The EMBO Journal vol.7 no.7 pp.2221 – 2228, 1988

Depletion of histone H4 and nucleosomes activates the *PHO5* gene in *Saccharomyces cerevisiae*

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Communicated by D.I.Meyer

We have previously constructed a yeast strain (UKY403) whose sole histone H4 gene is under control of the *GAL1* promoter. This yeast arrests in G_2 upon glucose treatment as a result of histone H4 depletion. The yeast *PHO5* gene contains phase nucleosomes covering promoter (UAS) sequences in the *PHO5* repressed state and it has been suggested that nucleosomes prevent the binding of positively acting factors to these UAS sequences. Using UKY403 we examined the length of polynucleosomes and nucleosome phasing in the *PHO5* upstream region by the use of micrococcal nuclease and indirect end-labeling. It was found that glucose arrest led to a severe disruption in *PHO5* chromatin structure and that most nucleosomes had their position altered or were lost from the *PHO5* promoter region. Cells undergoing nucleosome depletion synthesized large quantities of accurate *PHO5* transcripts even under repressive, high inorganic phosphate conditions. Histone H4 depletion did not appear to affect the repression or activation of another inducible yeast gene, *CUP1*. Arrest with landmarks in early G_1 (in the cell division cycle mutant *cdc28*) or in various stages of G_2 (in *cdc15, cdc17* and *cdc20*) does not activate *PHO5*; nor does arrest due to chromosome topology changes (in *top2* or the *top1top2* topoisomerase mutants). *cdc14*, which has its arrest landmark at a similar point in the cell cycle as *cdc15*, does derepress *PHO5*. However, since it also leads to derepression of CUP1 it is probably functioning through an independent mechanism. Therefore, our data suggest that nucleosomes regulate *PHO5* transcription.

Key words: histone H4 / *Saccharomyces cerevisiae* / *PH05* regulation

Introduction

There is good evidence that chromatin structure may be an important factor affecting the regulation of higher eukaryotic genes. For example, it has recently been shown that the presence of a nucleosome at a transcription initiation site prevents RNA synthesis *in vitro* (Lorch *et al.*, 1987). Similarly, genes assembled into nucleosomes *in vitro* prior to addition of transcription factors cannot be transcribed. In contrast, exposure of the promoter to transcription factors prior to nucleosome assembly allows gene activity (Workman and Roeder, 1987). There is considerable evidence showing that the genes of higher eukaryotes become more sensitive to DNase I, suggesting a more open conformation, in the active state (Weintraub and Groudine, 1976; Garel and Axel, 1976). Also, these genes often contain nuclease sensitive and hypersensitive sites indicative of the induced state (Wu and Gilbert, 1981; Pederson *et al.*, 1984; Becke *et al.*, 1984; Benezra *et al.*, 1986). These data suggest that chromatin structure changes are correlated with transcription *in vitro* and *in vivo*.

Chromatin structure for most yeast genes shows distinctive differences from that of higher eukaryotes. Most yeast genes are always in a DNase I sensitive conformation (Lohr and Hereford, 1979). There appear to be few cases in which nuclease hypersensitive sites, indicative of nucleosome-free regions, differ before and after yeast gene activation. For example, in the region between the *GAL1* and *GAL10* promoters, the element containing all four UAS regions is equally accessible to nuclease, irrespective of the state of *GAL* gene activation (Lohr, 1984; Proffitt, 1985). This is consistent with other data showing that the hypersensitivity of putative gene control regions in yeast is similar under conditions of either activation or repression (Struhl, 1982; Szent-Georgyi *et al.*, 1987). Also, using an entirely independent approach utilizing regulated GAL-promoter histone gene fusions, we have shown that nucleosome depletion in yeast has little effect on the regulation of *CUP1, GAL1* and *GAL10* promoters (Han *et al.*, 1987; Kim *et al.*, 1988).

The yeast *PHO5* gene represents an exception, in that there are chromatin structure differences between its active and inactive states. This gene encodes an acid phosphatase which is repressed when the concentration of inorganic phosphate (Pi) is high in the medium and is induced under conditions of low Pi (Oshima, 1982). It has been shown that an array of nucleosomes is positioned precisely over the transcription controlling region in the repressed condition (Almer and Horz, 1986). Nucleosomes cover three of the four UAS sequences in the upstream *PHO5* region. Upon induction, nucleosomes in the promoter region are selectively removed uncovering all four UAS sequences. It has been postulated that partial gene activity of *PHO5* may occur by use of only one UAS element, while full activation requires nucleosome removal from all four elements (Almer *et al.*, 1986). If this is indeed the case, and nucleosomes prevent access of UAS sequences or other promoter sequences to constitutively produced positive controlling factors (Koren *et al.*, 1986), one might predict that removal of nucleosomes from the upstream region of *PHO5* by means of histone H4 repression could allow factor binding and therefore activation of *PHO5*. In this paper, we examine the effect of repressing histone H4 synthesis on the chromatin structure and activity of the *PHO5* gene in the yeast strain UKY403. We find that arrest of histone H4 mRNA synthesis, in the presence of *PHO5* repressing conditions (high Pi), leads to nucleosome loss from the *PHO5* promoter and strong activation of the *PHO5* gene.

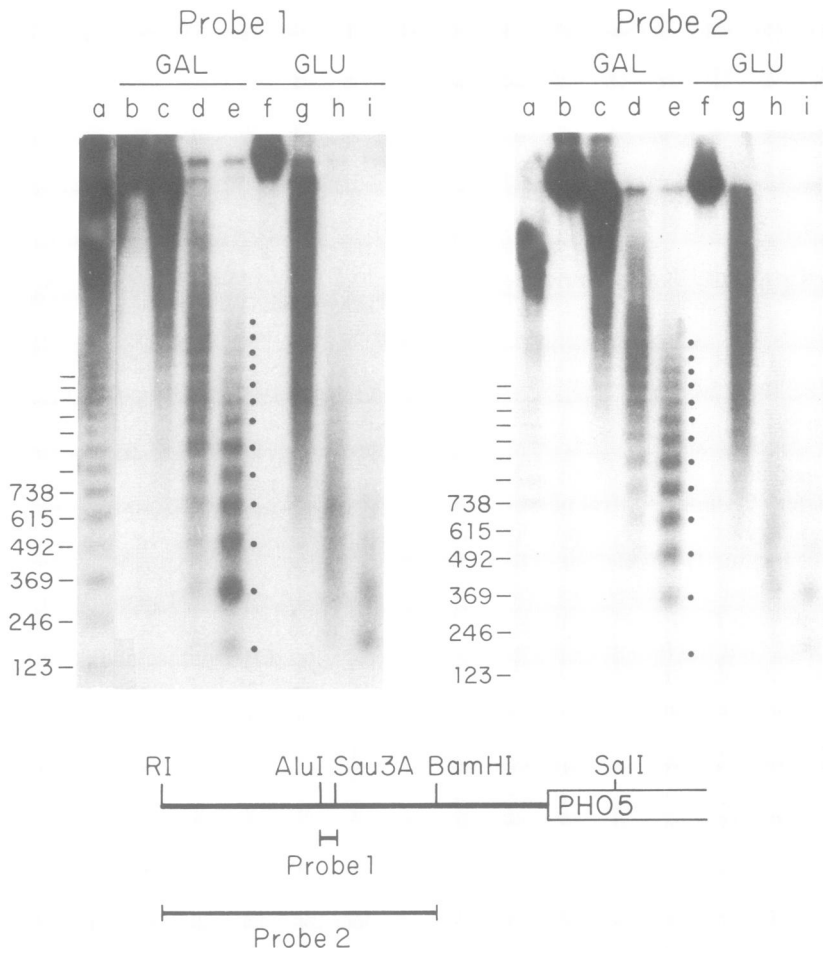

Fig. 1. Polynucleosomes are disrupted in the upstream region of the *PHO5* gene upon glucose arrest. Nuclei from UKY403 grown under the condition of *PHO5* repression (high Pi) were digested with 50 U/ml micrococcal nuclease for various lengths of time. DNA was isolated and separated in a 1.5% agarose gel, blotted and hybridized with ^{32}P-labeled probe 1 (*AluI*−*Sau*3A fragment, 150 bp). Radioactive counts were washed off with boiling water and the nylon filter was rehybridized to ^{32}P-labeled probe 2 (RI−*Bam*HI fragment, 2 kb). **Lanes b−e** display DNAs from cells grown on galactose; **lanes f−i** display DNAs from cells arrested in glucose for 4.5 h. The micrococcal nuclease digestion times for DNAs shown in lanes b, c, d and e (or f, g, h and i) were 0, 4, 8 and 20 min, respectively. **Lane a** contains the mol. wt marker DNA, with multiples of 123 bp, from Bethesda Research Labs (BRL).

Results

Polynucleosomes are disrupted in the PHO5-upstream region upon glucose arrest

We have shown in the yeast strain MHY102 that there is a complete round of DNA replication after H2B synthesis is repressed on glucose (Han *et al.*, 1987). This leads to cells which arrest in G_2 of the yeast cell cycle and whose DNA lacks approximately half the normal complement of nucleosomes. However, there is evidence of abnormal, non-stoichiometric histone/DNA association *in vivo* in histone H2B depleted cells (Han *et al.*, 1987). Since H3 and H4 alone can form nucleosome-like particles in the presence of DNA *in vitro* whereas H2A and H2B cannot (Simon *et al.*, 1978) we decided to study cells which were depleted of histone H4 instead. As shown in the accompanying publication (Kim *et al.*, 1988), the newly constructed yeast strain, UKY403, containing a repressible H4 gene, is similar to MHY102. UKY403 has had both its chromosomal H4 genes (H4-1 and H4-2) deleted. Histone H4 function is provided by an H4-2 gene under control of the *GAL1* promoter on a centromeric plasmid (pUK421). Every phenotypic landmark we have examined in UKY403 is similar to that of MHY102, as would be expected for deletions of essential proteins in the same complex. Shifting UKY403 from galactose to glucose shuts off histone H4 mRNA synthesis in <1 h. Arrest occurs at the same stage in G_2 and with the same inability to segregate replicated DNA to the new bud as in MHY102. Furthermore, an analysis of micrococcal nuclease treated nuclei from both strains shows a similar loss of nucleosomes as measured by micrococcal nuclease-resistant DNA ladders of total chromosomal DNA (Han *et al.*, 1987; Kim *et al.*, 1988).

To examine the spacing of nucleosomes and the length of polynucleosomes in the *PHO5* upstream promoter region, nuclei were isolated from UKY403 cells before and after glucose arrest and treated with varying amounts of micrococcal nuclease. The digested DNAs were then separated on agarose gels, transferred to nylon membrane and hybridized to two different ^{32}P-labeled DNA fragments taken from the upstream *PHO5* region. The experimental results are shown in Figure 1. Using probe 1, we see that the nucleosomal ladder upstream of *PHO5*, obtained prior to glucose arrest, is indicative of long polynucleosomes, consistent with the spacing of at least 13−14 nucleosomes

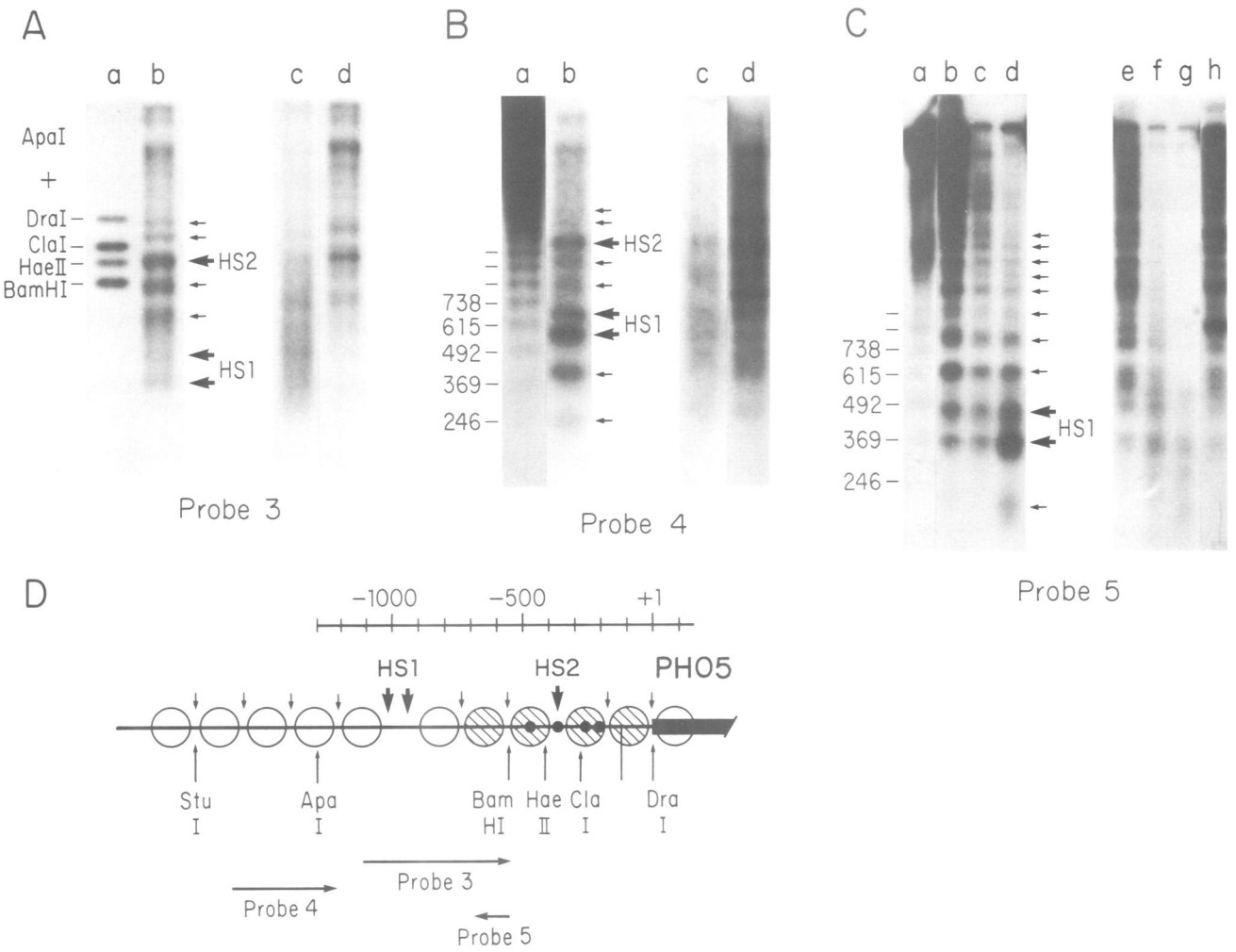

Fig. 2. Mapping nucleosomes by indirect end-labeling in the upstream region of the *PHO5* gene before and after glucose arrest. Micrococcal nuclease treated nuclei and DNAs were obtained as described in the legend to Figure 1. After the micrococcal nuclease cleaved DNAs were isolated, they were cut with various restriction enzymes, separated in agarose gels, blotted and hybridized with probes 3, 4 and 5. (**A**) Nuclei were cleaved for 10 min with micrococcal nuclease. DNA was isolated, recut with *Apa*I and probed with 500 bp DNA fragment probe 3 (*Sau*3A−*Bam*HI fragment, referred to as probe D by Almer and Horz, 1986). **Lane a**: reference markers generated by double restriction enzyme digestion of genomic UKY403 DNA cut with *Apa*I plus each of the second enzymes indicated; **lane b**: DNA from cells grown on galactose; **lane c**: DNA from 4.5 h glucose arrested cells; **lane d**: genomic DNA control. (**B**) Nuclei were digested with micrococcal nuclease for 10 min and treated as in (A). DNAs were cut with *Stu*I and probed with probe 4 (*Cla*I−*Sau*3A fragment, about 300 bp). **Lane a** contains mol. wt marker 123 bp ladder DNA (BRL); **lane b:** DNA from cells grown on galactose; **lane c**: DNA from glucose arrested cells; **lane d**: genomic DNA control. (**C**) DNAs were isolated from micrococcal nuclease treated nuclei, cut with *Bam*HI and probed with probe 5 (*Nde*I−*Bam*HI fragment, 120 bp, referred to as probe F by Almer and Horz, 1986). **Lane a:** 123 bp mol. wt marker DNA; **lanes b−d**: DNA from cells grown on galactose whose nuclei were cleaved with micrococcal nuclease for 4, 10 and 20 min respectively; **lanes e−g**: DNAs from glucose arrested cells whose nuclei were treated with micrococcal nuclease for 4, 10 and 20 min. Dark arrows refer to hypersensitive sites I and II previously described by Almer and Horz (1986). Their position was determined by distance from the restriction site used for indirect end-labeling. Lighter arrows refer to positions cleaved between nucleosomes as determined by Almer and Horz (1986) and by data in this figure. (**D**) The chromatin structure of the *PHO5* upstream region containing hypersensitive sites (HS), positioned nucleosomes and UAS elements (indicated by solid circles). Only five nucleosomes upstream of HS1 are indicated although probe 5 detects approximately eight contiguous nucleosomes upstream of HS1 (Figure 2C). Nucleosomes described as lost by Almer *et al.* (1986) during *PHO5* activation by low Pi are hatched. The location of probes 3, 4 and 5 are also indicated.

~165 bp apart. This arrangement is similar to that described for wild-type yeast (Lohr *et al.*, 1977). However, after glucose arrest for 4.5 h the pattern changes dramatically. We see greatly increased degradation of chromosomal DNA by micrococcal nuclease. Not only are the nuclease-resistant ladders much shorter (indicative of 3−4 nucleosomes), but at all nuclease concentrations there is evidence of altered nuclease sensitivity as shown by the increased background between bands (lanes h and i). Similar results are seen with probe 2. As described previously, arrest for long periods of time by other means does not lead to nucleosome loss (Han *et al.*, 1987). We conclude that polynucleosome length and nucleosome spacing in the *PHO5* upstream region are greatly disrupted upon glucose arrest in a similar manner to that of total chromatin reported previously (Han *et al.*, 1987; Kim *et al.*, 1988). We also conclude that, upon glucose arrest, nucleosomes are most likely lost from both chromatids in G_2, since a conservative assortment leaving all the nucleosomes on one strand would result in a similar pattern to that seen prior to glucose treatment. This is not consistent with the severe disruption in polynucleosome length evident in Figure 1.

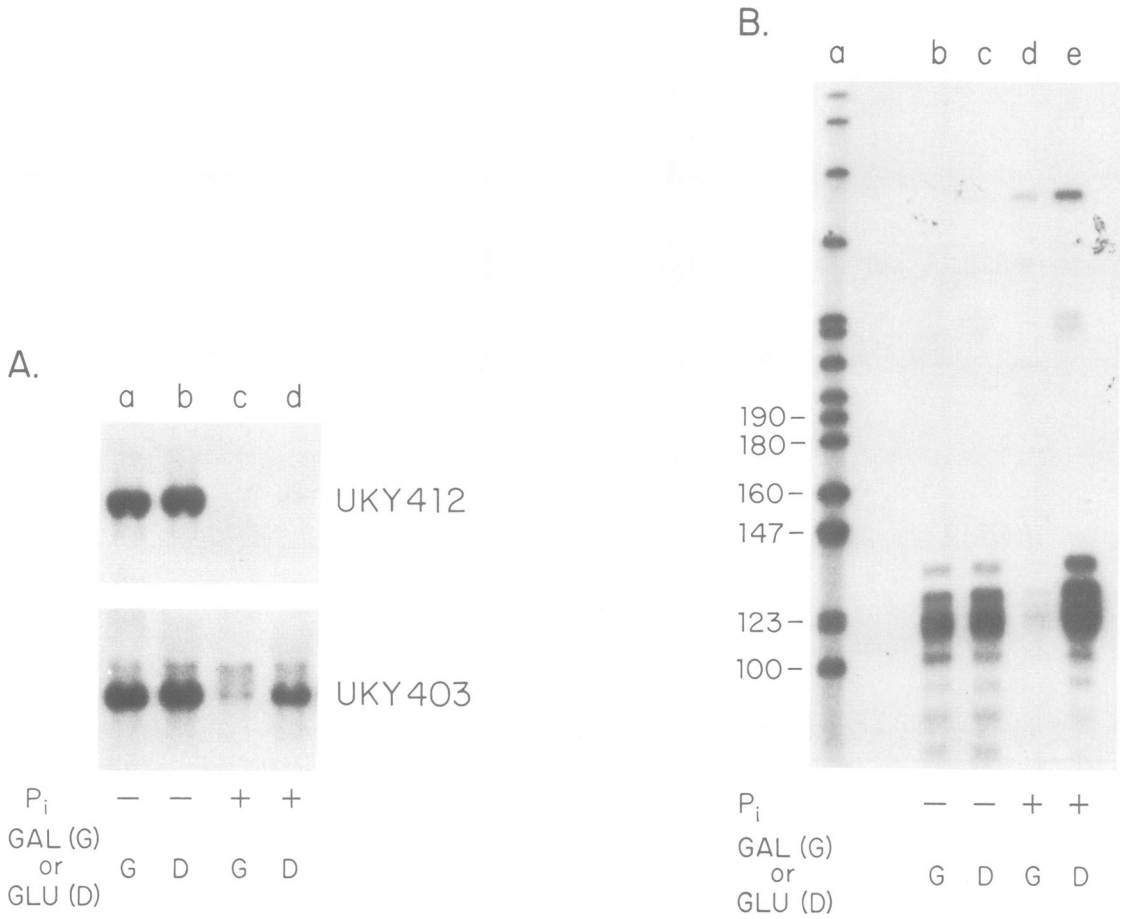

Fig. 3. Derepression of *PHO5* transcription in glucose arrested UKY403 cells. (**A**) Northern blot illustrating the synthesis of *PHO5* mRNA in UKY412 and UKY403 yeast strains. The two strains are isogenic containing disrupted H4-1 and H4-2 chromosomal genes. They differ in that UKY412 contains an episomal histone H4-2 gene attached to its own promoter on plasmid pUK499. UKY403 contains an H4-2 gene under the control of the *GAL1* promoter (Kim *et al.*, 1988). Cells grown in high Pi galactose based medium were then transferred and incubated further for 6 h in four different media: **lane a**, low Pi galactose medium; **lane b**, low Pi glucose medium; **lane c**, high Pi galactose medium; **lane d**, high Pi glucose medium. RNAs were isolated and separated by electrophoresis in 1% agarose (Han *et al.*, 1987). The RNAs were blotted and probed with 625 bp *Bam*HI–*Sal*I fragment labeled with ^{32}P (Meyhack *et al.*, 1982). Under conditions in which the *PHO5* gene is normally repressed (lane d), *PHO5* is seen to be activated as a result of glucose arrest and histone H4 depletion in UKY403 but not in UKY412 whose H4 gene is not under *GAL* control. (**B**) The transcription start sites of *PHO5* transcripts activated by histone H4 depletion appear normal. RNAs were isolated from UKY403 grown in galactose containing high Pi and transferred to the media described above. **Lanes b–e** display RNAs isolated from cells in growth conditions as described in lanes a–d (Figure 3A) and as shown in the figure. S1 nuclease mapping was done with a ^{32}P-labeled 350 bp *Cla*I–*Sal*I fragment. **Lane a** displays the mol. wt marker DNA generated by cutting PBR322 plasmid DNA with *Mps*I.

Identification of specific nucleosomes lost in the PHO5 promoter region upon glucose arrest

The position of individual nucleosomes has been precisely mapped in the *PHO5* upstream promoter region under conditions of gene activation and repression (Almer and Horz, 1986; Almer *et al.*, 1986). It was found that there is a specific pattern of nucleosome placement in the repressed *PHO5* promoter. This pattern, including the presence of nuclease hypersensitive (HS) sites, is shown in Figure 2. Upon induction by low Pi, the four nucleosomes shown hatched are removed, leaving all four UAS exposed in the promoter region. We wished to determine whether blocking H4 mRNA synthesis disrupts the individual nucleosomes positioned on the *PHO5* upstream sequence. UKY403 cells were examined before and after glucose arrest under conditions of PHO5 repression. Chromatin of the *PHO5* promoter region was analyzed by micrococcal nuclease treatment and subsequent mapping of the nuclease cuts by indirect end-labeling as described (Almer *et al.*, 1986). Nuclei from UKY403 cells were treated with micrococcal nuclease. The nuclease resistant DNAs were purified and cut with a restriction enzyme. These DNA samples were subjected to Southern blot analysis with specific probes from the *PHO5* upstream region. As shown in Figure 2A (lane b), B (lane b) and C (lane d), the use of different DNA probes for the *PHO5* upstream region allowed us to obtain a map of nuclease sensitive and hypersensitive sites defining nucleosome position prior to glucose arrest. The pattern we see is similar to that described by Almer and Horz (1986) for the upstream region of the repressed *PHO5* gene. Upon glucose arrest, the DNA bands representing nucleosome-protected sequences are largely, if not completely, lost (Figure 2A, lane c; B, lane c; and C, lanes e–f). The new, more diffuse bands which appear upon arrest may represent preferential cutting sites found in naked DNA (e.g. compare Figure 2A, lanes c and d). We conclude that glucose arrest, in the presence of repressive conditions (high Pi), results in the loss of phased nucleosomes over the *PHO5* upstream region.

PHO5 transcription is activated when histone H4 synthesis is repressed

We next asked whether regulation of *PHO5* was affected by histone H4 and nucleosome depletion. A control strain UKY412 was utilized which is isogenic to UKY403, except that its episomal H4-2 gene (in plasmid pUK499) is under control of its own wild-type promoter instead of the *GAL1* promoter in UKY403. As expected, this yeast strain does not arrest on glucose medium. UKY412 cells were grown logarithmically in galactose containing a high Pi concentration. This medium should have no effect on histone depletion and will repress *PHO5* transcription. Cells were then pelleted and transferred to the four different media shown in Figure 3A. These are low Pi media containing either galactose or glucose, and high Pi media containing galactose or glucose. RNA was isolated from logarithmically growing cells and probed by Northern blot hybridization to assay for expression of *PHO5*. To avoid possible cross hybridization of the PHO5 probe with *PHO3* coding sequences, a 625 bp *SalI*−*Bam*HI DNA fragment was used which includes the sequence coding for the 5′-untranslated end of the *PHO5* transcript (Meyhack *et al.*, 1982).

As shown in Figure 3A, low Pi activates *PHO5* transcription in UKY412 to high levels on either galactose or glucose (lanes a and b). Similarly the *PHO5* gene is repressed in high Pi in either carbon source (lanes c and d). A similar analysis of UKY403 cells shows activation of *PHO5* transcription in low Pi in either galactose or glucose (lanes a and b). Activation of *PHO5* appears unaffected by nucleosome depletion. Under conditions of high Pi, in galactose (lane c), there is repression of *PHO5*. This is expected, since nucleosome structure appears normal under these conditions. In contrast, in high Pi on glucose (lane d), conditions which deplete nucleosomes and should repress *PHO5*, we see activation of the *PHO5* gene. We conclude that histone H4 and nucleosome depletion leads to *PHO5* activation in the presence of high Pi repressive conditions.

We next wished to compare transcription start sites for *PHO5* transcripts produced after normal (low Pi) induction as opposed to activation through histone H4 loss. Therefore, S1 analysis was done by the methods described (Berk and Sharp, 1977) using a ^{32}P-labeled *Cla*I−*Sal*I 350 bp DNA fragment in probe excess. The hybridized complexes were then treated with S1 nuclease, denatured and electrophoresed on polyacrylamide as shown in Figure 3B. We find that the transcription start sites are similar whether induction occurs through nucleosome depletion or low Pi levels. The multiple start sites observed are also the same ones described previously by other investigators (Nakao *et al.*, 1986; Rudolph and Hinnen, 1987). This suggests that transcription initiation is relatively normal when induction of *PHO5* occurs through nucleosome depletion.

PHO5 transcription in cells arrested by means other than nucleosome depletion

Cells, arrested in G_2 by nucleosome depletion, activate the *PHO5* gene. Could this be a response to arrest in any stage of the cell cycle or to any change in chromosome structure? To address this question, we examined *PHO5* transcription in a variety of temperature sensitive (ts), cell division cycle (*cdc*) yeast mutants that arrest synchronously with landmarks in G_1 or G_2 of the cell cycle, or whose lethality is due to an event occurring during G_2. *PHO5* transcription was examined in the following mutants: *cdc28*, which arrests due to a defect in a kinase preventing DNA synthesis (DS); *cdc17* and *cdc20* whose diagnostic landmark occurs after DNA replication but before the medial stage of nuclear division (mND); *cdc14* and *cdc15* whose landmark is between medial (mND) and late (lND) nuclear division (Pringle and Hartwell, 1981); and the *top2* topoisomerase mutant which does not arrest synchronously, but whose lethal defect or execution point appears to occur during early mitosis (Holm *et al.*, 1985).

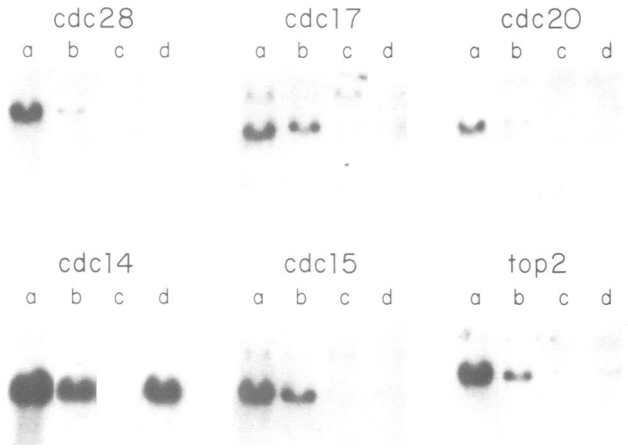

Fig. 4. *PHO5* transcription in temperature sensitive mutations arresting due to defects at different stages in the yeast cell cycle. Strains that arrest with landmarks in early G_1 (*cdc28*), early G_2 (*cdc17* and *cdc20*), and late G_2 (*cdc14* and *cdc15*) were chosen. The defect resulting in lethality of *top2* is believed to occur in early mitosis. The *top2* mutant does not arrest synchronously at the non-permissive temperature (Holm *et al.*, 1985). Each of the six strains were treated for 6 h under four different conditions: **lane a**, 23°C (permissive temperature), low Pi; **lane b**, 37°C (non-permissive temperature), low Pi; **lane c**, 23°C, high Pi; **lane d**, 37°C, high Pi. RNA was isolated and the Northern blot analysis was performed as in Figure 3. Of these mutations *cdc14* causes *PHO5* activation at the non-permissive temperature.

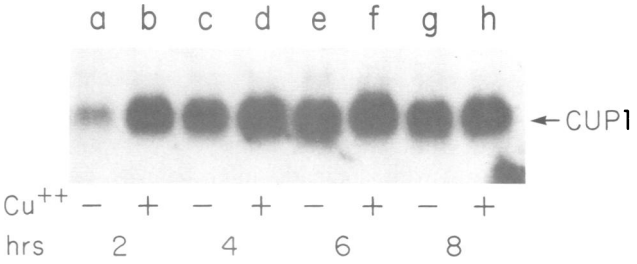

Fig. 5. *cdc14* also derepresses *CUP1* transcription at the non-permissive temperature. Northern blot analysis of *cdc14-1* RNAs isolated from cells grown for the periods of time shown at 37°C in the presence or absence of copper sulphate. Logarithmic cells grown in synthetic medium (SD) were shifted from 23 to 37°C. At the various times indicated, copper sulphate was added to one of two culture aliquots to 1 mM, and the samples were incubated further for 30 min. RNA isolation and Northern analysis were done as described (Han *et al.*, 1987). The 1.25 kb *Sau*3A fragment of pJW6 used as the probe for *CUP1* mRNA was described previously (Han *et al.*, 1987).

Cells were grown in high Pi medium and incubated for 6 h either at permissive temperature (23°C) or non-permissive temperature (37°C). RNA was isolated from each of these strains for Nothern blot analysis with *PHO5* ^{32}P-labeled DNA probe as described in Materials and methods.

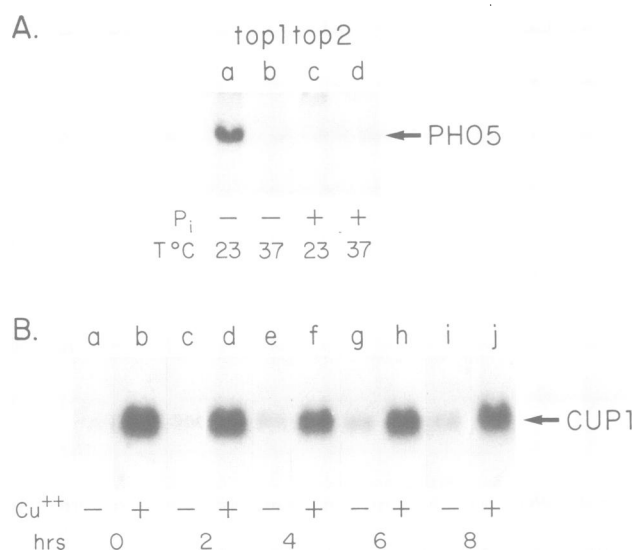

Fig. 6. Comparison of *PHO5* and *CUP1* transcription in a *top1top2* mutant strain. (**A**) Northern blot showing *PHO5* transcription in the *top1top2* mutant strain RS192. Cells were treated for 8 h under the four growth conditions described in Figure 4. **Lanes a** and **b**, cells in low Pi at 23 (permissive) and at 37°C (non-permissive) respectively: **lanes c** and **d**, cells in high Pi at 23 and 37°C, respectively. RNA isolation and Northern blot analysis were done as described in Figure 4. It is evident that no *PHO5* activation is seen under normal activating conditions (low Pi) at the non-permissive temperature of the *top1top2* mutant. (**B**) Northern blot showing normal *CUP1* regulation in the *top1top2* mutant at various times after arrest at the non-permissive temperature.

The results are shown in Figure 4. Lanes a and b represent growth in low Pi at 23 and 37°C, respectively. Lanes c and d show growth in high Pi at 23 and 37°C, respectively. Strains that arrest with landmarks in G_1 (*cdc28*), early G_2 (*cdc17, cdc20*) or late G_2 (*cdc15*) show no activation of *PHO5* in high Pi. Neither does arrest due to inactivation by *top2*. However we do see activation of *PHO5* in *cdc14* at the non-permissive temperature.

Histone H4 depletion is specific in its effect on *PHO5* derepression since *CUP1* is not derepressed even after long periods in glucose. To determine whether *cdc14* specifically derepresses *PHO5*, we analyzed the ability of the *CUP1* gene to respond to copper sulphate at the non-restrictive temperature of *cdc14*. Surprisingly, Northern blot analysis, using the *CUP1* DNA probe (Figure 5) shows that bringing *cdc14* to 37°C derepresses *CUP1* after several hours. Control experiments show no effect of temperature on *CUP1* activation in a non-*cdc14* strain (see below, legend to Figure 6). We conclude that the function of the *CDC14* product may involve repression of diverse genes. Therefore, *PHO5* activation is not simply a response to arrest in any part of the cell cycle, nor even to arrest in G_2 or to arrest as a result of changes in chromosome topology. *PHO5* derepression is occurring due to a specific event which appears to be different in nucleosome-depleted cells as opposed to *cdc14* at the non-permissive temperature.

We also examined *PHO5* activation under high Pi conditions in a *top1top2* double mutant (Figure 6A). Since yeast contains topoisomerses I and II which may complement each others functions (Brill *et al.*, 1987), we wished to determine the effect of a complete loss of topoisomerase activity on *PHO5* activation. We see no evidence of UAS activation under conditions of high Pi (lanes c and d) at either 23 or 37°C, confirming that changes in chromosomal topology need not lead to *PHO5* activation. While *PHO5* was activated by low Pi inducing conditions even at the non-permissive temperature in the *top2* mutant (Figure 4) the complete absence of topoisomerase activity, in the *top1top2* mutant at the non-permissive temperature, prevents *PHO5* activation (lane b). This is not due to a general inability of the *top1top2* mutant strain to support transcription and transcriptional activation since other genes are activated to high levels in this genetic background (Brill *et al.*, 1987) and the *CUP1* gene is regulated normally in the presence of copper sulphate even 8 h after bringing the strain to the non-permissive temperature (Figure 6B). We conclude that *PHO5* may be different in its dependence on topoisomerases for its activation.

Discussion

We find that histone H4 depletion leads to greatly increased nuclease sensitivity of chromatin in the region upstream of the *PHO5* gene. Micrococcal nuclease studies showed a decrease in the detected length of polynucleosomes from ~13–14 nucleosomes to 3–4. Indirect end-labeling data showed further that most if not all nucleosomes in the upstream promoter region are removed from their normal position after glucose arrest. We interpret these results to mean that (i) nucleosomes are being depleted from the upstream region of the *PHO5* gene, (ii) nucleosomes have been depleted from both chromosomal homologues in G_2, and (iii) those nucleosomes assembled prior to glucose arrest no longer occupy fixed positions. This could have occurred through a non-conservative mode of nucleosomal segregation during DNA replication in the absence of new nucleosome assembly. Alternatively, the remaining histone octamers present after histone arrest could have re-associated randomly to both chromosomes even if they were initially assorted onto one chromosome in a conservative fashion. We support the former model on the basis of experimental data in other eukaryotes showing that nucleosomal segregation is non-conservative (Cusick *et al.*, 1984).

Analysis of *PHO5* transcription upon H4-depletion shows that the *PHO5* gene is activated to a high level under usual conditions of *PHO5* repression (high Pi). Similar *PHO5* derepression is seen when H2B mRNA synthesis is arrested (data not shown) in the yeast strain, MHY102 (Han *et al.*, 1987). Furthermore, the transcripts have normal start points as determined by S1-nuclease digestion. Could the loss of nucleosomes from the *PHO5* promoter region have *directly* caused the activation of the *PHO5* gene? We believe that this is a strong possibility based on our knowledge of *PHO5* transcriptional regulation. From previous data (Bostian *et al.*, 1980; Toh-e *et al.*, 1981; Oshima, 1982; Lemire *et al.*, 1985) it has been proposed that transcription of the acid phosphatase (*PHO5*) mRNA is mediated by at least two positive-acting proteins (*PHO4* and *PHO2*) and two which are negative acting (*PHO80* and *PHO85*); all are produced constitutively at low levels in the repressed, high Pi condition. Inorganic Pi may act through the mediator *PHO81* gene product. In low Pi, *PHO81* appears to be required for the positively activating function of *PHO4* and/or *PHO2* which

act on a sequence just upstream of the *PHO5* coding region. Therefore, this model suggests a special role for *PHO4* and *PHO2* as positive activators which may act on the UAS sequences of the *PHO5* promoter region.

Almer *et al.* (1986) have found that during *PHO5* repression nucleosomes cover three of the four UAS sequences responsible for gene activation in the region of *PHO5*. All four sites are uncovered during activation. These authors have suggested that basal synthesis of *PHO5* mRNA occurs through the single uncovered UAS sequence under high Pi conditions. However, in low Pi medium, all four UAS sequences may be required for maximal activation by the positively acting proteins. Since nucleosome loss in our experiments leads to *PHO5* transcription in the presence of repressive high Pi conditions, this suggests that the four UAS sequences are now available for interaction with the constitutively produced *PHO4* and/or *PHO2* products. Alternatively, it is possible that uncovering the TATA promoter sequence upon nucleosome depletion leads to *PHO5* gene activation. In either case both our data and that of Almer *et al.* (1986) are consistent with a direct role for nucleosomes in repressing UAS function. It also raises the intriguing possibility that *PHO2* and/or *PHO4* contain an activity which removes the nucleosomes from the *PHO5* promoter thereby allowing *PHO5* activation.

Why *PHO5* activation is prevented at low Pi in the *top1top2* mutant at the non-permissive temperature is, at present, a mystery. While many prokaryotic genes are regulated by superhelical changes (Gellert, 1981; Drilica, 1984), no eukaryotic genes have been found to require topoisomerase for their activity to date (Brill *et al.*, 1987). The *PHO5* gene may represent a new class of eukaryotic gene sensitive to changes in promoter topology. Alternatively, if topoisomerases are necessary for the removal of nucleosomes, the inability of the *top1top2* mutant to activate *PHO5* in low Pi may reflect the repressive character of nucleosomes covering *PHO5* promoter sequences.

The data described above do not rule out the possibility that nucleosome depletion is an *indirect* cause of *PHO5* derepression. However, if this is the case, it is not a general response to cell cycle arrest, to arrest in G_2, nor to changes in chromosome superhelicity. Arrest of (ts) *cdc* yeast with landmarks in early G_1, early G_2 or late G_2 does not induce *PHO5* in the absence of low Pi conditions. Nor does the arrest which occurs due to topoisomerase denaturation, which would be expected to affect chromosomal topology.

It is interesting to note that, like histone H4 depletion in UKY403, *cdc14* induces *PHO5* under non-permissive conditions. However, *PHO5* is not activated at the non-permissive temperature of other *cdc* mutants analyzed. Even *cdc15* which has its diagnostic landmark at a very similar point in the cell cycle as *cdc14* (Pringle and Hartwell, 1981) fails to activate *PHO5* transcription. Surprisingly, while nucleosome depletion leads to *PHO5*, but not *CUP1* derepression, *cdc14* at the non-permissive temperature derepresses both genes. This suggests the intriguing possibility that *CDC14* may code for, or activate, a repressor protein involved in regulation of both *CUP1* and *PHO5*. We conclude that the effect of histone H4 and nucleosome depletion on *PHO5* derepression is a specific one and may reflect the novel use of nucleosomes as negative regulators of *PHO5* activity.

Materials and methods

Yeast strains and media

Yeast Strain List:

Strain	Mutations and Genetic Markers	Source
UKY403	*MATa ade2-101 his3-Δ200 leu2-3, 112 lys-801 trp1-Δ901 ura3-52 GAL+ thr tyr arg4-1 Δh4-1[HIS3+] Δh4-2[LEU2+]/pUK421 (CEN TRP1+ GAL-H4-2+)*	This lab.
UKY412	*MATa ade2-101 his3-Δ200 leu203, 112 lys-801 trp1-Δ901 ura3-52 GAL+ thr tyr arg4-1 Δh4-1[HIS3+] Δh4-2[LEU2+]/pUK499 (CEN URA3+ H4-2+)*	This lab.
A364A	*MATa ade1 ade2 ura1 lys2 tyr1 his7 gal1*	L.H.
185-3-4	cdc28-1(ts) *MATa ade1 ade2 ura1 his7 tyr1 lys2 gal*	SC
7-41	cdc14-1(ts) *MATa ade1 ade2 ura2 his7 tyr1 lys2 gal*	SC
17-17	cdc15-1(ts) *MATa ade1 ade2 ura2 his7 tyr1 lys2 gal*	SC
----	cdc17-1(ts)	C.M.
127	cdc20-1(ts) *MATa ade1 ade2 ura2 his7 tyr1 lys2 gal*	SC
RS191	top2-1(ts) *MATa ade2 ura3-1 his3-11 trp1-1 leu2-3, 112* can	R.S.
RS192	isogenic with RS191, contains also *top1-8[LEU2+]*	R.S.

SC: Yeast Genetic Stock Center, Department of Biophysics and Medical Physics, University of California, Berkeley; L.H.: Lee Hartwell; C.M.: C.McLaughlin; R.S.: R.Sternglanz. All cdc mutations are described by Pringle and Hartwell (1981). Wild-type yeast contains two H4 gene copies (H4-1 and H4-2) (Smith and Andresson, 1983). Both copies have been replaced in UKY403 with selectable marker genes and the strain rescued with the centromeric plasmid, pUK421, containing the H4-2 gene under *GAL1* control (Kim *et al.*, 1988). UKY412 is isogenic with UKY403 except that the plasmid pUK421 is replaced by the plasmid pUK499. pUK499 differs from pUK421 in that the H4 gene is attached to its own wild-type promoter.

The phosphate (Pi) concentrations in unprecipitated YEPD were measured by the method described by Ohnishi and Grall (1978) and were determined to be ~50 mM. Preparation of low Pi media was done as described by Rubin (1973) with the following modifications: 10 g yeast extract and 20 g peptone were dissolved in 300 ml H_2O; 10 ml 1 M $MgSO_4$ and 10 ml concentrated NH_4OH were added to the solution to precipitate inorganic phosphate. After filtration, the eluant was diluted 3-fold. After pH adjustment and autoclaving, 1/10 volume of 20% glucose or galactose was added. To measure the percentage of phosphate removed, a trace amount of radioactive ^{32}P was added to the media before precipitation. Radioactive counts were determined by scintillation counting before and after precipitation. Normally, we found that ~97–99% of free Pi can be removed from the medium by this method. For low Pi media, K_2HPO_4 was added back to the medium after precipitation and adjusted to a concentration of 0.2 mM. For high Pi media, K_2HPO_4 was added to a final concentration of 7.5 mM. For nuclease analysis, YEPD or YEPG (plus 100 μg/ml adenine to each) were used as high Pi media (Almer and Horz, 1986).

Micrococcal nuclease analysis of yeast chromatin

Nuclei were isolated as described (Almer and Horz, 1986), except that the first step of differential centrifugation was repeated once. DNA concentrations of the nuclei samples were measured by the method of Labarka and Paigen (1980). Digestion of nuclei with micrococcal nuclease and DNA purification were as described by Almer and Horz (1986), with the following modifications: a micrococcal nuclease digestion buffer containing 1 M sorbitol, 20 mM Pipes, pH 6.8, 5 mM $CaCl_2$ and 150 mM NaCl was used; the nuclei were resuspended in digestion buffer with a DNA concentration of 100 μg/ml. Micrococcal nuclease was added to 50 U/ml. Southern blot analysis was performed as described (Maniatis *et al.*, 1982). DNA fragments which were derived from the *PHO5* upstream region and used as probes were: probe 1, *Eco*RI–*Bam*HI fragment (~2 kb), isolated from plasmid pBR322(HIS3)/PHO3PHO5 (Meyhack *et al.*, 1982); probes 2–5 were isolated from a plasmid which has the *Cla*I–*Bam*HI (800 bp) subcloned in pBR322 (Almer and Horz, 1986). Probe 2, *Alu*I–*Sau*3A fragment (150 bp); probe 3, *Sau*3A–*Bam*HI fragment (500 bp); probe 4, *Cla*I–*Sau*3A fragment (300 bp); probe 5, *Nde*I–*Bam*HI fragment (120 bp).

The probes were labeled with [α-^{32}P]ATP by the method described (Feinberg and Vogelstein, 1982).

RNA analysis

RNA isolation and Northern analysis were described previously (Han *et al.*, 1987). The DNA probe for the *PHO5* transcript was the *Bam*HI–*Sal*I fragment (625 bp) and was isolated from plasmid pBR322(HIS3)/PHO3PHO5 (Meyhack *et al.*, 1982). S1 analysis of PHO5 transcript was as described by Nakao *et al.* (1986), except the total RNA was used and the S1 digestion was carried at 23°C for 30 min with 100 U S1 nuclease per sample containing 100 μg RNA. The RNAs used for S1 analysis were isolated separately from those used for Northern blot analysis. The low Pi and high Pi media in this experiment were prepared as described by Meyhack *et al.* (1982).

Acknowledgements

The authors are especially thankful to Dr W.Horz for providing *PHO5* probes and to Dr R.Sternglanz for *top1* and *top2* mutant yeast. We are indebted to the Biotechnology and Education Research Program for use of equipment. This work was supported by Public Health Service grants GM31336 and GM23674 from the National Institutes of Health.

References

Almer,A. and Horz,W. (1986) *EMBO J.*, **5**, 2681–2687.
Almer,A., Rudolph,H., Hinnen,A. and Horz,W. (1986) *EMBO J.*, **5**, 2689–2696.
Benezra,R., Cantor,C.R. and Axel,R. (1986) *Cell*, **44**, 697–704.
Becker,P., Renkawitz,R. and Schutz,G. (1984) *EMBO J.*, **3**, 2015–
Berk,A.J. and Sharp,P.A. (1977) *Cell*, **12**, 721–732.
Bostian,K.A., Lemire,J.M., Cannon,L.E. and Halvorson,H.O. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 6541–6545.
Brill,S.J., Dinardo,S., Voelkel-Meiman,K. and Sternglanz,R. (1987) *Nature*, **326**, 414–416.
Cusick,M.E., Depamphilis,M.L. and Wassarman,P.M. (1984) *J. Mol. Biol.*, **178**, 249–271.
Drilica,K. (1984) *Microbiol. Rev.*, **48**, 273–289.
Feinberg,A. and Vogelstein,B. (1983) *Anal. Biochem.*, **132**, 6–13.
Garel,A. and Axel,R. (1977) *Proc. Natl. Acad. Sci. USA*, **73**, 3966–3970.
Gellert,M. (1981) *Annu. Rev. Biochem.*, **50**, 879–910.
Han,M., Chang,M., Kim,U. and Grunstein,M. (1987) *Cell*, **48**, 589–597.
Holm,C., Goto,T., Wang,J.C. and Botstein,S. (1985) *Cell*, **41**, 553–563.
Kim,U., Han,M., Kayne,P. and Grunstein,M. (1988) *EMBO J.*, **7**, 2211–2219.
Koren,R., LeVitre,J. and Bostian,K.A. (1986) *Gene*, **41**, 271–280.
Labarka,C. and Paigen,K. (1980) *Anal. Biochem.*, **102**, 344–352.
Lemire,J.M., Willcocks,T., Halvorson,H.O. and Bostian,K.A. (1985) *Mol. Cell. Biol.*, **5**, 2131–2141.
Lohr,D. (1984) *Nucleic Acids Res.*, **12**, 8457–8474.
Lohr,D. and Hereford,L. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4283–4288.
Lohr,D., Corden,J., Tatchell,K., Kovacic,R.T. and Van Holde,K.E. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 79–83.
Lorch,Y., LaPointe,W. and Kornberg,R.D. (1987) *Cell*, **49**, 203–210.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Nakao,J., Miyanohara,A., Toh-e,A. and Matsubara,K. (1986) *Mol. Cell. Biol.*, **6**, 2613–2623.
Ohnishi,S.T. and Grall,R.S. (1978) *Anal. Biochem.*, **88**, 247–256.
Oshima,Y. (1982) In Strathern,L.N., Jones,E.W. and Broach,J.R. (eds), *The Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 159–180.
Pederson,D.S., Shupe,K. and Gorovsky,M.A. (1984) *Nucleic Acids Res.*, **12**, 8489–8507.
Pringle,J.R. and Hartwell,L.H. (1981) In Strathern,J.N., Jones,E.W. and Broach,J.R. (eds), *The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 97–142.
Proffitt,J.H. (1985) *Mol. Cell. Biol.*, **5**, 1522–1524.
Rubin,G.M. (1974) *Eur. J. Biochem.*, **41**, 197–202.
Rudolph,H. and Hinnen,A. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1340–1344.
Simon,R.H., Camerini-Otero,R.D. and Felsenfeld,G. (1978) *Nucleic Acids Res.*, **5**, 4805–4818.
Smith,M. and Andresson,O.S. (1983) *J. Mol. Biol.*, **169**, 663–690.
Struhl,K. (1982) *Cold Spring Harbor Symp. Quant. Biol.*, **47**, 901–910.
Szent-Gyorgyi,C., Finkelstein,D.B. and Garrard,W.T. (1987) *J. Mol. Biol.*, **193**, 71–80.
Toh-e,A., Inouye,S. and Oshima,Y. (1981) *J. Bacteriol.*, **145**, 221–232.
Weintraub,H. and Groudine,M. (1976) *Science*, **193**, 848–856.
Workman,J.L. and Roeder,R.G. (1987) *Cell*, **51**, 613–622.
Wu,C. and Gilbert,W. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 1577–1580.

Received on March 15, 1988; revised on April 19, 1988