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# Chromatin transitions during activation and repression of galactose-regulated genes in yeast

**Giacomo Cavalli and Fritz Thoma[1]**

Institut für Zellbiologie, ETH-Hönggerberg, 8093 Zürich, Switzerland
[1]Corresponding author

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**To study the fate of nucleosomes during transcription, a yeast gene '*GAL-URARIB*' was constructed which is tightly regulated by the *GAL1* promoter and shows in its inactive state a series of positioned nucleosomes that are sensitive for monitoring structural changes by micrococcal nuclease. Upon transcriptional activation, nucleosome positions were lost, but a residual nucleosomal repeat with an altered repeat length and no changes in psoralen accessibility measured by a band shift assay indicated that nucleosomes were present but rearranged on the transcribed gene. When chromatin was prepared 10 or 50 min after glucose repression, nucleosomes were repositioned in a large fraction of the population by a rapid process which most likely did not depend on histone synthesis or DNA replication. However, complete regeneration of the inactive structure and repeat length was observed after one cell generation (2.5 h) suggesting that in this step some missing histones were replaced. The results are consistent with a local dissociation of nucleosomes at the site of the polymerase followed by a rapid reassembly into nucleosomes behind it. The data are further supported by analysis of the chromosomal *GAL1*, *GAL7* and *GAL10* genes.**

*Key words:* chromatin structure/gene/transcription/nucleosome/yeast

## Introduction

Packaging of eukaryotic DNA into nucleosomes, chromatin fibres and higher order structures restricts the accessibility of the DNA sequence for regulatory proteins and polymerases. While recent reports substantiated the role of positioned nucleosomes in the regulation of transcription and replication (for reviews see Grunstein, 1990; Thoma, 1991; Kornberg and Lorch, 1992), the fate of nucleosomes when transcribed by RNA polymerase II is controversial and the structural transitions that lead to the regeneration of an inactive structure after termination of transcription escaped our attention. Taking advantage of the tight regulation of glucose-repressed genes in yeast, we followed the transcription-dependent structural transitions with respect to the presence of nucleosomes and nucleosome positioning using nuclease digestion and psoralen cross-linking as independent approaches.

In nucleosomes, the DNA is wrapped in two turns around a histone octamer and sealed from the outside by histone H1. Yeast, which is the organism of choice for this work, has no histone H1 and a short nucleosomal repeat of ~160 bp (Thomas and Furber, 1976; Lohr *et al.*, 1977; Bernardi *et al.*, 1991). Nucleosomes are dynamic structures. They can dissociate into DNA and histone components, undergo folding–unfolding transitions *in vitro* and change positions by sliding along the DNA *in vitro* and *in vivo* thereby modulating the accessibility of the DNA sequences (for reviews, see Thoma, 1992; Van Holde *et al.*, 1992). These properties are reflected by the fact that nucleosome positions *in vivo* are determined by at least three parameters, namely DNA sequences (in histone–DNA interactions) (Thoma and Simpson, 1985; Straka and Hörz, 1991), boundaries (flanking structures) (Thoma, 1986; Fedor *et al.*, 1988; Roth *et al.*, 1990) and chromatin folding (Thoma and Zatchej, 1988). Furthermore, nucleosomes may be selectively removed from promoter regions by interactions with transcription factors. These structural changes can be very fast and may occur in the absence of DNA replication (Schmid *et al.*, 1992). Competitive reconstitution experiments showed differences in nucleosome formation between various sequences, artificial 'bent' DNA being superior to a sequence of a sea urchin 5S ribosomal RNA gene (5S DNA) and to bulk nucleosomal DNA (Shrader and Crothers, 1989, 1990). Nucleosome formation on 5S DNA inserted in yeast minichromosomes suggested a hierarchical array of positioning signals of different strength (Thoma and Simpson, 1985). Hence, nucleosome stability and mobility might depend on differential affinities of sequences for histones.

The fate of nucleosomes during transcription elongation is not clear. For steric reasons it seems impossible that a transcription complex might follow the path of the double helix when it is folded on the nucleosome surface. Hence, some sort of structural alterations or alternatively dissolution of nucleosomes must occur (Thoma, 1991). The available data from *in vitro* transcription of single nucleosomes support both possibilities (Lorch *et al.*, 1987, 1988; Losa and Brown, 1987; Clark and Felsenfeld, 1992). The evaluation of the *in vivo* data is more problematic due to limitations of quantification in the experimental approaches (for a critical discussion see Reeves, 1984). A loss of nucleosomes was most clearly demonstrated for ribosomal RNA genes heavily transcribed by RNA polymerase I. The evidence is based on independent techniques, namely on electron microscopy, a loss of a nucleosomal repeat pattern by MNase digestion, enhanced accessibility to psoralen visualized in the electron microscope and by enhanced accessibility to psoralen measured by a band shift assay during gel electrophoresis (Sogo *et al.*, 1984; reviewed in Thoma and Sogo, 1988; Conconi *et al.*, 1989). The reports on transcriptional active RNA polymerase II genes scatter between nucleosome loss, presence of nucleosomes and altered nucleosomal structures (for reviews, see Kornberg and Lorch, 1992; Van Holde *et al.*, 1992). For example, one group of results favours the presence of nucleosomes on transcribed genes: nucleosome-like structures were observed in electron micrographs

between RNA polymerases of a transcription unit (Foe *et al.*, 1976) and transcripts were identified by psoralen cross-linking and electron microscopy in a nucleosomal region of SV40 minichromosomes (De Bernardin *et al.*, 1986). Using a nuclease digestion approach, a change from a nucleosomal repeat into a smear was observed in the yeast *GAL1* gene upon activation of transcription, but a residual repeat and a DNase I pattern were consistent with at least the presence of some nucleosomes on the template (Lohr, 1983). A second group of results suggests a loss of nucleosomes: a disruption of the nucleosomal repeat was observed for example after heat stimulation of the *Drosophila* heat shock genes (Wu *et al.*, 1979; Levy and Noll, 1981) or in the transcribed early histone genes of sea urchin (Wu and Simpson, 1985). While in transcriptionally active Balbani ring genes of Chironomus a nucleosomal repeat was detected and nucleosomes were observed by electron microscopy, hyperactivation of transcription produced a loss of nucleosomes and a loss of a nucleosomal repeat (Widmer *et al.*, 1984) suggesting that presence or absence of nucleosomes depends on the transcription rate. A third group of data supports an altered nucleosome structure which uncovers the previously shielded sulfhydryl groups of histone H3 (Prior *et al.*, 1983) or results in a 'half nucleosomal' cutting pattern by DNase I digestion (Lee and Garrard, 1991). Although these studies do not tell us the mechanisms of chromatin transcription at the site of the RNA polymerase, they strongly document that dramatic structural changes occur.

But what happens when transcription is turned off? When and how does regeneration of the inactive structure occur? A reformation of a nucleosomal repeat was observed 3 h after reversal from heat shock (Wu *et al.*, 1979), or on the *GAL1* gene when yeast cells entered the stationary phase (Lohr, 1983, 1984) and inhibition of RNA polymerase II transcription by $\alpha$-amanitin resulted in a rapid loss of the H3 sulfhydryl accessibility (Chen *et al.*, 1990). A detailed investigation of the transcription-dependent structural changes of the 87A7 heat shock locus in *Drosophila* cells revealed that the reestablishment of inactive preinduced chromatin structure occurred over a relatively short time and hence would not appear to require DNA replication (Han *et al.*, 1985). Chromatin analysis of sea urchin early histone genes during embryogenesis suggested that this highly active gene family is deficient in histones during transcription and that redeposition of histones following repression is succeeded by further remodelling of chromatin structure during later cell divisions (Wu and Simpson, 1985).

Based on the controversial structural situation of transcribed genes, we think that the extent of the structural perturbations during transcription as well as the regeneration of the inactive structure might be gene-specific and depend on several parameters such as the differential affinities of sequences for histones, the transcription rate, the RNA polymerase and the mode by which nucleosomes are positioned. We therefore addressed these topics by construction of an artificial gene in the yeast *Saccharomyces cerevisiae*, called *GAL-URARIB*, which has well-defined properties. It is heavily transcribed in galactose, tightly regulated and rapidly repressed by glucose, it has some precisely positioned nucleosomes that are sensitive to detect structural changes and nucleosome positioning depends on a boundary effect. We have studied the fate of nucleosomes and nucleosome positions using micrococcal nuclease digestions supplemented by an independent psoralen cross-linking assay in cells grown in glucose medium when the genes are repressed, in galactose medium when they are transcribed, and in a time course after repression with glucose. The studies on the artificial gene are supplemented and confirmed by investigations on the structurally more complex natural genes *GAL1*, *GAL7* and *GAL10*.

## Results

*GAL-URARIB* is a transcriptionally regulated gene which in contrast to many natural genes is characterized by a very clear chromatin structure and hence is most suitable to study structural changes upon transcriptional activation and repression (Figure 1). *GAL-URARIB* consists of the *GAL1* promoter, which is activated in medium containing galactose and repressed in glucose, a fragment of the *URA3* sequence of *S.cerevisiae* (*URA*), a sequence of the *Dictyostelium discoideum* ribosomal RNA gene (*RIB*) and the 3′ end of *URA3* as transcription terminator (U3′). *GAL-URARIB* was integrated in the *LEU2* locus in chromosome III of a yeast strain YM262 (Johnston and Davis, 1984) to give strain GCY5. GCY5 is characterized by a relative slow growth rate in selective and complete media containing galactose (~3 h). This property allows to proceed through the critical phase of chromatin preparations (~80 min up to lysis of spheroplasts) within a fraction of the time required for a cell cycle.

### *Transcriptional regulation of GAL-URARIB, GAL1 and GAL10 genes*

To investigate transcriptional regulation of *GAL-URARIB*, RNA was extracted from cells grown in glucose or galactose and analysed by Northern hybridization with probes for the 5′ region (URA) or the central part (RIB) of the gene (Figures 1 and 2a). While no transcript was detected when cells were grown in glucose, two major transcripts of 1.45 and 1.7 kb were detected by both probes when cells were grown in galactose. This shows that the gene was tightly repressed in glucose and activated in galactose as expected for genes driven by the *GAL1* promoter (St John and Davis, 1981). The 1.45 kb RNA is the most abundant transcript and is probably created by processing of the 1.7 kb species at the end of the RIB sequences (the RIB sequences contain the external transcribed spacer and the beginning of the coding region of 17S RNA). The efficiency of glucose repression was estimated by half-life analysis of RNA after shifting cells from galactose to glucose (Figure 2a). The RNA levels of *GAL-URARIB* decayed rapidly with little RNA left after 20 min. Plotting RNA levels versus time indicated a half-life of 6.5 min for the 1.45 kb transcript. The rapid decay showed that glucose repression on *GAL-URARIB* was complete within few minutes, consistent with previous reports of *GAL1-10*-driven genes (Adams, 1972; Nam and Fried, 1986). RNA analysis of the *GAL1* and *GAL10* genes showed the expected sizes of 1.7 kb for *GAL1* and 2.3 kb for *GAL10* (St John and Davis, 1981) (Figure 2), rapid repression of transcription in glucose and half-lives of ~8.5 and 5 min, respectively.

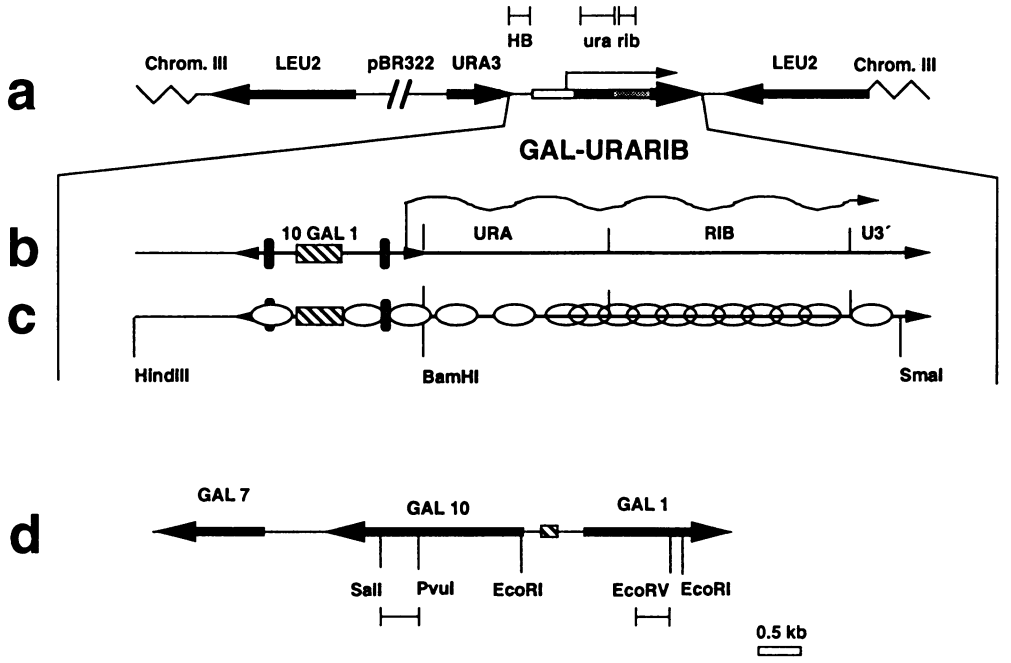


**Fig. 1.** The *GAL-URARIB* gene integrated in the *LEU2* locus of chromosome III. (**a**) *GAL-URARIB* was cloned in YIp28 and integrated in the *LEU2* locus. The map is shown to scale, as well as the probes used in this study (HB, ura and rib). (**b**) The *GAL-URARIB* gene consists of the divergent *GAL1-10* promoter, a 624 bp fragment from the yeast *URA3* gene (URA), a 830 bp sequence from the coding region of *D.discoideum* rDNA (RIB) and a 260 bp fragment from *URA3* 3′ end (U3′). The expected RNA transcript (wavy arrow), the non-nucleosomal region containing the $UAS_{GAL}$ (box) and the *GAL1* and *GAL10* TATA boxes (hatched ovals) are shown. (**c**) The chromatin structure of the inactive gene: positioned nucleosomes in the 5′ region and at the 3′ end of the gene (separate ovals) and random or multiple positions (overlapping ovals). The *Hin*dIII site used for indirect end-labelling and the *Bam*HI and *Sma*I sites used for psoralen analysis are indicated. (**d**) The *GAL1-GAL10-GAL7* region is shown (adapted from Citron and Donelson, 1984). The relevant restriction sites and DNA fragments used as probes (bars) are indicated.

### *Chromatin structure of the repressed GAL-URARIB gene*

Chromatin structure of the inactive *GAL-URARIB* gene was studied in cells grown in glucose, in which the gene is repressed. Crude nuclei were isolated and chromatin was digested by micrococcal nuclease (MNase). The DNA fragments were run on an agarose gel, blotted onto nitrocellulose membrane and hybridized to a probe spanning the central region of the gene (RIB). The results showed a clear nucleosomal repeat, extending for at least 15 bands. Hence, *GAL-URARIB* and its flanking regions are organized in nucleosomes (Figure 3a, glucose lanes). The repeat length for fragments corresponding to the monomer up to the tetramer, which all originate from the coding region of the gene, was ~180 bp. The repeat length of genomic chromatin measured on the same gel was ~160 bp (not shown) consistent with earlier observations (Lohr *et al.*, 1977; Bernardi *et al.*, 1991). This difference reflects the locus-specific nucleosome arrangement of *GAL-URARIB*.

Nucleosome positioning was studied using the indirect end-labelling method. The cutting sites for MNase in chromatin were compared with those in protein-free DNA. Protection of 140–200 bp against cutting was interpreted as precisely positioned nucleosomes (Figure 4). The *GAL1-10* promoter region showed three positioned nucleosomes (Figure 4a) and a region around $UAS_{GAL}$ with no strong cutting sites for MNase. This region was previously shown to be non-nucleosomal (Lohr *et al.*, 1987; Fedor *et al.*, 1988). Our observation confirms the earlier data and shows that the artificial construct did not affect promoter structure. Precisely positioned nucleosomes characterized by strong protection were located in the 5′ end of the transcribed region of *GAL-*

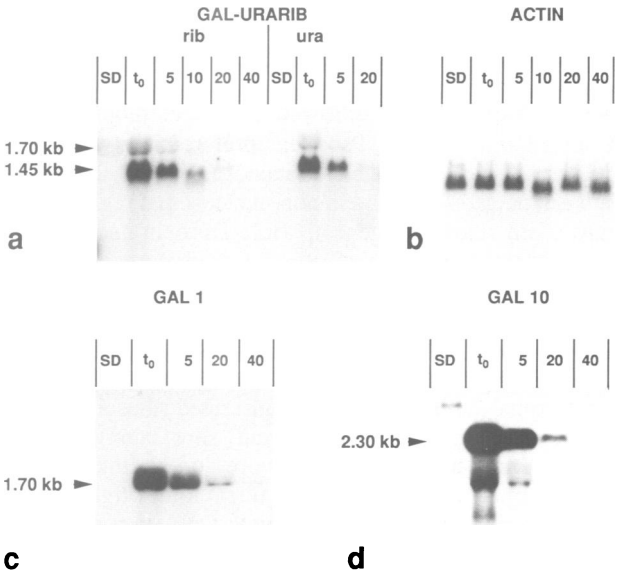


**Fig. 2.** Rapid repression of RNA synthesis in glucose. Northern analysis is shown from cells grown in glucose (SD lane), galactose ($t_0$ lane) or shifted from galactose to glucose for 5, 10, 20 or 40 min. (**a**) RNA of *GAL-URARIB*. The same transcripts (1.7 and 1.45 kb) were detected by the *RIB* probe (rib) and the *URA* probe (ura). (**b**) Analysis of the same RNA with a 500 bp probe from the actin gene. The levels of actin transcript are the same in glucose and galactose (Schultz *et al.*, 1987) and they were used as internal standard for correction of the loading errors in the calculation of half-lives. (**c**) *GAL1* gene detected by a 395 bp *Eco*RV–*Apa*I probe from the coding region. (**d**) *GAL10* RNA detected by a 416 bp *Sal*I–*Pvu*I probe. The sizes of the major transcripts are indicated. The *GAL-URARIB*, *GAL1* and *GAL10* transcripts decay rapidly after shifting the cells from galactose to glucose, whereas actin RNA levels remain constant.

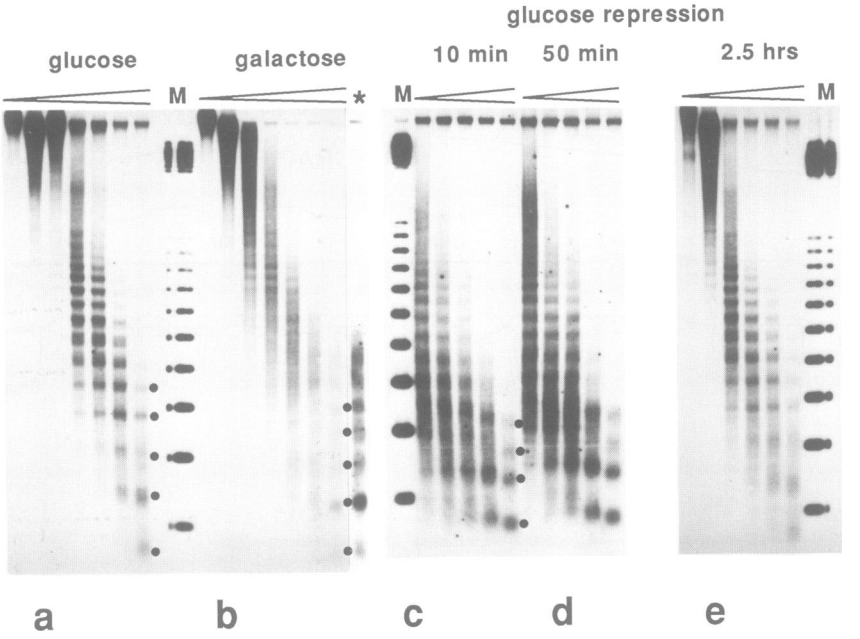


**Fig. 3.** Nucleosomal repeat analysis in *GAL-URARIB* reveals a transcription-dependent chromatin transition in *GAL-URARIB* and a rapid refolding after glucose repression. Crude nuclei were prepared from cells grown in glucose (**a**), galactose (**b**), or 10 min (**c**), 50 min (**d**) and 2.5 h (**e**) after shifting cells from galactose to glucose. Chromatin was digested with increasing amounts of MNase (<), DNA was purified, run on 1% agarose gels, blotted to nitrocellullose membranes and hybridized to the rib probe. A DNA ladder consisting of multiples of 256 bp was used for calibration (M lanes). Broad bands (dots) correspond to 1–5 nucleosomes ('nucleosome repeat'). The asterisk indicates a longer exposure of the last lane in panel b.

*URARIB*. Towards the 3′ end, protection was less clear, consistent with multiple or random nucleosome positions. This indicates that positioning at the 5′ end might be directed by a boundary function in the *GAL1-10* promoter (Fedor *et al.*, 1988).

As an alternative assay for chromatin structure, we applied psoralen cross-linking followed by a gel mobility shift (Conconi *et al.*, 1989). Psoralens preferentially react with and cross-link the double-stranded DNA in the linker between nucleosomes and in non-nucleosomal regions (e.g. transcribed ribosomal genes), while DNA in nucleosomes is not affected. Binding of psoralen leads to a retardation of DNA during electrophoresis. The extent of gel retardation is a function of the amount of psoralen cross-linking. Hence, nucleosomal substrates lead to a low retardation due to reactions in linker DNA (f band, fast band), while non-nucleosomal substrates such as transcribed ribosomal genes lead to a strong retardation (s band, slow band).

Cells grown in glucose were cross-linked with 4,5′,8-trimethylpsoralen and ultraviolet light (UV, predominantly 366 nm) and genomic DNA was extracted. DNA was cut with *Bam*HI and *Sma*I to release the *GAL-URARIB* coding region, electrophoresed alongside deproteinized DNA either non-cross-linked or cross-linked after purification. Cross-linking was then reversed with UV light (predominantly 254 nm) and DNA was blotted and hybridized with the *RIB* probe. The results showed a low retardation (f band) consistent with a nucleosomal structure for the *GAL-URARIB* gene in the repressed state (Figure 5a). As a positive control to demonstrate that the cross-linking procedure does detect non-nucleosomal chromatin, the same DNA was cut by *Eco*RI and two fragments of the coding region of the ribosomal genes were probed. One strong band showed low gel retardation (f band) while a minor fraction was retarded (smear above the strong band) and represents ribosomal genes that lost nucleosomes (Figure 5b). This is consistent with results obtained by Dammann *et al.* (1993) who showed that under similar growth conditions the majority of the ribosomal genes was inactive, slightly retarded and displayed a nucleosomal structure, whereas a minor fraction was active, non-nucleosomal and gave a higher retardation. They also demonstrated that the extent of cross-linking and band shift was sensitive to growth conditions.

### *Nucleosomes are present but rearranged on the transcribed GAL-URARIB gene*

Crude nuclei from cells grown in galactose were digested by MNase. When the nucleosomal repeat was analysed, a few bands were seen in the upper region of the gel for fragments >1.8 kb (Figure 3b) indicating a regular spacing of nucleosomes in the flanking regions. Below 1.8 kb, where the probe predominantly detects structures in the coding region of *GAL-URARIB*, the nucleosomal repeat changed into a smear and a residual nucleosomal pattern was found only at higher levels of digestion. However, the repeat length under these conditions was ~160 bp, ~20 bp shorter than in glucose (compare dots in Figure 3a and b). The altered repeat length for the residual repeat indicated that some nucleosomes were present during transcription but they were rearranged. Since a probe for the *RIB* sequences was used, the results emphasize a rearrangement on the *RIB* sequences. (Due to cross-hybridization with another *URA3* sequence in the genome no *URA* sequences could be used as probes.)

Mapping of nucleosome positions demonstrated that the nucleosome arrangement on the 5′ end of the transcribed *GAL-URARIB* was dramatically altered (Figure 4b). The cutting patterns of MNase in chromatin and control DNA were similar. The loss of protection of DNA cutting sites is consistent either with a loss of nucleosomes, or with a

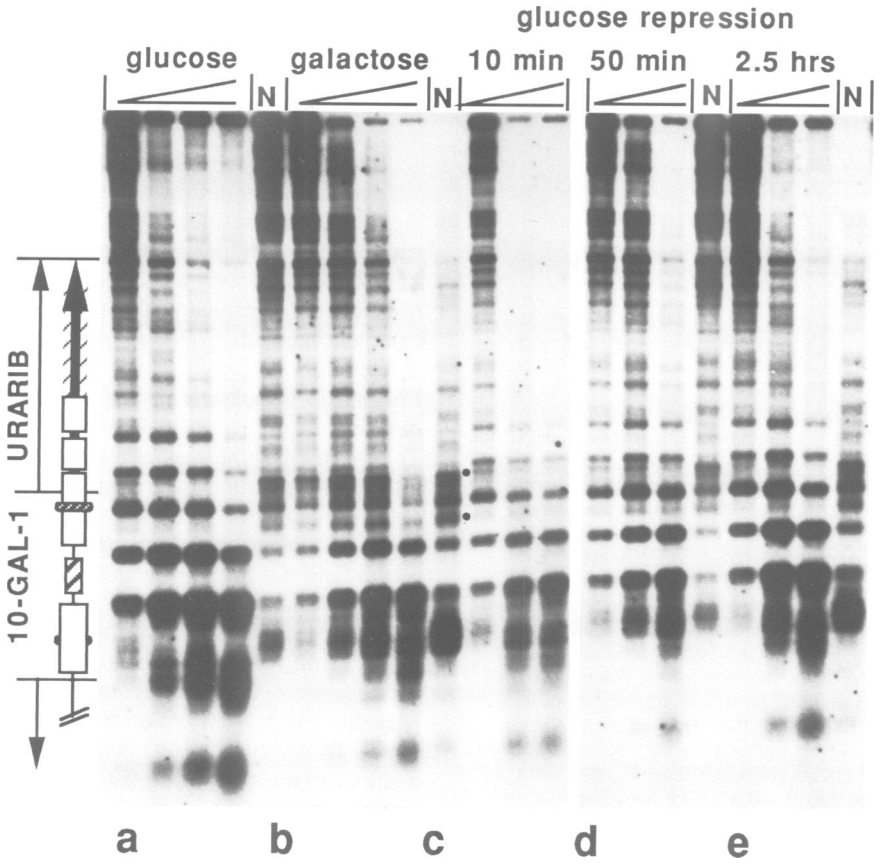


**Fig. 4.** Transcription-dependent loss of nucleosome positions and rapid repositioning after glucose repression on *GAL-URARIB*. Nucleosome positioning was analysed by MNase digestion and indirect end-labelling. Purified DNA from MNase-digested nuclei or naked DNA (N lanes) digested with MNase (the same DNAs as for Figure 3) were restricted with *Hin*dIII, run on an agarose gel, blotted and hybridized to the probe HB. Protections of 140–200 bp were interpreted as positioned nucleosomes. The non-nucleosomal region around $UAS_{GAL}$ (hatched box) and the transcripts directed by the promoter on the *GAL1* side (URARIB) and *GAL10* side (thin arrows) are indicated. Positioned nucleosomes (open boxes) were observed on the promoter and the 5′ end of the coding region of *GAL-URARIB* in glucose. The TATA boxes (hatched ovals) of the *GAL1* and *GAL10* genes are located between two nucleosomes and in a nucleosome, respectively (**a**). Positions were lost in galactose (**b**). Positioning was largely reestablished after 10 min (**c**) and 50 min (**d**) and was complete after 2.5 h (**e**).

rearrangment or randomization of nucleosomes along the DNA or with an alteration of nucleosomal structure that enhances the nuclease accessibility. To test whether nucleosomes were lost during transcription, we applied the psoralen-dependent gel retardation assay. If nucleosomes were lost as in transcribed ribosomal genes, the DNA is more accessible and psoralen cross-linking should produce a clear band shift and a retardation higher than in the repressed state. Cells grown in galactose were cross-linked and gel retardation of the *GAL-URARIB* fragment was analysed (Figure 5a). The results, however, showed a low retardation which was indistinguishable from the gel retardation of the inactive gene (f band). This strongly indicated that the transcribed *GAL-URARIB* gene was largely nucleosomal and that there was no dramatic loss of nucleosomes during transcription. This conclusion presumes that RNA polymerase II behaves similarly to RNA polymerase I and does not efficiently protect DNA against psoralen cross-linking (Sogo *et al.*, 1984).

### *Rapid repositioning of nucleosomes after glucose repression*

Since the transition from positioned nucleosomes in glucose to loss of positioning in galactose was clearly detectable and since transcription can be repressed within a few minutes, it was possible to study the kinetics of regeneration of the inactive structure after gene repression. Cells were shifted from galactose to glucose and chromatin was prepared 10 min after the shift. A nucleosomal repeat was reestablished throughout the transcribed region at all levels of digestion with MNase (upper and lower parts of the gel, Figure 3c). However, in the lower part of the gel, the pattern remained more smeared than the glucose pattern and the repeat length remained altered. This result suggested that chromatin was restructured to some extent but not completely under these conditions, in particular on the *RIB* sequences. Mapping nucleosome positions (Figure 4c) revealed a similar protection of cutting sites as in long-term repressed cells (cells grown in glucose, Figure 4a) and showed that nucleosomes were repositioned in the promoter region and in the 5′ part of the *GAL-URARIB* gene. However, protection was not quite complete (dots in Figure 4c) indicating that a fraction of the population was not yet properly regenerated.

To determine the time needed for complete regeneration of the inactive structure on *GAL-URARIB*, chromatin was analysed 50 min, 2.5 or 18 h after the shift from galactose to glucose. After 50 min no changes were observed compared with 10 min, neither in the repeat (Figure 3d), nor in the positioning pattern (Figure 4d). However, after 2.5 h when the cell number was doubled, both the

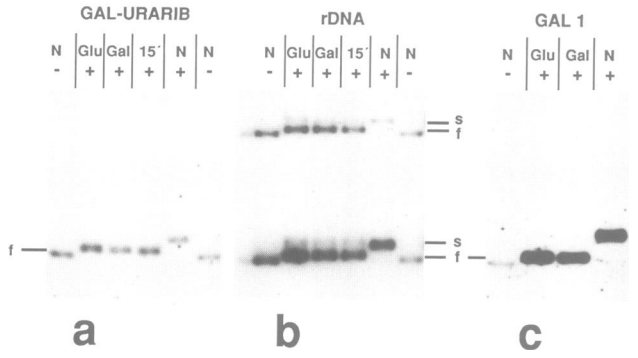


**Fig. 5.** Analysis of chromatin structure by psoralen cross-linking: nucleosomes are present during transcription in *GAL-URARIB* and *GAL1*. Cells grown in glucose (Glu), galactose (Gal), or 15 min after shifting cells from galactose to glucose (15′) were cross-linked with trimethylpsoralen. DNA was purified, digested with the appropriate restriction enzymes, run on an agarose gel, blotted and hybridized. Migration of the bands was compared with naked DNA not cross-linked (N−) or cross-linked (N+). The gel retardation of a DNA fragment depends on the amount of psoralen cross-linked. Nucleosomal chromatin gives a slightly retarded band (fast band, f), whereas nucleosome free chromatin gives a heavily retarded band (slow band, s). For *GAL-URARIB* analysis DNA was digested by *Bam*HI and *Sma*I and the *RIB* probe was used to detect the 1.7 kb fragment from the coding region (**a**). For *GAL1* analysis, DNA (from an independent experiment) was digested by *Eco*RI giving a 1.9 kb fragment detected by a 395 bp probe from the coding region (**c**). For the yeast ribosomal genes, DNA was digested by *Eco*RI and a 3.6 kb *Xba*I fragment was used as a probe, which allows to detect the 1.9 and 2.8 kb fragments of the coding region (**b**).

nucleosomal repeat and nucleosome positions were again similar to those of long-term repressed genes (Figures 3e and 4e). The repeat showed the same lengths and indirect end-labelling showed that the positions were completely restored. The same result was obtained 18 h (~9 generations) after the shift (not shown). It must be pointed out that the extent of protection in nucleosome positions and the extent of the residual repeats and smears were somewhat variable in individual preparations, but always showed the same tendencies with no exceptions. Furthermore, similar results were obtained when GCY5 cells were grown in complete medium (YPD and YPG, not shown).

### *Chromatin transitions in the GAL1, GAL7 and GAL10 genes*

To test whether the results were a specific property of the artificial construct, the chromatin structures of the chromosomal *GAL1*, *GAL7* and *GAL10* genes were also analysed. Previous reports showed a clear nucleosomal repeat for *GAL1* in the inactive state and positioned nucleosomes in the *GAL1-10* promoter region, while the control region lacked a nucleosomal structure (Lohr, 1984; Fedor *et al.*, 1988). A smeary pattern was generated by MNase for *GAL1* in galactose, but a DNase I cutting periodicity suggested presence of some nucleosomes (Lohr, 1983). Our MNase results confirm the earlier observations and extend the analysis by psoralen cross-linking, mapping nucleosome positions in the whole locus and by the regeneration analysis after repression.

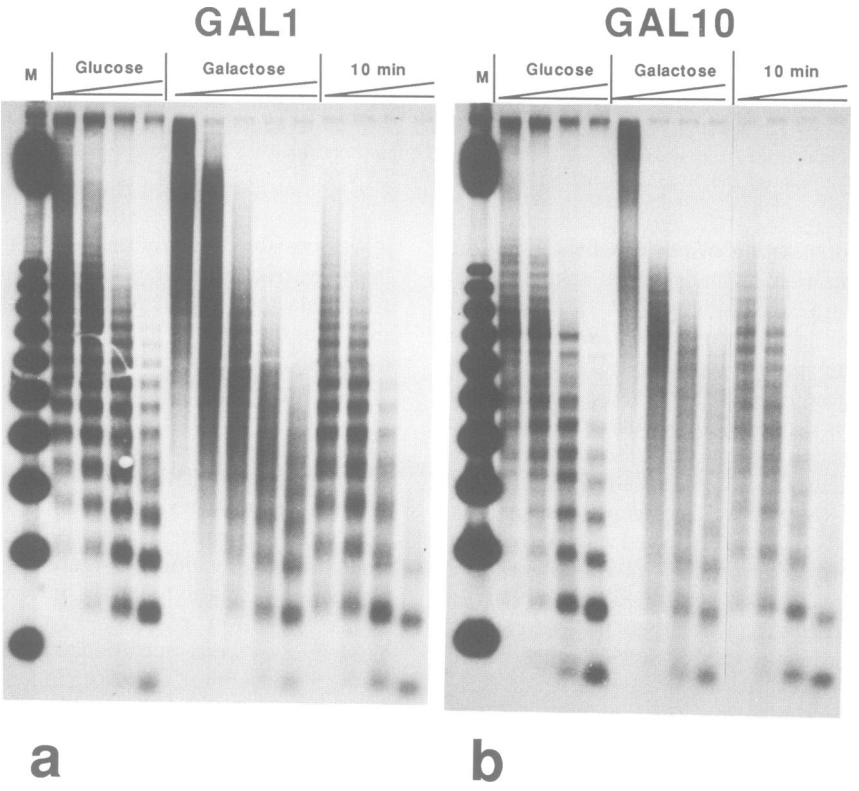


**Fig. 6.** Nucleosomal repeats of *GAL1* and *GAL10* genes. Nucleosomal repeat was analysed for *GAL1* (**a**) and *GAL10* (**b**) in glucose, galactose and 10 min after shifting cells from galactose to glucose, as indicated. The 395 bp *Eco*RV−*Apa*I and the 416 bp *Sal*I−*Pvu*I probes were used, which span the central part of the respective coding regions. The same DNAs were used as for Figures 3 and 4. A DNA ladder consisting of multiples of 256 bp was used for calibration (M lanes).

Psoralen analysis of *GAL1* chromatin showed no differences between glucose and galactose (Figure 5c). In both cases a 1.9 kb *Eco*RI fragment containing the *GAL1-10* promoter and ~1.2 kb *GAL1* coding region (Figure 1b) yielded a low gel retardation (f band), consistent with an overall nucleosomal structure for *GAL1* in the inactive and in the active state. Thus, no significant loss of nucleosomes was detected during transcription. The inactive *GAL1* gene showed a clear nucleosomal repeat with a repeat length of ~170 bp (Figure 6a). Mapping MNase cutting sites at low resolution indicated an array of positioned nucleosomes in the coding region (top of Figure 7b). However, mapping at higher resolution revealed that protection was not complete. Doublets or triplets of bands indicated that positioning was not precise and that overlapping positions might exist (dots in Figure 7a, Glu lanes). This property of *GAL1* chromatin makes it difficult to follow the fate of nucleosomes during transcription and regeneration of the inactive structure. Nonetheless, in galactose, a residual repeat was observed in a smeared background (Figure 6). When nucleosome positioning was analysed, chromatin and naked DNA gave a similar cutting pattern indicating that some nucleosomes were present but positions were lost during transcription (Figure 7a, Gal and N lanes). Ten minutes after shifting cells from galactose to glucose the repeat and positions were re-established to some extent. No further changes were observed in nucleosome positioning after 50 min and after one cell generation (2.5 h) the pattern was similar to the original one of glucose grown cells (Figure 7a).

The structural transitions on the *GAL10* gene are also summarized in Figures 6 and 7. The *GAL10* gene showed a nucleosome repeat in glucose with a repeat length of ~160 bp. The repeat was disrupted in galactose at low levels of digestion, but it was evident at higher levels of digestion. Ten minutes after repression, the repeat was re-established (Figure 6). Mapping at low resolution revealed an array of positioned nucleosomes in the coding region (Figure 7a). At higher resolution, nucleosomes appeared to be precisely positioned towards the 5′ end of the gene (dots in Figure 7b), while doublets of bands indicated overlapping positions towards the 3′ end (Figure 7c). The interpretation was somewhat hampered, since some cutting sites in linker DNA coincided with strong cutting sites in naked DNA. Upon transcription, protection was lost at some places and chromatin lanes were similar to DNA lanes (Figure 7a and b, compare Gal and N lanes), while other positions appeared to be less affected. The nucleosomal repeat and positions were re-established to a large extent 10 and 50 min after repression and no more dramatic changes were observed between 50 min and 2.5 h. Hence, the structural alterations during transcription and repression observed in the *GAL10* gene appeared to be less dramatic than in *GAL-URARIB* and might relate to the decreased sensitivity of the assay in the *GAL10* gene or to different transcription rates or different chromatin properties.

Most interestingly, the nucleosome mapping data show

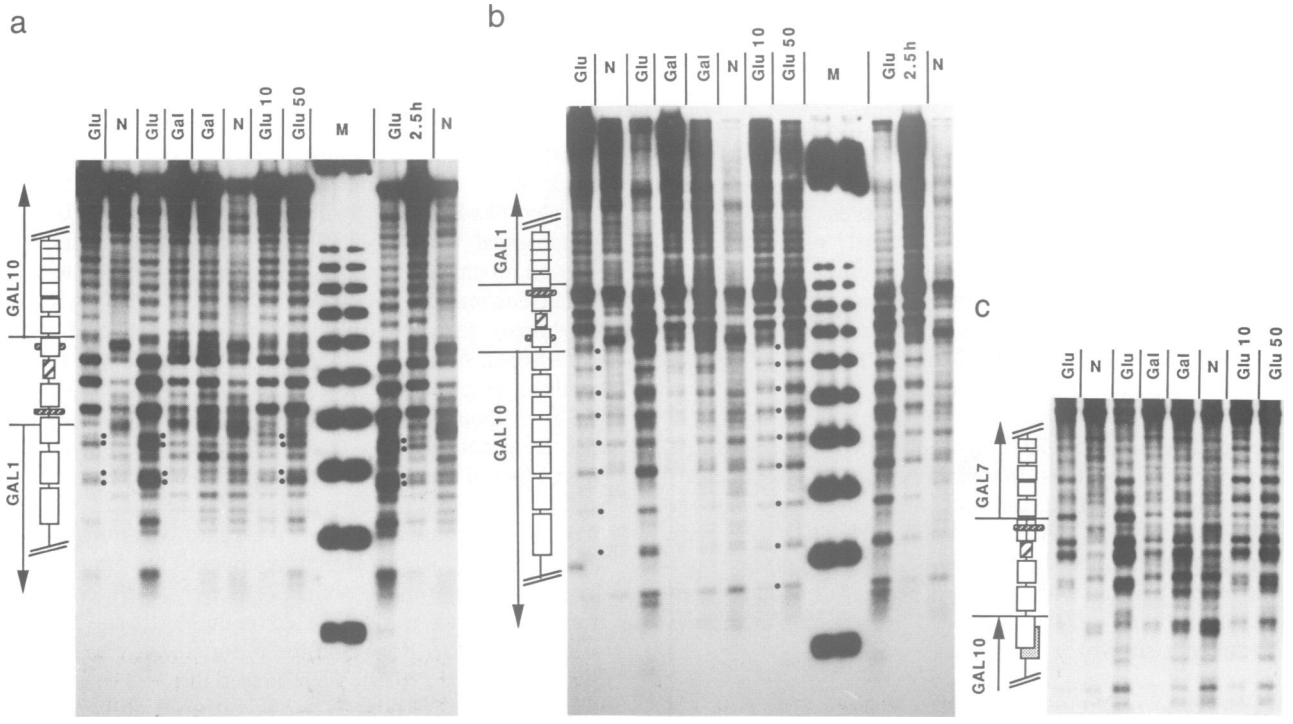


**Fig. 7.** Nucleosome positioning in the *GAL1-GAL10-GAL7* locus. Nucleosome positioning was analysed by MNase digestion and indirect end-labelling. The same DNAs as for Figures 3, 4 and 6 were used. (**a**) Samples were digested with *Eco*RV and hybridized with the 395 bp *Eco*RV−*Apa*I fragment to display positions on the *GAL1* gene on the bottom of the gel and on *GAL10* on the top part of the gel. Lanes from cells grown in glucose (Glu), galactose (Gal) and from cells shifted from galactose to glucose for 10 min (Glu 10), 50 min (Glu 50) or 2.5 h (Glu 2.5 h) are shown. A simplified interpretation of the chromatin structure in glucose is drawn with positioned nucleosomes (open boxes). $UAS_{GAL}$, the *GAL1* and *GAL10* TATA boxes are indicated as in Figure 4. Dots indicate that multiple positions might occur in *GAL1*. (**b**) Samples were digested with *Sal*I and hybridized with the 416 bp *Sal*I−*Pvu*I probe. (**c**) Samples were digested with *Pvu*I and hybridized with the 416 bp *Sal*I−*Pvu*I probe. A transparent box covering the *GAL7* TATA box indicates incomplete protection. Overlapping boxes indicate multiple positions.

that regeneration of the *GAL10* promoter structure required one cell generation. In the inactive state of the *GAL10* and *GAL-URARIB* genes, protection of a strong MNase cutting site showed that a precisely positioned nucleosome covers the region of the TATA box (Figures 7a, b and 4). This cutting site becomes accessible in galactose and remains accessible after 10 and 50 min of repression indicating that some factors might have been bound which prevented nucleosome formation. Replication might be required to clear the promoter region and re-establish the inactive structure.

Similar chromatin transitions from positioned nucleosomes in glucose to loss of positioning in galactose and repositioning of nucleosomes 10 min after shifting cells from galactose to glucose were observed when positioning over the *GAL7* gene was analysed (Figure 7c). This shows that rapid repositioning is not an exclusive property of genes driven by the *GAL1-10* promoter.

## Discussion

### *GAL-URARIB*

To study chromatin transitions at the nucleosome level, a gene was required that is tightly regulated with respect to activation and repression, heavily transcribed and should have positioned nucleosomes. Although mapping of MNase cutting sites by indirect end-labelling showed the presence of positioned nucleosomes in the 5′ region of the inactive *GAL1* gene (Lohr, 1983), we found that the *GAL1* and the *GAL10* genes were less well suited for chromatin analysis, since the nucleosomes appeared not to be precisely positioned or the cutting sites in the linker between nucleosomes coincided with strong cutting sites in protein-free DNA, hence reducing the sensitivity of the mapping assay (Figures 6 and 7). The artificial construct *GAL-URARIB* integrated in the chromosome sufficiently fulfils both criteria. First, the 5′ part is organized in precisely positioned nucleosomes with tight protection of MNase cutting sites. Secondly, we have shown that its transcription is tightly regulated, it is heavily transcribed in galactose, tightly repressed in glucose and glucose repression acts within a few minutes.

Upon induction of transcription, nucleosome positioning was lost on the chromosomally integrated *GAL-URARIB* gene. In contrast, Fedor and Kornberg (1989) reported, on a similar construct, only an enhanced susceptibility to MNase and (methidiumpropyl-EDTA)-iron(II) (MPE) without loss of nucleosome positions. In their construct *URA3* sequences were fused to the *GAL* promoter in a high copy number *TRP1ARS1* plasmid. We assign this difference to a copy number effect, since the *GAL-URARIB* gene placed on a centromere plasmid with only 3–5 copies per cell appeared less susceptible than the integrated construct (Cavalli and Thoma, not shown).

### *Nucleosome positioning on GAL-URARIB, GAL1 and GAL10*

Nucleosomes can be positioned *in vivo* by three mechanisms: sequence-dependent histone–DNA interactions, boundaries or flanking structures, and chromatin folding. By fusing *URA3* sequences to the *GAL1-10* promoter on small circular minichromosomes and by genetic manipulations of sequences in the upstream activating sequence ($UAS_G$), it was demonstrated that nucleosome positioning was dominated by a boundary effect from $UAS_G$ and that the decay in positioning towards the 3′ end was a consequence of statistical positioning limited by the boundary (Fedor *et al.*, 1988). Subsequent work identified GRF2 as an abundant yeast protein that binds $UAS_G$ and creates the nucleosome-free region (Chasman *et al.*, 1990). The precise positioning of nucleosomes in the *GAL1-10* promoter and in the 5′ region of *GAL-URARIB* as well as the loss of positioning towards the 3′ end of *GAL-URARIB* was consistent with a boundary effect. In contrast to the artificial constructs, the *GAL1* and the *GAL10* genes showed positioned nucleosomes throughout the coding region. Although positioning was not precise (double bands indicating overlapping positions) no obvious decay or randomization of positioning towards the 3′ end was recorded. This phenomenon has been observed for the *URA3* gene which was shown to be organized by strong boundaries at the 5′ and 3′ ends (Bernardi *et al.*, 1992). Therefore *GAL1* and *GAL10* are candidates for yeast genes that behave as structural and functional units organized by 5′ and 3′ boundaries (Bernardi *et al.*, 1992).

### *Nucleosomes are present on genes transcribed by RNA polymerase II*

Although we have observed dramatic structural changes by activation of transcription, a combination of three criteria suggests that nucleosomes are present during transcription by RNA polymerase II. (i) In all cases a residual nucleosomal repeat was detected. This is consistent with the earlier observations of a residual nucleosomal repeat and a DNase I pattern on the transcribed *GAL1* gene (Lohr, 1983). This assay, however, does not allow determination of whether all genes are transcribed and contain nucleosomes or whether the nucleosomes originate from a fraction that is not transcribed. Since the repeat length was altered in *GAL-URARIB*, the repeat does not represent an inactive fraction of the population, but must originate from genes that are transcribed or have been transcribed recently. (ii) The psoralen band shift assay allows us to distinguish between nucleosomal and non-nucleosomal fractions. This assay showed no enhanced accessibility to psoralen due to a loss of nucleosomes in *GAL-URARIB* and in *GAL1*, while the ribosomal gene controls showed the expected strong nucleosomal band containing the inactive copies and retarded bands of genes that had lost nucleosomes (Dammann *et al.*, 1993). Although a quantification with respect to fraction of lost nucleosomes is not yet established, we estimate that a loss of half of the nucleosomes per gene would create a band shift that should not escape detection. It seems also unlikely that only a minute fraction of the genes was transcribed at a given time, since the structural changes are dramatic with respect to loss of nucleosome positioning. Furthermore the transcription rate is high. Based on the levels and half-lives of *GAL-URARIB* RNA and the reported *URA3* RNA half-lives (Bach *et al.*, 1979), we estimated that ~8–18 *URARIB* transcripts were produced per minutes which corresponds to ~4–9 polymerases present on the gene or one polymerase every 400–170 bp. Furthermore, we assume that RNA polymerase II behaves similar to RNA polymerase I and does not efficiently protect DNA against psoralen cross-linking (Sogo *et al.*, 1984). (iii) The rapidity at which the inactive chromatin is regenerated after repression of transcription argues in favour of histones being present on or close to the transcribed template. Regeneration by newly synthesized histones might require much longer (see below).

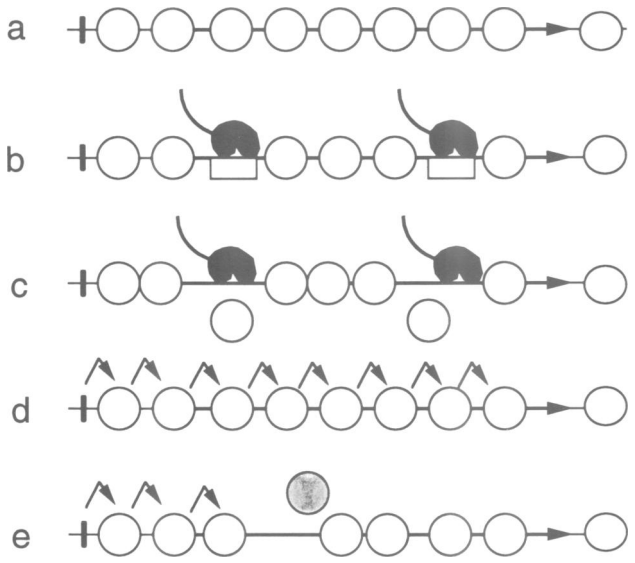


**Fig. 8.** Transcription-dependent structural transitions. (**a**) Inactive chromatin. (**b**) Transcription without displacement of histone octamer. Histones remain associated in a structurally altered nucleosome (box). Nucleosomes that are not transcribed remain at their positions. (**c**) Histones are displaced by transcription and reassemble randomly behind the polymerase. Nucleosome spacing and positioning is altered. (**d**) Regeneration of the inactive structure, e.g. by a boundary, if all nucleosomes are present (rapid phase). (**e**) Regeneration by replacement of missing histones (filled circle) (slow phase). Bent arrows illustrate the boundary effect. The boundary (black bar) positions the first nucleosome, the first nucleosome affects positioning of the second nucleosome etc.

The results are consistent with the presence of nucleosomes on the transcribed gene. However, what happens when an RNA polymerase meets the nucleosome? These mechanisms have been reviewed in detail (Thoma, 1991; Clark and Felsenfeld, 1992; Van Holde *et al.*, 1992), but only two extreme hypotheses are mentioned here. For steric reasons it seems impossible that the transcription complex follows the double helical path of DNA when it is wrapped on the outside of a histone octamer. One hypothesis suggests that nucleosomes might unfold into a linearized nucleoprotein complex and the processing polymerase might transcribe through the open nucleosome while the DNA rotates between the polymerase and the histones (Thoma, 1991). Given that such an unfolding–folding transition does not affect nucleosome positioning (e.g. by additional sliding of histones), one would expect the same nucleosome positions in the transcribed and non-transcribed state as it was reported when a nucleosome positioned on a 5S rDNA (5S nucleosome) was transcribed *in vitro* (Figure 8b; Losa and Brown, 1987). This apparently was not the case in *GAL-URARIB*, since an altered repeat length was observed and the nucleosome positions were lost.

A second hypothesis suggests that nucleosomes dissociate in front of the polymerase and reassociate behind it (Figure 8c). When the 5S nucleosome was located on a long DNA molecule, the nucleosome was displaced during *in vitro* transcription by SP6 polymerase and reformed at random sites—preferentially behind the polymerase—on the same DNA molecule (Clark and Felsenfeld, 1992). Such a histone transfer mechanism is favoured by the building up of positive supercoils in front of the polymerase and negative supercoils behind it. Our observations are consistent with a local dissociation and rapid reformation of nucleosomes close to the transcription site *in vivo*. Both, the observation of an altered repeat of the residual nucleosomes in *GAL-URARIB* and the loss of nucleosome positioning reflect a rearrangement or randomization of nucleosomes on the transcribed *GAL-URARIB* gene. Furthermore, the slow regeneration suggests that some histones were lost after dissociation and had to be replaced later in the cell cycle (see below).

### *Regeneration of inactive chromatin*

Our observations suggest two phases which might reflect regeneration in two fractions of the population. Only minutes after glucose repression the inactive structure was restored to a large extent both with respect to the presence of nucleosomes (a repeat) and with respect to nucleosome positioning. Complete restoration was observed after one generation. The rapidity by which the first step of this process occurs is consistent with the observation that a significant amount of nucleosomes was still present on the transcribed genes and further suggests that neither histone synthesis nor DNA replication was required. Although we cannot technically control for protein synthesis and DNA replication during the chromatin preparation procedure, the fact that chromatin preparations after 10 and 50 min yield similar results, also argues against an influence of these two processes.

Since nucleosome positioning *in vivo* is determined by the DNA sequence, boundaries and chromatin folding, we wonder how positioning occurs after repression of transcription. As discussed above, nucleosome positioning in the inactive *GAL-URARIB* might be established by a boundary function of GRF2 bound in $UAS_{GAL}$ (Fedor *et al.*, 1988). Since GRF2 is present when cells are grown in glucose and galactose (Chasman *et al.*, 1990), our results imply that GRF2 could act as an active boundary during the regeneration process (Figure 8d). Since nucleosomes were already present on the transcribed gene, positioning might occur by either sliding of histones on the DNA or local dissociation and reassembly at the right position. This mechanism hence shows similarity to the rearrangement of nucleosomes observed after DNA repair synthesis (Smerdon, 1991) and a similar process might position nucleosomes after they are assembled during replication. Knowing that the *URA3* gene is a structural and functional unit determined by boundaries at the 5′ and 3′ ends (Bernardi *et al.*, 1992), the repositioning data support the idea that the boundaries may serve an important role in the organization of the local structure of genes after it was disturbed by transcription or DNA repair.

Rapid repositioning of nucleosomes was also observed in the native *GAL1* and *GAL10* genes and in the *GAL7* gene, although no putative GRF2 binding sites were reported in the *GAL7* promoter (Chasman *et al.*, 1990). While chromatin structures in the 5′ regions of *GAL1* and *GAL10* might be organized by GRF2 in the divergent promoter region, the determinants of the 3′ regions and of the *GAL7* gene are not known.

The *GAL10* TATA site maintained its nuclease sensitivity after repression for 10 and 50 min. Since this site is located between the GRF2 binding site and the downstream positioned nucleosomes, this observation might argue against

a positioning mechanism by GRF2. Alternatively, one might postulate that this region is structured in an altered nucleosome that can pass the boundary effect of GRF2 to the downstream region, but its structure is still altered by transcription factors left behind after repression of transcription. Since regeneration of the inactive structure takes a cell cycle, it seems likely that DNA replication might be required to remove those factors and to regenerate the inactive structure.

We have observed that a minor fraction of chromatin was restored only after 2.5 h which was sufficient time for one round of replication. We think that this observation might reflect a chromatin maturation process. Our assays do not exclude that some nucleosomes or individual histones might have been lost during transcription. Hence, those genes in the population that are missing nucleosomes are unable to regenerate the inactive structure and give rise to the additional cutting observed by MNase. During the slow regeneration phase, the missing proteins might be replaced and reassembled into nucleosomes (Figure 8e). The lack of some histones is also consistent with a transient dissociation of nucleosomes during transcription, while not all histones manage to reassociate with the DNA after passage of the polymerase. While in the galactose preparations the smear was prominent in the middle of the gels (*GAL-URARIB*), after repression the smear was prominent in the lower part, reflecting the local structure of the *RIB* sequences (Figure 3). This suggests that the *RIB* sequences are less rapidly regenerated than the *URA* sequences on the 5′ end of *GAL-URARIB*. Since *in vitro* reconstitution experiments demonstrated that the efficiency of nucleosome formation is sequence-dependent (Shrader and Crothers, 1989), this effect might be due to a presumptive lower affinity of ribosomal sequences compared with *URA3* sequences. Alternatively, the regeneration process by a boundary in the 5′ region might reorganize the 5′ URA region prior to the 3′ *RIB* region.

The observation of a two phase regeneration process is consistent with a report on the regeneration of inactive chromatin on the early histone genes of sea urchin (Wu and Simpson, 1985). In that case, the heavily transcribed genes were deficient in nucleosomes (tested by MNase digestion) and the slow reformation of the nucleosomal repeat suggested that redeposition of histones following repression was succeeded by further remodelling of chromatin structure during later cell divisions. On the other hand it is interesting to note that the re-establishment of the preinduced chromatin structure of the *Drosophila* 87A7 heat shock locus was observed within 1 h and hence would not require DNA replication, but this process was dependent on protein synthesis, since it was blocked by protein synthesis inhibitors (Han *et al.*, 1985). Hence the heat shock gene might have lost more nucleosomes during transcription than the *GAL* genes reported here. Furthermore, we observed some variability in the extent of structural transitions in individual experiments. It therefore seems possible that some genes or transcription conditions might be found in yeast that lead to a dissociation of a large fraction of nucleosomes. Preliminary data on a *RIBURA* sequence integrated into the ribosomal locus show that this sequence indeed is depleted of nucleosomes when transcribed by RNA polymerase I (G.Cavalli and F.Thoma, unpublished results).

## Materials and methods

### *Plasmids, strains and media*

Plasmids pBM150 (Johnston and Davis, 1984), pNN77, pNN78 (St John and Davis, 1981) and pYA301 were used to isolate the *GAL1-10* promoter and probes for analysis of *GAL1*, *GAL7*, *GAL10* and the actin genes. The yeast strain YM262 (*MATα ura3-52 his3-200 ade2-101 lys2-801 tyr1-501*) (Johnston and Davis, 1984) was a gift of M.Johnston. GCY5 is YM262 with vector YIpGC1 integrated in the *LEU2* locus using *URA3* as a marker gene. GCY5 was grown at 30°C in 0.67% yeast nitrogen base without amino acids, uracil 20 mg/l, adenine 20 mg/l, L-histidine 20 mg/l, L-lysine−HCl 30 mg/l, L-tyrosine 30 mg/ml. 2% glucose (SD medium) or 2% galactose (SG medium) were present as carbon sources (Sherman *et al.*, 1981).

### *Construction of GAL-URARIB*

The *GAL-URARIB* gene was constructed by several subcloning steps as an *Eco*RI cassette to give plasmid pGC6. It contains the following elements. (i) The *GAL1-10* promoter as *Eco*RI−*Bam*HI fragment of pBM150. (ii) The *Bam*HI site is followed by 27 bp polylinker containing *Sfi*I and *Not*I sites and *Bam*HI ends (GATCCACTAGTGGCCTATGCGGCCGCGgatc; Boehringer). (iii) A fragment of the *URA3* sequence (from nucleotides 295−905; Rose *et al.*, 1984) was amplified by PCR using oligoprimers with *Sfi*I sites to give a 624 bp *Sfi*I fragment (*URA*) and inserted in the *Sfi*I site of the polylinker. (iv) A sequence from the transcribed region of *Dictyostelium discoideum* rDNA (nucleotides 1067−1888 representing the external transcribed region and part of the 17S rRNA region; Ozaki *et al.*, 1984) was amplified by PCR using oligoprimers with *Not*I sites to give a 830 bp *Not*I fragment (*RIB*) and inserted in the *Not*I site of the polylinker. (v) The 3′ end of *URA3* (U3′) as a 260 bp *Sau*3A−*Hin*dIII fragment attached to the *Bam*HI end of the polylinker. The *Hin*dIII site at the 3′ end was made blunt (Klenow) and changed into an *Eco*RI site by the addition of an *Eco*RI linker (CCGGAATTCCGG, Boehringer). The *Eco*RI fragment containing *GAL-URARIB* was blunt-ended and inserted in the blunt-ended *Bam*HI site of YIp28 (Botstein *et al.*, 1980) to give YIpGC1. YIpGC1 was linearized with *Asp*718 and integrated in *LEU2* by transformation of YM262 (Ito *et al.*, 1983). Integration as a single copy was confirmed by Southern analysis.

### *RNA analysis*

Cells were grown in galactose medium to an $OD_{600}$ of 0.65, collected by centrifugation and resuspended in the same volume of glucose medium prewarmed to 30°C. A 50 ml aliquot ($t_0$) was immediately removed, harvested by centrifugation for 15 s in an SS34 rotor at 6000−8000 r.p.m. and frozen in crushed dry ice. The remaining cells were further incubated at 30°C. After 5, 10, 20 and 40 min aliquots of 50 ml were harvested and frozen. Total RNA was extracted (Jensen *et al.*, 1983), run on 1% agarose gels containing formaldehyde (Thomas, 1983), blotted on Biodyne A membrane according to manufacturer's instructions (Pall) and hybridized to the appropriate probes (see figure legends). Amounts of transcripts were quantified by scanning autoradiographic films (Fuji RX exposed without enhancer screens) with a Shimadzu CS-930 scanner. The values were standardized with respect to the actin transcript levels. Half-life analysis was according to Parker *et al.* (1991).

### *Chromatin analysis by micrococcal nuclease*

Yeast cells were grown to $OD_{600}$ of 0.4−1.0. For shifting experiments, cells were harvested by centrifugation and resuspended in glucose medium prewarmed to 30°C. Crude yeast nuclei were prepared as described by Almer and Hörz (1986) and Bernardi *et al.* (1991) except that large amounts of zymolyase (100 000 U/g; Seikagaku Kogyo Co., Tokyo, Japan, 1 mg/ml) and incubation at 37°C were used to speed up spheroplasting. The nuclear pellet was resuspended in buffer A (20 mM Tris−HCl pH 8, 150 mM NaCl, 5 mM KCl, 1 mM EDTA and 1 mM PMSF) and MNase digestion (*Staphylococcus* nuclease, Boehringer) was in presence of 5 mM $CaCl_2$ at 37°C using different enzyme concentrations (7−150 units/ml for glucose chromatin, 2−75 units/ml for galactose chromatin). Control DNA was extracted from an aliquot of undigested nuclei, dissolved in buffer A and digested by MNase. Detection of the nucleosomal repeat by agarose gel electrophoresis, blotting and hybridization and indirect end-labelling to map nucleosome positions were as described by Thoma *et al.* (1984) and Bernardi *et al.* (1991). Radioactive probes were prepared using oligolabelling kits (Pharmacia).

### *Chromatin analysis by psoralen cross-linking*

A method developed by Conconi *et al.* (1989) and modified for yeast *S.cerevisiae* by Dammann *et al.* (1993) was used.

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