The histone variant H2A.Z interconverts two stable epigenetic chromatin states

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The nucleosomes occupying the chromosomal start sites of transcription contain the histone H2A variant H2A.Z in place of H2A. Upon galactose induction, nucleosomes are evicted from the GAL1 locus in Saccharomyces cerevisiae cells. H2A.Z (which is encoded by the HTZ1 gene in S. cerevisiae) is required for the eviction of the GAL1 promoter nucleosome and for the transcriptional activation of the GAL1 gene; however, histones are also important for transcriptional repression and we asked in the present paper if H2A.Z also plays a role in the glucose repression of the GAL1 promoter. With the help of a fusion of the URA3 ORF (open reading frame) to the GAL1 promoter, we were able to detect two different epigenetic transcription states of the GAL1 promoter in glucose-grown cells lacking H2A.Z: a repressed state that is occupied by a H2A-containing nucleosome and a derepressed state that is nucleosome-free. These two chromatin states are inherited stably through many cell divisions. According to the model described in the present paper, the role of H2A.Z is to facilitate the addition and removal of promoter nucleosomes and to prevent the formation of unfavourable stable epigenetic chromatin structures, which are not in accordance with the environmental conditions.

Key words: chromatin, epigenetics, histone, nucleosome, repression, transcription.

INTRODUCTION

The smallest building blocks of eukaryotic chromatin are the nucleosomes. Nucleosomes are complexes of 146 bp of DNA wrapped around a histone octamer, which consists of two copies of each of the four core histones H2A, H2B, H3 and H4 [1,2]. A nucleosome containing the histone H2A variant H2A.Z in place of histone H2A marks the 5’ end of genes [3–5], and the incorporation of H2A.Z into nucleosomes by the Swr1 complex is important for proper gene expression and genome stability [6,7]. The transcription status of a gene and its chromatin status influence each other [8,9]. For example, upon galactose induction nucleosomes are evicted from the GAL1 promoter, and Saccharomyces cerevisiae cells lacking H2A.Z are defective for this eviction and for the transcriptional activation of the GAL1 gene [10]. Epigenetic changes influence the phenotype without altering the genotype. They consist of changes in the properties of a cell that are inherited, but they do not represent a change in the DNA sequence. The epigenetic inheritance of a transcription state in the absence of the original signal has been termed ‘transcriptional memory’, which is considered a mechanism that persists only in the short-term (i.e. one or two cell divisions) [11–16]. Although transcriptional activation creates a nucleosome structure which allows the cell to respond to a second activation event faster, this nucleosome structure persists only for one or two cell divisions [17]. Another feature of this memory is the passage of signal-transducing proteins that persist inside the cell for approximately six divisions before being diluted out and which allow the cell to respond faster to a second gene induction event during this short time-span [15,17].

The S. cerevisiae GAL genes have been a paradigm for eukaryotic transcriptional regulation for the last decades [18]. Glucose causes repression of the GAL genes by Gal80 and Mig1 [19], whereas galactose triggers transcriptional activation of the GAL genes by the activator Gal4 [20]. When cells are grown with glucose, Mig1 binds to an upstream silencer and recruits the general repressor Tup1 to repress gene expression, whereas Gal80 binds to Gal4 and blocks its activation function [18]. Upon the switch to galactose medium, Snf1 phosphorylates Mig1, causing its translocation from the nucleus to the cytoplasm, whereas Gal3 sequesters Gal80 in the cytoplasm, leaving Gal4 free to activate the GAL genes, which are required for the utilization of galactose [21,22].

The histone variant H2A.Z, which is found in the nucleosome occupying the start site of transcription, is known to play an important role in the activation of gene expression. However, histones are also important for transcriptional repression, and we wanted to know if H2A.Z also plays a role in transcriptional repression. With the help of a fusion of the GAL1 promoter to the URA3 ORF (open reading frame), we have been able to show that H2A.Z is required to establish nucleosome occupancy and transcriptional repression of the GAL1 promoter in all glucose-grown cells. The GAL1 promoter is occupied by an H2A.Z-containing nucleosome and in the absence of H2A.Z the GAL1 promoter remains nucleosome-free in some cells, whereas it is occupied by an H2A-containing nucleosome in the others. Consequently, the GAL1 promoter is derepressed when it is nucleosome-free and repressed when it is occupied by a nucleosome. The two different chromatin states are inherited stably through many generations and less than 1% of cells are able to switch to the other state. Once switched, the chromatin status remains stable in those cells under non-selective conditions. There was no difference in DNA sequence of the GAL1 promoter in the nucleosome-occupied repression-competent glucose-grown ΔHTZ1 cells and in the nucleosome-free repression-deficient glucose-grown ΔHTZ1 cells, indicating that the GAL1 promoter

Abbreviations used: ChIP, chromatin immunoprecipitation; FOA, 5-fluoro-orotic acid; F plate, FOA-containing glucose plate; G plate, uracil-depleted galactose plate; HA, haemagglutinin; K plate, lysine-depleted uracil-containing glucose plate; ORF, open reading frame; SGD, Saccharomyces Genome Database; U plate, uracil-depleted glucose plate; W plate, tryptophan-depleted uracil-containing glucose plate.

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Figure 1  The repression status of the GAL1 promoter in ΔHTZ1 cells is stable

Cells of the indicated genotype were transformed with the LYS2-marked single-copy plasmid Pgal1-Ura317 expressing URA3 under the control of the GAL1 promoter. Cells were 10-fold serially diluted and titrated on to K, F, U, G plates. Lines 1–6 represent the first set of titrations. In subsequent titration rounds, cells were taken from the indicated plates of the previous rounds, 10-fold serially diluted and titrated on to the depicted plates. Lines 7–12 represent the second round of titrations, lines 13–18 represent the third round of titrations, lines 19–23 represent the fourth round of titrations and lines 27–30 represent the tenth round of titrations. See Supplementary Figure S1 for the complete set of titrations and Supplementary Figures S2–S4 for the titration schemes (at http://www.BiochemJ.org/bj/439/bj4390487add.htm). All plates were incubated for 3 days at 28°C. wt, wild-type.

exists in two different stable epigenetic chromatin states in glucose-grown ΔHTZ1 cells.

MATERIALS AND METHODS

Plasmids, strains and media

Pgai1-Ura317 and Pgai1-Ura314 were generated by cloning the GAL1–URA3 fusion from Pgai1-Ura304 into RS317 and RS314 respectively [24]. Scoops of approximately ten transformants were tested for their GAL1 transcription status on the respective reporter plates (see Figure 1). The URA3 ORF was integrated into the chromosomal GAL1 locus by transforming cells with the AgeI-cut TRP1-marked Pgai1-Ura304 integrative plasmid [23]. Individual colonies were picked from tryptophan-depleted glucose plates, restreaked on the same plates and tested for their GAL1 transcription status on the respective reporter plates (see Figure 2). DNA sequencing of genomic DNA-derived PCR fragments were used to confirm the integration events for all transformants. YCplac111-HTZ1 is an HTZ1-containing derivative of the LEU2-marked single-copy vector YCplac111 [25]. Endogenous H2B was tagged with three HA (haemagglutinin) epitopes and ten histidine residues by transforming the cells with the HpaI-cut LEU2-marked Yplac128-HTB1-HA3-H10 integrative plasmid, a derivative of YCplac111-HTA3-H10 [26] containing HTB1. Media were prepared as described previously [27]. See Supplementary Table S1 for the sequences of the PCR primers and Supplementary Table S2 for the genotype of the strains at http://www.BiochemJ.org/bj/439/bj4390487add.htm.

DNA quantification

DNA was prepared as described previously [28] and quantified by real-time PCR using the SYBR Green PCR Master Mix (Applied Biosystems).

mRNA quantification

*S. cerevisiae* cells were cultured in synthetic complete 2% (w/v) glucose medium at 28°C. At a D600 value of 1, the cells were collected by centrifugation (805 g for 5 min at 22°C). Galactose induction was performed by resuspending the cells in 2% galactose medium and incubation for the indicated amount of time. Total RNA was isolated using the RNAeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. cDNA was generated by reverse transcription PCR using the Taqman® MicroRNA Reverse Transcription Kit (Roche Applied Biosystems). Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). The mRNA decay assay was performed as described previously [29], with a final thiolutin (Sigma–Aldrich) concentration of 3 μg/ml.
ChIP (chromatin immunoprecipitation)

Yeast cells were cultured as described above. Chromatin cross-linking was performed with 1% formaldehyde for 20 min at 28°C with gentle agitation. Yeast cells were harvested, resuspended in 1 ml of yeast lysis buffer [100 mM Tris/HCl (pH 7.5), 50 mM KCl, 1 mM EDTA and 0.1% Nonidet P40] containing 2 mM PMSF and transferred to a screw cap tube containing acid-washed glass beads. Cells were broken using a Mini beadbeater. Yeast lysates were collected and centrifuged in a microcentrifuge at 4°C at 15,871 g for 10 min. Pellets were resuspended in 500 μl of yeast lysis buffer and sonicated. Sonication was performed using a cup-horn sonicator (Vibra-Cell™ from Sonics) at 40% maximal amplitude for 30 s for a total of 10 times. Sonicates were centrifuged in a microcentrifuge at 4°C at 15,871 g for 20 min and the supernatant transferred to new microfuge tubes. The DNA concentration of the supernatant was determined using a spectrophotometer (Nanodrop ND-1000), at a wavelength of 260 nm. The chromatin solution was then subjected to immunoprecipitation. This was performed by incubating samples with anti-HA beads (Sigma) overnight at 4°C followed by washing of the beads. A total of 15 washes were carried out in the following order: four times with yeast lysis buffer, four times with yeast lysis buffer containing 0.5 M NaCl, four times with ChIP wash buffer [10 mM Tris/HCl (pH 8), 0.25 M LiCl, 1 mM EDTA, 0.5% Nonidet P40 and 0.5% sodium deoxycholate] and three times with TE buffer [10 mM Tris/HCl (pH 7.5) and 1 mM EDTA]. Next, the chromatin DNA bound to the beads was eluted with ChIP elution buffer [50 mM Tris/HCl (pH 7.5), 10 mM EDTA and 1% SDS] by heating at 65°C for 10 min. Reverse cross-linking was then performed using 20 mg/ml pronase (Roche) and the ChIP products were extracted with 1:5 phenol/chloroform (Bio-Rad Laboratories) and purified with ethanol. Finally, real-time PCR was then carried out to quantify the amount of DNA present.

RESULTS

Transcriptional activation causes short-term memory

Defects in the transcriptional activation of the S. cerevisiae GAL1 promoter are manifested as growth defects on galactose plates, but defects of its transcriptional repression status do not display any growth phenotypes. Previously, we had generated a fusion of the GAL1 promoter to the URA3 ORF [23], that displays defects in the glucose repression status of the GAL1 promoter as growth on U plates (uracil-depleted glucose plates) and lack of growth on F plates [FOA (5-fluoro-orotic acid)-containing glucose plates], since Ura3, which encodes the enzyme orotidine-5′-phosphate decarboxylase, is required for the biosynthesis of uracil and also converts
FOA into toxic fluorouracil. *S. cerevisiae* cells transformed with the *LYS2*-marked single-copy vector Pgal1-Ura317 (that contains a fusion of the *GAL1* promoter to the *URA3* ORF) grew on F plates and on G plates (uracil-depleted galactose plates), but not on U plates (Figure 1, lines 3 and 4 and Supplementary Figure S1 at http://www.BiochemJ.org/bj/439/bj4390487add.htm), reflecting the carbon-source regulation of the *GAL1* promoter. The cells grew on the F plates and not on the U plates as *URA3* was not expressed due to the glucose repression of the *GAL1* promoter, but they grew on the G plates as galactose induction of the *GAL1* promoter caused expression of *URA3*. In wild-type cells grown with glucose, Mig1 represses *GAL1* by recruiting Tup1 to the *GAL1* promoter. However, Mig1 activates *GAL1* in the absence of Tup1 [30], and *TUP1*-deleted cells grew on the U plates, but not on the F plates (Figure 1, lines 1 and 2), indicating that the Δ*TUP1* cells expressed Ura3.

When wild-type *S. cerevisiae* cells were picked from the G plates (Figure 1, line 3) and titrated for a second round on a F plate, they failed to grow (Figure 1, line 9), indicating that the cells were killed by large amounts of Ura3 enzyme still present in the cells from their growth under activating conditions (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/439/bj4390487add.htm for the titration scheme). When the wild-type cells, which had been grown under activating conditions on a G plate for the first round (Figure 1, line 3) and under non-selective conditions on a K plate (lysine-depleted uracil-containing glucose plate) for a second round (Figure 1, line 9), were titrated for a third round, they grew on the F plates (Figure 1, line 15), indicating that the repression of the *GAL1* promoter had been restored and that the Ura3 enzyme had been diluted out during the estimated 25 divisions each cell had undergone on the second plate [31]. Our observations here are reminiscent of the short-term memory of a previous activated state of transcription [14,15].

### A mixed population of repression-deficient and -competent ΔHTZ1 cells

*S. cerevisiae* cells lacking H2A.Z (ΔHTZ1) are known to be defective for the transcriptional activation of the *GAL1* promoter [10], but since histones also play a role in transcriptional repression we asked if ΔHTZ1 cells are also defective for the transcriptional repression of the *GAL1* promoter. ΔHTZ1 cells containing the Pgal1-Ura317 plasmid grew on both F and U plates (Figure 1, lines 5 and 6), indicating a mixed population of cells, a minority of which repressed the *GAL1* promoter and a majority of which failed to do so. When the ΔHTZ1 cells were transformed additionally with a single-copy vector containing the *HTZ1* gene, they grew like wild-type cells (Supplementary Figure S5B, line 2 at http://www.BiochemJ.org/bj/439/bj4390487add.htm), indicating that the absence of H2A.Z had caused the difference in growth. When H2A.Z-lacking cells were picked from the F plate (Figure 1, line 5) and titrated for a second round, they grew on the F plate, but not on the U plate (Figure 1, line 12; see Supplementary Figure S3 at http://www.BiochemJ.org/bj/439/bj4390487add.htm for the titration scheme). The reverse was found for cells taken from the U plate (Figure 1, line 5), which grew on the U plate, but not on the F plate (Figure 1, line 11; see Supplementary Figure S4 at http://www.BiochemJ.org/bj/439/bj4390487add.htm for the titration scheme), indicating that the mixed population of cells observed during the first round had been sorted out in the second round.

One possible explanation for the growth differences is that the ΔHTZ1 cells had acquired chromosomal mutations during their growth under selective conditions. The first three rounds of the experiment were repeated with diploid BY4743ΔWΔHTZ1 cells and the result was the same as for the haploid cells (Supplementary Figure S5A), indicating that the differences in growth had not been caused by host mutations. In order to further confirm this, the ΔHTZ1 cells from U and F plates (Figure 1, line 5) were cured of the plasmid and retransformed with the original Pgal1-Ura317 plasmid. The cured cells behaved as in the first round of titrations (Supplementary Figure S5B, lines 3 and 4), indicating that no chromosomal host mutations had occurred during growth under selective conditions.

Another possible explanation for the growth differences is that mutations had occurred in the Pgal1-Ura317 plasmid during growth under selective conditions. The Pgal1-Ura317 plasmids from ΔHTZ1 cells grown under selective conditions on U and F plates (Figure 1, line 5) were isolated and the *GAL1–URA3* fusions were sequenced. The sequences on the plasmids isolated from cells grown on U and F plates were identical, indicating that the difference in growth on the U and F plates had not been caused by mutations in the *GAL1* promoter nor in the *URA3* gene. It was, however, found that there were eight changes in the DNA sequence of the *GAL1* promoter as compared with the sequence published in the SGD (Saccharomyces Genome Database; http://www.yeastgenome.org/), but these changes had already been present in the vector DNA prior to transformation as they originated from the S288C strain (GenBank® accession number NC_001134.7) and the A.T.C.C. genomic DNA library #77162, which had been used to clone the *GAL1* promoter. When the experiment was repeated with a vector containing the *GAL1* sequence published in the SGD, which can be found in the BY4742ΔW strain and in the A.T.C.C. genomic DNA library #37323, the results were comparable (Supplementary Figure S5C), indicating that the eight changes in the DNA sequence of the *GAL1* promoter did not significantly affect glucose repression.

A final possible explanation for the growth differences is that the copy number of the plasmid was higher in the cells picked from the U plate than in the cells picked from the F plate. However, quantitative real-time PCR indicated that the ratio of the episomal S288C *GAL1* promoter to the chromosomal BY4742ΔW *GAL1* promoter was the same in the cells picked from the U and F plates (Supplementary Figure S6 at http://www.BiochemJ.org/bj/439/bj4390487add.htm), indicating that the copy number of the Pgal1-Ura317 plasmid in the cells on the U and F plates was the same.

### The transcription status of the *GAL1* promoter in ΔHTZ1 cells is stable

The memory of the activated transcription status of the *GAL1* was lost after the growth on one plate under repressing conditions (Figure 1, lines 24–26), and we asked if the two different transcription states that had been observed for the *GAL1* promoter in glucose-grown ΔHTZ1 cells would persist through several rounds of unselected growth. Glucose repression-competent ΔHTZ1 cells, which had been selected for by growth on the F plate (Figure 1, line 5) and which had been titrated on a K plate for a second round of unselected growth (Figure 1, line 12), grew on the F plate, but not on the U plate in the third round (Figure 1, line 18). In contrast, glucose repression-deficient ΔHTZ1 cells, which had been selected for by growth on the U plate (Figure 1, line 5) and which had been titrated on a K plate for a second round of unselected growth (Figure 1, line 11), grew on the U plate, but not on the F plate in the third round (Figure 1, line 17), indicating that both transcription states were stable. In the fourth round of titrations, the transcription status of the *GAL1–URA3* fusion remained stable.
under non-selective conditions for the vast majority of cells, with less than 1% of the first-round F plate cells losing glucose repression and forming colonies on the U plate (Figure 1, line 22), and less than 1% first-round U plate cells restoring glucose repression and forming colonies on the F plate (Figure 1, line 23). The few cells that had switched their transcription status were included in subsequent titration rounds, and the switched transcription status was also found to be stable (Figure 1, lines 29 and 30 and Supplementary Figure S1). During eight rounds of growth under non-selective conditions, corresponding with an estimated 200 cell divisions [31], the transcription status of the \textit{GAL1}–\textit{URA3} gene fusion remained stable for the vast majority of cells (Figure 1, line 28), and the experiment was terminated. The long-term persistence of the transcription status observed in the absence of H2A.Z suggests that the function of H2A.Z is to allow for the conversion between two stable epigenetic transcription states.

The transcription status of the chromosomal \textit{GAL1} promoter is also stable

The experiments presented in Figure 1 employed an episomal \textit{GAL1} promoter, which might have a different chromatin structure than the \textit{GAL1} promoter that is present on the chromosome. Therefore the \textit{URA3} ORF was integrated into the chromosomal \textit{GAL1} locus of wild-type, \textit{TUP1}-deleted and \textit{HTZ1}-deleted BY4742Δ\textit{W} cells. All integration events were confirmed by DNA sequencing. All colonies picked for wild-type cells grew on F, but not U, plates (Figure 2, lines 3 and 4), whereas all colonies picked for \textit{TUP1}-deleted cells grew on U, but not F, plates (Figure 2, lines 1 and 2). For the \textit{HTZ1}-deleted cells, two of eight colonies picked grew predominantly on the U plate (Figure 2, lines 5 and 6), whereas the remaining six colonies grew predominantly on the F plate (two of these six colonies are shown in Figure 2, lines 7 and 8). Wild-type cells displayed the same short-term memory of transcriptional activation that had been observed for the single-copy vector (Figure 2, lines 27–29), whereas \textit{ΔHTZ1} cells displayed the same long-term persistence of the transcription status that had been observed for the single-copy vector (Figure 2 and Supplementary Figure S7 at http://www.BiochemJ.org/bj/439/bj4390487add.htm).

A possible explanation for the growth differences is that mutations had occurred in the \textit{GAL1}–\textit{URA3} locus during growth under selective conditions. The genomic DNA of \textit{ΔHTZ1} cells grown on U and F plates was isolated and the \textit{GAL1}–\textit{URA3} gene fusion was amplified by PCR and sequenced. No changes in DNA sequence were found for cells of the same strain from U and F plates, indicating that the difference in growth on the U and F plate had not been caused by a mutation in the \textit{GAL1} promoter nor in the \textit{URA3} gene. The experiment had been performed with the \textit{GAL1} promoter sequence of the S288C strain. When the experiment was repeated with an integrative vector containing the \textit{GAL1} promoter sequence of BY4742 cells, the results were comparable (Supplementary Figure S8 at http://www.BiochemJ.org/bj/439/bj4390487add.htm), indicating that the changes in the DNA sequence of the \textit{GAL1} promoter did not significantly affect glucose repression nor the epigenetic inheritance of the transcription status. During nine rounds of growth under non-selective conditions, corresponding with an estimated 225 cell divisions [31], the transcription status of the \textit{GAL1}–\textit{URA3} fusion remained stable for the vast majority of cells (Figure 2, line 31), and the experiment was terminated. Note that the cells in Figure 2 lines 21 and 24 had not been exposed to U and F plates prior to the titration, indicating that their different \textit{URA3} expression status had not been ectopically induced by growth under selective conditions, but that the growth on the U and F plates reflects the transcription status of the \textit{GAL1} promoter at the time of the integration of the \textit{URA3} ORF into the \textit{GAL1} locus.

**Figure 3** The growth on U and F plates reflects the transcription status of the \textit{GAL1} promoter

(A) The indicated strains were grown in glucose liquid medium to a \(D_{600}\) value of 1 and induced in galactose liquid medium for the indicated number of hours. Total RNA was isolated and the amount of \textit{GAL1} mRNA relative to \textit{ACT1} mRNA was determined by quantitative real-time PCR. Wild-type (WT) BY4742Δ\textit{W} cells grown with glucose were designated as 1 and the results are means \(±\) S.D. for three replicate experiments. (B) Wild-type BY4742Δ\textit{W} cells were grown in glucose liquid media to a \(D_{600}\) value of 1 and induced with galactose liquid medium for 24 h in the presence (block) and absence of thiolutin. The cells that had been induced in the absence of thiolutin were switched to fresh thiolutin-containing glucose (Glu) and galactose (Gal) media. Cells were harvested at the indicated number of minutes after they had been switched to fresh medium and total RNA was isolated. The amount of \textit{GAL1} mRNA relative to \textit{ACT1} mRNA was determined by quantitative real-time PCR. The value determined for cells grown with glucose was designated as 1 and the results are means \(±\) S.D. for three replicate experiments. (C) The depicted strains were picked from the indicated plates and grown with glucose to a \(D_{600}\) value of 1. Total RNA was isolated and the amount of \textit{URA3} mRNA relative to \textit{ACT1} mRNA was determined by quantitative real-time PCR. Wild-type BY4742Δ\textit{W} cells were designated as 1 and the results are means \(±\) S.D. for three replicate experiments.

**Growth on U and F plates reflects the \textit{GAL1} transcription status**

Growth on reporter plates indicates a certain transcription status; however, this has to be confirmed by the determination of mRNA levels. Reverse transcription coupled with real-time PCR was used to determine the expression level of \textit{GAL1} mRNA relative to \textit{ACT1} mRNA in glucose-grown and galactose-induced wild-type and \textit{ΔHTZ1} cells. Figure 3(A) shows that an 8-h galactose induction caused a 2800-fold increase in \textit{GAL1} mRNA relative to \textit{ACT1} mRNA in wild-type cells, but only a 500-fold increase in \textit{ΔHTZ1} cells consistent with the previously reported galactose activation defect of cells lacking H2A.Z [10]. However, only a
1.8-fold increase of \( \text{GAL1} \) mRNA in \( \Delta \text{HTZ1} \) cells grown with glucose as compared with glucose-grown wild-type cells was measured, which did not confirm the glucose repression defect of cells lacking H2A.Z that had been indicated by their growth on the U plates. The most probable explanation is that \( \text{GAL1} \) mRNA, like the mRNAs from other glucose-repressed genes, is stable when the cells are grown with galactose, but rapidly degraded when cells are switched to a glucose medium [32]. In order to show that this was indeed the case, we induced BY4742 \( \Delta \text{W} \) cells in liquid galactose medium for 24 h in both the presence and absence of the transcription inhibitor thiolutin [29]. In the presence of thiolutin, the level of \( \text{GAL1} \) mRNA relative to \( \text{ACT1} \) was the same as in glucose-grown cells (Figure 3B, block), whereas in the absence of thiolutin the level of \( \text{GAL1} \) mRNA relative to \( \text{ACT1} \) mRNA was more than 1000-fold higher than in glucose-grown cells (Figure 3B, 0 min). When the cells that had been induced with galactose medium for 24 h in the absence of thiolutin were switched to fresh galactose medium containing thiolutin, \( \text{GAL1} \) mRNA remained unchanged, indicating that \( \text{GAL1} \) mRNA is stable in cells grown with galactose. However, when the cells were shifted to glucose medium containing thiolutin, \( \text{GAL1} \) mRNA was degraded rapidly, explaining why the glucose repression defect of the \( \text{GAL1} \) gene of cells lacking H2A.Z had not been detected previously [33–36].

Reverse transcription coupled with real-time PCR was used to determine the expression level of \( \text{URA3} \) mRNA relative to \( \text{ACT1} \) mRNA in strains expressing \( \text{URA3} \) from the \( \text{GAL1} \) chromosomal locus that had been picked from the different plates in Figure 2. The depletion of \( \text{TUP1} \) caused a 160-fold increase in \( \text{URA3} \) mRNA (Figure 3C), reflecting the growth of \( \Delta \text{TUP1} \) cells on U plates. No increase in \( \text{URA3} \) mRNA was observed for the \( \text{BY4742} \Delta \text{W} \Delta \text{HTZ1#1} \) strain when the cells had been picked from the W or F plate, whereas a 65-fold increase was observed when the cells had been picked from the U plate. A 50- and 60-fold derepression of \( \text{URA3} \) mRNA was observed for the \( \text{BY4742} \Delta \text{W} \Delta \text{HTZ1#3} \) strain when the cells had been picked from the W or U plate respectively, whereas no increase was observed when the cells had been picked from the F plate, indicating that the growth on the U and F plates reflected the transcription status of the integrated \( \text{GAL1–URA3} \) fusion. Note that the cells labelled W in Figure 3(C) had not been exposed to U or F plates prior to the isolation of RNA, demonstrating that the difference in the transcription status of the \( \text{GAL1} \) promoter could not have been ectopically induced by growth under selective conditions.

Galactose induction evicts nucleosomes from the \( \text{GAL1} \) locus

ChIP can be used to determine nucleosome occupancy of chromosomal DNA in vivo. An H2A.Z-containing nucleosome occupies the +1 position at the \( \text{GAL1} \) promoter (Supplementary Figure S9 at http://www.BiochemJ.org/bj/439/bj4390487add.htm) [4,37]. Histone H2B was chromosomally tagged with three HA epitopes and ten histidine residues, and ChIP coupled with real-time PCR was used to determine the chromatin status of the \( \text{GAL1} \) locus. Consistent with previous reports [4,37], a nucleosome was detected at the \( \text{GAL1} \) promoter which was evicted when cells were induced for 4 h with galactose (Figure 4A). Similar results were observed for the \( \text{GAL1} \) ORF (Figure 4B), indicating that the entire \( \text{GAL1} \) locus becomes nucleosome-free upon galactose induction. In \( \Delta \text{HTZ1} \) cells grown with glucose, histone H2B was still detected at the \( \text{GAL1} \) promoter indicating that a histone H2A-containing nucleosome occupied the \( \text{GAL1} \) promoter in the majority of \( \Delta \text{HTZ1} \) cells (Figure 4A). The \( \text{GAL1} \) promoter

![Figure 4](image)

**Figure 4** Galactose induction evicts nucleosomes from the \( \text{GAL1} \) locus

Endogenous H2B of the indicated strains was C-terminally tagged with three HA epitopes and ten histidine residues. Cells were grown in glucose liquid medium to a \( D_{600} \) value of 1 and induced in galactose liquid medium for 4 h. Cross-linked proteins were precipitated with anti-HA beads. Quantitative real-time PCR was used to determine the percentage immunoprecipitation for the \( \text{GAL1} \) promoter (A) and the \( \text{GAL1} \) ORF (B) relative to the percentage immunoprecipitation obtained for the unrelated \( \text{ZDS1} \) promoter. Results are means ± S.D. for three replicate experiments.

nucleosome remained intact in most \( \Delta \text{HTZ1} \) cells when they were induced for 4 h with galactose (Figure 4A), presumably reflecting the \( \text{GAL1} \) activation defect of cells lacking H2A.Z.

**Nucleosome occupancy reflects the transcription status of the \( \text{GAL1} \) locus**

The titration results presented in Figures 1 and 2 indicate that there is a mixture of glucose repression-competent and -deficient \( \Delta \text{HTZ1} \) cells. The nucleosome ChIP signal obtained for the \( \text{GAL1} \) promoter in glucose-grown \( \Delta \text{HTZ1} \) cells shown in Figure 4(A) might have been due solely to the glucose repression-competent cells in the mixture, whereas the \( \text{GAL1} \) promoter in the glucose repression-deficient cells could have been nucleosome-free. The integration of the \( \text{URA3} \) ORF into the \( \text{GAL1} \) locus allows for the separation of the mixture into glucose repression-competent and -deficient cells. We repeated the ChIP experiments with glucose-grown cells that had been picked from F and U plates. Figure 5(A) shows that the \( \text{GAL1} \) promoter of repression-competent \( \Delta \text{HTZ1} \) cells (that had been picked from the F plate of Figure 2, lines 6 and 8) was nucleosome-occupied, whereas the \( \text{GAL1} \) promoter of repression-deficient \( \Delta \text{HTZ1} \) cells (that had been picked from the U plate of Figure 2, lines 6 and 8) was nucleosome-free. Similar results were obtained for the \( \text{URA3} \) ORF (Figure 5B), suggesting that the stable \( \text{URA3} \) transcription status of the cells picked from the F and U plates had been caused by two different stably inherited chromatin structures at the \( \text{GAL1–URA3} \) locus, an active one that was nucleosome-free and an inactive one that was nucleosome-occupied.

**DISCUSSION**

The term ‘epigenetic memory of transcription’ has been used to describe the increase in the speed of galactose induction of \( \text{GAL1} \) mRNA if the cells had previously been exposed to galactose...
The experiments presented in Figure 1, lines 24–26, and in Figure 2, lines 27–29, indicate that the URA3 expression level in glucose-grown cells is higher if the cells had been exposed to galactose in the previous titration round, which is reminiscent of this phenomena. Subsequently, however, it has been reported that the epigenetic memory had been due to Gal1 protein present in the cells that had been exposed to galactose rather than to the memory of a particular transcriptional state [15]. This is fully consistent with the results described in the present paper, as the increase in growth on the uracil-depleted plates and the reduction in growth on the FOA-containing plates of cells that had been exposed to galactose can be explained by the temporary persistence of Ura3 protein in the cell following their switch to a glucose medium. The experiments described in the present paper with regards to the stable transcription states of cells lacking H2A.Z are very different, as they describe the persistence of a particular stable transcription state rather than the memory of a different previous state.

Previous work had identified the histone variant H2A.Z, which occupies the +1 nucleosome at the GAL1 promoter as a positive factor for transcription as transcriptional activation of the GAL1 gene is reduced in cells lacking H2A.Z [36]. We have shown in the present paper that, in addition, H2A.Z is also required to establish transcriptional repression of the GAL1 promoter. This effect has been overlooked by previous studies, as transcriptional activity is conventionally measured by determining the steady state levels of mRNA and the GAL1 mRNA, which is stable in cells grown with galactose, is rapidly degraded in cells grown with glucose. The results regarding the instability of GAL1 mRNA in cells grown with glucose in the present paper are fully consistent with the reported kinetics of glucose shut-down experiments for the GAL1 mRNA with cells pregrown under inducing conditions [37]. The GAL1 mRNA disappears within minutes upon the exposure of the cells to glucose, which can only be explained by mRNA degradation.

We failed to detect significant levels of GAL1 mRNA in glucose-grown ΔHTZ1 cells (Figure 3A), and we believe that this was due to the degradation of GAL1 mRNA in glucose-grown cells. Moderately elevated levels (66-fold) of URA3 mRNA were detected in repression-deficient glucose-grown ΔHTZ1 cells that contained the URA3 ORF inserted at the GAL1 locus. We and others have also failed to detect significant levels of GAL1 mRNA in glucose-grown ΔTUP1 cells, and the role of Tup1 in the glucose repression of the GAL1 promoter had been established with the help of GAL1–lacZ reporter fusions [36, 38]. The discrepancy between the published GAL1 mRNA and GAL1–lacZ results for ΔTUP1 cells, as well as the quick disappearance of GAL1 mRNA in galactose-grown cells following their switch to a glucose medium [37] suggested strongly that GAL1 mRNA is not stable in glucose-grown cells. Therefore we tested the stability of GAL1 mRNA in glucose-grown cells, and we found that GAL1 mRNA was indeed rapidly degraded once the cells were exposed to glucose (Figure 3B).

In glucose-grown wild-type cells, a H2A.Z-containing nucleosome occupies the +1 position at the GAL1 promoter (Figure 6A). In the absence of H2A.Z, there is a mixed population of cells (Figure 6B). In the majority of glucose-grown cells lacking H2A.Z, the GAL1 promoter is occupied by a histone H2A-containing nucleosome. Transcriptional repression is intact in those cells and if they contain a fusion of the GAL1 promoter to the URA3 ORF, the cells grow on glucose plates containing FOA, whereas they fail to grow on glucose plates lacking uracil. However in a minority of glucose-grown cells lacking H2A.Z the GAL1 promoter is nucleosome-free. Transcriptional repression cannot be established in those cells and they grow on glucose plates lacking uracil, whereas they fail to grow on glucose plates containing FOA. It is important to note that transcriptional derepression produces much less mRNA than transcriptional activation. Although transcriptional activation by Gal4 increases the level of GAL1 mRNA by 2800-fold, the amount of

![Figure 5](image-link) Nucleosome occupancy reflects the repression status of GAL1

Endogenous H2B of the indicated strains that had been picked from the depicted plates was C-terminally tagged with three HA epitopes and ten histidine residues. Proteins of glucose-grown cells were cross-linked and precipitated with anti-HA beads. Quantitative real-time PCR was used to determine the percentage immunoprecipitation for the URA3-linked GAL1(S288C) promoter (A) and the URA3 ORF (B) relative to the percentage immunoprecipitation obtained for the unrelated ZDS1 promoter. Results are means ± S.D. for three replicate experiments.

![Figure 6](image-link) H2A.Z is required to establish glucose repression in all cells

(A) Schematic drawing depicting the chromatin status of the GAL1 promoter in wild-type cells grown with glucose and with galactose. In all wild-type cells grown with glucose, the GAL1 promoter is repressed and occupied by an H2A.Z-containing nucleosome, whereas in all wild-type cells grown with galactose, the GAL1 promoter is activated and nucleosome-free. (B) Schematic drawing depicting the chromatin status of the GAL1 promoter in glucose-grown cells lacking H2A.Z. In the majority of glucose-grown ΔHTZ1 cells, the GAL1 promoter is repressed and occupied by a histone H2A-containing nucleosome, whereas in a minority of glucose-grown ΔHTZ1 cells, the GAL1 promoter is derepressed and nucleosome-free. In the absence of H2A.Z, both transcription states and chromatin states are stably inherited through many cell divisions.
transcriptional derepression observed for URA3 mRNA in ΔHTZ1 cells expressing URA3 under the control of the GAL1 promoter was only some 50–65-fold.

We do not believe that the growth on U plates induces the GAL1 promoter, and we do not believe that growth on FOA-containing plates represses the GAL1 promoter, as Ppr1 (pyrimidine pathway regulation 1), the activator of the URA3 gene, has no effect on the GAL1 promoter. Rather we believe that there is a mixture of repression-deficient and -competent H2A.Z-lacking cells that can be separated on uracil-depleted plates and on FOA-containing plates once the URA3 ORF has been placed under the control of the GAL1 promoter. We would like to point out that the respective selection into glucose repression-deficient ΔHTZ1 cells on uracil-depleted plates and into glucose repression-competent ΔHTZ1 cells on FOA-containing plates was carried out only for the experiments featuring the episomal GAL1 promoter presented in Figure 1, where scoops of approximately ten transformants had been subjected to 10-fold serial dilutions prior to the titrations. For the experiments with the chromosomal GAL1 promoter presented in Figure 2, individual colonies had been picked from uracil-containing FOA-lacking plates, which did not select for or against the expression of the GAL1–URA3 reporter (the plates had lacked only tryptophan to select for the integration of the URA3 ORF into the GAL1 locus). The colonies had been amplified on tryptophan-lacking plates and the titrations on uracil-depleted and on FOA-containing plates served only to determine which colonies consisted of glucose repression-deficient ΔHTZ1 cells (colonies 3 and 4; Figure 2, lines 5 and 6) and which colonies consisted of glucose repression-competent ΔHTZ1 cells (colonies 1 and 2; Figure 2, lines 7 and 8), but the transcriptional state could not have been caused by selective conditions as the cells had not been exposed to selective conditions (Figure 2, compare lines 21 and 24; WW means that the cells had never been exposed to either the uracil-depleted or FOA-containing media).

The percentage of H2A.Z-lacking cells whose GAL1 promoter is nucleosome-free can be estimated from the 10-fold serial dilutions of cells presented in Figures 1 and 2. The results presented in Figure 1 suggest that a histone H2A.Z-containing nucleosome occupies the episomal GAL1 promoter in approximately 1% of ΔHTZ1 cells, whereas 99% of the episomal GAL1 promoter remains nucleosome-free. The results presented in Figure 2 indicate that an H2A-containing nucleosome occupied the chromosomal GAL1 promoter in approximately 75% of ΔHTZ1 cells, whereas 25% remained nucleosome-free. The inheritance of these two chromatin states of the GAL1 promoter on both the episome and the chromosome is completely stable. Only very few cells are able to switch to the other state and once switched, they remain in the new state. The chromatin state is not restricted to the GAL1 promoter nucleosome, but also extends to the URA3 ORF which also exists in an active nucleosome-free state and in an inactive nucleosome-containing state. Since H2A.Z is also required for the removal of the GAL1 promoter nucleosome upon galactose induction, it is actually required for both the removal and the addition of the nucleosome at the GAL1 promoter. According to the model described in the present paper, the role of H2A.Z is to facilitate the addition and removal of promoter nucleosomes and to prevent the formation of unfavourable stable epigenetic chromatin structures, which are not in accordance with the environmental conditions.

AUTHOR CONTRIBUTION

Jin Zhao, Wee Leng Siew, Weiqi Sun and Norbert Lehming performed the experiments and analysed data. Norbert Lehming wrote the paper.

ACKNOWLEDGEMENTS

We thank L.M. Chew for excellent technical assistance and Dr I. Albert (Pennsylvania State University, University Park, PA, U.S.A.) for permission to use Supplementary Figure S9.

FUNDING

This work was supported by the Academic Research Fund [AcRf Tier 1 grant numbers T13-0902-P16 and P223-URC1-06].

REFERENCES


H2A.Z interconverts chromatin states

SUPPLEMENTARY ONLINE DATA

The histone variant H2A.Z interconverts two stable epigenetic chromatin states

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Supplementary Figures S1–S9 and Supplementary Tables S1 and S2 are on the following page.

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The transcriptional status of the GAL1 promoter in ΔHTZ1 cells is stable

Cells of the indicated genotype were transformed with the LYS2-marked single-copy plasmid Pgal1-Ura317 expressing URA3 under the control of the GAL1 promoter. Cells were 10-fold serially diluted and titrated on to K, F, U, G plates. Lines 1–6 represent the first set of titrations. In subsequent titration rounds, cells were taken from the indicated plates of the previous rounds, 10-fold serially diluted and titrated on to the depicted plates. Lines 7–12 represent the second round of titrations, lines 13–18 represent the third round of titrations, lines 19–23 represent the fourth round of titrations, lines 24–30 represent the fifth round of titrations, lines 31–38 represent the sixth round of titrations, lines 39–46 represent the seventh round of titrations, and lines 50–53 represent the tenth round of titrations. The letters indicate the growth history, e.g. line 30 'ΔHTZ1 from UKKF' shows the fifth round of titrations. ΔHTZ1 cells picked from the U plate of the first set (line 5) were grown for two rounds of titrations under non-selective conditions on K plates (lines 11 and 17) before being picked from the F plate of the fourth round (line 23). See Figures S2–S4 for the titration schemes. All plates were incubated for 3 days at 28°C.
First round: 10-fold serial dilutions of wild-type *S. cerevisiae* BY4742 cells containing the Pgal1-Ura317 plasmid which expresses Ura3 under the control of the GAL1 promoter were 10-fold serially diluted and titrated on to K, F, U and G plates and incubated for 3 days. The cells grew on K, F and G, but not on U, plates, reflecting the carbon-source regulation of the GAL1 promoter, which is repressed when the cells are grown with glucose and which is active when the cells are grown with galactose as the carbon source. Second round: cells were taken from the G plate, 10-fold serially diluted, titrated on to the indicated plates and incubated for 3 days. The cells grew well on K and G plates, residually on U plates and failed to grow on F plates, indicating that the cells had accumulated large amounts of Ura3 enzyme during growth under activating conditions on the G plate, which allowed them to grow for a few cell divisions on the plate and which killed them on the F plate. Third round: cells which had been grown for the first round on the G plate and for the second round on the K plate were 10-fold serially diluted, titrated on to the indicated plates and incubated for 3 days. The cells grew exactly as in the first round, indicating that no stable chromatin state had been established during the growth on the G plate in the first round and that the Ura3 enzyme had been diluted out during the growth on the K plate in the second round.

First round: 10-fold serial dilutions of BY4742 ΔWΔHTZ1 cells containing the Pgal1-Ura317 plasmid were titrated on to the indicated plates and incubated for 3 days. The cells grew well on the K, U and G plates, and less well on the F plate, indicating a mixed population of cells, a minority of which repressed the GAL1 promoter and a majority of which failed to do so. Second round: cells were taken from the F plate, 10-fold serially diluted, titrated on to the indicated plates and incubated for 3 days. The cells grew well on the K, F and G plates, whereas they failed to grow on the U plate, indicating that all cells now repressed the GAL1 promoter when grown with glucose. Growth on the G plate indicates that the URA3 gene had not been damaged by growth on the F plate and that also the galactose induction of the GAL1 promoter was still intact. Third round: cells which had been grown for the first round on the F plate and for the second round on the K plate were 10-fold serially diluted, titrated on to the indicated plates and incubated for 3 days. The cells grew exactly as in the second round, indicating that the derepression status of the GAL1 promoter was stable and that all cells expressed Ura3. Fourth and fifth rounds: cells which had been grown for the first round on the U plate and for the subsequent rounds on K plates were 10-fold serially diluted, titrated on to the indicated plates and incubated for 3 days. A few colonies were observed on the F plates, indicating that while the GAL1 promoter was still derepressed in most cells as they expressed Ura3, a few cells had switched the expression status of the GAL1 promoter and now repressed the URA3 gene.
J. Zhao and others

Figure S5 Growth differences are not caused by mutations

(A) Diploid BY4743ΔW and BY4743ΔWΔHTZ1 strains (which are LYS +) were transformed with the TRP1-marked single-copy plasmid Pgal1-Ura314 that expressed URA3 under the control of the GAL1 promoter. Diploid cells were 10-fold serially diluted and titrated on to the depicted plates (lines 1–4). In the second round of titrations (lines 5 and 6), cells were taken from the indicated plate of the first round, 10-fold serially diluted and titrated on to the depicted plates. In the third round of titrations (lines 7 and 8), cells were taken from the indicated plate of the second round, 10-fold serially diluted and titrated on to the depicted plates. All plates were incubated for 3 days at 28 °C. (B) BY4742ΔW cells were transformed with the LEU2-marked single-copy vector YCplac111 (line 1). BY4742ΔWΔHTZ1 cells were transformed with YCplac111 containing the HTZ1 gene (line 2). BY4742ΔWΔHTZ1 cells from the U plate of Figure 1 of the main text, line 5, were cured of the plasmid and transformed with YCplac111 (line 3). BY4742ΔWΔHTZ1 cells from the F plate of Figure 1 of the main text, line 5, were cured of the plasmid and transformed with YCplac111 (line 4). All strains were made competent and transformed with the Pgal1-Ura317 plasmid. Dilutions of cells (10-fold) were titrated on to K, F, U and G plates and incubated at 28 °C for 3 days. (C) Cells of the indicated genotype were transformed with the LYS2-marked single-copy vector Pgal1-Ura317 expressing URA3 under the control of the GAL1 promoter of BY4742 (odd lanes) or S288C (even lanes). Cells were 10-fold serially diluted and titrated on to K, F, U, and G plates. Lines 1–6 represent the first set of titrations. In the second round of titrations (lines 7–14), cells were taken from the indicated plate of the first round, 10-fold serially diluted and titrated on to the depicted plates. All plates were incubated for 3 days at 28 °C.

Figure S6 The copy number of the Pgal1-Ura317 plasmid was not affected

DNA was isolated from cells that had been picked from the indicated plates of Figure 1 of the main paper. The ratio of episomal GAL1 promoter (S288C) to chromosomal GAL1 promoter (BY4742) was determined by quantitative real-time PCR. No template was detected with DNA isolated from untransformed BY4742ΔWΔHTZ1 cells and the primer pair for the episomal GAL1 promoter, and no template was detected with the Escherichia coli-purified Pgal1-Ura317 plasmid and the primer pair for the chromosomal GAL1 promoter, indicating that the primer pairs were specific for their respective templates.
Figure S7  Transcription status of the chromosomal GAL1 promoter is stable in ΔHTZ1 cells

The URA3 gene was integrated into the GAL1 chromosomal locus of BY4742ΔW cells of the indicated genotype with the help of the TRP1 marker. Cells were 10-fold serially diluted and titrated on to W, F, U and G plates. Lines 1–8 represent the first set of titrations. In subsequent titration rounds, cells were taken from the indicated plates of the previous rounds, 10-fold serially diluted and titrated on to the depicted plates. Lines 9–19 represent the second round of titrations, lines 20–30 represent the third round of titrations, lines 31–37 represent the sixth round of titrations, lines 38–44 represent the seventh round of titrations, lines 45–51 represent the tenth round of titrations and lines 52–57 represent the eleventh round of titrations. W and G plates were incubated for 3 days at 28°C, whereas U and F plates were incubated for 6 days at 28°C.
Figure S8  Glucose repression was not affected by strain variations

The URA3 gene was integrated into the GAL1 chromosomal locus with the help of the TRP1 marker. The integrative plasmid contained either the GAL1 promoter sequence from BY4742 or from S288C as indicated. All integration events were confirmed by DNA sequencing. 10-fold serial dilutions of cells were titrated on to W, F, U, and G plates. W and G plates were incubated for 3 days at 28 °C, whereas U and F plates were incubated for 6 days at 28 °C.

Figure S9  Nucleosome map of the GAL1 promoter

The nucleosome map of the GAL1 promoter was taken from http://h2az.atlas.bx.psu.edu/?chrom=chr2&feature=278750&zoom=1000&plot=comp-id&submit=Display%21&min_fit=0 &img_size=1000. The top panel shows the position of the H2A.Z-containing nucleosome N278796 and the panel below shows the positions of all nucleosomes of the GAL1 promoter. Permission to reproduce this Figure has been given by Dr Istvan Albert (Pennsylvania State University, University Park, PA, U.S.A.).
**Table S1  Sequences of PCR primers used**

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**Table S2  Genotypes of strains used**

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