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Distinct activated and non-activated RNA polymerase II complexes in yeast

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We used a transcriptional run-on assay in permeabilized yeast cells to study the distribution of RNA polymerase II (pol II) complexes before and after activation by Gal4. Polymerases were found engaged on the gene at the 5′ end before activation, but only appeared at the 3′ end after activation. Mutations of the pol II C-terminal domain (CTD), the CTD kinase Kin28 and the holoenzyme subunit Srb2 all inhibited the formation of 3′ polymerases in response to activator. However, these mutations did not inhibit the establishment of polymerases at the 5′ end. The differences between 3′ and 5′ ternary complexes suggest that they represent qualitatively distinct 'activated' and 'non-activated' forms of polymerase. The results implicate CTD phosphorylation in a switch from 'non-activated' transcription, which is confined to the 5′ end, to an 'activated' mode that traverses the length of the gene.
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Introduction

The mechanism by which transcriptional activators stimulate RNA synthesis by polymerase II (pol II) is a major problem in eukaryotic gene expression. One aspect of activator function is the stimulation of the frequency of initiation relative to basal transcription. This function is thought to be mediated by recruiting the pol II holoenzyme (Barberis *et al.*, 1995; Hengartner *et al.*, 1995) and general factors such as transcription factor (TF) IID to the promoter (Klein and Struhl, 1994; Tjian and Maniatis, 1994; Zawel and Reinberg, 1995). Activation is almost certainly more complex than simply stimulating initiation because a frequently observed rate-limiting step *in vivo* occurs after initiation, at the promoter clearance or elongation steps (Rougvie and Lis, 1990; Krumm *et al.*, 1995). Certain activators also stimulate these post-initiation events (reviewed in Lis and Wu, 1993; Bentley, 1995), but the mechanism underlying this aspect of activator function is quite unclear. A fundamental question which remains is whether activation is simply an increase in the frequency of the same events which occur during basal transcription or, alternatively, whether there is some additional qualitative difference between activated and basal transcription.

Genetic analysis in yeast has identified a number of polypeptides required for activators to stimulate transcription. For example, some mutants of TATA-binding protein (TBP) specifically inhibit activated transcription from certain promoters (T.K.Kim *et al.*, 1994; Arndt *et al.*, 1995; Stargell and Struhl, 1995). In addition, the C-terminal domain (CTD) of the pol II large subunit is required for the function of certain activators in yeast and higher eukaryotes (Allison and Ingles, 1989; Scafe *et al.*, 1990; Gerber *et al.*, 1995). The CTD is composed of repeats of the heptad sequence Tyr–Ser–Pro–Thr–Ser–Pro–Ser which become hyperphosphorylated after the polymerase has been recruited to the promoter (Payne *et al.*, 1989; Lu *et al.*, 1991). Yeast cells with fewer than 8–11 of the 27 repeats are inviable (Nonet *et al.*, 1987b; West and Corden, 1995), and strains with 11–14 repeats are unable to stimulate transcription fully in response to activators such Gal4 and a Gal4(1–147)–Gcn4 fusion protein (Allison and Ingles, 1989; Scafe *et al.*, 1990).

The *SRB* genes were isolated as suppressors of a CTD truncation (Nonet and Young, 1989). These genes code for components of a large complex called the 'mediator', which interacts with core pol II via the CTD to form a 'holoenzyme' (Thompson *et al.*, 1993; Y.J.Kim *et al.*, 1994; Koleske and Young, 1994). The 'mediator' confers responsiveness to activators *in vitro* (Y.J.Kim *et al.*, 1994; Koleske and Young, 1994), and the deletion of *SRB2* and other *SRB*s weakens activation by Gal4 *in vivo* (Liao *et al.*, 1995).

The 'mediator' is a powerful enhancer of CTD phosphorylation by the general transcription factor TFIIH (Y.J.Kim *et al.*, 1994), which is a complex of nine subunits required for both pol II transcription and nucleotide excision repair (Drapkin and Reinberg, 1994). In some, but not all, preparations TFIIH co-purifies with the pol II holoenzyme (Y.J.Kim *et al.*, 1994; Koleske and Young, 1994). The kinase subunit of yeast TFIIH is a cyclin-dependent kinase which is the product of the *KIN28* gene (Simon *et al.*, 1986; Valay *et al.*, 1993; Feaver *et al.*, 1994). The level of CTD phosphorylation and the steady-state levels of several mRNAs were rapidly reduced following the transfer of *kin28* temperature-sensitive (ts) mutants to the non-permissive temperature (Cismowski *et al.*, 1995; Valay *et al.*, 1995). The holoenzyme contains a second cyclin-dependent kinase encoded by *SRB10* which may also phosphorylate the CTD (Liao *et al.*, 1995). Both these kinases are necessary for normal pol II transcription *in vivo*, but their exact roles are unclear.

Elegant experiments in *Drosophila* have shown that polymerases stalled near the start site of the quiescent *Hsp70* gene have hypophosphorylated CTDs, but that after heat shock, when elongation resumes, most polymerases

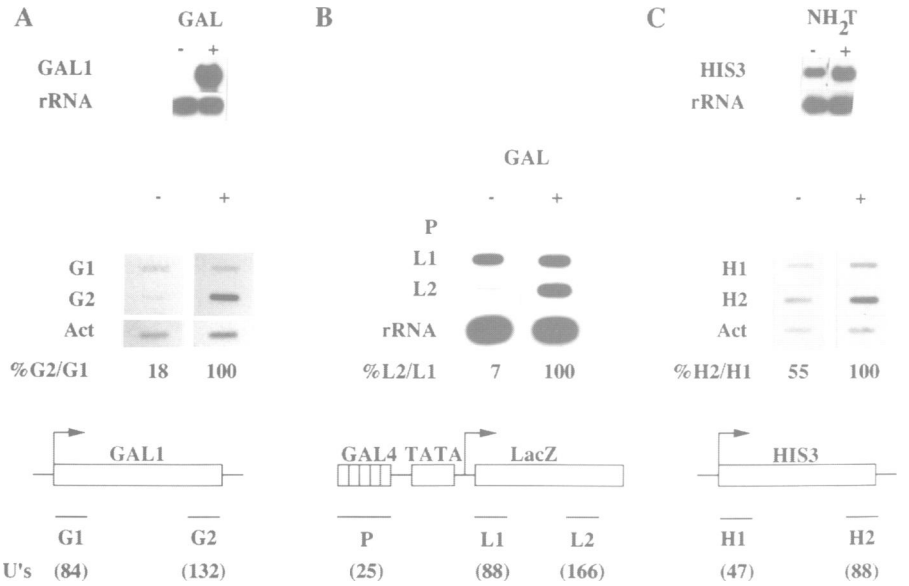

Fig. 1. Activation alters the distribution of pol II complexes along a gene. (**A**) Northern blot (top) and run-on (bottom) analyses of chromosomal *GAL1* expression in strain Y1114 (see Table I), – and + galactose. Run-on hybridization was to probes G1 and G2 (see map) and actin (Act). (**B**) Run-on assay of *Gal5–CYC1–lacZ* on a multicopy plasmid in strain Z96 using probes P, L1 and L2 (see map) and an rRNA probe. (**C**) Northern blot (top) and run-on (bottom) analyses of chromosomal *HIS3* expression in Y1114, – and + 20 mM aminotriazole (NH_2T). Northern blots were reprobed for rRNA as a loading control. Percentage 3′/5′ polymerase densities (G2/G1, L2/L1 and H2/H1) were calculated after compensating for the number of U residues (Us) complementary to each probe and normalizing to actin (A) or rRNA (B and C). Polymerase density ratios were adjusted to 100% for induced conditions.

positioned at both the 5′ and 3′ ends of the gene have hyperphosphorylated CTDs (O'Brien *et al.*, 1994). The intriguing correlation between elongation and CTD phosphorylation does not resolve the query as to whether this modification is a cause or a consequence of relieving the polymerase arrest. To address this question *in vivo* requires the analysis of cells where pol II phosphorylation is disrupted.

We have studied how the CTD, Kin28 and Srb's affect transcription *in vivo* by mapping pol II ternary complexes before and after activation in wild-type and mutant yeast cells. We used a run-on assay in permeabilized whole cells (Elion and Warner, 1986), which yields a 'snapshot' of the distribution of polymerases on a gene. Because it is detecting polymerases engaged on a gene at a particular instant, the run-on assay is a more direct measure of transcriptional activity than an analysis of steady-state RNA populations. The run-on assay has not been applied previously in a system which allows the analysis of mutants in the transcriptional apparatus. Our analysis in yeast identified two types of polymerase complex, 'activated' and 'non-activated', which are distributed differently along the gene and are affected differently by mutations in the transcriptional apparatus.

Results

Activators in yeast alter the distribution of pol II along a gene

Pausing and premature termination of transcription cause a drop in polymerase density on a gene which can be detected by a run-on assay. In higher eukaryotes this assay led to the discovery of blocks to elongation in many genes (Bentley, 1995). We adapted the run-on method, developed originally to investigate pol I transcription in yeast (Elion and Warner, 1986), to measure the distribution of transcribing pol II molecules. Yeast cells were permeabilized with 0.5% Sarkosyl, which prevents initiation and permits elongation after stripping most chromatin proteins from the DNA template. The Sarkosyl-resistant pol II ternary complexes were permitted to elongate a short distance and incorporate [^{32}P]UTP into nascent transcripts. The labelled RNA was hybridized to strand-specific probes, corresponding to 5′ and 3′ regions of the gene, and RNase-resistant hybrids were quantified. The run-on signal is proportional to the density of polymerases in a particular region of the gene, averaged over the population of permeabilized cells. The assay does not measure initiation rates, only the distribution of preinitiated polymerases (see Discussion). Relative polymerase densities were calculated from the hybridization signals after correction for the differences in U content of the probes and normalization to the signal from actin or ribosomal RNA. These calculations are based on the assumption that in the presence of Sarkosyl all polymerases elongate at the same average rate. One limitation of the run-on technique is its relatively limited resolution. As a result of this limitation we are unable to distinguish whether or not polymerases detected in the most 5′ region of a gene have cleared the promoter.

Initially we investigated activation of the chromosomal *GAL1* gene by the Gal4 protein in the presence of galactose. A Northern blot analysis showed a >59-fold stimulation of *GAL1* mRNA relative to rRNA when galactose was added to a culture (Figure 1A, top). Polymerase density was determined in two regions of the gene by hybridization of nascent run-on products to the G1 and G2 probes, centred at positions +129 and +1336 relative to the start site (Figure 1A, bottom). Surprisingly, the run-on revealed polymerases at the 5′ end of the gene even before transcription was activated, as shown by the

hybridization of nascent RNAs to the G1 probe. However, very few polymerases reached the 3′ end in the absence of galactose, as assessed by hybridization to G2. The addition of galactose had no significant effect on polymerase density at the 5′ end of the gene, but it caused a 5.5-fold increase in 3′ polymerase density relative to the actin control (Figure 1A, bottom, compare – and + panels). Similar results were obtained in five independent experiments. We do not fully understand why the increase in run-on signal is substantially less than the increase in mRNA level measured by Northern blotting; however, the 5.5-fold stimulation of the 3′ G2 signal is a minimum value because of inaccuracy in the measurement of the low signal above background in the absence of galactose. The value of the G2:G1 ratio after correcting for U content was 1.74 in galactose compared with 0.31 in raffinose. The unexpected G2:G1 value greater than unity is presumably a result of more efficient hybridization to the G2 probe. To help compare polymerase distributions before and after activation in different experiments, we normalized the ratio of 3′:5′ polymerase density to 100% in the presence of galactose. The relative G2:G1 polymerase density ratios in Figure 1A are therefore given as 100 and 18% in the presence and absence of galactose, respectively.

To test whether the effect of galactose on 3′:5′ polymerase distribution was specific to *GAL1* or a general property of activation by Gal4, we studied a *lacZ* reporter gene driven by the *CYC1* promoter. This fusion gene has been characterized extensively and multiple start sites of transcription have been mapped within a 50 base region of the *CYC1* sequence (Hahn *et al.*, 1985). This fusion gene with five upstream Gal4 binding sites (*Gal5–CYC1–lacZ*) was introduced into yeast on a multicopy plasmid. A run-on analysis was performed with two single-stranded probes (L1 and L2) centred 330 and 2040 bases from the start site of transcription. When cells were grown in raffinose-containing medium (non-activated), a high run-on signal was observed in the 5′ (L1) region of the *lacZ* gene (Figure 1B) but not in the 3′ (L2) region. The high run-on signal on *lacZ* relative to *GAL1* or *HIS3* reflects the high copy number of the reporter. Similar results were obtained in the presence of glucose (data not shown). Very few polymerases were detected upstream of the *CYC1* start site with probe P (Figure 1B). This control eliminates the possibility that a significant portion of the run-on signal from *lacZ* is the result of polymerases which initiate incorrectly or read around the plasmid from other promoters. When galactose was added to the culture, the 5′ run-on signal normalized to rRNA increased by only 1.4-fold, whereas the 3′ signal increased by 19.6-fold. As a result, the net ratio of 3′:5′ polymerases (L2:L1) increased by 14.0-fold (from 7 to 100%). We conclude that pol II is engaged at the 5′ ends of both the *GAL1* and *Gal5–CYC1–lacZ* genes prior to transcriptional activation, although no steady-state mRNA was detectable under these conditions (Figures 1A and 6A). When transcription was activated by Gal4, the profile of polymerases on both genes shifted from a strong bias in favour of the 5′ end to a more equal distribution between the 5′ and 3′ ends.

The *HIS3* gene, unlike *GAL1*, has a significant constitutive level of transcription which is increased further by the Gcn4 activator in response to amino acid starvation or treatment with aminotriazole. The addition of aminotriazole caused a 3-fold increase in the *HIS3* mRNA level relative to rRNA, in agreement with published results (Figure 1C, top; Hill *et al.*, 1986). A run-on analysis was carried out with two probes (H1 and H2) centred 115 and 760 bases from the start site, respectively. When *HIS3* expression was activated, we reproducibly detected in five experiments an almost 2-fold increase in the density of polymerases over the H2 region relative to H1. In the experiment shown in Figure 1C (bottom), the normalized H2:H1 ratios were 55 and 100% before and after aminotriazole induction respectively. In summary, the activation of transcription by Gcn4, like that by Gal4, caused a shift in the overall distribution of polymerases towards the 3′ end of the gene.

Transcriptional run-on signals are abolished by the rpb1-1 mutation

To confirm that both the 5′ and 3′ polymerase complexes detected under non-activated and activated conditions, respectively, do indeed contain pol II, we analysed the *Gal5–CYC1–lacZ* gene in a *rpb1-1* mutant strain. This mutation in the pol II large subunit causes rapid inactivation of the enzyme when the temperature is raised to 37°C (Nonet *et al.*, 1987a). Cells were transferred to 37°C for 1 h and then a run-on analysis was performed as usual at 25°C. Almost all the transcription signal detectable by the run-on assay was eliminated at the non-permissive temperature in both raffinose- (non-activated) and galactose-containing (activated) medium (Figure 2A). The run-on signals were also inhibited by the addition of 10 μg/ml α-amanitin (Jerome and Jaehning, 1986; data not shown). We conclude that the signals detected by the run-on assay at both ends of the gene under activated and non-activated conditions result from pol II transcription.

The Gal4 activation domain is required for polymerases to reach the 3′ end

We tested whether the effect of Gal4 on polymerase distribution required its activation domains by comparing the full-length protein with its N-terminal DNA-binding domain. For this experiment we studied an integrated *CYC1–lacZ* reporter gene with three upstream Gal4 binding sites (Figure 2B) in the yeast strain HF7c, which does not express Gal4 or Gal80 (Feilotter *et al.*, 1994). The Gal4 DNA-binding domain (residues 1–147) and full-length Gal4 were expressed constitutively from the alcohol dehydrogenase (ADH)1 promoter on plasmid vectors. Northern blotting showed that full-length Gal4 significantly stimulated the production of *lacZ* mRNA relative to Gal4(1–147) (Figure 2B, top, compare lane 1 with lane 2). We detected pol II engaged at the 5′ end of the *lacZ* sequences by hybridization to the L1 probe, even when only the Gal4(1–147) DNA-binding domain was expressed (Figure 2B, bottom). Very few polymerases were detected at the 3′ end, as indicated by the small amount of hybridization to the L2 probe. A similar result was obtained in the absence of any Gal4-binding protein (data not shown). Therefore the establishment of Sarkosyl-resistant, engaged polymerase complexes at the 5′ end of this reporter gene appears to be an intrinsic property of the *CYC1* promoter. However, this property is not unique to the *CYC1* promoter because identical results were obtained with a *LexAop4–GAL1–lacZ* reporter, pSH18–34 (Estojak

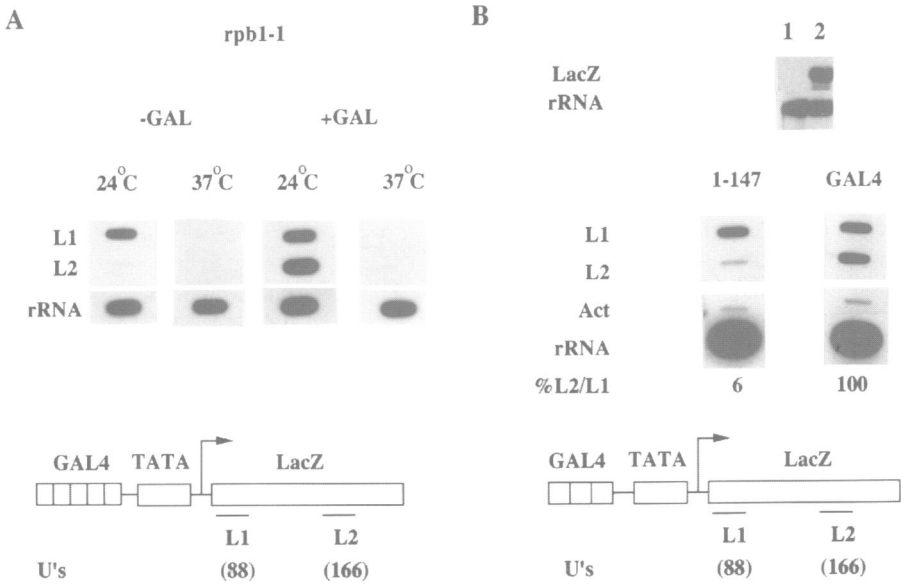

Fig. 2. Effects of the *rpb1-1* mutation and different Gal4 proteins on *Gal5–CYC1–lacZ* transcription. (**A**) Run-on analysis as in Figure 1B of *Gal5–CYC1–lacZ* and rRNA control in the *rpb1-1* strain, Y260 (see Table I), – and + galactose (GAL), at the permissive (24°C) and non-permissive (37°C) temperatures. (**B**) Northern blot (top) and run-on (bottom) analyses of an integrated GAL *UAS–CYC1–lacZ* gene in strain HF7c expressing Gal4(1–147) (lane 1) or full-length Gal4 (lane 2) from plasmids pGBT9 and pCL1, respectively (see Materials and methods). Actin and rRNA controls are shown.

et al., 1995), in the presence of *lexA* DNA-binding domain (P.Atadja, J.Archambault and D.L.Bentley, unpublished data).

In contrast to Gal4(1–147), intact Gal4 gave rise to an approximately equal distribution of polymerases between the 5′ and 3′ (L1 and L2) regions of the *lacZ* gene. Gal4 activation caused a 1.5-fold increase in the density of 5′ polymerase and a 28.5-fold increase in the density of 3′ polymerase relative to Gal4(1–147). The net result of Gal4 activation was therefore a 19.0-fold elevation of the normalized 3′:5′ (L2:L1) polymerase density ratio from 6 to 100% (Figure 2B). Similar results were obtained in four independent experiments. Gal4(1–147)–VP16, like intact Gal4, also stimulated the L2:L1 ratio when it activated transcription of the *Gal5–CYC1–lacZ* gene (data not shown). Gal4(1–147)–VP16 expression in HF7c was not highly toxic, as it is in some other strains (Berger *et al.*, 1992; see also Xiao *et al.*, 1995). We conclude that the change in distribution of polymerases along a gene which occurs in the presence of Gal4 requires an activation domain. Furthermore, the expression of Gal4 in the absence of the Gal80 repressor closely mimicked the natural response to galactose shown in Figures 1 and 2A.

In summary, the experiments in Figures 1 and 2 show that a run-on assay in permeabilized yeast cells can be used to detect pol II engaged on genes both before and after transcriptional activation. Activation by Gal4 caused a change in polymerase distribution along the gene which requires the activation domain. In the non-activated state, polymerases were confined to the 5′ end of the gene, but when transcription was activated, polymerases were able to reach the 3′ end efficiently, resulting in a more equal distribution between the two ends of the gene.

Run-on analysis of transcription in a TBP mutant strain

We wanted to confirm that the polymerase complexes detected by the run-on assay were dependent on TBP and

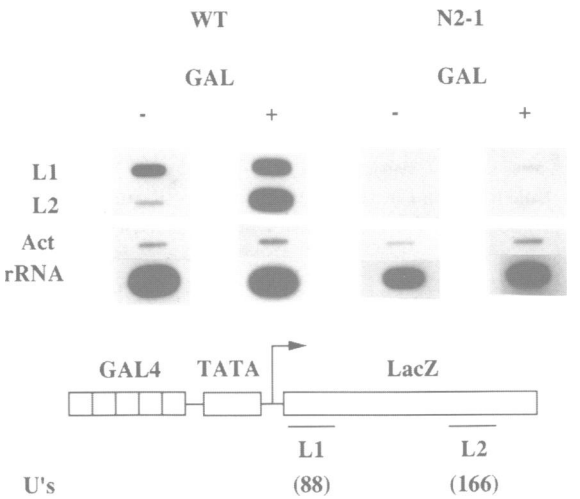

Fig. 3. N2-1 mutant of TBP abolishes 5′ and 3′ polymerase complexes. Run-on analysis of *Gal5–CYC1–lacZ* transcription in the N2-1 TBP mutant and the isogenic wild-type strain BYΔ2 (WT) – and + galactose (GAL). Actin and rRNA controls are shown.

were not some form of artefactual pol II transcription. The transcription of *Gal5–CYC1–lacZ* was analysed in the TBP mutant, N2-1, which has amino acid substitutions at positions 138 and 139 and is defective for interaction with TFIIA (Stargell and Struhl, 1995). This mutant is severely compromised in its response to galactose. In the presence of galactose, we did not detect any significant run-on signals from either the 5′ or 3′ (L1 or L2) regions of the *lacZ* gene in the N2-1 mutant (Figure 3, right panel). Surprisingly, in raffinose-containing medium, the N2-1 mutation also virtually abolished the run-on signal normally observed at the 5′ end of *lacZ*. Similar results were obtained in two independent experiments. In contrast, an isogenic strain carrying the wild-type TBP gene produced high *lacZ* run-on signals at the 5′ end prior to activation and at both the 5′ and 3′ ends after galactose

induction (Figure 3, left panel). The constitutive transcription of actin and *HIS3* (data not shown) was unaffected by the N2-1 mutation, in agreement with previous results based on steady-state RNA analyses (Stargell and Struhl, 1995). We conclude that the N2-1 mutant of TBP prevents all initiation on the *Gal5–CYC1–lacZ* gene, whereas constitutive transcription from genes such as actin and *HIS3* is unaffected. Other examples of the promoter-specific effects of TBP mutants on constitutive transcription have been reported previously (Arndt *et al.*, 1995). In summary, all the polymerases detected on the *Gal5–CYC1–lacZ* gene by the run-on assay were dependent on TBP, and therefore we presume they arose from bona fide initiation events.

The effect of CTD truncation on activation by Gal4

We used the run-on assay to investigate how a partial deletion of the pol II CTD affected activation by Gal4. Previously it was demonstrated that, in a strain (C6) which retains only 12.7 of the 27 heptad repeats in the CTD, transcriptional activation by galactose was only 40% of the wild-type level (Scafe *et al.*, 1990). To study this defect in detail, we introduced the *Gal5–CYC1–lacZ* plasmid into the truncated CTD strain (ΔCTD; Scafe *et al.*, 1990) and its isogenic wild-type (WT) counterpart, and performed run-on analyses (Figure 4). In a control experiment, no *lacZ* signal was observed in the wild-type strain lacking the *Gal5–CYC1–lacZ* plasmid (Figure 4A, panel C). When cells were grown in raffinose (–GAL), almost identical (<5% difference) run-on signals relative to rRNA were detected at the 5′ end of the *lacZ* gene in the wild-type and ΔCTD strains (Figure 4A, panels –GAL). Very few polymerases were detected at the 3′ end of *lacZ* in either strain, so the L2:L1 polymerase density ratios were low: 7 and 5% for WT and ΔCTD, respectively. We conclude that the CTD truncation had no effect on the establishment of engaged polymerases at the 5′ end of the gene in the absence of an activation signal.

In the presence of galactose, however, the distribution of polymerases differed significantly between the wild type and partial CTD deletion mutant. The 5′ (L1) run-on signal was stimulated 5.4-fold relative to rRNA by galactose in the wild type and 2.3-fold in the mutant. In contrast, the 3′ run-on signal (L2) was stimulated 70-fold by galactose in the wild type but only 7.5-fold in the ΔCTD strain (Figure 4A, +GAL). In two independent experiments there was, on average, a 4.9-fold lower L2:L1 ratio in the ΔCTD strain than in the wild type when cells were exposed to galactose. Under these conditions, the 3′:5′ polymerase density ratio on the chromosomal *GAL1* gene (G2:G1) was reduced from 100% in the wild type to 14% in the ΔCTD strain (Figure 4B). The CTD truncation also modestly reduced the *GAL1* 5′ run-on signal by 40% relative to the wild type in the presence of galactose. In summary, the major effect of partial CTD deletion was a reduction in the number of polymerases reaching the 3′ end of the gene when transcription was activated by Gal4.

Mutation of SRB2 prevents the formation of 3′ but not 5′ polymerase complexes

Srb2 is a subunit of the pol II holoenzyme (Thompson *et al.*, 1993; Y.J.Kim *et al.*, 1994) that is required for full Gal4 activation *in vivo* (Liao *et al.*, 1995). The *Gal5–*

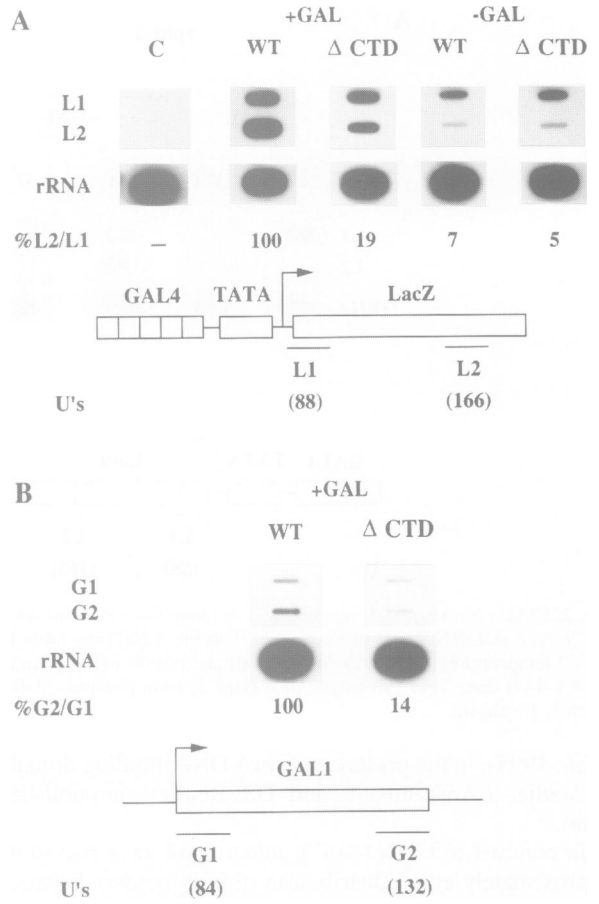

Fig. 4. CTD truncation preferentially inhibits the formation of 3′ polymerase complexes. (**A**) Run-on assay of the *Gal5–CYC1–lacZ* reporter in the wild-type, Z96 (WT), and the CTD truncated strain, C6 (ΔCTD) in the presence (+) or absence (–) of galactose (GAL). rRNA controls used for normalization of the run-on signals are shown. (**B**) Run-on assay of the chromosomal *GAL1* gene as in Figure 1A in the Z96 (WT) and C6 (ΔCTD) strains in the presence of galactose.

CYC1–lacZ reporter plasmid was introduced into an *SRB2* deletion strain (*srb2Δ1*; Koleske *et al.*, 1992) and an isogenic wild-type strain (*SRB2*) for run-on analysis (Figure 5). Both strains had high levels of polymerase at the 5′ end and low levels at the 3′ end of *lacZ* before the addition of galactose, as indicated by hybridization to L1 and L2 respectively (Figure 5, – panels). The addition of galactose had very little effect on the transcription of *Gal5–CYC1–lacZ* in the *srb2Δ1* mutant in three independent experiments. In the experiment shown in Figure 5 (right), the L2:L1 polymerase density ratio increased from 2 to 6%, but this difference is well within experimental variation caused by inaccuracy in quantitation of the low L2 signals above background. In contrast, a significant increase in L2:L1 ratio from 10 to 100% was observed in the *SRB2* strain on adding galactose (Figure 5, left). We conclude that after galactose induction there is a >15-fold difference (6 versus 100%) between the wild-type and *srb2Δ1* strains in the 3′:5′ polymerase density ratio (L2:L1) on the *lacZ* gene. The L2:L1 ratio in the presence of galactose was also reduced greatly relative to wild type in a strain deleted for the Srb10 kinase subunit of the holoenzyme (Liao *et al.*, 1995; P.Atadja and D.L.Bentley, unpublished results). These *srb* mutants therefore behave

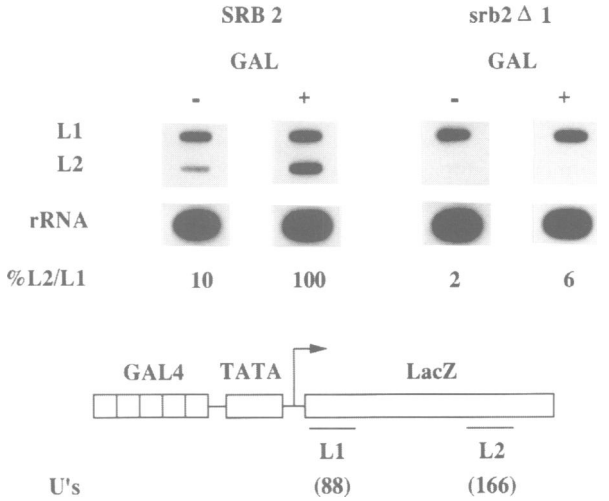

Fig. 5. The deletion of *SRB2* prevents the formation of 3′ polymerase complexes in response to Gal4. Run-on assay of the *Gal5–CYC1–lacZ* gene – and + galactose (GAL) in wild-type strain, Z424 (SRB2), and the *SRB2* deletion strain, Z425 (*srb2*Δ1). rRNA controls are also shown.

similarly to the CTD truncation, although their effects are more extreme. In both cases the generation of pol II complexes at the 3′ end of the gene was specifically inhibited, while establishment of complexes at the 5′ end was relatively unaffected.

Activation by Gal4 is defective in a kin28 mutant

The experimental results shown in Figure 4 demonstrate a role for the CTD in the generation of polymerase complexes which travel to the 3′ end of the *lacZ* gene in response to Gal4. The *KIN28* gene product is the kinase subunit of yeast TFIIH (Tfb) which can phosphorylate the CTD (Feaver *et al.*, 1994). If CTD phosphorylation is involved in activation by Gal4, then a *kin28* mutation may cause the same transcriptional defect as a CTD truncation because both would reduce the number of phosphates at the C-terminus of the large subunit. The transcription of *Gal5–CYC1–lacZ* was analysed in a mutant, *kin28 ts3*, and an isogenic wild-type strain (Valay *et al.*, 1993). Northern blotting of *lacZ* mRNA in the mutant at the permissive temperature showed normal induction by galactose (Figure 6A, lanes +) compared with the wild-type (wt) strain (Figure 6A, compare lanes 2 and 4 with lanes 1 and 3). When cells were induced with galactose for 3 h at the permissive temperature (30°C) and then transferred to the non-permissive temperature (37°C) for 1 h, the *lacZ* mRNA level was reduced by at least 10-fold in the mutant cells relative to the wild type (Figure 6A, compare lane 4 with lane 6).

A run-on analysis of *lacZ* transcription was carried out in the *kin28 ts3* and wild-type strains at 30 and 37°C in the presence and absence of galactose (Figure 6B). When galactose was added to the cultures at 30°C both the *kin28 ts3* and *KIN28* wild-type strains showed >20-fold increases in 3′ polymerase density, with little effect on 5′ polymerase density (<1.5-fold). The net effect of galactose induction in both strains was an increase in the L2:L1 ratio from 5 to 100%. At 37°C in the absence of galactose the *kin28 ts3* and wild-type cells both had high 5′ and

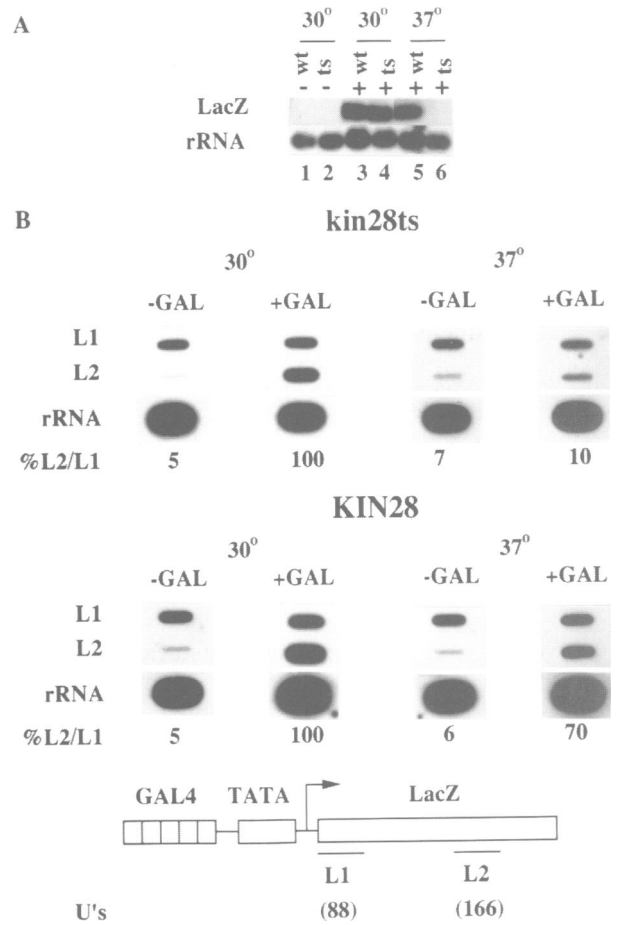

Fig. 6. Inactivation of Kin28 prevents polymerases from reaching the 3′ end in response to Gal4. (**A**) Northern blot of *lacZ* mRNA in the absence (–) or presence (+) of galactose in the wild-type strain GF262-2 (wt) or the *kin28ts3* strain (ts) at the permissive (30°C) and non-permissive (37°C) temperatures. Blots were rehybridized to an rRNA probe as a loading control. Cells were grown for 3 h in 2% galactose and harvested 60 min after shifting to 37°C. (**B**) Run-on assay of *Gal5–CYC1–lacZ* in the wild-type *KIN28* and mutant *kin28 ts3* strains – and + galactose (GAL) at 30 and 37°C. Cells were grown as in (A).

low 3′ run-on signals which were not significantly different from those observed at the permissive temperature (<10% difference). We conclude that the formation of engaged ternary complexes at the 5′ end of the gene prior to activation was not significantly inhibited by disabling Kin28.

When the *kin28 ts3* cells were induced with galactose for 3 h at 30°C and then shifted to 37°C for 1 h, the run-on signal at the 5′ end of the *lacZ* gene (normalized to rRNA) was reduced by only 10% on average (n = 2). On the other hand, the signal at the 3′ end was reduced by >85% (Figure 6B, top). The net result was that the normalized 3′:5′ polymerase density ratio declined from 100 to 10% when the *kin28 ts3* cells were shifted to 37°C, whereas in isogenic *KIN28* cells it declined from 100 to 70%. In summary, inactivation of the Kin28 kinase inhibited the formation of polymerase complexes, which extend to the 3′ end of the gene in the activated state, with little effect on the complexes confined to the 5′ end in the non-activated state.

Discussion

Analysis of pol II transcription by a run-on assay in yeast

We have used a run-on assay to examine the activation of pol II transcription in wild-type and mutant yeast cells. The run-on assay is based on the extension of nascent RNAs in permeabilized whole cells and gives a 'snapshot' view of the distribution of pol II ternary complexes on a gene. The advantages of this method over steady-state RNA studies like nuclease protection or Northern blotting are that it minimizes the effects of differential RNA stability and permits the detection of transcription close to the start site where the RNAs produced are very short. The criterion for the detection of polymerases by this method is that they be resistant to 0.5% Sarkosyl, which is used for permeabilization. Initiation is completely inhibited by this concentration of Sarkosyl, but transcription complexes with as few as two phosphodiester bonds are resistant (Cai and Luse, 1987). The hybridization signals obtained in a run-on experiment are proportional to the average number of polymerases occupying a given region of the transcription unit (polymerase density), assuming equal elongation rates for all polymerases in the *in vitro* reaction. In this study, we used the assay simply to compare the polymerase densities in two segments of the gene: one close to the start site and the other close to the 3′ end. Run-on signals from the *Gal5–CYC1–lacZ* reporter gene were only observed downstream of the correct start site (Figure 1B) and were inhibited almost totally by the mutations in the pol II large subunit (*rpb1-1*) and TBP (N2-1). These controls show that the assay detects genuine pol II transcription and not an artefact caused by permeabilization of the cells.

Although there are clear advantages to the run-on assay for the investigation of transcription *in vivo*, caution must be exercised in interpreting the results (Krumm *et al.*, 1992; Strobl and Eick, 1992). The hybridization signals reflect polymerase occupancy in a given region of the gene, but they do not distinguish whether the polymerases are actively elongating or stalled at the instant when the cells are permeabilized. In *Drosophila* and mammalian nuclei, stalled polymerases resume elongation in a run-on reaction in the presence of Sarkosyl (Rougvie and Lis, 1988; Krumm *et al.*, 1992), and it is likely that the same applies in yeast. If, as in many genes of higher eukaryotes (Rougvie and Lis, 1990; Krumm *et al.*, 1995), a large fraction of templates is occupied by polymerases stalled near the 5′ end, then a high run-on signal will be generated in this region even though there is little or no active initiation. As a consequence, the 5′ run-on signal is not a reliable measure of initiation rate. The comparison of 5′ with 3′ run-on signals is, however, an informative measure of post-initiation events (promoter clearance and elongation) *in vivo*. By applying the run-on assay to yeast mutants, we have been able to assess the role of several components of the transcriptional apparatus in post-initiation events.

Pol II is stably engaged on genes prior to activation in yeast

Using the run-on assay, we consistently observed pol II engaged at the 5′ end of *GAL1* and *GAL–UAS–lacZ* reporter genes prior to induction by galactose. This observation indicates that pol II can be recruited to the promoter and initiate transcription in the absence of an activation signal. Our results resemble the observations made originally on the *Drosophila Hsp70* gene, which also has polymerases engaged at the 5′ end before transcription is activated by heat shock (Rougvie and Lis, 1988). By analogy with *Hsp70* and many other genes in higher eukaryotes (Krumm *et al.*, 1995), the 5′ polymerases observed in yeast in the non-activated state may be stalled elongation complexes. However, *in vivo* permanganate footprinting of several yeast genes, including *GAL1*, did not detect polymerases paused downstream of the start site (Giardina and Lis, 1993). Paused polymerases may not have been detected by this method if they are located beyond the region analysed or if they are not arrested at a small number of discrete positions. Alternatively, the 5′ polymerase complexes in yeast may not be arrested; they could correspond to actively transcribing polymerases that terminate prematurely and are released from the template in the 5′ region. We conclude from these results that a post-initiation block to transcription, either premature termination or promoter-proximal pausing, can be an important rate-limiting step in pol II transcription in yeast.

Activation alters the distribution of ternary complexes along a gene

While pol II complexes were engaged constitutively at the 5′ ends of Gal4-responsive genes, significant amounts of polymerase were only detected at the 3′ ends when transcription was activated. The ability of Gal4 to stimulate transcription which extends to the 3′ end of the gene in yeast is shared with a number of factors in higher eukaryotes, including heat shock factor (HSF) (Lis and Wu, 1993), human immunodeficiency virus tat (Kao *et al.*, 1987; Laspia *et al.*, 1990) and fusion proteins containing the p53, E2F, E1a and VP16 activation domains (Yankulov *et al.*, 1994; Blau *et al.*, 1996). Gcn4 and Gal4–VP16 (data not shown) also demonstrated this ability in yeast. Taken together, these data imply that the regulation of the ability to traverse the length of the gene is a highly conserved feature of pol II function *in vivo*. Gal4 is bound to its cognate DNA-binding sites *in vivo* even in the absence of galactose, but the Gal80 repressor prevents it from activating transcription (Johnston, 1987). Gal80 may inhibit the formation of polymerase complexes which reach the 3′ end but not complexes confined to the 5′ end. In summary, our results demonstrate a significant effect of the Gal4 activator on the ability of polymerases to reach the 3′ end of a gene. However, for the reasons outlined above, the fact that we did not observe large increases in the 5′ run-on signal in response to Gal4 does not necessarily argue against an additional strong effect on the initiation rate.

'Activated' and 'non-activated' polymerase complexes are distinguished by mutations in SRB2, CTD and KIN28

We investigated the effects of mutations in the transcriptional apparatus on activated and non-activated transcription using the run-on assay. Partial deletion of the CTD, conditional inactivation of Kin28 and deletions of *SRB2* and *SRB10* (P.Atadja and D.L.Bentley, unpublished data)

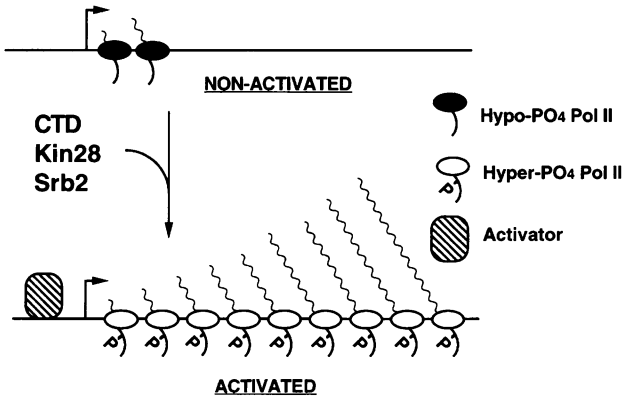

Fig. 7. Model for a switch between distinct 'non-activated' and 'activated' forms of pol II ternary complex involving CTD phosphorylation in the presence of an activator such as Gal4. The model is based on data in this paper and that of O'Brien *et al.* (1994). 'Non-activated' polymerases are confined to the 5′ end and do not require full-length CTD, Kin28 or Srb2. 'Activated' polymerases require these components and are able to travel to the 3′ end efficiently.

all inhibited the formation of polymerase complexes that travelled to the 3′ end of the gene when transcription was activated. In contrast, these mutations did not prevent the establishment of stably engaged polymerases at the 5′ end in the non-activated state. The clear differences between the properties of 5′ and 3′ ternary complexes strongly suggest that they are biochemically distinct. Not only are they distributed differently on the gene but, more importantly, they are affected differently by mutations in the CTD, Kin28 and Srb's. On the basis of this evidence, we propose there are qualitatively distinct 'activated' and 'non-activated' forms of polymerase complex.

We do not know if the generation of 'activated' and 'non-activated' pol II complexes is a universal phenomenon in yeast. This bimodal pattern of transcription may be restricted to highly regulated promoters like *GAL1* and *Gal5–CYC1*. The sequence of the TATA element, for example, could influence whether initiation by 'non-activated' polymerases occurs in the absence of activator. Previously, TATA-dependent differences in the processivity of transcription (Lu *et al.*, 1993; Yankulov *et al.*, 1994) and the response to activators have been reported (Harbury and Struhl, 1989). It may also be significant that TBP is bound constitutively *in vivo* to the *CYC1* promoter we analysed (Chen *et al.*, 1994).

One possibility is that 'activated' and 'non-activated' complexes result from the recruitment of different forms of pol II to the promoter. In this respect, it is worth noting that several species of pol II have been isolated from yeast cells, including core polymerase and at least two types of holoenzyme, which differ in whether or not they contain TFIIH and TFIIB (Y.J.Kim *et al.*, 1994; Koleske and Young, 1994). We favour the alternative model that a single form of polymerase is recruited to the promoter, followed by a modification which switches it from the 'non-activated' to the 'activated' state in the presence of a factor such as Gal4. The effects of CTD deletion and inactivation of Kin28 suggest that phosphorylation of the CTD is involved in such a switch (see Figure 7). The 'mediator', which includes Srb's, is a strong enhancer of CTD phosphorylation (Y.J.Kim *et al.*, 1994). Therefore *SRB2* deletion, like the other two mutations, may affect transcription by reducing CTD phosphorylation.

The role of the CTD and TFIIH in transcriptional activation

The function of CTD phosphorylation in pol II transcription remains unresolved. However, there are several ways it could affect whether or not polymerases reach the 3′ end of the gene. Phosphorylation could trigger the release of polymerases that are paused close to the start site (Usheva *et al.*, 1992; Lis and Wu, 1993). It could also inhibit the premature termination of transcription. CTD phosphorylation might aid elongation through chromatin, which is a formidable barrier to pol II *in vitro* (Izban and Luse, 1991). Genetic experiments suggest a functional connection between CTD phosphorylation and chromatin because mutations which suppress a CTD truncation phenotype occur in the genes for histone H3 and Sin1, a high mobility group-like chromatin protein (Peterson *et al.*, 1991; Kruger *et al.*, 1995). Furthermore, mutants in the global transcriptional regulator Sin4, which modulates chromatin structure (Jiang and Stillman, 1992), are co-lethal with *kin28 ts3* at the permissive temperature (Valay *et al.*, 1995).

Although a minimum length of CTD is essential for viability in yeast, it is dispensable for transcription *in vitro* in some cases. The CTD is not required for transcription from the adenovirus major late promoter in HeLa cell extracts, although it is required for transcription from the dihydrofolate dehydrogenase (DHFR) promoter (Kang and Dahmus, 1993; Akoulitchev *et al.*, 1995). The TFIIH CTD kinase is not needed for initiation at either the adenovirus major late promoter or the DHFR promoter *in vitro*, but it is necessary for the production of DHFR run-off transcripts, suggesting involvement in a step after initiation on certain promoters (Akoulitchev *et al.*, 1995; Makela *et al.*, 1995). In addition, inhibitors of TFIIH kinase inhibited pol II promoter clearance and elongation in HeLa nuclear extract (Yankulov *et al.*, 1995; Jiang *et al.*, 1996). These data are consistent with the correlation between CTD phosphorylation and the release of paused polymerases, which occurs when the *Drosophila Hsp70* gene is activated by heat shock (Weeks *et al.*, 1993; O'Brien *et al.*, 1994). What has remained unclear is whether CTD phosphorylation is a cause or a consequence of the active elongation which follows a heat shock stimulus. Our results demonstrate that in yeast a full-length CTD, Kin28 and Srb's are actually necessary *in vivo* for polymerases to travel efficiently beyond the 5′ portion of the gene, although they are not essential for loading onto the 5′ end. The results suggest the model (Figure 7) that CTD phosphorylation is required to generate polymerase complexes which are competent to traverse the full length of the gene with high efficiency.

Materials and methods

Strains and growth conditions

Saccharomyces cerevisiae strains are listed in Table I. For galactose induction, cultures were grown overnight in SC medium plus 2% raffinose without tryptophan, leucine or uracil, as necessary, to maintain the plasmids. They were diluted to 2–6×10^6/ml in the same medium, and galactose was added to 2%. Cells were harvested 3 h later for a

Table I. Strains used in the experiments

Strain	Genotype	Source
Y1114	*MAT***a** *HMLa HMRa ho-Bgal HIS3 leu2 trp1 ura3*	K.Nasmyth
HF7c	*MAT***a** *ura3 52 his3Δ200 lys2 trp1 leu2 gal4-542 gal80-538 LYS::GAL1-HIS3 URA3::* $GAL4_{17mer\times3}$*–CYC1–lacZ*	Feilotter *et al.* (1994)
Z96	*MATα his3Δ200 ura3-52 leu2 rpbΔ297::HIS3 [pRP214]*	Scafe *et al.* (1990)
C6	*MATα his3Δ200 ura3-52 leu2 rpb1-187::HIS3 [pRP112Δ104]*	Scafe *et al.* (1990)
GF262-2	*MATα leu2 trp1 ura3 his3*	Valay *et al.* (1993)
kin28ts3	*MATα leu2 trp1 ura3 his3 kin28-ts3*	Valay *et al.* (1993)
Z424	*MATα his3Δ200 leu2 ura3-52 trp1Δ1*	Koleske *et al.* (1992)
Z425	*MATα his3Δ200 leu2 ura3-52 trp1Δ1 srbΔ1::HIS3*	Koleske *et al.* (1992)
N2-1	*MAT***a** *ura3-52 trp1Δ1 spt15Δ [pTBP-N2-1; TRP1]*	Stargell and Struhl (1995)
BYΔ2	*MAT***a** *ura3-52 trp1Δ1 spt15Δ [pTBP-WT; TRP1]*	Stargell and Struhl (1995)
Y260	*MAT***a** *ura3-52 rpb1-1*	Nonet *et al.* (1987a)

Northern blot or run-on analysis. *HIS3* was induced with aminotriazole (20 mM; Hill *et al.*, 1986) for 18 h.

Plasmids

Gal5–CYC1–lacZ was made by inserting a *Xba*I–*Xho*I fragment containing five Gal4 binding sites from GAL/E1bTATA (Lillie and Green, 1989) at position –178 of the multicopy *CYC1–lacZ* fusion vector A256 carrying *URA3* (Guarente, 1983).

pCL1 (Fields and Song, 1989) is a *LEU2* YCp50 derivative which expresses full-length Gal4 from the ADH1 promoter. pGBT9 (Bartel *et al.*, 1993) is a 2 μm-based TRP1 plasmid which expresses Gal4(1–147) from the ADH1 promoter. pVZ–*GAL1* contains bases 4379–4846 of *GAL1* (Citron and Donelson, 1984) amplified by PCR and inserted into the *Bam*HI–*Eco*RI sites pVZ (Henikoff and Eghtedarzadeh, 1987) for riboprobe synthesis. pVZ–*HIS3* contains the 1.2 kb *Bam*HI–*Pst*I fragment (–447 to +707) in pVZ for riboprobe synthesis. pVZ–*lacZ* contains the *Pvu*II fragment of bases +106 to +2664 relative to the ATG in pVZ for riboprobe synthesis. pSCR5 contains the 1.6 kb *Nhe*I–*Bcl*I fragment spanning the 3′ end of 16S, 5.8S and the 5′ end of 26S rRNA in Bluescript SK^+. This plasmid was a gift from R.Reeder. pBS KS^- Δ*Pvu*II is Bluescript KS^- lacking most of the *lacZ* sequences. It was made by excising the *Pvu*II fragment.

Northern blotting

Total RNA was isolated by vortexing cells with glass beads in a 1:1 mix of extraction buffer (0.1 M EDTA, 0.1 M NaCl, 0.05 M Tris–HCl, pH 8.0, 0.5% SDS) and phenol:chloroform (1:1). The aqueous phase was re-extracted with phenol:chloroform twice and precipitated with 2.5 M ammonium acetate and 2.5 volumes of ethanol. 3 μg were electrophoresed on 1.2% formaldehyde agarose gels. Antisense riboprobes for *GAL1*, *HIS3* and *lacZ* were made from pVZ–*GAL1*, –*HIS3* and –*lacZ*, respectively. A random-primed 1.66 kb *Pst*I–*Sac*I fragment derived from pSCR5 was used as the probe for rRNA.

Transcriptional run-on analysis

Run-on reactions were performed as described previously (Elion and Warner, 1986) with minor modifications. The cultures (6×10^7 cells) were chilled on crushed ice, washed once in 5 ml 10 mM Tris–HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, and permeabilized in 0.5% Sarkosyl in 1 ml water for 15 min. The cells were then resuspended in a 120 μl reaction containing 50 mM Tris–HCl, pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM dithiothreitol, 0.5 mM each of ATP, GTP and CTP, 100 μCi [^{32}P]UTP (800 Ci/mmol), 1 U/μl RNAguard (Pharmacia), 10 mM phosphocreatine and 1.2 μg/μl creatine kinase. The reaction was incubated at 25°C for 8 min, and terminated by the addition of α-amanitin to 10 μg/ml. This was followed by DNase I and proteinase K digestion (Roberts and Bentley, 1992). RNA was isolated as described above and prehybridized to 12.5 μg single-stranded pBS KS^- Δ*Pvu*II for 1 h before hybridization to slot-blot filters containing single-stranded probes (3.0 μg) as described previously (Roberts and Bentley, 1992). After washing, the filters were digested with 1 μg/ml RNase A in 0.3 M NaCl, 10 mM Tris–HCl, pH 7.5, 5 mM EDTA for 30 min at 37°C. All run-on results shown were confirmed by at least two independent experiments with each strain. The data were quantified by a PhosphorImager (Molecular Dynamics) using the volume integration function of the Imagequant program. All values were normalized for U content of the RNA sequence and for the level of actin or rRNA control signal. Figures are from autoradiograms exposed for 2–4 days.

Run-on hybridization probes

P is a *Pst*I–*Bam*HI fragment containing five Gal4 binding sites from Gal4/E1bTATA (Lillie and Green, 1989) cloned into M13mp18. H1 is the 256 base *Asu*II–*Msc*I fragment of *HIS3* (–25 to +231) cloned into the *Sma*I site of M13mp19. H2 is the 234 base *Asp*718–*Xho*I fragment of *HIS3* (+646 to +880) cloned into the *Asp*718–*Sal*I site of M13mp19. G1 is a 258 base PCR fragment comprising bases +1 to +258 relative to the ATG of *GAL1* (Citron and Donelson, 1984). It was cloned into the *Bam*HI–*Eco*RI site of M13mp19. The PCR primers were 5′-CCGGAATTCCGGATGACTAAATCTCATTCAGAAG-3′ and 5′-CGCGGATCCCTCGTTCAAAACTTTGACGGCGC-3′. G2 is a 467 base PCR fragment of bases +1102 to +1569 relative to the ATG of *GAL1* cloned into the *Bam*HI–*Eco*RI site of M13mp19. Primers were 5′-CCGGAATTCCTCGCGAAGAATTCACAAGAGAC-3′ and 5′-CGCGGATCCGCTGCCCAATGCTGGTTTAGAGAC-3′. The actin probe is a 1.1 kb *Asp*718–*Xho*I fragment from pYA301 (Gallwitz and Sures, 1980) cloned into the *Sal*I–*Asp*718 site of M13mp18. The rRNA probe is a 1.66 kb *Pst*I–*Sac*I fragment from pSCR5 cloned into M13mp19.

The L1 5′ *lacZ* probe is the rescued single-stranded KS^- Bluescript plasmid which contains bases –215 to +207 of *lacZ* relative to the ATG interrupted at position +15 by a 207 base polylinker region. This probe is homologous to approximately positions +128 to +550 relative to the start site of transcription in the *CYC1–lacZ* fusions.

The L2 3′ *lacZ* probe is a 784 base *Mlu*I fragment corresponding to positions +1316 to +2100 relative to the ATG or approximately +1651 to +2435 relative to the start site of transcription of the *CYC1–lacZ* fusion. It was inserted into the *Sma*I site of M13mp18.

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