The EMBO Journal vol.7 no.7 pp.2211 – 2219, 1988

Effects of histone H4 depletion on the cell cycle and transcription of *Saccharomyces cerevisiae*

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Communicated by D.I.Meyer

We have constructed a yeast strain (UKY403) in which the sole histone H4 gene is under control of the *GAL1* promoter. This allows the activation of H4 mRNA synthesis on galactose and its repression on glucose. UKY403 cells, pre-synchronized in G_1 with *alpha*-mating factor, have been used to show that glucose treatment results in the loss of approximately half the chromosomal nucleosomes. This depletion is only partially reversible when the H4 gene is reactivated on galactose. It was found that the resultant lethality manifests itself first in S phase, the period of nucleosome assembly, but leads to highly synchronous arrest in G_2 and a virtually complete block in chromosomal segregation. Histone H4-depleted chromatin was analyzed for its efficiency as a template for all three RNA polymerases. Using pulse-labeling, we find no evidence for altered transcription by RNA polymerase I (25S, 18S and 5.8S rRNAs) or RNA polymerase III (5S rRNA, tRNAs). Northern blot analysis was used to measure levels of RNA polymerase II transcripts. There was little effect on the activation or repression of the *CUP1* chelatin gene. While there may be some decrease in the level of certain mRNAs (e.g. *HIS4, ARG4*) other message levels (*HIS3, TRP1*) show little change upon glucose repression. Therefore, nucleosome loss certainly does not have a general effect on transcription.

Key words: histone H4/*Saccharomyces cerevisiae*/cell cycle/transcription

Introduction

The nucleosome octamer contains two molecules each of core histones H2A, H2B, H3 and H4 around which is wrapped ~146 bp of DNA (McGhee and Felsenfeld, 1980). In higher eukaryotes, there is also a fifth histone, H1, which may be involved in compaction of chromatin and repression of genes during terminal differentiation (Schlissel and Brown, 1984; Weintraub, 1984). This protein does not appear to be present in yeast (Certa *et al.*, 1984). However, yeast differs from higher eukaryotes in that its chromosomes are not highly condensed. Also, since all yeast genes must have the potential for activity in successive cell cycles, yeast probably lacks the ability to permanently inactivate genes.

There is evidence for different functions for individual core histones. For example, histone protein sequences vary considerably in their rates of evolutionary divergence. The histone H2B sequence is relatively divergent while histone H4 is certainly one of the most conserved proteins known in evolution (McGhee and Felsenfeld, 1980; Wells, 1986). H3 and H4, which form a tetramer, can interact with DNA to form a nucleosome-like particle *in vitro*; histones H2A and H2B cannot (Simon *et al.*, 1978). Finally, there is increasing evidence that unlike H3 and H4, H2A and H2B have the ability to disassociate and transfer between nucleosomes *in vivo* (Jackson and Chalkley, 1981; Smith *et al.*, 1984).

To investigate the function of core histones during the yeast cell cycle, we previously constructed a yeast strain (MHY102) whose sole histone H2B gene was under the control of the GAL10 promoter and could therefore be repressed in the presence of glucose. Using asynchronous cells, we showed that H2B loss led to a disruption in chromosomal segregation. However, a full round of DNA synthesis and relatively normal RNA synthesis continued in the absence of new H2B (Han *et al.*, 1987). Given the more crucial role of histone H4 in assembly of the nucleosome we extended the analysis here to histone H4-depleted cells. A yeast strain (UKY403) was constructed in which the sole functional H4 gene in the cell was fused to the *GAL1* promoter on a centromeric plasmid. Utilizing UKY403 cells pre-synchronized in G_1 with *alpha*-mating factor we addressed the following questions regarding H4 depletion during the yeast cell cycle. (i) When during the cell cycle is histone H4 first required for cell viability and when does the H4-depleted cell arrest? (ii) How is polynucleosome assembly altered by repression of H4 mRNA synthesis and will nucleosomes assemble on non-replicating as well as replicating DNA? (iii) How is transcription by RNA polymerases I, II and III affected by their use of altered chromosomal templates?

Results

Histone H4-2 transcription is under control of the GAL1 promoter in UKY403 cells

Yeast contains two gene copies (H4-1 and H4-2) for histone H4. While each of these genes codes for the same amino acid sequence, they are unlinked genetically and differ from each other in surrounding non-coding sequences (Smith and Andresson, 1983; Smith and Murray, 1983). A yeast strain was previously constructed which has histone H4-1 and H4-2 genes replaced by the marker genes *HIS3* and *LEU2* respectively (P.Kayne, U.-J.Kim and M.Grunstein, in preparation). In the yeast UKY403, their function has been replaced by an episomal H4-2 gene which had its own promoter removed and replaced by the inducible *GAL1* promoter (Figure 1A). This element is activated in galactose-containing medium and repressed in glucose-containing medium (to be referred to here as simply galactose and glucose for brevity). In the experiments described below, we have chosen to concentrate our experiments on UKY403 cells which were pre-synchronized in G_1 with *alpha*-mating

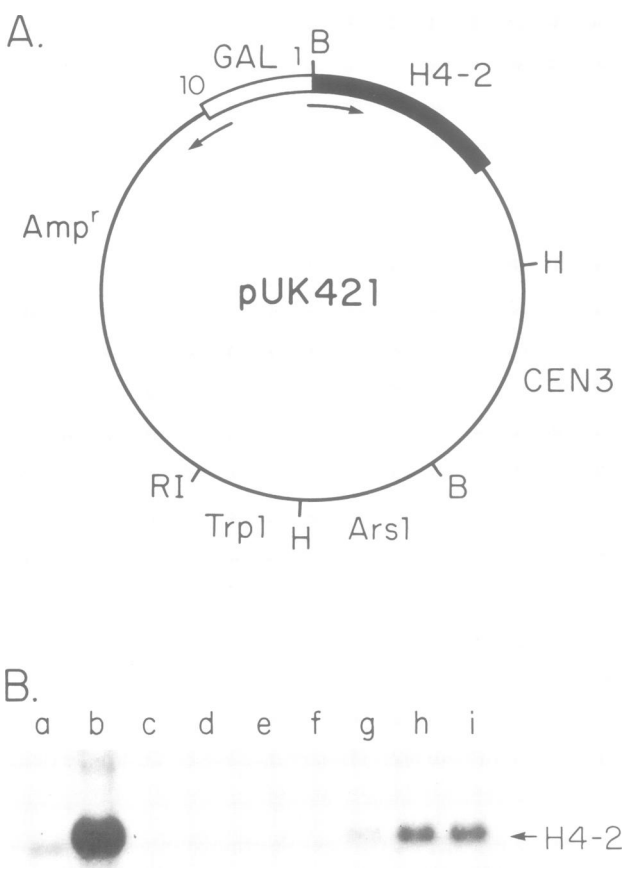


Fig. 1. (**A**) Map of plasmid pUK421 containing the H4-2 gene under control of the *GAL1* promoter. The plasmid also contains a centromeric DNA fragment *CEN3* and the selectable marker gene *TRP1*. Symbols for restriction enzyme sites: B, *Bam*HI; H, *Hin*dIII; RI, *Eco*RI. (**B**) Northern blot analysis of H4-2 transcripts in UKY403 cells to illustrate the activation of the *GAL1*-H4-2 fusion gene on galactose, its repression on glucose and reactivation on galactose. The cells were grown in galactose medium YEPG (**lane b**), and synchronized by adding *alpha*-factor for 2 h. Glucose (2%) was added, and the *alpha*-factor arrest was continued for an additional 2 h (**lane c**). Then the cells were washed and grown in YEPD glucose medium, and aliquots of the cells were taken after 1, 2 and 4 h in glucose (**lanes d, e, f** respectively). An aliquot of the cells was taken after 3 h in YEPD medium, washed and grown in YEPG for 1, 3 and 6 h (**lanes g, h, i** respectively). RNA was isolated from cells as described by Zitomer *et al.* (1979). Six micrograms of RNA were loaded per well, electrophoresed, blotted and hybridized to ^{32}P-labeled H4-2 gene-specific probe as described in Materials and methods. **Lane a** contains RNA from a wild-type control strain [D585-11C (*MATa, lys1*$^-$)] containing both H4-1 and H4-2 genes under control of their own promoters.

factor. This allows the analysis of biochemical changes, induced by glucose arrest, in a more homogeneous group of cells.

Figure 1B shows the results of carbon source regulation of the histone H4-2 gene. Total cellular RNAs were isolated from UKY403 cells before and after glucose treatment. The RNAs were electrophoresed on agarose gels and transferred to nitrocellulose for Northern blot hybridization to a ^{32}P-labeled H4-2 DNA probe. Compared to H4 mRNA expression in a wild-type control strain (D585-11C, lane a) there is a much larger quantity of H4 message synthesized from the *GAL1* promoter (lane b). However, when UKY403 cells in galactose are synchronized in G_1 with *alpha*-factor and then treated with glucose for ≥ 1 h, new H4 mRNA is no longer synthesized, and the remaining H4 mRNA is degraded (lanes c–f). To determine whether the glucose-repressed H4 gene could be reinduced to activity, UKY403 cells were transferred from a 2-h treatment in glucose (lane e) and followed for 1, 3 or 6 h in galactose (lanes g–i). It is evident that H4 mRNA synthesis resumes in galactose. While the H4 mRNA level is not as high as that overproduced on galactose prior to glucose treatment (lane b), it is still higher than the H4 mRNA level seen in a strain containing two normal H4 genes (compare lane h and lane a). We conclude that galactose and glucose will respectively activate and repress H4 mRNA synthesis in the pre-synchronized UKY403 cells and that the *GAL1* promoter can be reactivated on galactose after its repression by glucose.

Growth of UKY403 cells upon glucose arrest

When asynchronous UKY403 cells are treated with glucose, they undergo first cycle arrest after less than one (0.7) round of cell division (Figure 2A). The cells arrest uniformly after ~3 h as budded cells containing a single large bud and a G_2 quantity of DNA as determined by flow cytometry (data not shown). If budded cells are treated with the DNA staining dye, 4′,6-diamidino-2-phenylindole (DAPI), it is found that >90% of the budded cells contain a single nucleus after ~2 h of glucose treatment. However, with increased time in glucose, DNA segregates to the bud in ~20% of the budded cells (Figure 2B). These results are similar to those found in the yeast MHY102 upon repression of H2B mRNA synthesis (Han *et al.*, 1987) and argue strongly that histones are required for the process of chromosomal segregation to the bud.

One possible reason for the ability of certain cells to segregate DNA after glucose treatment may lie in the timing of glucose addition with respect to the cell cycle. If the pool of histone H4 protein is large enough and the cells are close enough to S phase when nucleosome assembly with newly replicated DNA occurs, one round of chromatin assembly may occur in those cells even after glucose addition. If this is indeed the case, it would be predicted that pre-synchronization of UKY403 cells with *alpha*-mating factor in G_1 (Pringle and Hartwell, 1981) followed by glucose arrest would prevent most of the cells from segregating chromosomes. UKY403 cells were therefore synchronized with *alpha*-factor and allowed to incubate in glucose containing *alpha*-factor in order to deplete the cell of remaining H4 mRNA. When *alpha*-factor was washed away and the cells were released into glucose, we found that virtually all the cells arrested with a single large bud in G_2 and that >97% could not segregate DNA to the bud even after long periods in glucose (inset to Figure 2A and Table I). This further supports our contention that histone H4 is required for the process of chromosomal segregation. Similar data was obtained upon histone H2B depletion in pre-synchronized MHY102 cells (M.Han and M.Grunstein, unpublished).

Lethality resulting from glucose arrest occurs first in S phase

While histone H4 synthesis is required for the process of chromosomal segregation in mitosis, it would seem unlikely that the lethal defect resulting from H4 loss occurs as late

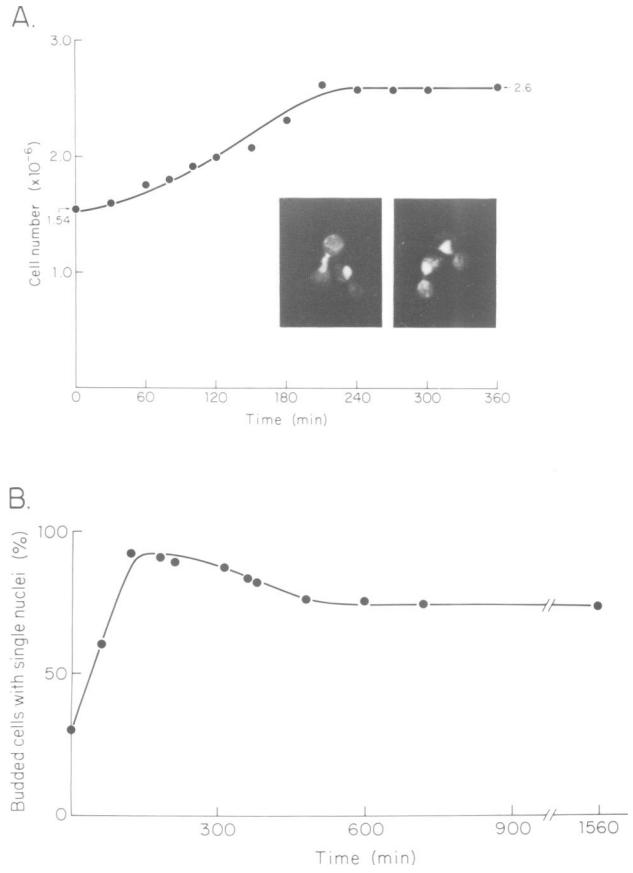


Fig. 2. (**A**) Growth curve of UKY403 cells in glucose medium shows first cycle arrest. Cells were grown in YEPG medium and 2% glucose was added when the culture reached 1.54×10^6 cells/ml. Photos (inset) show DAPI-stained nuclei of the terminally arrested cells illustrating the defect in chromosomal segregation. (**B**) Percentage of cells with a single nucleus amongst cells containing a single large bud. Glucose was added to asynchronously growing UKY403 cells in YEPG. Aliquots of cells were taken at indicated time points for DAPI staining, and the stained nuclei were viewed through 100× or 40× Zeiss Neofluor lenses on a Nikon UFX-11 microscope equipped for epifluorescence.

as mitosis. For example, new nucleosome assembly occurs concomitant with DNA replication in S phase. Therefore, it seems reasonable that defective chromatin formed in S phase would be the primary lethal defect resulting eventually in aborted chromosomal segregation. We have previously shown that arrest resulting from histone H2B repression is irreversible. When cells are transferred from galactose to glucose, the biochemical changes induced by arrest prevent growth when cells are transferred back to galactose (Han *et al.*, 1987). When asynchronous MHY102 cells were treated in this manner, we found that the percentage of cells reaching lethality was similar to those reaching the terminal phenotype at all time points. Similar results are obtained when histone H4 mRNA synthesis is repressed (data not shown). One interpretation of this result is that lethality is associated with reaching the terminal phenotype, or mitosis. However, since these experiments started with asynchronous cells, those reaching the point of lethality are not necessarily those reaching mitosis. Consequently, we undertook a similar analysis in which we started with synchronous cells.

UKY403 cells treated with *alpha*-factor in galactose, as described in Materials and methods, were released into glucose. Their progress during the cell cycle until final arrest was monitored visually and by flow cytometry. As shown in Figure 3A, such cells pass synchronously from G_1 to G_2 in ~60 min. Cells treated in this manner with glucose were transferred back to galactose plates at various points in the cell cycle and the resultant colonies were counted. As shown in Figure 3B, lethality is first evident as the cells exit from G_1 and pass through S phase and is largely complete by the time the cells reach G_2. This period of lethality coincides with the period during which nucleosomes are assembled on newly replicated DNA.

Table I. UKY403 cells, pre-synchronized with *alpha*-factor and treated with glucose, arrest with a single large bud and contain a single G_2 nucleus

Hours in glucose	Cells with single nuclei (%)			Cells with two nuclei (%)	Others (%)	Total number of cells counted
	⊙⊙	⊙⊙	⊙⊙	⊙⊙	⊙	
2	6.6	65	27.4	0.6	0.4	485
6	11.9	58.1	28.6	0.9	0.5	444
14	36	42.5	17.6	1.5	0.7	405

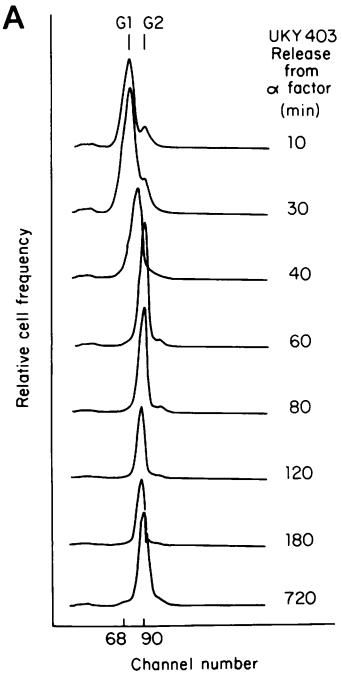
Described are various morphologies of budded cells and the position of their nuclei as determined by DAPI staining. UKY403 cells were synchronized at G_1 by *alpha*-factor as described in Materials and methods. The cells were released from *alpha*-factor arrest, grown in glucose medium (YEPD) and taken after 2, 4 and 14 h in glucose.

Nucleosomes will assemble on non-replicating DNA in G_2

We have shown in Figure 3A that pre-synchronized UKY403 cells will arrest in G_2 after ~60 min in glucose. Even if they are kept in glucose for longer periods of time, no further replication occurs under conditions of histone H4 repression (Figure 3A). Similar results were observed under conditions of histone H2B repression when assayed by both flow cytometry and DNA labeling (Han *et al.*, 1987). Therefore, since H4 mRNA synthesis can be induced even after glucose arrest (Figure 1), we wished to determine whether reactivation of H4 will allow nucleosomes to form on pre-existing DNA. Nuclei were isolated from pre-synchronized UKY403 cells before and after glucose addition and treated with micrococcal nuclease. DNA was purified and electrophoresed on agarose gels stained with ethidium bromide to visualize protected DNA bands. Since the nucleosome protects attached DNA from digestion, the ladder of bands is indicative of polynucleosome length. We find that the average number of ethidium-bromide-stained bands visualized prior to glucose arrest is indicative of ≥11 nucleosomes (Figure 4). This decreases to approximately three nucleosomes after arrest. Also, there is evidence of greatly increased nuclease sensitivity at all nuclease concentrations judging from the smearing seen between bands in lanes e and f. This suggests that nucleosomes are lost after H4 mRNA repression, causing decreased protection of DNA. When these arrested yeasts are returned to galactose, the number of bands indicative of nucleosomes increases to approximately eight. However, there is still evidence of increased nuclease sensitivity between these bands. These data argue that nucleosomes do reassemble, albeit incompletely, when the H4 gene is reactivated after glucose arrest.

Superhelical density measures the presence of nucleosomes on plasmid DNA since, in a covalently closed episomal

molecule, a nucleosome induces a single superhelical turn (Worcel *et al.*, 1981; Wang, 1982). We wished to use superhelicity as an independent measure of chromatin reassembly upon resumption of histone H4 mRNA synthesis. Therefore, DNA was isolated from UKY403 cells arrested for different periods in glucose and also from cells transferred from glucose back to galactose. The DNA was electrophoresed on chloroquine–agarose gels as described previously (Han *et al.*, 1987), transferred to nitrocellulose paper for Southern blot analysis and hybridized to ^{32}P-labeled pUC13 plasmid containing pBR322 sequences found also in pUK421. Prior to glucose arrest, superhelicity of pUK421 plasmid shows greatest density at band positions 13 and 14 (Figure 5A). After arrest the peak of greatest density shifts to bands 5–7. This represents a decrease in superhelical density of ~50–60%. When cells that have been arrested in this manner at G_2 are transferred back to galactose, superhelical density increases again, although the peak is much broader and does not reach density levels achieved earlier. Since the induction of transcription might displace nucleosomes (Almer *et al.*, 1986), we also examined the 2 μ plasmid (Figure 5B) which contains no galactose-inducible promoter. It is evident that similar changes in superhelical density are seen with either pUK421 or 2 μ plasmids. Control experiments show no changes in plasmid superhelical density due to changes in carbon source in a strain which is isogenic with UKY403 except that its histone H4 gene is not under *GAL* control (data not shown). We conclude that nucleosomes will reassemble on non-replicating DNA *in vivo*. However, assembly appears to be incomplete under the conditions employed here.



Effects of nucleosome depletion on transcription by RNA polymerases I, II and III

The phasing of nucleosomes has been implicated in the regulation of eukaryotic transcription (Gross and Garrard, 1987). However, we do not know to what extent polynucleosome structures are necessary for accurate transcription and transcriptional control *in vivo*. The *CUP1* chelatin gene is activated by the presence of Cu^{2+} in the medium and repressed in its absence (Fogel and Welch, 1982). We have previously found that H2B depletion has no obvious effect on either the repression of the *CUP1* gene in the absence of Cu^{2+}, nor on its activation when Cu^{2+} was present (Han *et al.*, 1987). Since histones H3 and H4 are more central to the assembly and stability of the nucleosome we wished

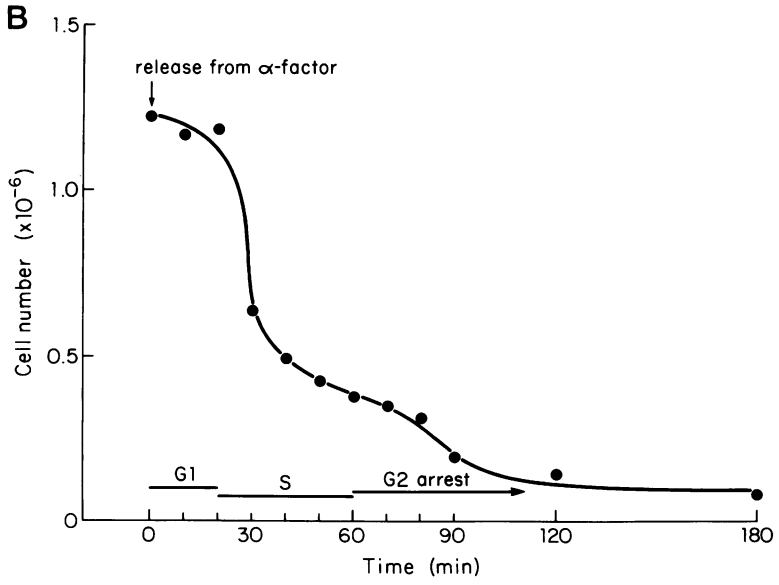


Fig. 3. (**A**) Flow cytometry of UKY403 cells synchronized in galactose with *alpha*-factor and transferred to glucose medium shows arrest in G_2. The cells were grown and synchronized as described in Materials and methods. Samples were taken at various times after the release from *alpha*-factor arrest. Channel numbers were 68 and 90 for G_1 and G_2 cells respectively as determined by the use of haploid and diploid cell controls (Han *et al.*, 1987). (**B**) Mapping the stage of the cell cycle when histone H4 mRNA repression is found to be lethal. Cells were synchronized with *alpha*-factor as described in Materials and methods, released into YEPD and samples were removed at the intervals shown. These were diluted, plated on YEPG plates and assayed for colony formation after 2 days of growth at 30°C. Approximately 500 cells were plated per Petri dish. Lethality is first evident as the cells exit G_1 and enter S phase and is largely complete by G_2.

to re-examine this question using the histone H4 gene under *GAL1* control and analyzing for transcription by all three RNA polymerases of yeast.

UKY403 cells were arrested in glucose medium for 4 h, and pulse-labeled with ^{32}P for 30 min (Brill *et al.*, 1987). RNA was isolated and electrophoresed in 2.5% polyacrylamide−0.5% agarose to separate 25S and 18S rRNAs, the products of RNA polymerase I. An aliquot of this RNA was also electrophoresed in 10% polyacrylamide for separation of 5S RNA and tRNAs, products of RNA polymerase III. As shown in Figure 6 (A and B), there is no evidence of different amounts of ^{32}P-labeled RNAs of any of these species 4 h after glucose arrest. The 5.8S RNA species seen in Figure 6B is also the product of RNA polymerase I transcription, since it results from processing of 35S ribosomal precursor RNA which gives rise to 25S, 18S and 5.8S RNAs (Warner, 1982). Therefore, neither RNA polymerase I or III transcription rates appear to be altered by the use of nucleosome-depleted chromatin as template.

We examined *CUP1* transcription in H4-depleted UKY403 cells as one measure of RNA polymerase II activity. Pre-synchronized cells were analyzed for their ability to be induced by Cu^{2+} before and after glucose arrest. As seen in Figure 7, the *CUP1* gene is activated to similar levels prior to glucose arrest and as long as 8 h after glucose treatment. Steady-state mRNA levels of certain other yeast genes (*HIS3, HIS4, ARG4* and *TRP1*) involved in amino acid metabolism (Figure 8) were then examined. The average half-life for yeast mRNAs is ~22 min (Chia and McLaughlin, 1979). Since the cells used in Figure 8 were arrested for 6 h in glucose prior to RNA isolation for Northern blot analysis, the steady-state RNA levels we see in Figure 8 are likely to reflect continuing new RNA synthesis. For each of these genes we compared mRNA

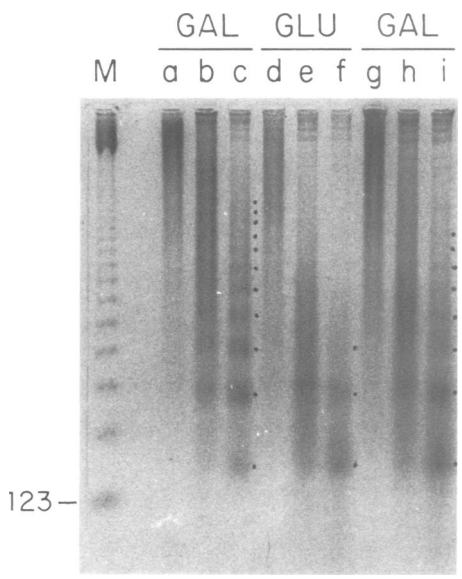


Fig. 4. Micrococcal nuclease digestion of nuclei isolated from UKY403 cells to illustrate the effect of histone depletion on nucleosome protected DNAs. **Lanes a−c**: DNAs from nuclei isolated before glucose arrest in YEPG galactose medium and treated with micrococcal nuclease (50 U/ml/100 μg DNA) for 4, 10 and 20 min respectively. **Lanes d−f**: similarly digested DNAs from nuclei isolated from cells transferred to glucose (YEPG + 2% glucose) for 4 h. **Lanes g−i**: DNAs isolated from nuclei of cells transferred to galactose for 3 h after 4 h in glucose. Digested DNAs were electrophoresed in 1.5% agarose gels stained with ethidium bromide. **Lane M** contains a mol. wt marker (123 bp ladder from BRL). Prior to glucose arrest at least 11 nucleosome-protected DNA bands are evident. This decreases to approximately three bands in glucose and is increased to approximately eight bands when these cells are transferred back to galactose.

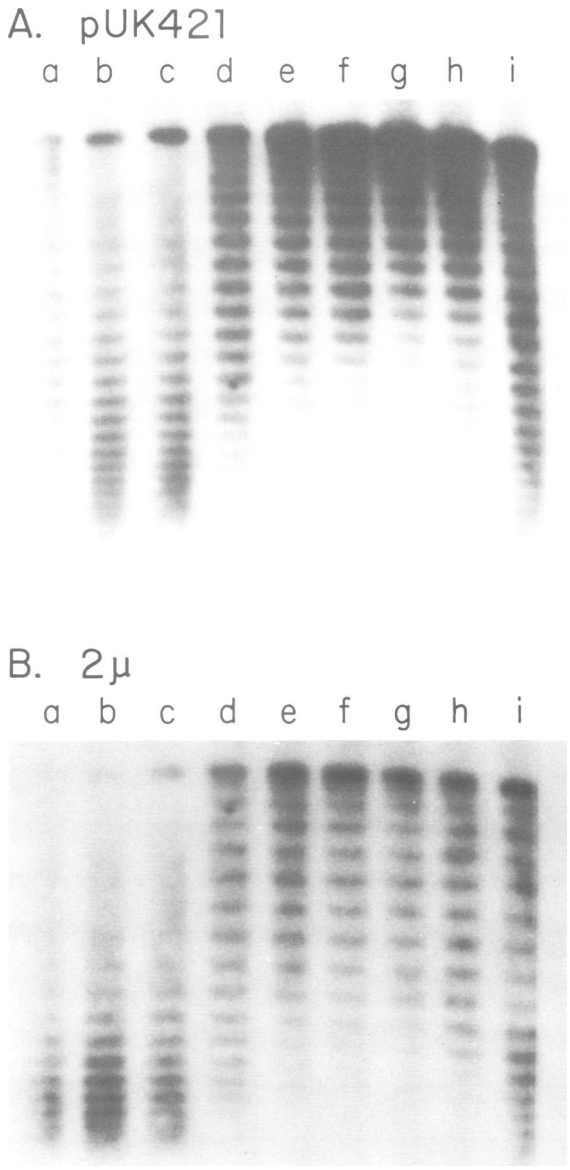


Fig. 5. Superhelicity of covalently closed episomal DNAs in UKY403 cells before and after glucose arrest. Synchronously growing cells were sampled at indicated time points, plasmid DNAs were isolated and electrophoresed in chloroquine−agarose gels and subjected to Southern blot analysis with ^{32}P-labeled DNA. The lanes shown contain DNAs from asynchronously growing cells, in YEPG medium (**lane a**); synchronized by *alpha*-factor for 2 h (**lane b**); treated with 2% glucose for 2 h in the presence of *alpha*-factor (**lane c**); after washing and growing in YEPD medium for 1, 3 and 5 h (**lanes d−f** respectively); removed after 3 h of arrest in YEPD medium and switched back to YEPG medium for 1, 2 and 4 h (**lanes g−i** respectively). (**A**) Plasmid pUK421 was detected by hybridization to ^{32}P-labeled pUC13 DNA (Messing, 1983). (**B**) 2*u* circle DNA was detected by ^{32}P-labeled M13mp2YE17 RF DNA (from J.Campbell). For both plasmids we see a decrease in superhelical density upon histone H4 and nucleosome depletion. This is partially restored upon reactivation of the H4-2 gene in galactose.

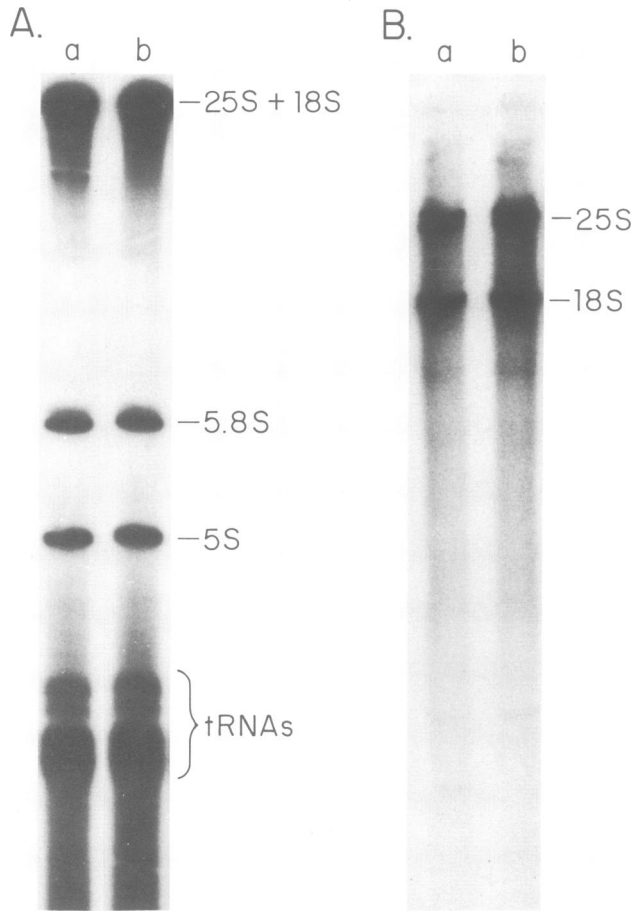


Fig. 6. UKY403 cellular RNAs pulse-labeled *in vivo* with inorganic P_i. ^{32}P-labeled RNAs extracted from cells, pulse-labeled for 30 min, were electrophoresed in a 10% polyacrylamide gel (**A**) or 2.5% polyacrylamide−0.5% agarose composite gel (**B**) and autoradiographed. Approximately 3 μg RNA was loaded per lane. RNA from UKY403 cells grown in (YEPG) galactose (**lane a**). RNA labeled after the cells were depleted of histone H4 by growing in YEPG + 2% glucose for 4 h (**lane b**). RNA labeling before or after glucose treatment is similar for all RNAs seen here.

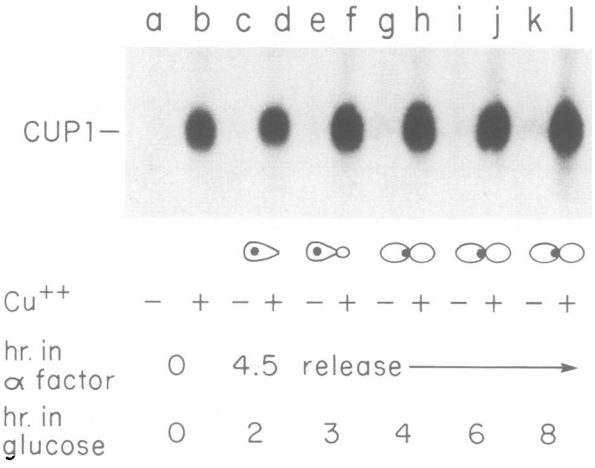


Fig. 7. Induction of the *CUP1* gene before and after the depletion of histone H4. UKY403 cells were grown in (YEPG) galactose and synchronized as described in Materials and methods. RNAs were isolated from cells under the conditions shown and ~4 μg RNA was loaded per well, electrophoresed and transferred to nitrocellulose for hybridization to *CUP1* DNA probe (Materials and methods). For the induction of *CUP1* transcription, 1 mM $CuSO_4$ was added to the culture for 30 min. Histone H4 depletion appears to have no obvious effect on either the uninduced or induced *CUP1* mRNA levels.

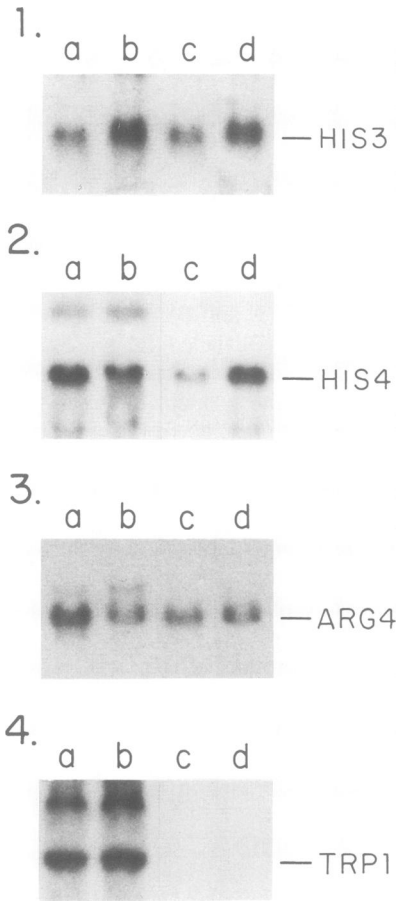


Fig. 8. The effect of histone H4 depletion on mRNA levels of genes involved in amino acid metabolism. RNAs were isolated for Northern blot analysis from UKY403 cells in galactose (**lane a**) or after transfer to glucose for 6 h (**lane b**). Similarly RNAs were isolated from UKY412 grown in galactose (**lane c**) or glucose (**lane d**). Fifteen micrograms of RNA were loaded per lane. The probes used for each of the mRNAs shown are described in Materials and methods. While UKY403 arrests on glucose, UKY412 grows on both galactose and glucose media since its sole episomal H4-2 gene is under control of the wild-type H4-2 promoter. UKY412 serves as a control for effects of glucose on mRNA levels. These experiments illustrate no major effect of H4 depletion on the steady-state levels of these mRNAs.

levels in UKY403 before and after glucose arrest (lanes a and b respectively). We also compared mRNA levels before and after glucose treatment (lanes c and d) in UKY412. This yeast is isogenic with UKY403 except that its episomal H4 gene is under the control of its own wild-type promoter and consequently this yeast will not arrest in glucose. Since UKY412 is *trp*$^-$ (see Materials and methods), this yeast serves as a control only for the other three genes in Figure 8. In comparing mRNA levels before and after glucose arrest in UKY403 and UKY412, we find evidence of some changes in RNA levels. For example, there is an increase in *HIS3* transcript in UKY403 when shifting from galactose to glucose. However, this is likely due to the change in carbon source and not to nucleosome depletion (since this increase is also evident in UKY412). Some decrease in mRNA level is seen for *HIS4* and *ARG4*, while no obvious change is seen for *TRP1* upon arrest of UKY403. Therefore, we conclude that while some mRNA levels may change upon nucleosome depletion, there is clearly no global change in the synthesis of RNAs produced by polymerases I, II or III.

Discussion

Comparing effects of H4 and H2B depletion on the yeast cell cycle

The data presented in this paper indicate that the effects of H2B repression in the yeast MHY102 (Han *et al.*, 1987) and of H4 repression in UKY403 are very similar with regard to their cell cycles. In both cases, cells arrest at G_2 containing chromatin partially depleted of nucleosomes and inhibited from chromosomal segregation. As would be expected for proteins which are part of the same important complex, the synthesis of both histones is necessary for nucleosome and chromatin structure. The steps involved in constructing MHY102 and UKY403 preclude their being isogenic strains. Nevertheless some differences seen between them may be significant. The effects of H4 repression on the cell cycle were more immediate. UKY403 cells arrested in ~2 h after addition of glucose. MHY102 cells in which H2B synthesis was repressed arrested at the terminal phenotype after 4–5 h. UKY403 cells arrested after 0.7 rounds of cell division, while MHY102 cells completed a full round of division before arresting in G_2. This may be due to the more central role of H4 in nucleosome assembly and structure (Simon *et al.*, 1978; McGhee and Felsenfeld, 1980).

Lethality resulting from histone H4 depletion is most evident in S phase

When histone H4 production is repressed, UKY403 cells go on to arrest in G_2 unable to complete chromosomal segregation. Therefore, histones should first be required for viability somewhere between nucleosome assembly in S and chromosomal segregation in G_2. We have shown that the repression of histone H4 mRNA synthesis by glucose is first lethal to UKY403 yeast cells in S phase. Also, we have shown that the chromatin defect induced by nucleosome loss is only partially reversible once H4 mRNA synthesis resumes. This suggests that the lethal defect caused by nucleosome depletion in S is an irreversibly altered chromatin structure. However, it may be that lethality occurs somewhat earlier in S than shown in Figure 3B. While there is significant H4 mRNA pool after glucose is added to the cells in G_1 (Figure 1B), excess H4 protein could have been synthesized prior to glucose addition. Furthermore, while the other core histone mRNAs are not synthesized appreciably in G_1, there may still be a small stable pool of other core histones remaining from previous cell cycles. The possible presence of a core histone pool is based on the observation of some nucleosome assembly upon reactivation of H4 on galactose (Figures 4 and 5). This could delay the apparent period of lethality and may explain why a small fraction of the lethality observed in Figure 3B occurs as late as G_2.

Polynucleosome assembly in vivo

The arrest of histone H4 mRNA synthesis by glucose results in general nucleosome loss as determined by micrococcal nuclease digestion of chromatin and superhelical density of UKY403 episomes. We estimate from plasmid pUK421 and 2*u* superhelical measurements that 50–60% of nucleosomes are lost upon repression of H4 mRNA synthesis. Previous experiments utilizing histone H2B depletion through glucose arrest showed a loss of ~45% of nucleosomes under similar conditions (Han *et al.*, 1987). The greater change in superhelical density upon H4 repression could reflect the more central role of H4 in nucleosome formation, although it is not expected that >50% of nucleosomes may be lost if a full round of histone free DNA replication occurs upon glucose arrest. Among other possibilities, the greater change in superhelical density could reflect an instability of remaining nucleosomes when H4 is depleted. This is a possibility since there is evidence that remaining nucleosomes may slide or be otherwise unstable after glucose arrest depletes the cell of histone H4 (Han *et al.*, 1988, accompanying paper).

Table II. Yeast srains and genotypes used

Strain	Genotype
UKY403	*MATa,ade2-101(ochre),his3-Δ200,leu2-3,-112,lys-2-801 (amber),trp1-Δ901,ura3-52,GAL$^+$,thr$^-$,tyr$^-$,arg4-1, Δh4-1,[HIS3$^+$],Δh4-2[LEU2$^+$]/pUK421(TRP1$^+$,GAL1-H4-2$^+$)*
UKY412	*MATa,ade2-101(ochre),his3-Δ200,leu2-3,112,lys2-801 (amber),trp1-Δ901,ura3-52,GAL$^+$,thr$^-$,tyr$^-$,arg4-1, Δh4-1,[HIS3$^+$],Δh4-2[LEU2$^+$]/pUK499(URA3$^+$,H4-2$^+$)*
D585-11C	*MATa,lys1*

In line with data utilizing higher eukaryotic cells (Leffak, 1983), yeast nucleosomes can reassemble on DNA which is no longer replicating. This must mean that there are some pools of other core histones which can assemble with the newly synthesized H4 protein once the cells are shifted from glucose to galactose. We find, however, that reassembly is incomplete. The resultant DNAs produce shorter ladders when treated with micrococcal nuclease and still have an abnormally low superhelical density, suggesting that the chromatin formed contains fewer than the wild-type number of nucleosomes. This may provide a clue to the continuing inviability of yeast cells which are shifted from glucose to galactose.

There are several possible explanations for the lack of complete nucleosome assembly on non-replicating DNA. When the H4 gene is reactivated on galactose there is no similar reactivation of the other core histone genes which are normally inactive in G_2. Hence assembly of nucleosomes may stop once the pools of available core histones are depleted. Alternatively, once nucleosomes are depleted as a result of H4 repression, it may be difficult for assembly of new nucleosomes to occur accurately. This may be due to sliding of residual nucleosomes (Han *et al.*, 1988) and to the inability of newly synthesized nucleosomes to assemble accurately between existing ones.

Effects of nucleosome depletion on transcription

The template used for transcription *in vivo* is chromatin. Glucose-treated UKY403 cells lose approximately half their nucleosomes. Yet our data show no dramatic effect of glucose arrest in UKY403 on transcription by all three RNA polymerases. Nor is there evidence that activation or repression of the *CUP1* gene is altered when nucleosomes are removed. This could be due to the function of the residual nucleosomes; however, those which remain are likely to be displaced after glucose arrest (Han *et al.*, 1988). This suggests that if remaining nucleosomes still allow function, they may do so from displaced positions. It is possible that many yeast genes may not require an ordered, phased chromatin structure for proper gene regulation. Support for

this view comes from the data of Lohr and Hereford (1979) showing that a large fraction of yeast genes are in a perpetually open, DNase-I-sensitive conformation. This contrasts with the globin genes of higher eukaryotes which appear to be DNase I sensitive only when active (Groudine and Weintraub, 1982; Weintraub *et al.*, 1982) and with at least one yeast gene (*PHO5*). This gene has an ordered, phased nucleosome array which is clearly perturbed upon activation (Almer *et al.*, 1986). This may explain why transcription of *PHO5* is strongly affected by nuclesome loss in UKY403 as described in the accompanying paper (Han *et al.*, 1988).

Materials and methods

Yeast strains and media

Table II shows the strains of yeast used and their genotypes.

Methods used in recombinant plasmid construction have been described by Maniatis *et al.* (1982). Yeast and bacterial transformation and selection were done as described previously (Wallis *et al.*, 1983). For the construction of pUK421, a 1.5-kb *Hin*dIII fragment containing the histone H4-2 gene (Smith and Murray, 1983) was digested with *Bal*31 to trim the promoter, and inserted into M13mp10 for sequencing. Sequencing showed that the H4-2 promoter region including the TATA box (Smith and Andresson, 1983) was completely removed, leaving 31 bp of leader sequence upstream from the translation initiation codon. *Eco*RI blunt-*Bam*HI fragment from the M13mp10 subclone was inserted into *Bam*HI−*Sal* blunt site of the yeast vector pBM258 (Johnston and Davis, 1984) such that the H4-2 structural gene was placed under the control of the *GAL1* promoter. The restriction fragment containing the *GAL1*−H4-2 fusion gene from the resulting plasmid (pUK420) was subcloned into a shuttle vector pTSB1-1 (obtained from T.Schuster). The resulting plasmid was designated pUK421 (Figure 1). This plasmid was transformed into the yeast strain 44A (P.Kayne, U.-J.Kim and M.Grunstein, in preparation) in which H4-1 and H4-2 genes in the genomic background have been replaced by *HIS3* and *LEU2* marker genes by gene replacement techniques (Rothstein, 1983) and which contained an episomal copy of the H4-2 gene on a *CEN3,URA3*$^+$ plasmid. *TRP1* transformants were grown in SG-trp medium, and the *URA* plasmid was lost by screening for *ura*$^-$,*TRP*$^+$ colonies. The resulting strain in which histone H4 synthesis is completely under the control of *GAL1* promoter was designated UKY403. UKY412 strain is isogenic to UKY403 except that it harbors a centromeric plasmid pUK499, carrying *URA3* and the H4-2 gene under the control of its own wild-type promoter, instead of pUK421. Construction of PKD4-2b (*MATa ade2-101(ochre), his3200, leu2-3,112, lys2-801(amber), trp1 901, ura3-52, thr*$^-$, *tyr*$^-$, *arg4-1, GAL*$^+$), a parental strain of UKY403, is described elsewhere (P.Kayne, U.-J.Kim and M.Grunstein, in preparation).

Media and chemicals

YEPD and SD media containing 2% dextrose have been described (Sherman *et al.*, 1974). YEPG and SG media are identical to YEPD and SD except that 2% galactose rather than dextrose is added as a carbon source. DAPI and *alpha*-mating factor were purchased from Sigma.

Growth and synchronization

UKY403 cells were grown in YEPG medium. *alpha*-Factor was added to 10 μg/ml at a cellular concentration of 3−5 × 10^6 cells/ml. The cells were arrested at G_1 for 2 h, then 2% glucose was added to this culture and the cells were arrested in *alpha*-factor for a further 2 h to turn off the synthesis of H4 mRNA before the cells could undergo DNA replication. Then the cells were washed with YEPD three times to remove *alpha*-factor, and grown in YEPD. Synchronization for other strains was done in YEPD without changing medium. In certain cases, cells were not easily arrested due to differences in batches of *alpha*-factor. In those situations *alpha*-factor was added twice at 5 μg/ml at 2-h intervals.

Flow cytometry and DAPI staining

Cells were taken at desired time points, washed once in cold distilled water, fixed in 70% ethanol for 30 min and subjected to flow cytometry or DAPI staining as reported previously (Han *et al.*, 1987).

Micrococcal nuclease digestion of yeast nuclei

Yeast nuclei were prepared and digested with micrococcal nuclease as described in the accompanying paper (Han *et al.*, 1988). About 10 μg of the digested DNA was electrophoresed in a 1.5% agarose gel. The nucleosomal ladders were visualized by ethidium bromide staining.

Superhelical density of plasmid DNAs

Yeast plasmid DNA was isolated as described by Abraham *et al.* (1983). The DNA was electrophoresed on a 0.7% chloroquine−agarose gel (Shure *et al.*, 1977), transferred to a nitrocellulose filter and hybridized to either ^{32}P-labeled pUC13 (Messing, 1983) or M13mp2YE17 RF DNA which contains a fragment of 2 μ circle DNA and exposed to X-ray film (Kodak, AR) at −70°C under an intensifying screen (Du Pont, Chronex III).

In vivo labeling and electrophoresis of ribosomal and tRNAs

UKY403 cells either growing logarithmically in YEPG or arrested in 2% glucose + YEPG for 4 h were labeled with 100 μCi/ml inorganic ^{32}P for 30 min. Cells were washed with cold 0.2 M LiCl twice, resuspended in the same solution and extracted with an equal volume of phenol with vigorous shaking for 30 min at 65°C. The aqueous phase was extracted twice with phenol, twice with chloroform and RNA was precipitated by adding 2.5 vol of ethanol. Equal amounts of RNA (3 μg) were loaded onto either 10% polyacrylamide−7 M urea gel or 2.5% polyacrylamide−0.5% agarose composite gel (Peacock and Dingman, 1968) with 4 M urea. The gels were fixed in 20% methanol/10% acetic acid for 2−3 h, dried and exposed for autoradiography.

Northern blot hybridization

Yeast total RNA was extracted as described (Zitomer *et al.*, 1979). Between 6 and 8 μg RNA was loaded in each lane and electrophoresed in a formaldehyde gel in 1 × MOPS buffer (pH 7.0) and transferred to nitrocellulose filters as described by Maniatis *et al.* (1982). The filters were hybridized to ^{32}P-labeled (Feinberg and Vogelstein, 1983) DNA fragments in 7% SDS, 0.5% bovine serum albumin, 1 mM EDTA at 65°C overnight. The filters were washed twice in 2 × SSC, 0.1% SDS at 65°C for 20 min, twice in 0.1 × SSC, 0.1% SDS at 65°C for 20 min and exposed to X-ray film. The probe for *CUP1* mRNA was described previously (Han *et al.*, 1987). The 1.1-kb *Hin*dIII−*Eco*RI fragment containing the H4-2 gene (Smith and Murray, 1983) was used to probe histone H4 mRNA. The 1.5-kb *Eco*RI fragment from YRp7 (Struhl *et al.*, 1979), the 3.0-kb *Hin*dIII fragment from pYe(Arg4)-114 (from J.Carbon's laboratory), the 791-bp *Pst*I−*Dde*I *HIS3* fragment from pMH203 (Han *et al.*, 1987) and a 1.7-kb *Eco*RI−*Sal*I fragment from pFW45 (constructed by F.Winston and obtained from K.McEntee) which contains a 1.4-kb *Bgl*II−*Sal*I *HIS4* gene fragment were used to probe the transcripts of *TRP1, ARG4, HIS3* and *HIS4* genes respectively.

Acknowledgements

We are grateful to Drs J.Carbon, S.Fogel, L.Johnson, M.Johnston, K.McEntee, T.Schuster, M.Smith and K.Struhl for bacterial or yeast strains and Roger Bohman for flow cytometry analysis. We are indebted to the Biotechnology and Education Research Program for use of equipment. This work was supported by Public Health Service Grants GM23674 and GM31336 from the National Institutes of Health.

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Received on March 13, 1988; revised on April 19, 1988