Evidence for distinct mechanisms facilitating transcript elongation through chromatin *in vivo*

Arnold Kristjuhan and Jesper Q Svejstrup*

Cancer Research UK London Research Institute, Clare Hall Laboratories, South Mimms, Hertfordshire, UK

The mechanism and kinetics of RNA polymerase II transcription and histone acetylation were studied by chromatin immunoprecipitation in yeast. Our results indicate that a significant fraction of polymerases starting transcription never make it to the end of a long *GAL-VPS13* fusion gene. Surprisingly, induction of *GAL* genes results in substantial loss of histone–DNA contacts not only in the promoter but also in the coding region. The loss of nucleosomes is dependent on active transcript elongation, but apparently occurs independently of histone acetylation. In contrast, histones in genes previously shown to require the histone acetyltransferases *Gcn5* and *Elp3* for normal transcription do not lose DNA contacts, but do become acetylated as a result of transcription. Together, these results suggest the existence of at least two distinct mechanisms to achieve efficient transcript elongation through chromatin: a pathway based on loss of histone–DNA contacts, and a histone acetylation-dependent mechanism correlating with little or no net loss of nucleosomes.

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Introduction

The eukaryotic genome is packaged into chromatin, which presents a constant barrier to transcription and other cellular processes that require access to DNA. In order to contend with chromatin, the basal transcription machinery is assisted by numerous factors that increase the plasticity of nucleosomal DNA. The first group of chromatin-remodelling factors are enzymes that covalently modify the N-termini of histones (histone acetyltransferases (HATs), histone methyltransferases, etc.) to help make nucleosomes mobile or support the binding of other factors, while proteins in the second group, the ATP-dependent chromatin remodellers, change the positions of nucleosomes to expose DNA for the transcription machinery (Workman and Kingston, 1998; Narlikar *et al*, 2002). Acetylation of histones is one of the best-characterized general mechanisms for increasing the accessibility of chromatin and allowing factors to gain access to DNA. For

example, it has been shown that many transcriptional activators recruit HATs to increase acetylation of histones at promoters (Hassan *et al*, 2001a; Kadam and Emerson, 2002), and we have previously shown that the steady-state level of H3 acetylation in the coding region of genes has a positive correlation with gene activity (Kristjuhan *et al*, 2002). Although hyperacetylation of histones generally indicates higher transcriptional activity, not all active promoters are hyperacetylated and the need for acetylation differs substantially between genes (Deckert and Struhl, 2001; Kristjuhan *et al*, 2002; Kurdastani *et al*, 2004). In addition to 'targeted' recruitment to genes, HATs and histone deacetylases (HDACs) also have a role in the maintenance of a global histone acetylation–deacetylation equilibrium in cells (Wittschieben *et al*, 2000). This mechanism has been proposed to be responsible for rapid restoration of a normal histone acetylation pattern when the signal for targeted acetylation or deacetylation has been removed (Vogelauer *et al*, 2000; Katan-Khaykovich and Struhl, 2002).

Acetylated histone tails can be recognized by chromatin remodelling complexes, which in turn can lead to a full opening of chromatin (Hassan *et al*, 2001a, b). For example, nucleosomes in the *PHO5* and the *PHO8* promoter are first hyperacetylated by Gcn5, and then remodelled by the SWI/SNF complex to achieve full activation of the gene (Reinke *et al*, 2001; Reinke and Horz, 2003). Theoretically, remodelling of chromatin can occur in two ways: nucleosomes can be pushed away from the target DNA sequence, assuming that they can be accommodated in neighbouring regions of DNA (remodelling *in cis*). Alternatively, they may be temporally removed from DNA altogether (remodelling *in trans*). Recent studies of the *PHO* genes have shown that removal of nucleosomes from a promoter in response to induction of transcription can indeed take place (Boeger *et al*, 2003; Reinke and Horz, 2003), and that this removal occurs *in trans*, with the help of chromatin assembly factor Asf1 (Adkins *et al*, 2004; Boeger *et al*, 2004). The extent and generality of nucleosome loss in response to transcriptional activation has not been reported, and whether it is restricted to promoters is also unknown. However, in the case of *PHO5* and *PHO8*, only promoter nucleosomes, not coding region nucleosomes, are lost (Adkins *et al*, 2004).

Transcription-related histone chaperones such as Spt6 and FACT are capable of binding histones and assembling chromatin (Belotserkovskaya *et al*, 2003; Kaplan *et al*, 2003), suggesting that they may be involved in disassembling and reassembling chromatin during transcript elongation. Nevertheless, the basic mechanisms employed by the transcription machinery to contend with nucleosomes in the coding region of activated genes *in vivo* are still poorly understood (Svejstrup, 2003).

In this study, we followed the dynamics of transcription and histone acetylation over the entire locus of two galactose-inducible genes, as well as three constitutive genes whose normal expression requires the HATs Gcn5 and Elp3. Our

*Corresponding author. Cancer Research UK, Clare Hall, Blanche Lane, South Mimms, Hertfordshire EN6 3LD, UK. Tel.: +44 1707 62 5960; Fax: +44 207 269 3801; E-mail: j.svejstrup@cancer.org.uk

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results shed new light on the mechanisms underlying transcriptional activation and transcript elongation through chromatin.

Results

Transcriptional induction of a *GAL-VPS13* fusion gene

The *Saccharomyces cerevisiae* genome contains about 6000 genes with an average size of less than 1500 bp (Cherry *et al.*, 1997). The very compact genome and short genes mean that discriminating between chromatin modification events originating from promoters and coding regions is difficult. To try and overcome the problem of too short genes, we constructed a model yeast strain where a galactose-inducible promoter was inserted in front of the long (9.5 kb) *VPS13* gene at its natural locus (Figure 1A). In this strain, transcription of the synthetic *GAL-VPS13* gene can be induced by galactose, and the transcriptional unit itself is long enough to distinguish between chromatin modification caused by transcriptional initiation and elongation, without considerable influence from events elsewhere in the gene or at other genes nearby.

Cells carrying the *GAL-VPS13* gene were grown to mid-log phase in YP-raffinose and induced by adding galactose to the growth medium. Samples were taken at different time points up to 2 h of induction and acetylation of histones was analysed by chromatin immunoprecipitation (ChIP) assays. We also used the ChIP assay to study the kinetics of transcriptional induction by measuring RNA polymerase II (RNAPII) density in the promoter and coding region of the *GAL-VPS13* gene. An RNAPII ChIP approach has previously been used to study transcription and it has been shown that the level of RNAPII density in a gene is in good correlation with the rate of its transcription (Kulish and Struhl, 2001; Kristjuhan *et al.*, 2002; Pokholok *et al.*, 2002; Bryant and Ptashne, 2003).

RNAPII was already detectable on the promoter of *GAL-VPS13* 4 min after adding galactose, but it took about 1 h until recruitment of polymerase reached its maximal level (Figure 1B). The kinetics of TBP recruitment to the *GAL-VPS13* promoter was similar to that of RNAPII (data not shown). Accumulation of RNAPII at the end of the gene (8.3 kb from the transcriptional start site, and more than 2 kb from the beginning of the next gene) followed the same kinetics as observed on the promoter, although the

rate of increase in density, as well as the final density level, was lower. This may suggest that several of the polymerases loaded onto this long gene never make it to the end. This

Figure 1 Kinetics of RNAPII recruitment to *GAL-VPS13* and *GAL1* upon galactose induction. (A) Schematic representation of the synthetic *GAL-VPS13* locus on chromosome XII. Vertical arrows indicate the locations of promoter (prom.) and coding region (2.2, 5.5, and 8.3 kb) PCR probes that were used in ChIP assays. (B) The amount of RNAPII on the promoter and in the coding region of *GAL-VPS13* was analysed by ChIP. Co-immunoprecipitated DNA was quantified by real-time PCR with the appropriate primers. (C) Kinetics of *VPS13* mRNA accumulation upon induction of the *GAL-VPS13* gene measured by real-time RT-PCR with primers close to the 5' end of the transcript. (D) As (C), but with primers specific for the 3' end (8.3 kb) of the *VPS13* transcript. (E) Recruitment of RNAPII to the indigenous *GAL1* promoter was analysed by ChIP and real-time PCR. All samples were also analysed with primers specific for the constitutively expressed *FBA1* gene and this result was used as a 'loading control' to normalize results from the *GAL-VPS13* and *GAL1* genes. RNAPII level (panels B and E) or *VPS13* mRNA level (panels C and D) in uninduced conditions (time point 0 min) was set to 1 and other time points show the fold of difference relative to this uninduced sample.

possibility was further supported by the observation that the level of RNAPII occupancy decreased gradually with increased distance from the transcription start site as measured by ChIP of RNAPII also at 2.2 and 5.5 kb from the transcription start site (Figure 1B).

In order to further investigate this hypothesis, we used reverse transcriptase-PCR (RT-PCR) on RNA from formaldehyde crosslinked cells to monitor the production of 5' and 3' sequences of the *VPS13* transcript during the time course of induction (Figure 1C and D). The overall kinetics of accumulation of the transcript precisely reflected the kinetics of RNAPII appearance on the gene (compare Figure 1B with Figure 1C and D), confirming the reliability of RNAPII recruitment by ChIP as an indicator of ongoing transcription. More importantly, as predicted from the RNAPII recruitment data, a substantial amount of 'aborted transcription' was indicated also at the mRNA level. At full induction, the amount of 5' sequences of the *VPS13* transcript increased about 120-fold, while full-length mRNA (measured by the accumulation of 3' sequence of *VPS13* transcript) increased only about 30-fold compared to noninduced conditions (compare Y-axis in Figure 1C and D). This suggests that a considerable fraction of polymerases loaded onto the *GAL-VPS13* gene never reach the end of the coding region.

We noticed that full induction of the *GAL-VPS13* promoter was considerably slower than reported previously for a similar type of induction of the *GAL1* promoter (Bryant and Ptashne, 2003). To investigate whether the kinetics of induction of *GAL-VPS13* was specific for this long fusion gene or simply representative of *GAL* activation in our strain background, we also tested the kinetics of induction of the indigenous *GAL1* gene (Figure 1E, and data not shown). Analysis of RNAPII ChIP samples with *GAL1*-specific probes did not reveal any significant difference in kinetics or fold of activation between the endogenous *GAL1* gene and the long *GAL-VPS13* fusion gene (compare with Figure 1B).

Nucleosomes are lost from *GAL-VPS13* and *GAL1* loci upon induction

As a consequence of activation-induced chromatin remodeling, the number of nucleosomes in the promoter region of the *PHO5* gene is reduced dramatically (Boeger *et al*, 2003; Reinke and Horz, 2003). Because removal of histones could be a general mechanism to achieve high levels of transcription, we tested whether a loss of nucleosomes was also detectable upon induction of *GAL* genes. A yeast strain expressing FLAG-tagged histone H2B was induced with galactose and the relative amounts of histones H2B and H3 on the *GAL1* locus were determined by ChIP assays using

anti-FLAG and anti-H3 C-terminal antibodies, respectively. As observed at the *PHO5* promoter (Boeger *et al*, 2003; Reinke and Horz, 2003), histones lost contact with the *GAL1* promoter in response to gene activation (Figure 2A).

Figure 2 Loss of histone-DNA contacts at the active *GAL1* and *GAL-VPS13* loci. The amount of histone H3 (grey bars) and H2B (white bars) on the *GAL1* promoter (A) or coding region (B) was determined by ChIP assays with anti-FLAG and anti-H3 C-terminal antibodies, respectively. Co-immunoprecipitated DNA was quantified by real-time PCR and all samples were normalized according to the amount of histones H3 and H2B in a nontranscribed region on chromosome IV (see Materials and methods). The level of histones under uninduced conditions (time point 0 min) was set to 1 and other time points show the amounts of histones relative to the uninduced sample. (C, D) The relative amount of histone H3 at the promoter (C) or in the coding region (D) of *GAL-VPS13* was determined by ChIP assays with H3 C-terminal antibody.

Surprisingly, histones also lost contact with DNA in the coding region of the *GAL1* gene (Figure 2B). Because both H2B and H3 were lost in a coordinated manner, we favour the idea that entire nucleosomes, rather than individual histone dimers, were 'lost' from the locus. In support of this idea, loss of histone DNA contacts was also observed in the *GAL-VPS13* gene where transcriptional activation led to a reduction of H3 levels in the coding region similar to what was found for *GAL1* (compare Figure 2D and B). At fully induced conditions (1–2 h after adding galactose), the detectable amount of H3 dropped up to four-fold compared to uninduced conditions, and the loss of H3 signal was in good correlation with the increase of RNAPII transcription through the region (compare Figure 1B with Figure 2D, for example). *GAL1* seemed to lose histone H3–DNA contacts to an even higher degree than *GAL-VPS13* in the promoter region, although the difference was minor (compare Figure 2A and C). The reason for this difference is unknown, but it could be envisaged that the small intergenic region between the divergently transcribed, highly active *GAL1* and *GAL10* genes would be even more prone to nucleosome loss than the unidirectional promoter region of the *GAL-VPS13* fusion gene.

These data, combined with those previously reported for the *PHO5* promoter (Boeger *et al.*, 2003; Reinke and Horz, 2003), suggest that loss of histone–DNA contact may be a common consequence of gene activation. Significantly, our results also indicate that histones can lose contact with DNA not only in promoters but also in the coding regions of actively transcribed genes.

Transcription-dependent nucleosome loss in the coding region of *GAL1*

One of the hallmarks of chromatin remodelling at the *PHO5* promoter is that it occurs in a transcription-independent manner (Fascher *et al.*, 1993). Similarly, loss of histone–DNA contacts in the *GAL*-induced genes might conceivably be brought about by a transcription-independent mechanism. To address whether the loss of histone–DNA contacts in the *GAL1* gene required transcription, we used a strain expressing a temperature-sensitive allele of the largest RNAPII subunit, Rpb1. At the restrictive temperature, the *rpb1-1* strain rapidly ceases transcription due to loss of RNAPII polymerization activity (Nonet *et al.*, 1987), as well as loss of recruitment of the polymerase to promoters (Schroeder *et al.*, 2000). The *rpb1-1* strain and its wild-type counterpart were induced at 25 and 37°C, and the dynamics of RNAPII recruitment and loss of H3 was monitored by ChIP assays. As shown in Figure 3A, both wild-type and *rpb1-1* cells activated *GAL1* transcription at 25°C, but only wild-type cells were able to do so at 37°C. Importantly, the loss of histone–DNA contacts was dependent on active RNAPII transcription, because only wild-type but not the *rpb1-1* strain lost histone H3 at 37°C in the *GAL1* coding region (Figure 3B; similar data for the promoter are not shown). These data indicate that the high level of transcription at the *GAL1* gene displaces nucleosomes, or at least leads to a substantial loss of histone–DNA contacts.

Acetylation of histones upon galactose induction of the *GAL-VPS13* gene

Transcription of genes is facilitated by protein complexes that covalently modify chromatin. HATs are the best characterized

Figure 3 Loss of histone–DNA contacts in the coding region of *GAL1* requires active transcription. Wild-type (WT) and *rpb1-1* strains were induced at 25 and 37°C and the kinetics of RNAPII recruitment (A) or loss of histone H3 (B) were determined by ChIP before (0 min) and after (15 and 90 min) galactose induction. The results were normalized as described for Figure 2.

of such factors. In order to evaluate the role of histone acetylation in transcription of the long fusion gene, we analysed the status of H3 and H4 acetylation at *GAL-VPS13* during induction (Figure 4). Because of the observed loss of histone–DNA contacts, the results from ChIP assays with acetyl-H3- and acetyl-H4-specific antibodies were normalized according to the total level of H3 present at the corresponding time point (see Figure 2C and D). Upon induction of transcription, acetylation of histone H3, but not H4, increased at the promoter of *GAL-VPS13* throughout the time course and reached a maximal level (three- to four-fold above uninduced conditions) within 15 min of induction (Figure 4A).

Surprisingly, we did not observe any significant increase in histone acetylation in the coding region of *GAL-VPS13* during gene induction (Figure 4B). This indicates that H3 and H4 are either not acetylated in a transcription-dependent manner in the coding region, or that if they are, such acetylation is so transient that we cannot detect it by the ChIP assay. In the

Figure 4 Histone H3 and H4 acetylation at the *GAL-VPS13* locus during activation. The amount of acetyl-H3 (grey bars) and acetyl-H4 (white bars) at the promoter (A) or in the coding region (B) of the *GAL-VPS13* gene was determined by ChIP. The results were first normalized according to the amount of acetyl-H3 or acetyl-H4 in a nontranscribed region on chromosome IV, and then normalized to reflect the number of nucleosomes present at the corresponding position of the *GAL-VPS13* gene at the relevant time point of induction (adjustment for the loss of nucleosomes).

latter case, one might assume that inactivation of HDACs would help to preserve a putative hyperacetylated state of histones. To address this possibility, we used HDAC inhibitors (TSA and sodium butyrate) but failed to observe an effect on histone acetylation levels (data not shown). We also analysed histone acetylation in cells lacking specific HDACs (using *rpd3*, *hos2*, and *rpd3 hos2* strains). Although the steady-state levels of H3 and H4 acetylation in these strains were generally higher than in wild-type cells, we were still unable to detect any transcription-dependent increase in H3 or H4 acetylation in the coding region of the *GAL-VPS13* gene (data not shown).

To reveal the HAT(s) responsible for H3 acetylation in the *GAL-VPS13* promoter, we constructed a strain carrying the inducible *GAL-VPS13* gene in combination with deletion of the genes encoding two transcription-related HATs, *GCN5* and *ELP3*. Gcn5 is the catalytic subunit of SAGA, a major tran-

scription-related HAT complex in yeast (Grant *et al*, 1997), while Elp3 is the catalytic subunit of Elongator, a HAT complex originally isolated with hyperphosphorylated, elongating RNAPII (Otero *et al*, 1999). Histone H3 is the primary target for both of these complexes and significant redundancy in their function has been demonstrated (Grant *et al*, 1997; Wittschleben *et al*, 2000; Kristjuhan *et al*, 2002, 2003; Winkler *et al*, 2002). Induction of *GAL-VPS13* in *elp3*, *gcn5*, and *elp3 gcn5* strains showed nearly wild-type levels of RNAPII recruitment both to the promoter and to the end of coding region (Figure 5A), with only a slight delay in the kinetics observed in the mutant strains, indicating no dramatic requirement for these factors for the induction of the gene. The loss of nucleosomes also occurred to a similar extent both in wild-type and the mutant strains (Figure 5B). As significant histone H4 acetylation was not detected in the locus in wild-type cells, and the *gcn5* and *elp3 gcn5* strains were deficient for H3 acetylation in the promoter of the gene (Figure 5C), the loss of histone-DNA contacts detected in this region may have occurred in an acetylation-independent manner. Another possibility was that the lack of *GCN5* and hence lack of H3 acetylation resulted in compensatory acetylation of H4. However, no evidence for increased histone H4 acetylation at the gene in *gcn5* or *gcn5 elp3* cells was obtained (data not shown).

Taken together, these data show that Gcn5 is the major HAT involved in acetylation of H3 in the promoter of the *GAL-VPS13* gene. Nevertheless, Gcn5 HAT activity (and the increase of promoter H3 acetylation in general) is apparently not important for efficient transcription of the gene, or for the observed loss of histone-DNA contacts.

Transcription-dependent changes in histone-DNA contacts and histone acetylation at *GCN5/ELP3*-dependent genes

The experiments presented above point to the removal of nucleosomes as a mechanism to achieve high levels of transcription. These data raised the question whether other genes, such as the majority of constitutively expressed genes, might also be subject to this type of chromatin reorganization as a consequence of transcription. Relevant in this regard, we have previously shown that the global level of histone H3 acetylation is extremely low in *elp3 gcn5*, and that expression of several genes is dramatically reduced in this strain. Most significantly, the expression levels of dramatically downregulated genes correlate very well with the level of H3 acetylation in their coding regions (Kristjuhan *et al*, 2002). The mechanism allowing transcription of these genes, in contrast to the *GAL*-induced genes, might thus conceivably involve acetylation of resident histones rather than, or in addition to, nucleosome eviction.

To test these possibilities experimentally, we first investigated whether the low level of expression of several genes in the *elp3 gcn5* strain resulted in a higher nucleosome density in the coding region of these genes in the mutant compared to wild type. The amounts of RNAPII and nucleosomes in the coding region of *BAT1*, *GDH1*, and *YGP1* in wild-type and *elp3 gcn5* cells were compared (Figure 6). As reported previously (Kristjuhan *et al*, 2002), there was a significantly reduced level of transcribing RNAPII in these genes in *elp3 gcn5* cells compared to the wild-type strain (Figure 6; 'RNAPII'). In contrast, ChIP analysis of the histone H3 levels indicated that

Figure 5 Induction of *GAL-VPS13* transcription in *elp3*, *gcn5*, and *elp3 gcn5*. (A) Recruitment of RNAPII to the promoter (pr.) and the end of the coding region (8.3 kb) of *GAL-VPS13* was analysed in WT, *elp3*, *gcn5*, and *elp3 gcn5* cells after 0, 15, and 90 min of induction. (B) As in (A), but histone H3. (C) Acetylation of histone H3 at the *GAL-VPS13* locus in WT, *elp3*, *gcn5*, and *elp3 gcn5* strains was determined at 0, 15, and 90 min time points of galactose induction. The results were normalized as described for Figures 2 and 4.

the nucleosome density in the middle of the coding region remained the same (Figure 6; 'Histone H3'). Two conclusions can be drawn from this result. Firstly, nucleosome levels in active genes are not necessarily directly correlated with the level of transcriptional activity, that is, transcription does not necessarily lead to loss of histone-DNA contacts. And

Figure 6 Nucleosome density at *BAT1*, *GDH1*, and *YGP1* is unaffected by *gcn5 elp3* mutation. The amount of RNAPII and histone H3 in the middle of the coding region of *BAT1*, *GDH1*, and *YGP1* in WT and *gcn5 elp3* cells was determined by ChIP. The graphs represent the relative levels of RNAPII and histone H3 in the *gcn5 elp3* strain with the corresponding values in the WT strain set as 1 (stippled line). Co-immunoprecipitated DNA was quantified by real-time PCR and all samples were normalized according to the amount of RNAPII and histone H3 in a nontranscribed region on chromosome IV (see Materials and methods).

secondly, the reduced gene expression observed in *gcn5 elp3* cannot be caused by an abnormally high density of nucleosomes in the coding region of the tested genes.

To further investigate nucleosome density in the coding region of the *GCN5/ELP3*-dependent constitutive genes, we also made deletions of the *BAT1*, *GDH1*, and *YGP1* promoters to reduce their transcription levels. Quantification of the corresponding transcripts confirmed that the production of mRNA from these genes was effectively shut down by deletion of the respective promoters (Figure 7A). As can be seen in Figure 7B, however, the level of histone H3-DNA contacts did not change significantly in the coding region of these genes in response to the abrogation of promoter-directed transcription.

Finally, we also investigated chromatin structure in the genes after abrogation of transcription via the temperature-sensitive *rpb1-1* allele, allowing measurements of nucleosome density and modification levels without altering the primary sequence of the DNA in the region. This approach also minimized the effect of transcription/modification at neighbouring genes. This complete transcriptional shut-off resulted in a slight but repeatedly observed increase in histone-DNA contacts over each of the three genes (Figure 7C), suggesting that some loss of histone-DNA contact may be a more widespread consequence of ongoing transcription. Significantly, cessation of transcription correlated with a more than three-fold decrease in histone H3 acetylation at *YGP1*, and approximately two-fold decreases at *BAT1* and *GDH1* (Figure 7D), indicating that transcription-associated histone acetylation indeed occurs in the coding region of these genes.

Discussion

In this study, we followed the dynamics of transcription and chromatin modification during the time course of gene activation. The key results were the following. Firstly, by comparing 'snapshots' of RNAPII density and mRNA production

in the beginning and end of a long test gene, we found that a significant proportion (at least half) of RNAPII molecules initiating transcription never make it to the end of this gene. Secondly, nucleosomes not only in the promoter but also in the coding region of *GAL* genes lose contact with DNA in the course of gene induction, and this 'nucleosome loss' requires transcription. Thirdly, histone H3 rapidly becomes

hyperacetylated in the *GAL* promoter, but not the coding region, as a result of activation. In contrast, no evidence of histone H4 acetylation was obtained. Fourthly, histone H3 promoter acetylation was dependent on *GCN5*. Nucleosome loss was observed also in cells lacking *GCN5* (and even in *gcn5 elp3*), suggesting that the loss of histone-DNA contacts in *GAL* genes is independent of H3 acetylation. Finally, histone loss and modification in response to transcription was studied in genes previously shown to require *Gcn5* and *Elp3* for normal transcription. Interestingly, these genes did not dramatically lose histone-DNA contacts in response to transcription, but the histones were acetylated in a transcription-dependent manner. Together, these data suggest that efficient transcription through chromatin can occur by distinct mechanisms.

Time course of transcriptional activation in a *GAL-VPS13* fusion gene

The use of a *GAL-VPS13* fusion gene made it possible to clearly distinguish between transcription-coupled events driven by transcriptional initiation and elongation. While this study was in progress, results obtained by Struhl and colleagues with a similar fusion gene were reported (Strasser *et al*, 2002; Mason and Struhl, 2003). Although the speed of *GAL* activation and repression is known to occur with different speeds in different strain backgrounds, it was somewhat surprising that activation of the *GAL-VPS13* fusion gene in our strains occurred in a more or less linear manner over a period of more than 1 h. This is in contrast to results reported by Bryant and Ptashne (2003), who found that recruitment of RNAPII to the *GAL1* gene in their strains reached plateau levels already 15–18 min after galactose addition. The reason for the slower activation kinetics observed in our strain background (the same kinetics were also observed at the indigenous *GAL1* gene) is unknown and unfortunately precluded rigorous measurements of the speed of elongation through the long *VPS13* coding region.

However, our experiments with the fusion gene yielded the interesting result that the level of polymerase detected at the promoter was significantly higher than that detected further

Figure 7 Transcription-associated histone H3 acetylation, but little nucleosome loss at the *GCN5/ELP3*-dependent genes. (A) The level of *BAT1*, *GDH1*, and *YGP1* mRNAs as determined by real-time RT-PCR in strains where the promoters of the indicated genes had been deleted. mRNA level in the wild-type strain was set as 1 (stippled line). Note that these genes are expressed at different levels compared to each other in wild-type cells (*BAT1* = 15 transcripts/cell; *GDH1* = 10 transcripts/cell; *YGP1* = 6 transcripts/cell) (Velculescu *et al*, 1997). (B) The amount of histone H3 at *BAT1*, *GDH1*, and *YGP1* in wild-type and promoter deletion strains was determined by ChIP. The level of histone H3 in the wild-type strain was set as 1 (stippled line). (C) The amount of histone H3 in the middle of the coding region of *BAT1*, *GDH1*, and *YGP1* in wild type and *rpb1-1* at 25 and 37°C was determined by ChIP. The fraction of histone H3 observed in the absence of transcription compared to in the presence of transcription (37°C/25°C ratio) is shown. No significant change occurred after temperature shift in the wild-type cells, and therefore the ratio observed in this strain was 1 (stippled line). (D) The amount of histone H3 acetylation in the middle of the coding region of *BAT1*, *GDH1*, and *YGP1* in wild type and *rpb1-1* at 25 and 37°C was determined by ChIP. The fraction of histone H3 acetylation in the absence of transcription compared to in the presence of transcription (37°C/25°C ratio) is shown, with the ratio obtained in the wild-type strain set as 1 as in (C) (stippled line). The results were normalized as described for Figures 2 and 4.

into the gene (at 2.2, 5.5, and 8.8 kb), suggesting either that RNAPII–DNA crosslinking efficiency is higher at the promoter than for elongating polymerases further into the gene, or that a substantial fraction of polymerases loaded onto the gene never make it to the end, presumably because they abort transcription prematurely. Quantitative RT–PCR measurements of RNA produced from the locus showed that up to four-fold more transcript RNA was produced in the 5′ end of the gene than at the 3′ end, supporting the idea that substantial abortive transcription occurred at this locus. This idea was further supported by the observation that substantial smearing below the full-length *VPS13* transcript was detected by Northern blot (data not shown). The idea that abortive transcription (in the beginning of the gene prior to promoter clearance) is a natural part of the transcription process has long been generally accepted based on *in vitro* experiments (Dvir, 2002), but data from living cells to support it have been lacking. Our data clearly indicate that even transcripts of substantial length can be aborted during transcription of certain genes. The process underlying such transcriptional abortion is unknown but is of great relevance to an understanding of basic transcription processes and their regulation.

Loss of histone–DNA contacts during activation

The chromatin transactions taking place at the yeast *PHO* and *GAL* genes have been the subject of several studies over the years (Almer and Horz, 1986; Almer *et al.*, 1986; Cavalli and Thoma, 1993; Fascher *et al.*, 1993). However, only with the availability of techniques such as ChIP has it become possible to make judgment on the fate of nucleosomes in the promoter of *PHO5* and *PHO8*, for example (Boeger *et al.*, 2003; Reinke and Horz, 2003; Adkins *et al.*, 2004; Boeger *et al.*, 2004). Thus, traditional tools for studying chromatin structure, such as nuclease digestion of chromatin in isolated nuclei, are capable of assessing whether chromatin structure has been perturbed, but not of distinguishing between loss of nucleosome positioning and loss of histone–DNA contacts. Indeed, others have reported substantial changes in chromatin structure at the *GAL1-10* locus after gene activation, as assayed by micrococcal nuclease digestion (Li and Smerdon, 2002). Our ChIP data indicate that these dramatic changes are due to histones losing contact with DNA in both the promoter and coding region of the genes. Interestingly, this is in apparent contrast to *PHO5* and *PHO8* where nucleosomes in the promoter, but not the coding region, are lost (Adkins *et al.*, 2004). Moreover, the loss of histone–DNA contacts we observed is dependent on active transcription. This also appears to be in contrast to the dramatic change in chromatin structure in the promoter of *PHO5*, which occurs even when the TATA box of the gene is removed so that transcription is abolished (Fascher *et al.*, 1993). In support of our data, loss of histone–DNA contacts in the coding region of *GAL* genes has also been observed by Bentley and co-workers (L Zhang and D Bentley, personal communication).

Cotranscriptional changes in histone acetylation

Studying the pattern of histone acetylation during activation of *GAL-VPS13* indicated that histone H3, but not H4, is hyperacetylated in the promoter in response to transcriptional induction. Deletion of the transcription-related HATs *ELP3* and *GCN5* revealed that Gcn5 is the major HAT

responsible for this acetylation. Fully induced levels of H3 acetylation in the promoter were achieved within 15 min, whereas final levels of RNAPII recruited were achieved much later, suggesting that recruitment of SAGA (Gcn5) and of RNAPII were independent events, as previously observed by others (reviewed by Cosma, 2002). Histone H3 acetylation in the promoter was not crucial for activation, however, as *gcn5* cells showed wild-type levels of gene activation despite a lack of increased promoter acetylation.

In contrast to the acetylation pattern in promoter regions, we did not detect any significant increase of H3 or H4 acetylation in the coding region of the genes. This result was surprising, because several observations predict a role for histone acetylation and deacetylation activities in the coding regions of the *GAL* genes during transcription. For example, the Elongator HAT complex and the histone deacetylase Hos2 are indeed present in the coding region of *GAL1* during activation (Wang *et al.*, 2002; Gilbert *et al.*, 2004), indicating a role for HATs and HDACs in transcript elongation. It is important to note, however, that although we were unable to detect changes in the level of histone acetylation in the coding region during activation, it is possible that these modifications are very dynamic and that hyperacetylation of histones occurs only transiently in coding regions (Svejstrup, 2003).

Genes do not invariably lose nucleosomes in a transcription-dependent manner

We previously reported that several genes are severely down-regulated in *gcn5 elp3* cells and that reduced transcription of these genes correlates with low histone H3 acetylation in their coding region (Kristjuhan *et al.*, 2002). We were therefore somewhat surprised that activation of the long *GAL-VPS13* fusion gene showed no requirement for *GCN5/ELP3*. The same lack of *GCN5/ELP3* requirement was observed for the *GAL1* gene (data not shown). In an attempt to shed light on potential differences in basic mechanisms underlying transcription through chromatin, we also studied transcription-dependent nucleosome density and acetylation in the *GCN5/ELP3*-dependent *BAT1*, *GDH1*, and *YGP1* genes. Because these genes are constitutively expressed at different levels, we compared their histone H3 density under normal conditions (where it would be presumed to be low if transcription generally leads to loss of histone–DNA contacts) to conditions where transcription was significantly reduced (by *gcn5 elp3* mutation, deletion of the TATA box or inactivation of RNAPII). Remarkably, none of these genes appear to lose nucleosomes as a consequence of transcription. However, they are acetylated in a transcription-dependent manner.

These data point to the existence of distinct mechanisms for enabling transcription through coding regions packed in nucleosomes. In the first, histones lose contact with DNA, and in the other, little or no net loss of histone–DNA contact occurs. These mechanisms correlate with transcription levels and histone H3 acetylation in a potentially telling manner: firstly, transcription levels at the *GAL* genes are very high, with a new polymerase initiating transcription approximately every 6–8 s (Iyer and Struhl, 1996). This in all likelihood results in a large number of polymerases on the coding region at the same time (theoretically one every 150–200 bp, if the rate of transcription is presumed to be 1500 bases/min). Indeed, RNAPII ChIP–reChIP–reChIP experiments with

strains concomitantly expressing three different, distinctly tagged versions of Rpb3 indicate that at least three polymerases can be present per 300–600 bp *GAL* coding region fragment under fully induced conditions (A Kristjuhan, unpublished). Such high levels of transcription/RNAPII density might thus not permit efficient re-establishment of histone–DNA contacts after polymerase passage, resulting in a net loss of nucleosomes.

However, transcription of the majority of genes occurs with a much lower density of transcribing polymerases, and transcription of such genes might thus occur without substantial loss of nucleosomes. Our data suggest that at least the *GCN5/ELP3*-dependent *BAT1*, *GDH1*, and *YGP1* genes fall into this category. The fact that these genes in contrast to *GAL1* and *GAL-VPS13* are acetylated in a transcription-dependent manner is in agreement with the idea that when nucleosome eviction does not occur, histone acetylation can become very important for transcription through chromatin.

Materials and methods

Yeast strains

All *S. cerevisiae* strains were congenic with strain W303 (Thomas and Rothstein, 1989). To create a *GAL-VPS13* fusion gene at the *VPS13* locus in chromosome XII, the *GAL10* promoter (500 bp fragment upstream from the *GAL10* ORF) was inserted in front of the *VPS13* gene by homologous recombination *in vivo*. The resulting *GAL-VPS13* locus contains all the regulatory elements of the *GAL10* promoter (the Gal4 binding sites, TATA box, transcriptional start site, and the 5' noncoding stretch of mRNA) followed by the 9435 bp long ORF of *VPS13* (Figure 1A). Growth of yeast strains is neither affected by the loss of *VPS13* function nor by its overexpression. The genotypes of the strains were as follows: *MATa GAL-VPS13::TRP1 TBP-3xHA::URA3* (JSY 679), *MATa GAL-VPS13::TRP1 TBP-3xHA::URA3 elp3Δ::LEU2* (JSY 681), *MATa GAL-VPS13::TRP1 TBP-3xHA::URA3 gcn5Δ::HIS3* (JSY 683), *MATa GAL-VPS13::TRP1 TBP-3xHA::URA3 elp3Δ::LEU2 gcn5Δ::HIS3* (JSY 685), *MATα GAL-VPS13::TRP1 bat1Δpromoter::URA3* (JSY1008), *MATα GAL-VPS13::TRP1 gdh1Δpromoter::URA3* (JSY1009), and *MATα GAL-VPS13::TRP1 ygp1Δpromoter::URA3* (JSY1010). The JR5-2A strain (W303, but *htb1-1 htb2-1*), expressing a plasmid-borne copy of FLAG-tagged H2B protein (Robzyk *et al*, 2000), was kindly provided by Dr Mary Ann Osley, while the strain carrying an *rpb1-1* mutation and its wild-type counterpart (Nonet *et al*, 1987) was kindly provided by Dr Richard Young.

Galactose induction of transcription

Cells were grown for 12–15 h in yeast extract-peptone (YP) medium containing 2% raffinose as a carbon source. Before induction with galactose, 25 ml of mid-log phase cells (density $0.5\text{--}1 \times 10^7$ cells/ml) were fixed in 1% formaldehyde as a 0 min time-point sample. Cells in mid-log phase were induced in a large batch (200 ml) by adding

galactose to the growth medium (final concentration 2%). At different time points after adding galactose, 25 ml aliquots were taken and fixed in 1% formaldehyde for ChIP assays.

Cells carrying the *rpb1-1* mutation were grown in YP-raffinose at 25°C to mid-log phase and shifted to 37°C for 30 min before galactose induction.

Chromatin immunoprecipitation assay and RT-PCR

ChIP assays were performed essentially as described previously (Kristjuhan *et al*, 2002). Antibodies directed against RNAPII (4H8), diacetyl-H3, and tetraacetyl-H4 were purchased from Upstate, anti-FLAG M2 antibody was purchased from Sigma, and antibody recognizing the C-terminus of histone H3 was a generous gift from Dr Alain Verreault. Co-precipitated DNA was analysed by quantitative real-time PCR using the ABI Prism[®] 7000 Sequence Detection System in standard conditions (40 cycles; 95°C 15 s + 60°C 1 min). Absolute[™] QPCR reagents (ABgene) were used in combination with FAM-labelled TaqMan[®] fluorogenic quantitation probes to detect sequences in the promoter or in the coding regions of the *GAL-VPS13* gene. Sequences of the *GAL1* gene were analysed by real-time PCR with Absolute[™] QPCR SYBR Green reagents (ABgene) without fluorescent probe. *GAL-VPS13* promoter-specific PCR primers amplify the sequence about 150 bp downstream from the TATA box and contain sequences unique to the *VPS13* gene in order to distinguish the *GAL-VPS13* promoter from the *GAL10* promoter. The coding region primers are specific for the sequences at 2.2, 5.5, and 8.3 downstream from the promoter of *GAL-VPS13*, and 1.2 kb downstream from the promoter of *GAL1* genes. Two control regions in the genome were used to compensate for possible fluctuations arising either from different amounts of starting material or possible loss of material during handling of samples. RNAPII ChIP of the ubiquitously and highly expressed *FBA1* gene was used for normalization of all RNAPII ChIPs. A probe in the middle of a 3.2 kb long ORF-free region on chromosome IV (between the *NRG1* and *HEM13* genes) was used to normalize all histone-related ChIPs. Because we observed a loss of histone H3 signal from *GAL-VPS13* and *GAL1* genes upon galactose induction, we also normalized all acetyl-histone-specific ChIP results (shown in Figures 4, 5, and 7) according to the total H3 signal (as detected with C-terminus-specific antibody) at the genes. Experiments were typically repeated 3–4 times; error bars in figures show standard deviations.

For detection of mRNA, cells were treated as described for RNA-immunoprecipitation assays (Gilbert *et al*, 2004). The amount of *VPS13* and *FBA1* mRNAs was then quantified by one-step real-time RT-PCR with Absolute[™] QPCR SYBR Green reagents (ABgene).

The sequences of all primers and fluorogenic probe oligos used in this study are available upon request.

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References

- Adkins MW, Howar SR, Tyler JK (2004) Chromatin disassembly mediated by the histone chaperone Asf1 is essential for transcriptional activation of the yeast PHO5 and PHO8 genes. *Mol Cell* **14**: 657–666
- Almer A, Horz W (1986) Nuclease hypersensitive regions with adjacent positioned nucleosomes mark the gene boundaries of the PHO5/PHO3 locus in yeast. *EMBO J* **5**: 2681–2687
- Almer A, Rudolph H, Hinnen A, Horz W (1986) Removal of positioned nucleosomes from the yeast PHO5 promoter upon PHO5 induction releases additional upstream activating DNA elements. *EMBO J* **5**: 2689–2696
- Belotserkovskaya R, Oh S, Bondarenko VA, Orphanides G, Studitsky VM, Reinberg D (2003) FACT facilitates transcription-dependent nucleosome alteration. *Science* **301**: 1090–1093
- Boeger H, Griesenbeck J, Strattan JS, Kornberg RD (2003) Nucleosomes unfold completely at a transcriptionally active promoter. *Mol Cell* **11**: 1587–1598
- Boeger H, Griesenbeck J, Strattan JS, Kornberg RD (2004) Removal of promoter nucleosomes by disassembly rather than sliding *in vivo*. *Mol Cell* **14**: 667–673
- Bryant GO, Ptashne M (2003) Independent recruitment *in vivo* by Gal4 of two complexes required for transcription. *Mol Cell* **11**: 1301–1309
- Cavalli G, Thoma F (1993) Chromatin transitions during activation and repression of galactose-regulated genes in yeast. *EMBO J* **12**: 4603–4613
- Cherry JM, Ball C, Weng S, Juvik G, Schmidt R, Adler C, Dunn B, Dwight S, Riles L, Mortimer RK, Botstein D (1997) Genetic and physical maps of *Saccharomyces cerevisiae*. *Nature* **387**: 67–73

- Cosma MP (2002) Ordered recruitment: gene-specific mechanism of transcription activation. *Mol Cell* **10**: 227–236
- Deckert J, Struhl K (2001) Histone acetylation at promoters is differentially affected by specific activators and repressors. *Mol Cell Biol* **21**: 2726–2735
- Dvir A (2002) Promoter escape by RNA polymerase II. *Biochim Biophys Acta* **1577**: 208–223
- Fascher KD, Schmitz J, Horz W (1993) Structural and functional requirements for the chromatin transition at the PHO5 promoter in *Saccharomyces cerevisiae* upon PHO5 activation. *J Mol Biol* **231**: 658–667
- Gilbert C, Kristjuhan A, Winkler GS, Svejstrup JQ (2004) Elongator interactions with nascent mRNA revealed by RNA immunoprecipitation. *Mol Cell* **14**: 457–464
- Grant PA, Duggan L, Cote J, Roberts SM, Brownell JE, Candau R, Ohba R, Owen-Hughes T, Allis CD, Winston F, Berger SL, Workman JL (1997) Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex. *Genes Dev* **11**: 1640–1650
- Hassan AH, Neely KE, Vignali M, Reese JC, Workman JL (2001a) Promoter targeting of chromatin-modifying complexes. *Front Biosci* **6**: D1054–D1064
- Hassan AH, Neely KE, Workman JL (2001b) Histone acetyltransferase complexes stabilize swi/snf binding to promoter nucleosomes. *Cell* **104**: 817–827
- Iyer V, Struhl K (1996) Absolute mRNA levels and transcriptional initiation rates in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* **93**: 5208–5212
- Kadam S, Emerson BM (2002) Mechanisms of chromatin assembly and transcription. *Curr Opin Cell Biol* **14**: 262–268
- Kaplan CD, Laprade L, Winston F (2003) Transcription elongation factors repress transcription initiation from cryptic sites. *Science* **301**: 1096–1099
- Katan-Khaykovich Y, Struhl K (2002) Dynamics of global histone acetylation and deacetylation *in vivo*: rapid restoration of normal histone acetylation status upon removal of activators and repressors. *Genes Dev* **16**: 743–752
- Kristjuhan A, Walker J, Suka N, Grunstein M, Roberts D, Cairns BR, Svejstrup JQ (2002) Transcriptional inhibition of genes with severe histone h3 hypoacetylation in the coding region. *Mol Cell* **10**: 925–933
- Kristjuhan A, Wittschieben BO, Walker J, Roberts D, Cairns BR, Svejstrup JQ (2003) Spreading of Sir3 protein in cells with severe histone H3 hypoacetylation. *Proc Natl Acad Sci USA* **100**: 7551–7556
- Kulich D, Struhl K (2001) TFIIS enhances transcriptional elongation through an artificial arrest site *in vivo*. *Mol Cell Biol* **21**: 4162–4168
- Kurdistani SK, Tavazoie S, Grunstein M (2004) Mapping global histone acetylation patterns to gene expression. *Cell* **117**: 721–733
- Li S, Smerdon MJ (2002) Nucleosome structure and repair of N-methylpurines in the GAL1-10 genes of *Saccharomyces cerevisiae*. *J Biol Chem* **277**: 44651–44659
- Mason PB, Struhl K (2003) The FACT complex travels with elongating RNA polymerase II and is important for the fidelity of transcriptional initiation *in vivo*. *Mol Cell Biol* **23**: 8323–8333
- Narlikar GJ, Fan HY, Kingston RE (2002) Cooperation between complexes that regulate chromatin structure and transcription. *Cell* **108**: 475–487
- Nonet M, Scafe C, Sexton J, Young R (1987) Eucaryotic RNA polymerase conditional mutant that rapidly ceases mRNA synthesis. *Mol Cell Biol* **7**: 1602–1611
- Otero G, Fellows J, Li Y, de Bizemont T, Dirac AMG, Gustafsson CM, Erdjument-Bromage H, Tempst P, Svejstrup JQ (1999) Elongator, a multisubunit component of a novel RNA polymerase II holoenzyme for transcriptional elongation. *Mol Cell* **3**: 109–118
- Pokholok DK, Hannett NM, Young RA (2002) Exchange of RNA polymerase II initiation and elongation factors during gene expression *in vivo*. *Mol Cell* **9**: 799–809
- Reinke H, Gregory PD, Horz W (2001) A transient histone hyperacetylation signal marks nucleosomes for remodeling at the PHO5 promoter *in vivo*. *Mol Cell* **7**: 529–538
- Reinke H, Horz W (2003) Histones are first hyperacetylated and then lose contact with the activated PHO5 promoter. *Mol Cell* **11**: 1599–1607
- Robzyk K, Recht J, Osley MA (2000) Rad6-dependent ubiquitination of histone H2B in yeast. *Science* **287**: 501–504
- Schroeder SC, Schwer B, Shuman S, Bentley D (2000) Dynamic association of capping enzymes with transcribing RNA polymerase II. *Genes Dev* **14**: 2435–2440
- Strasser K, Masuda S, Mason P, Pfannstiel J, Oppizzi M, Rodriguez-Navarro S, Rondon AG, Aguilera A, Struhl K, Reed R, Hurt E (2002) TREX is a conserved complex coupling transcription with messenger RNA export. *Nature* **417**: 304–308
- Svejstrup JQ (2003) Transcription. Histones face the FACT. *Science* **301**: 1053–1055
- Thomas BJ, Rothstein R (1989) Elevated recombination rates in transcriptionally active DNA. *Cell* **56**: 619–630
- Velculescu VE, Zhang L, Zhou W, Vogelstein J, Basrai MA, Bassett Jr DE, Hieter P, Vogelstein B, Kinzler KW (1997) Characterization of the yeast transcriptome. *Cell* **88**: 243–251
- Vogelauer M, Wu J, Suka N, Grunstein M (2000) Global histone acetylation and deacetylation in yeast. *Nature* **408**: 495–498
- Wang A, Kurdistani SK, Grunstein M (2002) Requirement of Hos2 histone deacetylase for gene activity in yeast. *Science* **298**: 1412–1414
- Winkler GS, Kristjuhan A, Erdjument-Bromage H, Tempst P, Svejstrup JQ (2002) Elongator is a histone H3 and H4 acetyltransferase important for normal histone acetylation levels *in vivo*. *Proc Natl Acad Sci USA* **99**: 3517–3522
- Wittschieben BO, Fellows J, Du W, Stillman DJ, Svejstrup JQ (2000) Overlapping roles for the histone acetyltransferase activities of SAGA and Elongator *in vivo*. *EMBO J* **19**: 3060–3068
- Workman JL, Kingston RE (1998) Alteration of nucleosome structure as a mechanism of transcriptional regulation. *Annu Rev Biochem* **67**: 545–579