml DNase I (lanes 1 to 3) or with 6.0, 3.0 and 1.0 U/ml DNase I (lanes 5 to 7). DNA was isolated, digested with BglII, separated in a 1.5% agarose gel, blotted and hybridized with a PvuII/XhoI restriction fragment as a probe. The three DNase I hypersensitive sites at high phosphate conditions, HS1, HS2, and HS3, are marked by asterisks in the gel. Lane 4 contains a mixture of restriction nuclease double digests of genomic DNA digested with BglII and EcoRV, HpaI, NheI, RsaI, HindIII or XhoI. The locations of these sites relative to the *PHO8* gene based on the sequence of Kaneko et al. (13) are shown on a map drawn at the bottom, with +1 referring to the *PHO8* initiation codon. The horizontal arrow denotes the probe. Hypersensitive sites at high phosphate (HS1, HS2, and HS3) and no phosphate conditions are shown (◆). The right half of the autoradiogram is a longer exposure of the same blot.

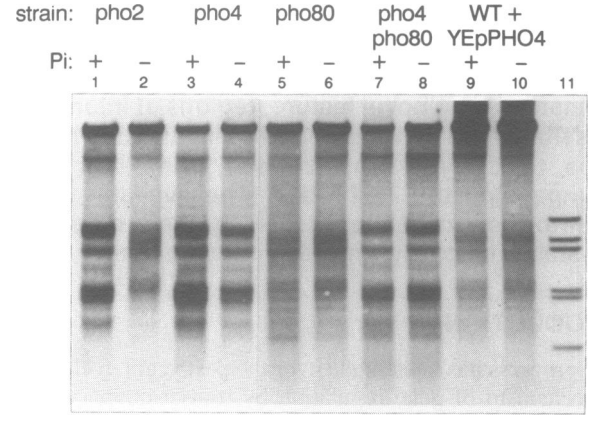

Figure 2. Role of different regulatory genes in the establishment of the chromatin structure at the *PHO8* promoter. Nuclei from different strains were analyzed as in Fig.1. DNaseI concentrations used were in the range from 1 to 5 U/ml. Marker fragments in lane 11 were as in Fig.1. The right half of the autoradiogram is a longer exposure of the same blot.

Modulation of the chromatin structure at the *PHO8* promoter depends on *PHO4* and *PHO80* but not *PHO2*

In order to demonstrate that the chromatin changes that we are observing at the *PHO8* promoter indeed reflect the physiological regulation of *PHO8* we analyzed the chromatin structure at the *PHO8* promoter of strains lacking either *PHO2, PHO4* or *PHO80*.

DNase I analyses show that the transition to the active chromatin structure indeed requires the presence of *PHO4* but not of *PHO2* (Fig.2, compare lanes 1 and 2, which look like wild type, with lanes 3 and 4). As expected, a *pho80* mutant shows active chromatin at both, no and high phosphate conditions (Fig.2, lanes 5,6). The *pho4* mutation is epistatic over the *pho80* mutation since a *pho4/pho80* double mutant looks like a pho4 mutant (Fig.2, lanes 7,8).

We had previously determined that overexpression of *PHO4* in wild type cells leads to a slight constitutivity in *PHO5* expression and an open chromatin configuration also at high phosphate conditions (8). This is also the case for the *PHO8* promoter as shown in lanes 9 and 10 of Fig.2. These results therefore demonstrate that the regulatory proteins PHO4 and PHO80 but not PHO2 are directly involved in the modulation of the chromatin structure at the *PHO8* promoter.

Accessibility to restriction nucleases at the *PHO8* promoter

Digestion of nuclei with restriction nucleases constitutes an alternative approach to monitor accessible regions in chromatin. It has the added benefit of yielding quantitative measurements aside from the fact that from knowledge of the DNA sequence the actual map positions of the restriction sites in question are precisely known.

Nuclei from cells in which *PHO8* was either repressed or induced were digested with a variety of different restriction nucleases and the accessibility of each site determined as described (4). Representative examples of this kind of analysis are shown in Fig.3. In each track, the large fragment signifies lack of cleavage of the site in question while the smaller fragment

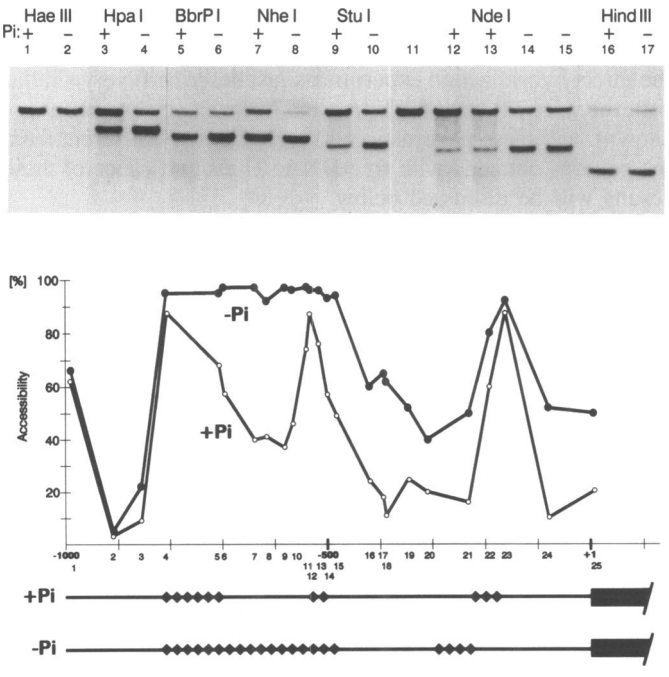

Figure 3. Accessibility of the *PHO8* promoter to restriction nucleases. Nuclei containing approximately 20μg of DNA were isolated from cells grown in either high phosphate (+Pi) or in no phosphate (−Pi) medium and digested in 200μl with restriction nucleases for 60 min at 37°C. DNA was isolated, cleaved with the appropriate enzyme(s), blotted and hybridized to suitable restriction fragments. Results for a number of restriction enzymes are shown at the top with those for NdeI fully documented in lanes 11−15 (11, no enzyme added; 12 and 14, 60 U added; 13 and 15, 200 U added). Secondary digestion was with BglII (+570) and EcoRV (−874), and an XhoI/PvuII (+54 to +370) restriction fragment was used as a probe. For the other enzymes results obtained with only the higher enzyme concentrations are shown. Accessibility for all restriction sites examined is shown underneath, as measured by determining the ratios of the band intensities in the autoradiograms. Restriction sites are designated by the numbers on the horizontal axis of the diagram as follows: EcoRI(1), Sau96I and *HaeIII(2)*, HinfI(3), MboII(4), HinfI(5), MboII(6), BanII or HgiAI(7), *HpaI* and HindII*(8)*, RsaI(9), BanII(10), Sau96I(11), *BbrPI(12)*, *NheI(13)*, CfoI(14), HindII(15), *StuI* and HaeIII*(16)*, HinfI(17), *NdeI(18)*, Sau96I(19), MboII(20), RsaI(21), MboII(22), *HindIII(23)*, HgiAI(24), HinfI(25). Sites underlined correspond to the examples shown at the top. The regions hypersensitive to DNase I at repressed and derepressed conditions deduced from Fig.1 are shown for comparison below.

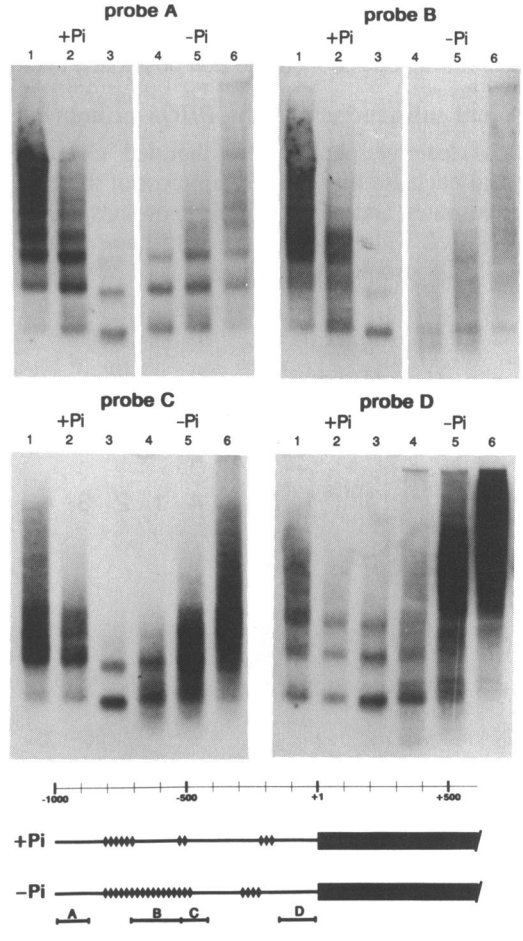

Figure 4. Nucleosomal substructure at the *PHO8* promoter. Nuclei isolated from cells grown in either high phosphate (+Pi) or no phosphate (−Pi) medium were extensively digested with 5, 15, and 40 U micrococcal nuclease per ml (lanes 1 to 3 and 6 to 4, respectively). DNA was isolated, separated in 2% agarose gels, blotted and hybridized with the following restriction fragments shown schematically underneath: (A) EcoRI/EcoRV (−989 to −874); (B) HinfI/NheI (−707 to −516); (C) NheI/StuI (−516 to −421); (D) CfoI/CfoI (−152 to −10). DNaseI hypersensitive regions (♦) at repressed and derepressed conditions are shown for comparison at the bottom.

is generated if the site had been accessible. The ratio of the two fragments was determined and plotted in a promoter map as shown in Fig.3. The pattern matches the results obtained with DNase I quite well. Full accessibility to restriction nucleases at high phosphate conditions was obtained in the regions shown to be hypersensitive. Also the conversion of HS1 and HS2 to a long hypersensitive site after phosphate starvation is paralleled by a change to full accessibility for those restriction sites located between HS1 and HS2.

In contrast to the full accessibility reached further upstream, the region between HS2 and HS3 becomes only about 60% accessible to restriction nucleases. A similar intermediate accessibility is observed at repressed conditions for the region between HS1 and HS2, and it should be noted that the accessibility of the sites between HS2 and HS3 is also significantly higher than what we find for standard nucleosomal arrays (4,15). The question therefore arises if the partial protection observed is due to the presence of nucleosomes or of nonhistone proteins. In order to address this point, we assayed for the presence of nucleosomes by hybridizing micrococcal nuclease digests directly with short probes from the regions in question. As opposed to indirect endlabel experiments, this method only monitors absence or presence of core particles on a given DNA and does not distinguish between positioned and non positioned nucleosomes.

Nucleosomal substructure at the *PHO8* promoter

Active and inactive nuclei were digested extensively with micrococcal nuclease thus converting most of the chromatin to mononucleosomes and short oligonucleosomes. These digests were hybridized without secondary restriction to short probes from the *PHO8* promoter region.

The presence of nucleosomes upstream of HS1 is shown with probe A in Fig.4. Importantly, there is no difference between active and inactive nuclei. This is clearly different when a probe from the region between HS1 and HS2 is used (Fig.4, probe B). Nuclei from cells grown in high phosphate media give a signal corresponding to a core particle. Bands corresponding to di- and trinucleosomes are diffuse, consistent with the presence of hypersensitive sites flanking this nucleosome shown schematically at the bottom. Different results were obtained when cells had been grown at no phosphate conditions. There is only a very weak mononucleosome signal consistent with the removal of the nucleosome located between HS1 and HS2.

When the DNA region downstream of HS2 is analyzed (Fig.4, probe C) a core particle is clearly detected at high phosphate conditions. The picture changes at no phosphate conditions. There is much more smearing, the mononucleosome signal although still present is much weaker. Almost the same kind of pattern is generated with a probe extending from −389 to −214 (not shown). That the chromatin structure around the TATA-box also changes upon derepression is shown with probe D in Fig.4. A clear nucleosomal signal at high phosphate conditions becomes much weaker upon derepression.

When the region between HS2 and HS3 was analyzed by indirect endlabeling with micrococcal nuclease (as opposed to the direct hybridization experiments just described) very similar patterns were obtained for active and for inactive chromatin (not shown), and these patterns in turn were not very different from the patterns obtained with free DNA. The significance of these results will be discussed below.

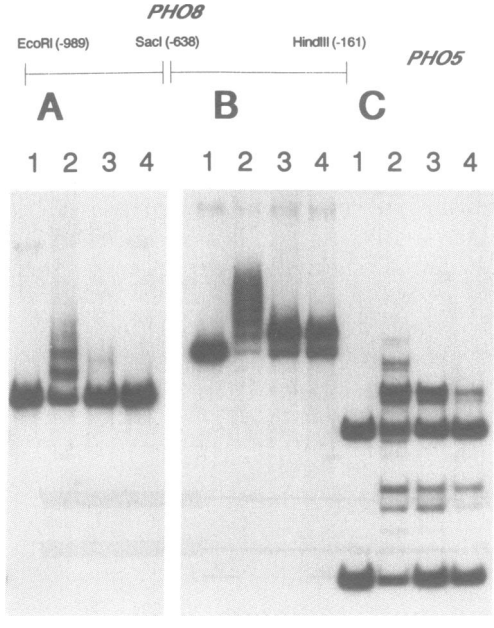

Figure 5. Analysis of PHO4 binding sites at the *PHO8* promoter by gel retardation. An EcoRI/SacI (−989 to −638) fragment (A) and a SacI/HindIII (−638 to −161) fragment (B), both from the *PHO8* promoter, or in (C) a BamHI/ClaI together with a ClaI/BstEII fragment from the *PHO5* promoter (upper and lower fragment, respectively, in lane 1) were incubated without PHO4 protein (lane 1) or with 3μg/ml PHO4 protein in the presence of different concentrations of poly(dI-dC) (10, 100, and 800 μg/ml in lanes 2−4, respectively) and subjected to gel electrophoresis as described in Materials and Methods.

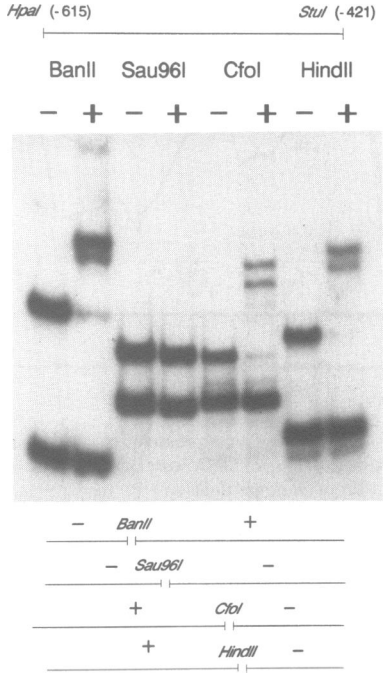

Figure 6. Localization of the high affinity PHO4 binding site at the *PHO8* promoter. A labeled HpaII/StuI (−615 to −421) fragment was digested with different restriction enzymes, each digest incubated without (−) or with (+) PHO4 protein (3μg/ml) in the presence of 10 μg/ml poly(dI-dC) and analyzed as in Fig.5. Shown underneath are the fragments generated by the different nucleases with those that bind PHO4 marked '+' and those which do not marked '−'. See text for actual positions of the restriction sites.

The *PHO8* promoter contains binding sites for PHO4

A search for PHO4 binding sites at the *PHO5* promoter using *in vitro* footprinting has uncovered two sites that were called UAS_p1 and UAS_p2 (7). They show very little actual similarity with each other and differ markedly in their affinity for PHO4. The only common feature is a short sequence (CACGT) which was considered a possible core element of the PHO4 binding site (7). No sequence with strong homology to either of these two sites was found at the *PHO8* promoter. However, one CACGT element was discovered at position −531. In addition, a sequence was found extending from position −732 to −742 which showed high homology (9 out of 11 bp) to a sequence partially overlapping the UAS_p1 site at the *PHO5* promoter. Interestingly, both of these sequences at the *PHO8* promoter are located within hypersensitive chromatin regions which makes them particularly good candidate target sites for an activating protein.

To get an overview of possible PHO4 binding regions we carried out gel retardation experiments using partially purified PHO4 protein expressed in E.coli (7). An EcoRI/SacI (−989 to −638) and a SacI/HindIII (−638 to −161) fragment covering the whole *PHO8* promoter upstream of the TATA-box were examined for PHO4 binding in the presence of different poly(dI-dC) concentrations (Fig.5). In the same experiment, two *PHO5* fragments, one containing the weaker, the other the stronger PHO4 binding site (small and large fragment, respectively, in Fig.5C, lane1). were also examined for comparison. As can be seen from Fig.5, both *PHO8* fragments did bind PHO4. Multiple bands were generated as previously observed in an analysis of PHO4 binding sites at the *PHO5* promoter (7), probably due to PHO4 oligomerization (11). However, retardation of the EcoRI/SacI fragment of the *PHO8* promoter was observed only at the lowest poly(dI-dC) concentration used (compare Fig.5A, lanes 2 and 3). At the same time binding to the other *PHO8* and also to the two *PHO5* fragments persisted and was still observed at the highest poly(dI-dC) concentration (Fig.5B and C). These results indicate that there may be a PHO4 binding site on the EcoRI-SacI fragment that is significantly weaker than the other ones.

To narrow down possible PHO4 binding sites, the two *PHO8* fragments were further cleaved with several enzymes and the subfragments examined by band shift analysis (Fig.6). The strong binding site (Site 2) must be located between BanII (−566) and CfoI (−500), probably around or very close to the Sau96I site at −538, since digestion with this enzyme abolished binding altogether (Fig.6). In analogous experiments it could be demonstrated that the weaker binding site (Site 1) is located between a HphI (−804) and a MboII site (−698) (not shown).

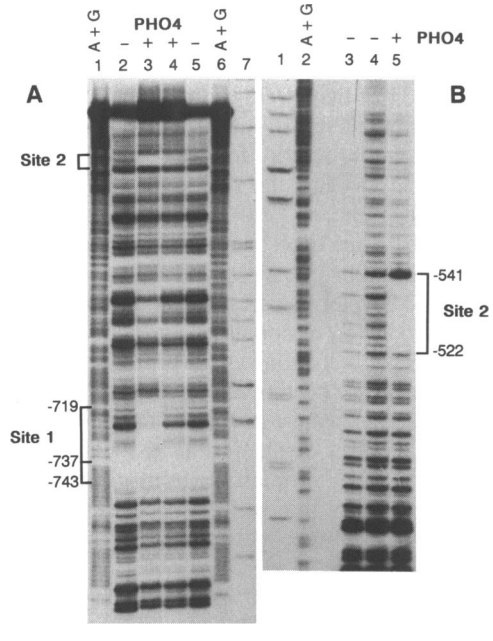

Figure 7. DNaseI footprint analysis of PHO4 binding sites on the *PHO8* promoter. An EcoRV/StuI fragment 5'-endlabeled either at the EcoRV end (A) or the StuI end (B) was used for DNase I footprinting. Poly(dI-dC) concentration in the reaction mixture was 10 μg/ml. **A.** The fragment was digested with 30 U/ml DNaseI in the absence of PHO4 (lanes 2 and 5) or presence of 15 μg/ml (lane 3) or 5 μg/ml (lane 4) PHO4 protein. Partial degradation at purine residues is shown in lanes 1 and 6 and a HpaII digest of pBR322 DNA in lane 7. **B.** Digestion was with 75 U/ml DNaseI (lane 3) or 30 U/ml (lane 4) in the absence of PHO4 or with 30 U/ml DNaseI in the presence of 15 μg/ml PHO4 (lane 5). Partial degradation at purine residues is shown in lane 2 and a HpaII digest of pBR322 DNA in lane 1. The regions protected against DNaseI are marked by brackets on the side.

Determination of PHO4 binding sites by in vitro footprinting experiments with DNaseI

To precisely define interactions of PHO4 with the *PHO8* promoter, DNase I footprinting experiments were performed. For that purpose we used an EcoRV/StuI (−874 to −421) fragment which contained both potential PHO4 sites according to the gel shift experiments. As can be seen from Fig.7A, lane 3, two clearly protected regions, Site 1 and Site 2, stood out. The upstream region, which corresponds to Site 1, extends from position −719 to −737/−743 (we cannot discriminate between −737 and −743 due to the lack of DNaseI cleavage in free DNA in this region). In addition, there is some weakening in band intensity further up in the gel in a region around −670/−680. Binding of PHO4 to that region must be be very weak, however, since it did not lead to a band shift under conditions that readily identified Site 1 as a PHO4 target (see above). Binding of PHO4 to Site 1 is in turn weaker than to Site 2: notice how protection at Site 1 is completely lost upon lowering the PHO4 concentration threefold, while protection at the downstream region (Site 2) is preserved under these conditions (compare lanes 3 and 4 of Fig.7A). To precisely define the boundaries of Site 2, the same fragment, labeled at the other end, was examined (Fig.7B). The protected region can be localized between position −522 and

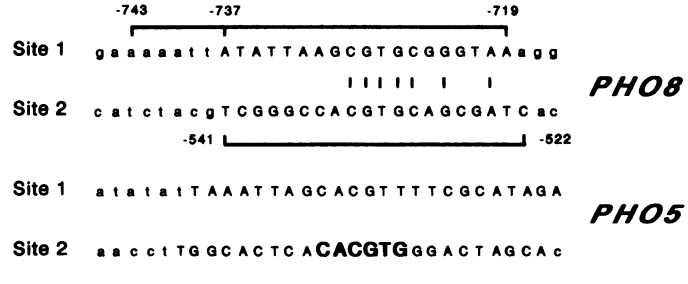

Figure 8. The PHO4 binding sites at the *PHO8* promoter. Site 1 and Site 2 at the *PHO8* promoter is shown and for comparison the two sites from the PHO5 promoter (7). Only the upper strands are shown for all four sites. For both promoters, Site 1 corresponds to the lower affinity site. Sequences protected against DNaseI digestion are indicated by upper case letters. The sequences are aligned with respect to the CACGTG core motif which is shown in bold letters for Site 2 of the *PHO5* promoter.

−541 with distinct enhancement of DNaseI cleavage at position −542 as is often observed in footprinting experiments at the boundary of a binding site. In conclusion these experiments fully confirm the results of the gel shift experiments concerning the location of the two binding sites and also demonstrate that PHO4 has a lower affinity for Site 1 than for Site 2.

DISCUSSION

Chromatin transition at the *PHO8* promoter upon derepression of the gene

Under conditions of *PHO8* repression, there are three hypersensitive sites at the *PHO8* promoter. Two of them correspond to binding sites for the PHO4 protein. The third hypersensitive site at the *PHO8* promoter does not contain a PHO4 binding site. The corresponding DNA region is highly likely to contain elements involved in the functioning of the *PHO8* gene. As yet unidentified regulatory proteins might bind to the DNA, or this region might be involved in the transmission of the activation signal which is likely to emanate at the further upstream sites.

In the repressed promoter, the hypersensitive sites appear to be surrounded by DNA organized in nucleosomes. At least one of these nucleosomes, located between HS1 and HS2, is released upon activation of the promoter to yield a continuous hypersensitive site. A short protected region ('cold spot') within that site is often visible around position −500 to −550 (e.g. see Fig.1, lanes 6 and 7). This most likely reflects binding of the PHO4 protein since we have been able to detect PHO4 interaction with its binding site at −530 also *in vivo*, when cells grown at no phosphate conditions were subjected to *in vivo* footprinting with dimethylsulfate (U. Venter and W.H., manuscript in preparation).

In contrast to the *PHO5* case not all of the *PHO8* promoter becomes hypersensitive when the gene is activated: the region between HS2 and HS3 and also the TATA-box region acquire intermediate accessibility to restriction nucleases and show only moderate DNaseI sensitivity.

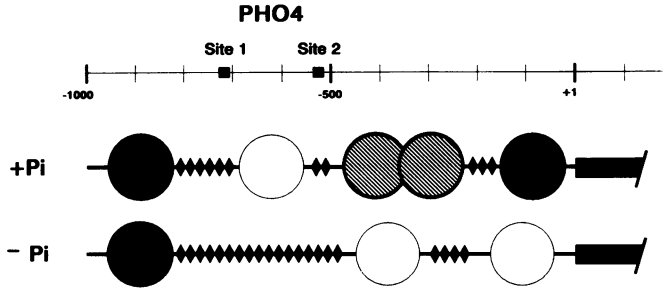

Figure 9. Model for the chromatin structure of the *PHO8* promoter under repressed and derepressed conditions and PHO4 binding sites. PHO4 binding sites deduced from DNaseI footprinting experiments are shown at the top. DNaseI hypersensitive regions (♦) are depicted in the schematic of the chromatin structure at high phosphate (+Pi) or no phosphate (−Pi) conditions underneath, together with stable nucleosomes (solid circles), slightly unstable nucleosomes (hatched circles), and highly labilized nucleosomes (open circles). The three categories are based on differential accessibility of the corresponding chromatin regions to restriction nucleases (Fig.3) and the core DNA assay of Fig.4. The two hatched nucleosomes overlap in the schematic since there is no evidence for their adopting identical positions in all cells.

PHO4 does have a pivotal role in this process of chromatin activation. Permanently inactive chromatin is observed in a *pho4* disruption mutant, while a constitutively open structure is the result of *PHO4* overexpression or of a *pho80* disruption. The PHO80 protein is thought to functionally inactivate PHO4 at high phosphate conditions, possibly by direct protein protein interactions (11).

PHO2 on the other hand does not seem to be involved in the chromatin modulation, the nuclease patterns in a *pho2* and a wild type strain are indistinguishable. This result complements the work of Kaneko et al. (13) who reported that *PHO2* was not required for induction of alkaline phosphatase enzyme activity in response to phosphate starvation.

What is the structural basis of chromatin regions showing intermediate accessibility to nucleases?

One unique feature of the *PHO8* promoter are regions showing intermediate sensitivity to DNaseI, both at high phosphate conditions (between HS1 and HS2) and after derepression of the *PHO8* gene (downstream of HS2 and at the TATA-box region). By the use of restriction nucleases we have been able to obtain more quantitative data. Accessibility is in the range from 40 to 60% for a variety of different nucleases in these regions. The region between HS2 and HS3 shows a significantly elevated accessibility to restriction nucleases also at high phosphate conditions (see Fig.3). It should be noted that these intermediate values are not properties of the nucleases used, since other sites for most of these nucleases located elsewhere at the *PHO8*, *PHO5* (4,5) or *TDH3* (15) promoter are either 5−10% or 90−100% accessible in the same digests of active nuclei. This is shown, for example, by the differential accessibility of the two RsaI sites at −581 and −231 of the *PHO8* promoter at both, high and no phosphate conditions (Fig.3B, site 9 and site 21).

What could be the reason for this unusual intermediate susceptibility? The fact that the region between HS1 and HS2 and likewise the entire *PHO5* promoter acquire 100% accessibility in the same digests argues against the presence of a sizable fraction of cells in the population that have not become derepressed yet. The intermediate accessibility could in principle be due to the association of nonhistone proteins with the DNA. We consider this unlikely, however, because it has been our experience that under the conditions employed here, only histones confer clear protection against restriction nuclease attack. At the same time, we have evidence for an association of histone octamers with the DNA in question from direct hybridization experiments of extensive micrococcal nuclease digests (Fig.4). From its size, the region between HS2 and HS3 could accommodate two regular core particles in the repressed promoter. However, a 20−30% accessibility to restriction nucleases which increases to 50% towards the 5′ edge is not consistent with a stable dinucleosome. Similarly, indirect endlabeling experiments with micrococcal nuclease yield patterns in this region which are not very different from the free DNA patterns, again arguing against a compact dinucleosome.

The organization of the region between HS2 and HS3 after *PHO8* derepression is even more unusual with a 50% accessibility to restriction nucleases throughout most of it. The core particle signal obtained from direct hybridization experiments of micrococcal nuclease digests is much weaker than before derepression, but it is clearly still there. How can these results be reconciled?

Invoking either absence or presence of standard core particles might be too schematic a concept. We have evidence for the *PHO5* promoter that the transition from a nucleosomal to a nonnucleosomal state proceeds in the absence of replication, i.e. on a preexisitng nucleosomal template (W.H., ms. in prep.). This implies that there might be intermediate states in the transition, and for *PHO8* we might be witnessing such intermediate states. Our results would be best explained by the presence of unstable nucleosomes or nucleosomes unfolded to various degrees, with changed properties such as not to confer full protection against restriction nucleases and micrococcal nuclease. That nucleosomes can indeed assume different conformations has been the outcome of several recent studies (16−18), for review see (19).

PHO4 binding sites at the *PHO8* promoter

Establishing a consensus sequence for a PHO4 binding site from previous results with the *PHO5* promoter was complicated by the fact that the two binding sites established by *in vitro* footprinting at the *PHO5* promoter (7) have little in common. They only share a CACGT motif which was proposed to be the core element of a *PHO4* binding site. The two *PHO4* binding sites found at the *PHO8* promoter are also dissimilar and are matched in only 7 of 19 positions (Fig.8). Comparing the two low affinity binding sites at *PHO5* and *PHO8* with each other (Site 1 in both cases) also reveals only little homology (7 out of 19 base pairs). Of the CACGT motif only CGT is conserved in *PHO8* (or CACG if one takes the complementary strand), but there is a homology in 9 out of 11 bp at the 5′-borders of these weak sites extending into the AT-rich region immediately upstream. Comparing the two high affinity sites from the *PHO5* and *PHO8* promoter again yields little overall homology (8 matches out of 15). Both sites do contain, however, the conserved palindrome CACGTG in the core region.

Hayashi and Oshima (20) recently investigated functionally important DNA elements at the *PHO8* promoter by deletion mutagenesis and PHO4 binding experiments. Two regulatory regions contributing to derepression were found, a proximal region (−548 to −502) and a distal one (−704 to −661). From gel shift experiments a PHO4 binding site was assigned to the CACGTG motif contained within this proximal region. This proximal element overlaps precisely with our HS2 region, and our footprinting experiments show indeed that PHO4 binds to the site proposed by Hayashi and Oshima. Their distal element partly overlaps with our HS1 region and is adjacent to our second (weaker) PHO4 binding site (Site 1), which, however, does not generate a band shift in the experiments of Hayashi and Oshima (20). At high PHO4 concentrations we actuallly see very weak protection in our footprint analyses also around −670/−680 (Fig.7A, lane 3). It is conceivable therefore that, *in vivo*, the distal regulatory region documented by Hayashi and Oshima requires the simultaneous presence of Site 1 and an even weaker PHO4 binding site located around −670/−680.

Chromatin structure and regulation of the *PHO8* promoter

PHO8 shares with other phosphatase genes regulated by *PHO4* the presence of a high affinity and a low affinity binding site in the promoter. In *PHO5* (5), as well as *PHO10* and *PHO11* (S.B. and W.H., ms. in prep.) the low affinity site is within a hypersensitive site at high phosphate conditions, while the high affinity site is contained within an adjacent positioned nucleosome. In the case *of PHO8,* the high affinity site is within a very short hypersensitive region, and we do not know yet if it is accessible *in vivo* under high phosphate conditions. In our scheme, transition to an open chromatin configuration is a prerequisite for derepression of these genes (21) and is initiated at the low affinity site. An advantage of using the low affinity site for this purpose might be to ensure that the transition is instigated only at substantial concentrations of active PHO4. Removal of the adjacent nucleosome would then free the high affinity PHO4 binding site leading in effect to cooperativity in the chromatin transition and promoter activation.

Fig.9 shows our model for the chromatin structure at the *PHO8* promoter under repressed as well as derepressed conditions. At high phosphate condtions, the PHO4 binding sites are within the two upstream hypersensitive regions which are separated by a very labile nucleosome (shown in white). Downstream of HS2 there is a partly destabilized nucleosomal arrangement (hatched nucleosomes). A third hypersensitive site (HS3) is located upstream of the TATA-box which is contained within a stable nucleosome (shown in black) under high phosphate conditions. HS3 might recruit additional yet unidentified transcription factors. The presence of both PHO4 binding sites in hypersensitive regions and also the presence of labilized nucleosomes at high phosphate conditions distinguishes *PHO8* from *PHO5* and might be reflected in the high basal activity of *PHO8* as opposed to *PHO5* (22, and unpublished).

Upon derepression of the gene, the nucleosome between HS1 and HS2 is displaced. PHO4 must be instrumental in this transition since disruption of *PHO4* abolishes the chromatin transition. In contrast to the DNA between HS1 and HS2, the region downstream of HS2 does not become completely devoid of nucleosomes at no phosphate conditions, and this seems to hold also for the TATA-box region. Instead unstable or unfolded nucleosomes persist (shown in white). This situation is quite different from the *PHO5* promoter where nucleosomes on the promoter disappear completely upon derepression (5). A reason for the difference might be the lack of a PHO2 binding site and an intranucleosomal PHO4 site in the *PHO8* promoter, since we have shown that *both, PHO2* and *PHO4* contribute to the removal of the corresponding nucleosome in the *PHO5* case (8).

As a novel feature, nucleosomes persist on the *PHO8* promoter, which, however, have unusual structural properties making them less compact. In this way, even with such unstable or unfolded nucleosomes persisting, there might be enough flexibility to allow the upstream sites to interact with the proximal promoter elements. The fact that the *PHO8* unlike the *PHO5* promoter does not open up completely upon starving cells for phosphate would be in keeping with the much lower degree of derepression of *PHO8* (22, and our unpublished results). It is intriguing that in the promoter mutagenesis experiments of Hayashi and Oshima (20) an inhibitory region extending from position −421 to −289 was detected which corresponds precisely to the unstable nucleosome persisting between HS2 and HS3 after derepression of the *PHO8* gene.

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