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Transcriptional termination signals for RNA polymerase II in fission yeast

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Transcription 'run-on' (TRO) analysis using permeabilized yeast cells indicates that transcription terminates between 180 and 380 bp downstream of the poly(A) site of the *Schizosaccharomyces pombe ura4* gene. Two signals direct RNA polymerase II (pol II) to stop transcription: the previously identified 3′ end formation signals located close to the poly(A) site and an additional downstream element (DSE) located at the region of termination. The downstream signal (135 bp) appears to act by pausing the elongating polymerase. TRO analysis indicates that elevated levels of transcribing polymerases accumulate over the DSE and that removal of this signal leads to transcription proceeding beyond the normal termination region. Furthermore, when inserted between two competing polyadenylation signals, this DSE increases the utilization of upstream poly(A) sites *in vivo*. We show that polymerase pausing over an extended region of template ensures termination of pol II transcription close to the poly(A) site.

Keywords: 3′ end formation/polyadenylation/RNA polymerase II/*Schizosaccharomyces pombe*/transcription

Introduction

A variety of cellular processes, ranging from correct gene expression to homologous recombination and chromosome transmission, are dependent on efficient transcriptional termination. Failure of the elongating polymerase to stop transcription before reaching the adjacent transcription unit can lead to a reduction in the expression of the downstream gene by destabilizing the binding of transcription factors to the promoter (Bateman and Paule, 1988; Henderson *et al.*, 1989). Such 'transcriptional interference' has also been shown to impede correct transmission of eukaryotic chromosomes (Hill and Bloom, 1987) and meiotic gene conversion (Rocco *et al.*, 1992). In yeast, where transcription units are densely packed on the compact genome, the requirement for efficient termination of transcription may be particularly significant (Oliver *et al.*, 1992).

The high level of the transcripts synthesized by RNA polymerase I (pol I) and RNA polymerase III (pol III) has facilitated the investigation of signals that direct their formation. Pol I transcription terminates some 10–20 bp upstream of the binding site for a sequence-specific protein Reb1p (reviewed in Reeder and Lang, 1994). *In vitro* assays in *Saccharomyces cerevisiae* have demonstrated that while binding of Reb1p mediates polymerase pausing, an additional T-rich element located upstream of the protein-binding site is also required to pause and release the polymerase (Lang and Reeder, 1993; Lang *et al.*, 1994; Jeong *et al.*, 1995). RNA pol III is capable of mediating transcriptional termination unassisted (Cozzarelli *et al.*, 1983), with four regions of the second-largest subunit of the yeast pol III being implicated in affecting termination efficiency (James and Hall, 1990; James *et al.*, 1991; Shaaban *et al.*, 1995). A string of T residues on the non-template strand constitute an RNA pol III termination signal (Allison and Hall, 1985). An *in vitro* investigation of the kinetics of pol III chain termination in *S.cerevisiae* demonstrated significant pausing of the polymerase over the T-rich element, followed by release of the enzyme from the template (Matsuzaki *et al.*, 1994).

A bipartite signal has been proposed to direct RNA polymerase II (pol II) termination in higher eukaryotes. A functional polyadenylation element acts as the upstream signal, with mutation of this highly conserved processing signal leading to reduced termination efficiency downstream (Whitelaw and Proudfoot, 1986; Logan *et al.*, 1987; Connelly and Manley, 1988; Lanoix and Acheson, 1988). Moreover the 'strength' of this processing signal correlates with termination efficiency (Edwalds-Gilbert *et al.*, 1993). The nature of the downstream signal, located in the region where pol II termination takes place, remains poorly defined. Various types of downstream element (DSE) including a distorted DNA template (Kerppola and Kane, 1990), *trans*-acting factors binding to the DNA (Connelly and Manley, 1989a,b; Ashfield *et al.*, 1994) and structure of the transcript (Pribyl and Martinson, 1988) have been implicated in mediating pol II termination. One of the functions of this DSE may be to pause the elongating polymerase (Enriquez-Harris *et al.*, 1991; Eggermont and Proudfoot, 1993) in a similar fashion to that observed for both pol I and pol III.

Polyadenylation signals also appear to direct termination of pol II transcription in *S.cerevisiae*. Pol II-mediated transcription into centromeres and autonomously replicating sequences (ARSs) dramatically affects plasmid stability in yeast. Relief of this type of 'transcriptional interference' has therefore been employed to identify putative pol II transcription terminators (Snyder *et al.*, 1988). Using this approach, various elements involved in *CYC1* polyadenylation have been shown to be necessary for correct plasmid maintenance *in vivo* (Russo and Sherman, 1989; Russo, 1995). *CYC1* transcriptional termination signals have also been investigated directly by transcription 'run-on' (TRO) analysis. An 83 bp fragment that encompasses the region necessary for correct *CYC1* polyadenylation has been shown to direct efficient termination (Osborne and Guarente, 1989). This 83 bp *CYC1*

fragment was also shown to be sufficient to direct transcriptional termination in an assay where levels of plasmid superhelicity were employed to measure transcriptional activity (Osborne and Guarente, 1988). The possible involvement of a 'rho-like' helicase function in pol II termination in yeast has been raised by the ability of the *Escherichia coli* rho protein to mediate efficient RNA pol II transcription arrest *in vitro* (Wu and Platt, 1993).

The role of signals located downstream of the poly(A) site in mediating transcriptional termination in yeast has not been addressed directly. An *in vitro* investigation has suggested that transcription stops beyond the site of polyadenylation of both the *GAL7* and *ADH2* genes in *S.cerevisiae* (Hyman and Moore, 1993). These results support previous *in vivo* data that implicate elements downstream of the *CYC1* processing signal in directing termination of transcription (Russo and Sherman, 1989).

It is apparent that the precise signals and mechanism required for transcriptional termination by RNA polymerase II are as yet poorly understood. We have used a TRO assay to map polymerase density at the 3′ end of the fission yeast *ura4* gene in order to identify the signals required for transcription termination. Using this assay, we show that a DSE together with a functional poly(A) signal are required for termination of transcription close to the poly(A) site in yeast. Moreover, TRO analysis and *in vivo* poly(A) site competition data indicate that the DSE functions by pausing the elongating polymerase in the region where transcription terminates.

Results

Nascent transcription at the 3′ end of the S.pombe ura4 gene

A TRO assay was employed to measure levels of polymerase density at the 3′ end of the *ura4* gene in *Schizosaccharomyces pombe*. The TRO assay involves brief incubation of detergent-permeabilized yeast cells in a transcription buffer containing [α-^{32}P]UTP, followed by the hybridization of the pulse-labelled partially hydrolysed RNA to immobilized strand-specific templates. During the 'run-on' reaction, polymerase complexes only elongate a short distance, incorporating [α-^{32}P]UTP into the growing RNA chain. As such, the 'run-on' signal is proportional to the density of active RNA polymerase complexes on the DNA template. Assuming polymerase molecules dissociate from the template following cessation of transcription, this assay can therefore be used to identify where transcription terminates, by locating where levels of polymerase density fall to zero.

'Run-on' analyses across the 3′ end of the *ura4* gene were performed in: (i) the *ura4* deletion strain (*h*$^+$, *ade6-704, ura4-D18, leu-32*) where the 1.76 kb *Hin*dIII fragment containing the *ura4* gene has been deleted from chromosome III (Grimm *et al.*, 1988); (ii) the wild-type strain 972 (*h*$^-$) carrying a single genomic copy of *ura4*; and (iii) where multiple copies of the *ura4* gene are present on the autonomously replicating plasmid pU (Figure 1A). TRO analyses in strains where one or more copies of the *ura4* gene are present revealed significant levels of incorporation of [α-^{32}P]UTP into *ura4* primary transcripts detected over antisense probes 1, 2 and 3. In contrast, only background levels were detected with the *ura4* gene removed (Figure 1B). While the level of *ura4* transcription was four to five times higher when the *ura4* gene was present on a plasmid, the pattern of transcript signals derived from the genomic and plasmid templates across the 3′ end of the *ura4* gene appeared very similar. The background level of signal detected over probe 4 is indicative of transcription of the wild-type *ura4* gene terminating close to the poly(A) site located in probe 3.

To demonstrate promoter specificity and to improve the sensitivity of the TRO assay, the 5′ end of the *ura4* gene was replaced by the promoter of the *nmt1* gene. The *nmt1* gene is highly expressed and easily regulated, being repressed in the presence of 2 μM thiamine [no message with thiamine, *nmt* (Maundrell, 1990)]. As shown in Figure 2, a TRO analysis was carried out on the *ura4* deletion strain transformed with the *nmt1–ura4* fusion plasmid (pNU). When grown in the absence of thiamine, high levels of *ura4* transcription were observed across antisense probes 1, 2 and 3. However, when thiamine was present in the growth media, only background levels of transcription were detected across the antisense probes. The relative level of nascent *ura4* transcription detected with pNU is ~8-fold greater (unpublished data) than that found with pU (Figure 1B; 3). As the level of nascent transcription falls close to background over probe 4, similar to that observed with the genomic and plasmid-borne copies of the intact *ura4* gene, it appears that the signals directing termination of *ura4* transcription are also recognized efficiently by polymerases initiating at the heterologous *nmt1* promoter. However, the level of signal over probe 3 is considerably higher in pNU than with the intact *ura4* gene (Figure 1B; 3, and Figure 2). It is possible that the strong *nmt1* promoter may generate more highly 'processive' polymerase complexes than the wild-type *ura4* gene, resulting in an increased polymerase density at the 3′ end of the *ura4* gene. A similar increase in polymerase density has been observed recently following activation of RNA polymerase II by Gal4 in *S.cerevisiae* (Akhtar *et al.*, 1996). We note that thiamine-mediated repression of the *nmt1–ura4* transcription unit coincides with the appearance of low level nascent transcripts over sense probes 1 and 2. These antisense transcripts are also detectable using the intact *ura4* gene (Figure 1B) and do not appear to be regulated by thiamine (unpublished data). Apparently, high levels of transcription generated by the *nmt1* promoter inhibit formation of these antisense transcripts. However, their functional significance is unclear.

Transcription terminates efficiently close to the ura4 poly(A) site

To define more accurately where transcription terminates at the 3′ end of the *ura4* gene, a series of shorter single-stranded M13 probes 1'–8' (100 nucleotides or less) were used to detect nascent transcripts (Figure 3). As a control for both the T content of these probes and their relative ability to hybridize to RNA, uniformly labelled RNA synthesized by T7 RNA polymerase was also tested (Strobl and Eick, 1992).

The level of nascent *ura4* transcription detected across these short probes declines dramatically after probe 4', falling to background levels over probe 8' in both pU and pNU (Figure 3). As the polyadenylation site of both *ura4* and *nmt1–ura4* transcripts has been mapped to a position 8 bp from the 3′ end of probe 1' [*ura4* (Humphrey *et al.*, 1994), *nmt1–ura4* (unpublished data)], transcription

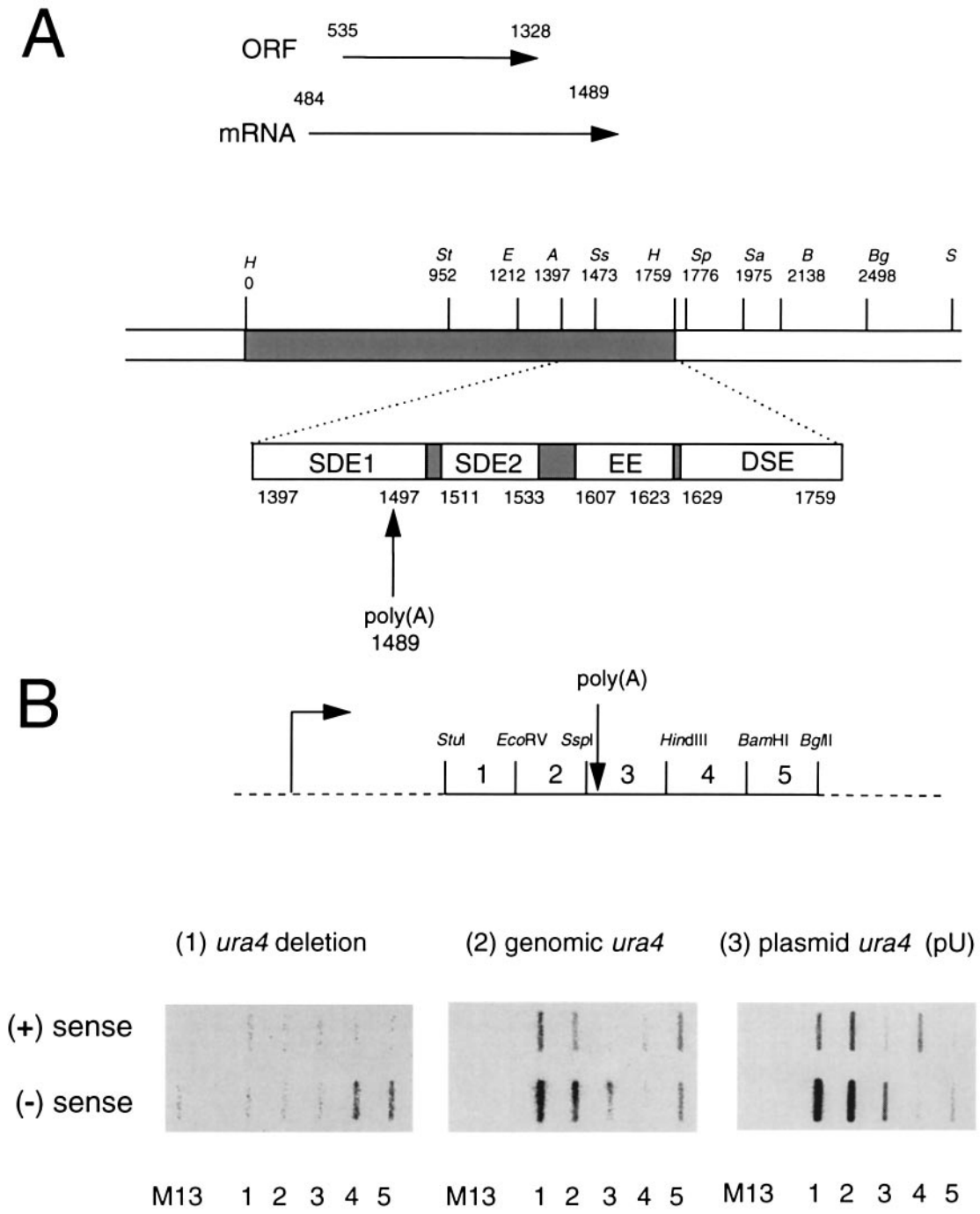


Fig. 1. Nascent transcription at the 3′ end of the *S.pombe ura4* gene. (**A**) Structure of the *ura4* gene showing the location of the ORF, mRNA and various 3′ end formation signals, SDE1, SDE2, EE and DSE. Restriction sites employed in the construction of M13 probes are also indicated: *H*, *Hin*dIII; *St*, *Stu*I; *E*, *Eco*RV; *A*, *Avr*II; *Ss*, *Ssp*I; *Sp*, *Spe*I; *Sa*, *Sau*3AI; *B*, *Bam*HI; *Bg*, *Bgl*II; *S*, *Sma*I. The shaded box represents the *Hin*dIII fragment removed in the *ura4* deletion strain. (**B**) TRO analysis across the 3′ end of the *ura4* gene showing the distribution of signal in the strains indicated. The location of the *ura4* poly(A) site with respect to a series of contiguous hybridization probes is also shown. The M13 probe with no insert controls for the background level of transcription.

clearly proceeds beyond the *ura4* polyadenylation site and terminates across probes 5'–8', 180–380 bp downstream.

Polyadenylation signals and a region located downstream of the poly(A) site are required for efficient termination of transcription

The decrease in transcription signals observed between probes 2 and 4 (Figures 1B and 2A) suggests that signals within this region were responsible for mediating transcriptional termination at the 3′ end of the *ura4* gene. A TRO analysis was therefore performed on a strain in which a 362 bp *Avr*II–*Hin*dIII fragment encompassing probe 3 had been deleted (pNUD). The high levels of transcripts hybridizing to probe 4 (162%), probe 5 (211%) and probe 6 (140%) demonstrate that signals within probe 3 are necessary to mediate efficient transcriptional termination (Figure 4A; 2).

In *S.cerevisiae*, polyadenylation signals are believed to

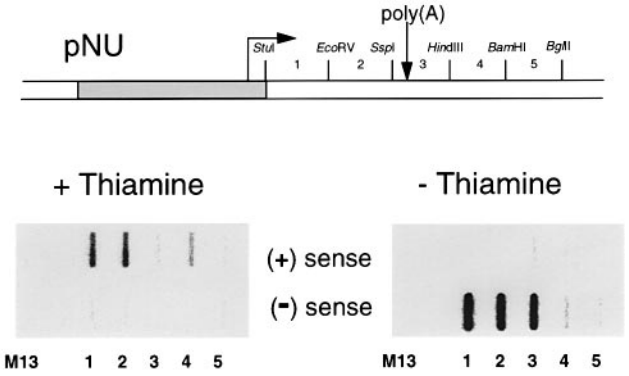


Fig. 2. Regulated *ura4* transcription. TRO signals at the 3′ end of the *ura4* gene in *S.pombe* transformed with pNU and grown in the presence or absence of thiamine. The shaded box in the pNU construct represents the replacement of the 5′ end of the *ura4* gene with the *nmt1* promoter.

be involved in directing pol II termination (Osborne and Guarente, 1988, 1989; Russo and Sherman, 1989; Russo, 1995). As three distinct sequence elements involved in *ura4* 3′ end formation: two site-determining elements (SDE1 and SDE2) and an efficiency element (EE) (Humphrey *et al.*, 1994), are located within the region covered by probe 3, it seemed likely that these elements were responsible for directing termination of pol II-mediated transcription in fission yeast. Previously we had generated specific mutations of these signals which destroy their ability to promote 3′ end formation activity (see Materials and methods and Humphrey *et al.*, 1994). Mutation of the three signals in combination (pNUM) resulted in a significant level of readthrough transcription beyond probe 3 (probe 4, 87%; probe 5, 63%; and probe 6, 12%), demonstrating that these 3′ end formation signals also play an important role in termination of *ura4* transcription (Figure 4A; 3).

While the level of transcriptional readthrough observed in the strain carrying mutations in the 3′ end formation signals (pNUM) indicates their role in transcriptional termination, the difference in the transcription profiles obtained with pNUM and pNUD suggested that an additional element(s) was still present within the probe 3 region of pNUM, which was affecting levels of transcription (Figure 4A; 2 and 3). To investigate whether sequences located downstream of the three previously identified elements play a role in *ura4* transcript termination, a TRO assay was performed on a strain in which the 135 bp region between the efficiency element and the 3′ end of probe 3 are deleted in combination with the mutations in the 3′ end formation signals (pNUM-DSE). The increased level of transcription readthrough detected over probes 4, 5 and 6 (118, 203 and 114% respectively) with the DSE removed indicates that the DSE is also involved in directing termination. Furthermore, removal of the DSE alone (pNU-DSE) resulted in elevated levels of transcription beyond probe 3. The readthrough transcripts observed over probe 4 (72%), probe 5 (35%) and probe 6 (10%) demonstrate that the DSE element is required for transcriptional termination close to the *ura4* poly(A) site (Figure 4A; 5). Another feature of these data that should be noted relates to the variation of signal over probe 3 for each of the specific mutations described above. For each mutation,

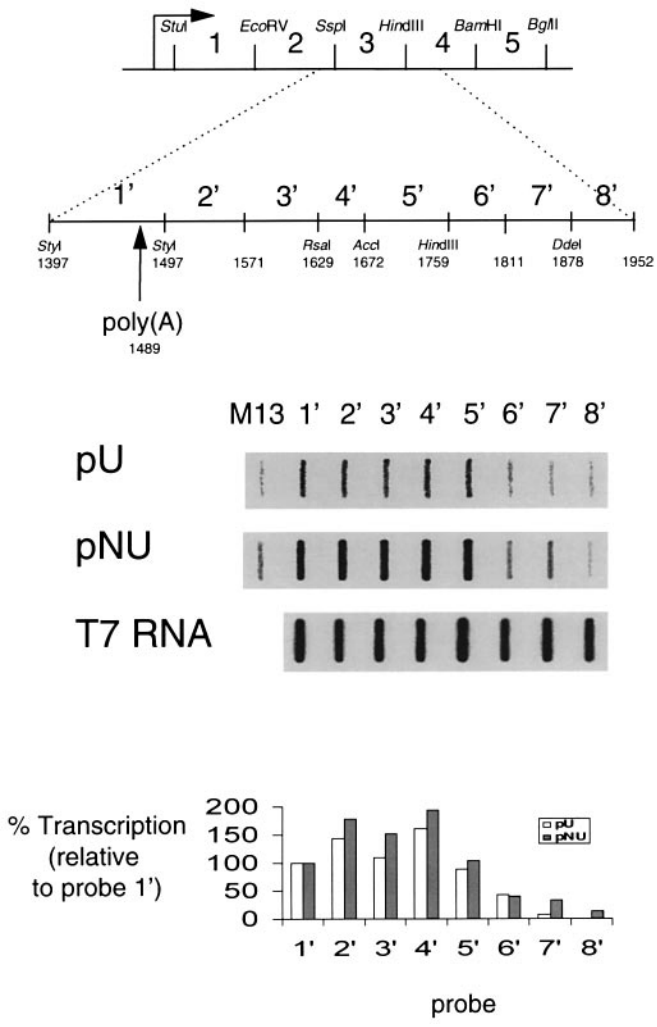
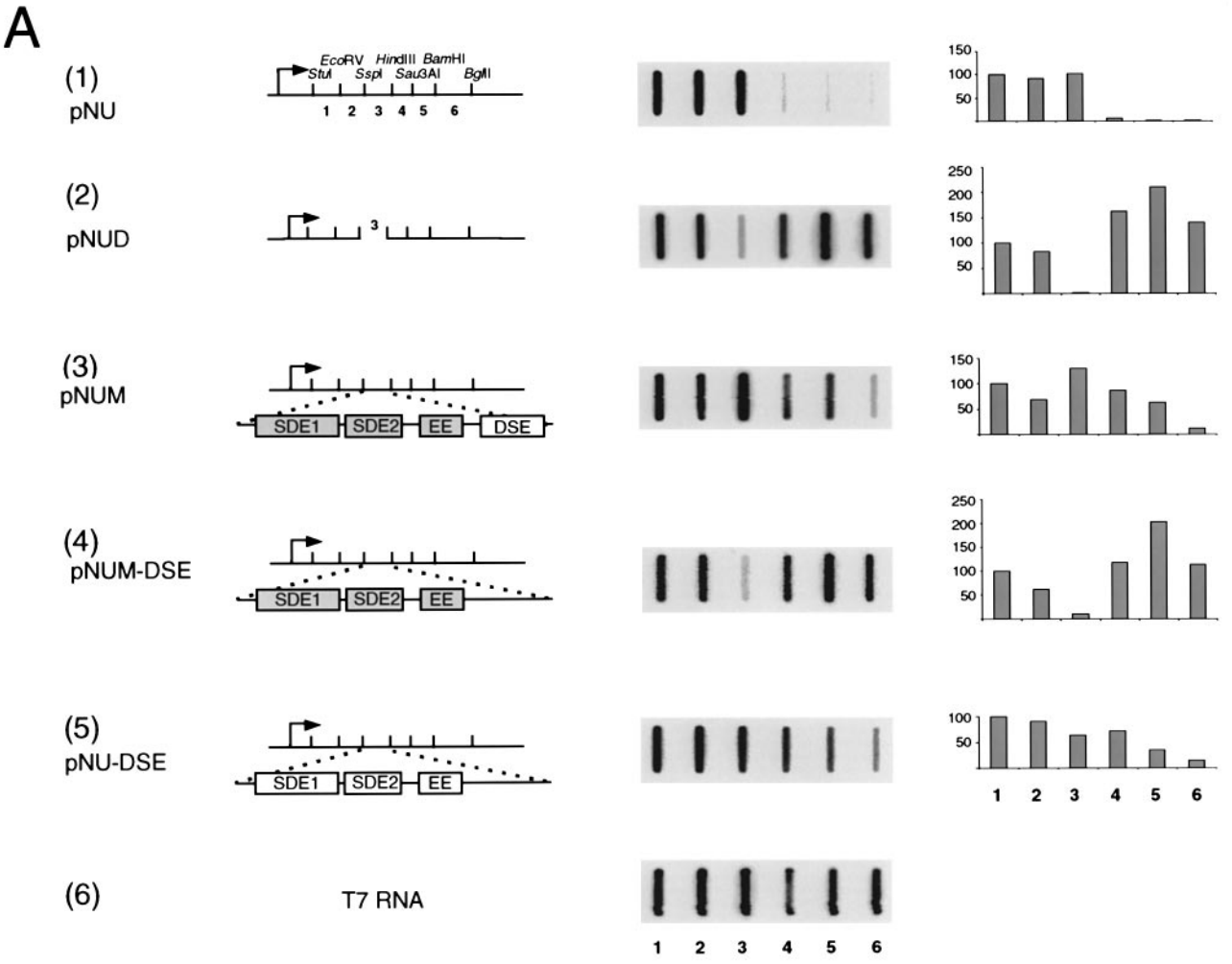


Fig. 3. *ura4* transcription proceeds beyond the poly(A) site. The arrangement of short probes 1'–8' is indicated with respect to the series of longer hybridization probes and the *ura4* poly(A) site. TRO signals in *S.pombe* transformed with pU and pNU are shown, as is the hybridization pattern observed across these probes with a uniformly labelled transcript generated by RNA polymerase T7. Transcription levels are corrected for M13 background signals, related to the hybridization signal produced with the uniformly labelled transcript, then plotted with respect to probe 1'.

a homologous version of probe 3 was employed. Even so, it is evident that for pNUM there is an enhanced signal for probe 3 contrasted by a greatly reduced signal with pNUM-DSE. These data can be interpreted as evidence for a pausing effect induced by sequences in the DSE region. This phenomenon is described in more detail below.

The effect of these mutations on the efficiency of *ura4* 3′ end formation *in vivo* was assessed by measuring levels of steady-state transcripts using Northern blotting. Deletion of the region covered by probe 3 resulted in the formation of a dramatically reduced level of longer steady-state transcripts, polyadenylated well downstream of the wild-type *ura4* poly(A) site (Figure 4B; 1 and 2). RT–PCR analysis revealed that these transcripts were polyadenylated at sites within probe 6 (unpublished data). The 'weak' nature of these downstream cryptic signals (unpublished data) may be responsible for the reduced steady-state levels observed. Surprisingly, in the strain carrying mutations in the three elements involved in *ura4* 3′ end formation



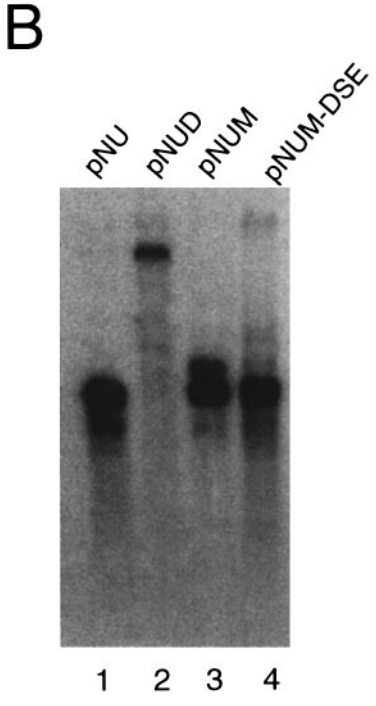


Fig. 4. Polyadenylation signals and a region located downstream direct termination of *ura4* transcription. (**A**) TRO signals at the 3′ end of the *ura4* gene in *S.pombe* transformed with constructs 1–5 are shown. The schematic composition of constructs transformed into the *ura4* deletion strain is indicated, with shaded boxes representing mutated forms of a given element. Homologous hybridization probes 1–6, incorporating mutations where necessary, were used to detect 'run-on' signals. The restriction sites employed in the construction of the M13 probes are indicated. The hybridization pattern produced with a uniformly labelled T7-generated transcript across the wild-type probes used with construct pNU (1) is also shown. Transcription levels are corrected for M13 background signals, related to the hybridization signal observed with the uniformly labelled transcript (6), then plotted with respect to probe 1. (**B**) Northern blot analysis: 10 µg of total RNA extracted from given constructs was separated on a 1.5% denaturing formaldehyde gel, transferred to a nylon membrane and hybridized with a *ura4*-specific 1.2 kb *Hin*cII–*Eco*RV random primer-labelled probe.

(pNUM), efficient polyadenylation of *ura4* transcripts was still observed close to the wild-type poly(A) site. Although the major site of polyadenylation had shifted slightly (RT–PCR unpublished results), the ability of this construct to direct polyadenylation in this region demonstrated the presence of an additional, cryptic signal with the capacity to mediate 3′ end formation. Removal of the DSE (pNUM-DSE) only slightly altered the pattern of 3′ end formation observed with pNUM, with ~10% of transcripts now polyadenylated at the distal sites observed in the deletion strain pNUD.

The steady-state RNA results for pNU and pNUD are consistent with the data from the *in vitro* TRO assay showing that the intact *ura4* signals direct efficient 3′ end formation while deletion of the 270 bp region covered by probe 3 results in transcriptional readthrough (Figure 4A; 1, 2, and B; 1 and 2). However, pNUM gave little detectable readthrough RNA, and removal of the DSE (pNUM-DSE) led to only a small increase in readthrough product (Figure 4B, lanes 3 and 4). In contrast, TRO analysis showed significant readthrough transcription for pNUM and almost full levels of readthrough for pNUM-DSE, equivalent to pNUD. It therefore appears that with pNUM-DSE most of the polymerases continue to elongate beyond probe 3 (Figure 4A; 4) even though the majority of steady-state transcripts are polyadenylated in the region spanned by probe 3. This difference may well be explained by the existence of a weak cryptic polyadenylation signal retained within probe 3 of this construct. Such an element may be insufficient to direct transcriptional termination, but have the capacity to compete as a processing signal with the distal polyadenylation region.

The downstream region functions as a transcriptional pause signal

As the DSE appears to affect both the ‘strength’ of 3′ end formation signals and level of active polymerases at the 3′ end of the *ura4* gene (Figure 4), the influence of this element was studied in more detail. TRO analyses indicate that a high level of active polymerases accumulates over the DSE. In addition, an analysis of steady-state RNA demonstrates that when placed between two competing polyadenylation signals the DSE promotes 3′ end formation at the upstream site. These findings, described below, are consistent with the DSE acting to pause elongating polymerases close to the *ura4* poly(A) site.

Short hybridization probes 1’–8’ (Figure 5) were used to examine the distribution of active polymerases in strains carrying mutations in the signals for *ura4* 3′ end formation. With the site-determining and efficiency elements mutated (pNUM), relatively high levels of *ura4* transcripts hybridize to probes 4’, 5’, 7’ and 8’. As the DSE spans probes 4’ and 5’ it can be seen that a relatively high polymerase density appears to accumulate over this element. These data also confirm the high signal previously observed for probe 3 with pNUM in Figure 4A.

The accumulation of transcript signals over the 5′ end of the DSE is orientation specific. When the orientation of the 5′ DSE is reversed (pNUM/r5′ DSE), the transcript signal over this region falls dramatically (Figure 5A; probe 4’S). Interestingly, signals over probe 5’, 7’ and 8’ remain high in the pNUM/r5′ DSE strain, indicating that high levels of polymerase density accumulate across this region independently of the signal within the 5′ DSE.

Difficulties encountered using ‘run-on’ analysis to measure polymerase density have stemmed from the failure of transiently paused polymerases to be represented adequately on the polymerase profile (Strobl and Eick, 1992; Eick *et al.*, 1994). The inability of such paused ternary complexes to elongate during the ‘run-on’ reaction has led to under representation of this class of polymerase in the overall profile. In order to circumvent such problems, we have performed the ‘run-on’ assay in a high salt transcription buffer in sarkosyl-permeabilized yeast cells. Under such conditions, paused polymerase complexes have been shown to switch to an elongation-competent status (Rougvie and Lis, 1988; Krumm *et al.*, 1992) ensuring a relatively complete polymerase profile.

The elevated level of polymerase density over DSE may well reflect a region where elongating polymerases accumulate in response to a transcriptional pause signal. The presence of both sarkosyl (0.5%) and KCl (250 mM) should ensure that such paused polymerases will elongate during the ‘run-on’ reaction and thus be represented in the resulting polymerase profile. However, it is also possible that high transcript signals could be produced by elevated rates of polymerase elongation across the DNA template. In an attempt to determine whether the high ‘run-on’ signals observed over the DSE were produced by either a relatively high density of active polymerases or by polymerases moving at an increased rate, a poly(A) site competition (PAC) assay was performed to assess the ability of the DSE to affect the level of 3′ end formation at two competing sites *in vivo*. In this PAC assay, two polyadenylation signals located on the same transcript compete as potential 3′ end processing sites. The level of polyadenylation at the two signals will depend on both the relative ‘strength’ of the signals, that is their ability to recruit the processing factors involved in cleavage and polyadenylation of the transcript, and the relative availability of the two signals for processing. The period that the upstream signal alone is available for processing will reflect the time taken for the transcribing polymerase to elongate between the two signals. If the DSE functions by pausing elongating polymerases, we would predict that placing it between two competing polyadenylation signals would effectively increase the period in which only the upstream signal was available for processing. We would therefore expect to observe elevated levels of 3′ end formation at the upstream polyadenylation site. If the high ‘run-on’ signals over the DSE are generated by polymerases moving relatively quickly across this region, we would not expect to observe such an increase in processing at the upstream signal.

To achieve poly(A) site competition, signals at the 3′ end of the *ura4* gene, and various derivative constructs, were introduced into the *nmt1* open reading frame (ORF), thus creating competition between the *ura4* and *nmt1* 3′ end formation signals (Figure 5B). These constructs were then transformed into a *nmt1* deletion strain, which lacks most of the *nmt1* ORF (*h*$^+$, *nmt1*::*ura4*, *ade6-704*, *ura4-D18*, *leu1-32*; Maundrell, 1990). While all *nmt1* transcripts are polyadenylated at the *nmt1* processing site in the absence of a competing 3′ end formation signal (Figure 5B; 1), insertion of the *ura4* 3′ end formation

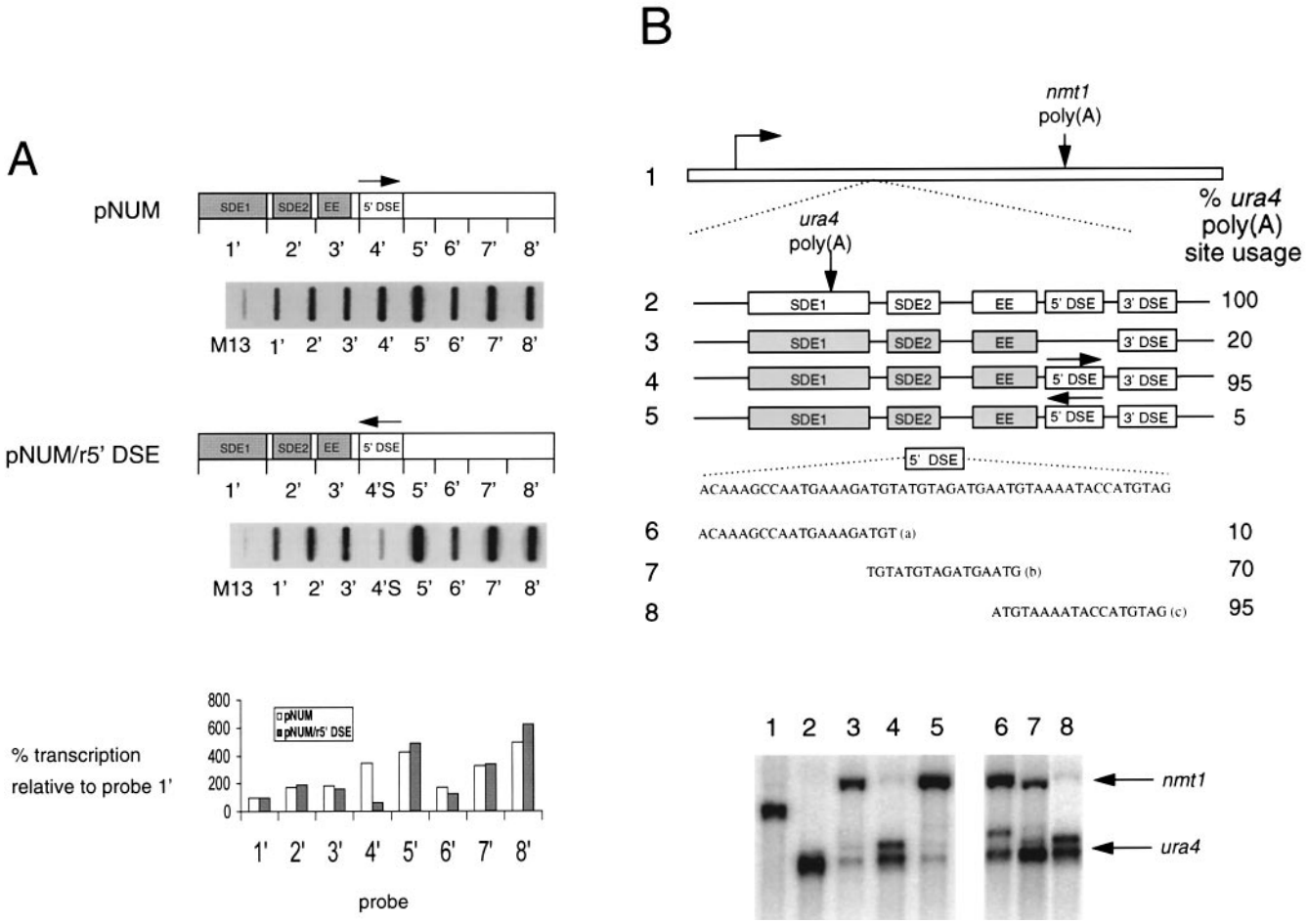


Fig. 5. The downstream region functions as a transcriptional pause signal. (**A**) TRO signals in *S.pombe* transformed with pNUM and pNUM/r5′ DSE with the series of short hybridization probes 1'–8' is shown. The schematic composition of constructs transformed into the *ura4* deletion strain is indicated. Shaded boxes indicate the mutated form of a given element and the reverse orientation of intact 5′ DSE. Homologous hybridization probes were used to detect 'run-on' signals incorporating mutations where appropriate. The sense probe (4'S) is used to detect transcript signals over the 5′ DSE fragment with the pNUM/r5′ DSE construct. Transcription levels are corrected for M13 background signals, related to the signal hybridization signal observed with the uniformly labelled transcript, then plotted with respect to probe 1'. (**B**) Poly(A) site competition assay. Constructs are schematically represented along with the ability of each fragment to form *ura4* 3′ ends. Northern blot analysis: 10 μg of total RNA extracted from given constructs was separated on a 1.5% denaturing formaldehyde gel, transferred to a nylon membrane and hybridized with a *nmt1*-specific 0.8 kb *Hin*dIII–*Stu*I random primer-labelled probe. The percentage 3′ end formation was calculated as the percentage of the smaller *ura4* 3′ end band to the total amount of RNA specific for the probe Northern blot analysis.

signals into the *nmt1* ORF switched the site of polyadenylation to the upstream *ura4* site, with no transcripts being detected at the distal *nmt1* processing site (Figure 5B; 2). Mutation of the *ura4* 3′ end formation elements resulted in polyadenylation at two distinct sites in the *ura4* region, demonstrating that this fragment retained signals capable of directing polyadenylation of most (95%) of the transcripts (Figure 5B; 4), in a fashion consistent with steady-state data described above (Figure 4B; 4). Removal of the 49 bp 5′ DSE (probe 4') considerably 'weakened' the strength of the *ura4* 3′ end formation signal, with the level of polyadenylation in this region falling from 95% to 20% (Figure 5B; 3). Moreover, this element was shown to function in an orientation-specific fashion in the PAC assay. When the orientation of the 5′ DSE was reversed, it failed to rescue levels of *ura4* 3′ end formation (Figure 5B; 5). Furthermore, the 3′ DSE, present in all of these constructs, has also been shown to promote 3′ end formation at upstream sites (unpublished data). These results are consistent with the DSE functioning to pause elongating polymerases thus elevating levels of 3′ end formation at upstream sites.

As such, it would seem unlikely that the high 'run-on' signal detected over the DSE is the result of polymerase complexes elongating particularly quickly across this region, but rather indicates that a relatively high density of active polymerase complexes accumulate across the DSE.

In an attempt to locate the functional region within the 49 bp 5′ DSE, three sub-fragments which span the 5′ DSE (a, 20; b, 16; and c, 19 bp) were assayed for their ability to increase polyadenylation at the upstream *ura4* signal. Clearly both the central (b) and 3′ (c) fragments promote considerable levels of 3′ end formation at the upstream *ura4* signal (Figure 5B, lanes 7 and 8), identifying two short *cis*-acting signals in the 5′ DSE with the capacity to pause the elongating polymerase.

The orientation-dependent correlation between the high 'run-on' signal observed over the 5′ DSE *in vitro* and the capacity of this element to promote utilization of an upstream processing site *in vivo* in the PAC assay, strongly

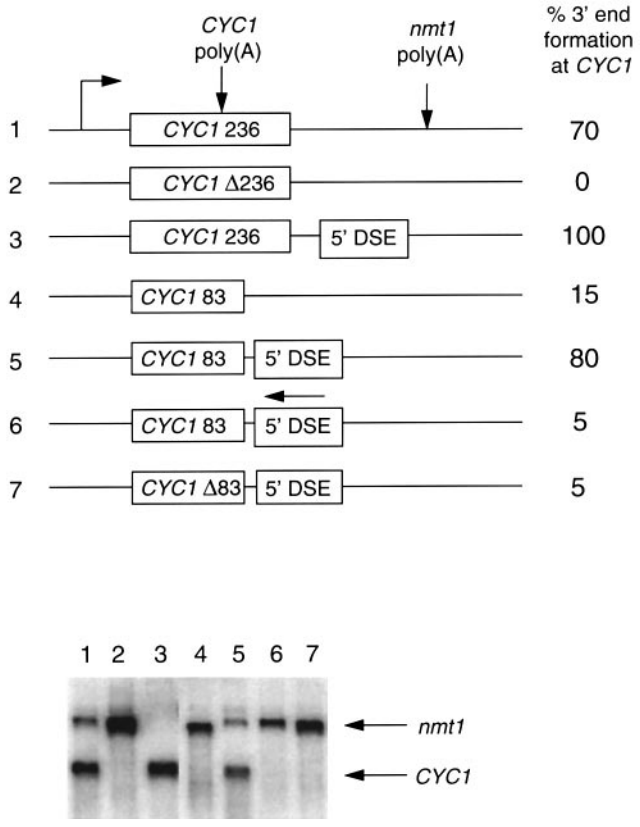


Fig. 6. The 49 bp 5′ DSE *ura4* pause element is sufficient to increase *CYC1* 3′ end formation in *S.pombe*. Poly(A) site competition assay. Constructs transformed into the *nmt1* deletion strain are schematically represented along with the ability of each fragment to form *CYC1* 3′ ends. Northern blot analysis: 10 μg of total RNA extracted from given constructs was separated on a 1.5% denaturing formaldehyde gel, transferred to a nylon membrane and hybridized with an *nmt1*-specific 0.8 kb *Hin*dIII–*Stu*I random primer-labelled probe. The percentage 3′ end formation was calculated as the percentage of the smaller *CYC1* 3′ end band to the total amount of RNA specific for the probe.

suggest that DSE is functioning as a transcriptional pause region.

The 49 bp 5′ DSE ura4 pause element is sufficient to increase CYC1 3′ end formation in S.pombe

In order to investigate whether the 5′ DSE signal was sufficient to mediate polymerase pausing, the effect of placing it in a PAC assay between two heterologous 3′ end formation signals was determined by Northern blot analysis.

The 83 bp *CYC1* fragment contains the signals sufficient for 3′ end processing in *S.cerevisiae in vivo* (Ruohola *et al.*, 1988). The 236 bp *CYC1* fragment, which has been shown to direct 3′ end processing *in vitro* (Butler and Platt, 1988), includes these signals and extends 98 bp 5′ and 55 bp 3′. Constructs *CYC1* Δ83 and *CYC1* Δ236 carry a 38 bp deletion which removes signals required for efficient polyadenylation (Zaret and Sherman, 1982; Butler and Platt, 1988). As found previously, the *CYC1* 236 fragment mediates efficient 3′ end formation, with the 38 bp deletion abolishing the ability of this region to direct polyadenylation in fission yeast (Figure 6; 1 and 2) (Humphrey *et al.*, 1991). Insertion of the *ura4* 5′ DSE between the *CYC1* 236 and *nmt1* polyadenylation signals increases 3′ end formation at the upstream *CYC1* location from 70% to 100% (Figure 6; 1 and 3). The *CYC1* 83 fragment works less efficiently in this PAC assay (15% level) but is significantly enhanced to an 80% level by the insertion of the 5′ DSE downstream (Figure 6; 4 and 5). As found previously, the effect of the 5′ DSE is orientation-dependent (Figure 6; 6). Moreover, introduction of the 5′ DSE downstream of the mutated signal *CYC1* Δ83 led to no detectable 3′ end formation at the upstream signal (Figure 6; 7). This shows that the 5′ DSE does not mediate 3′ end formation in isolation, but rather acts in concert with a functional polyadenylation signal to direct 3′ end formation. These results demonstrate that the 49 bp 5′ DSE is sufficient to elevate levels of 3′ end formation at both 'strong' (*CYC1* 236) and 'weak' (*CYC1* 83) upstream polyadenylation signals.

Discussion

We have used a TRO assay to measure levels of polymerase density at the 3′ end of the fission yeast *ura4* gene in order to investigate the signals that direct transcription termination. The major advantage of this assay is that it measures levels of polymerase density rather than assessing the affect of transcription on other events within the cell.

ura4 transcription arrests at a number of heterologous sites close to the major *ura4* poly(A) site. The finding that transcription terminates close to the 3′ end processing site is consistent with a previous RT–PCR analysis where no transcripts were detected >120 nucleotides beyond the *ura4* poly(A) site (Humphrey *et al.*, 1994). However, the exact site(s) of transcription termination differs in the two assays, with 'run-on' transcripts being detected some 60–320 nucleotides further downstream than in the steady-state analysis. It is probable that RT–PCR analysis was either not sensitive enough to detect the unstable primary transcripts, or that the assay was actually measuring minor species of processed *ura4* mRNA, present at low levels, such as those utilized in pNUM.

The results presented here demonstrate that as well as the 3′ end formation signals located close to the poly(A) site (Humphrey *et al.*, 1994), another distinct element located 140–270 bp downstream of the processing site is involved in directing termination of transcription. In *S.cerevisiae*, the involvement of downstream termination signals has not yet been resolved. Signals up to 54 nucleotides downstream of the *ADH2* poly(A) site appear to be required for efficient 3′ end formation when located in an intron (Hyman *et al.*, 1991). In addition an 83 bp fragment which encompasses the *CYC1* polyadenylation signals and extends only 20 bp beyond the poly(A) site has been shown to be sufficient to direct termination of transcription in a plasmid topology assay (Osborne and Guarente, 1988) and using TRO analysis (Osborne and Guarente, 1989). In contrast, this 83 bp *CYC1* fragment failed to alleviate transcriptional interference imposed on the yeast centromeric element *CEN3*, with a 249 bp *CYC1* fragment, which extends 100 bp beyond the poly(A) site, functioning to 'protect' the centromere (Russo and Sherman, 1989). While the results from this plasmid stability assay suggest that a downstream element may be involved in termination in yeast, it is also possible, as the authors point out, that the additional downstream sequence

is required to prevent steric hindrance between the 3′ end formation signals and those mediating centromere function. It has also been demonstrated using *S.cerevisiae* nuclear extracts, that pol II transcription extends beyond the poly(A) site of the *ADH2* and *GAL7* genes, with inefficient pausing and/or termination being observed at distinct sites downstream (Hyman and Moore, 1993). We show here that a transcription termination signal is located 140–270 bp downstream of the *S.pombe ura4* poly(A) site. Moreover, we show that this DSE is required for efficient termination, with removal of this region resulting in significant levels of transcription readthrough.

The process of RNA polymerase II transcriptional termination has been proposed to involve recognition of the polyadenylation signal and communication of this event to the elongating polymerase (Logan *et al.*, 1987; Connelly and Manley, 1988; Proudfoot, 1989). While the mechanism employed to convey this information to the polymerase complex is unknown, slowing the elongation rate of the polymerase after the poly(A) signal would effectively retain the transcribing complex close to the processing signal, presumably assisting their interaction. Furthermore, lowering the levels of nascent transcription beyond the poly(A) site would reduce the possibility of the elongating polymerase 'interfering' with the level of expression of genes located downstream (reviewed in Eggermont and Proudfoot, 1993). Indeed, the possibility that transcriptional pausing may be associated with pol II termination in mammalian genes has been demonstrated previously in this laboratory (Enriquez-Harris *et al.*, 1991).

We believe that this DSE functions by pausing elongating polymerases. The high polymerase density observed over this region in the 'run-on' assay is indicative of transcriptional pausing. Furthermore, the ability of this region to promote 3′ end formation at upstream signals when placed between two competing polyadenylation sites (Figures 5B and 6) is consistent with the DSE operating as a transcriptional pause signal. The DSE does not mediate polyadenylation (Figure 6, lane 7) or transcription termination in isolation (unpublished data), but rather appears to act in concert with an upstream 3′ end processing signal to mediate efficient termination of pol II-mediated transcription.

Multiple signals appear to act as transcriptional pause elements at the 3′ end of the *ura4* gene. As well as the pause signal located at the region of termination within the DSE (Figure 5A, probes 4' and 5', and 5B, lanes 7 and 8), other regions of high polymerase density are observed further downstream (probes 4, 5 and 6 in Figure 4A; 2 and 4, and probes 7' and 8' in Figure 5A). It therefore appears that an extended pause signal exists at the 3′ end of the *ura4* gene. A similar arrangement of multiple redundant elements, although not necessarily pause signals, has also been observed at the 3′ end of the mouse β^{maj} gene, where several redundant elements may act in concert to direct transcriptional termination (Tantravahi *et al.*, 1993). In yeast, where the arrangement of genes and other elements susceptible to transcriptional interference are densely packed on the compact genome, multiple pause elements may be employed to ensure efficient termination of pol II transcription.

It has been demonstrated previously from *in vitro* studies that yeast pol II can be paused at a pol I termination signal (Lang *et al.*, 1994). This raised the possibility that pause signals may also exist at the 3′ end of pol II-transcribed genes in yeast as found for both pol I and pol III. Our studies provide the first evidence for the presence of such pol II transcriptional pause signals which form an integral part of the termination signal.

Materials and methods

PCR primers

A list of oligonucleotide sequences used as PCR primers is given in Table I.

'Run-on' constructs

The plasmid pU was constructed by inserting a 3.4 kb fragment containing the *ura4* gene into the vector p*dLEU2*, a derivative of the *S.pombe* vector pIRT2 in which the *LEU2* gene has been replaced with a deleted version (*dLEU2*) of this selectable marker. The *dLEU2* gene has been used as a selectable marker in *S.cerevisiae* due to its ability to increase plasmid copy number (Erhart and Hollenberg, 1983). p*dLEU2* was constructed by substituting the wild-type *LEU2* gene in pIRT2 (Promega) with a 1.7 kb *Hpa*I–*Nru*I fragment from the vector pYEULlacZ (Macreadie *et al.*, 1991) which contains the deleted *dLEU2* gene. The presence of contiguous genomic sequence beyond the *ura4* poly(A) site was achieved by replacing the 0.3 kb *Avr*II–*Bam*HI* region from the vector p*URA4* with a 2.0 kb *Avr*II–*Sma*I fragment from pGC3 (Grimm *et al.*, 1988) to form pUL (* indicates Klenow 'fill-in' of a given restriction site prior to ligation). pURA4 was formed by introducing a 1.7 kb *Bam*HI*–*Hin*dIII* fragment containing the *ura4* gene from pON140 (Humphrey *et al.*, 1991) into the *Sma*I site of pIRT2. The 3.4 kb *Sal*I fragment containing the *ura4* gene from pUL was inserted into the unique *Sal*I site of p*dLEU2* to produce pU. Construction of pNUT involved replacing the 5′ end of the *ura4* gene with the *nmt1* promoter. This was accomplished by introducing a 2.7 kb *Stu*I–*Sal*I fragment from pUL at the *Bam*HI* site of pREP1 (Maundrell, 1990). A 5 kb *Pst*I–*Sst*I fragment containing this *nmt1–ura4* fusion construct and the putative *nmt1* 'terminator' region was then inserted into p*dLEU2* restricted with *Pst*I and *Sst*I to produce pNUT. pNUD was constructed by removal of the 270 bp *Avr*II–*Hin*dIII fragment from pNU. As this *Hin*dIII site is not unique, this involved replacing the 1.1 kb *Avr*II*–*Bgl*II region of pNU with a 800 bp *Hin*dIII*–*Bgl*II fragment. A 3.0 kb *Hin*dIII*–*Sal*I* fragment from pUL was inserted into the *Eco*RI* site of pGEM7Zf(+) (Promega) to form pUG. Restriction with *Xho*I* linearized this plasmid immediately 5′ to the *Hin*dIII site. After restriction with *Bgl*II, a 800 bp fragment (X*Bg800) was ligated into pNU cut with *Avr*II* and *Bgl*II, producing pNUD. Mutation of the 3′ end formation signals SDE1, SDE2 and EE was achieved by performing a PCR-based mutagenesis of the SDE2 signal on a template already carrying mutations in SDE1 and EE described previously as del C and del DS (Humphrey *et al.*, 1994). To achieve this, PCR was performed with the primers 5′ *ura4* and 3′ SDE2 and 5′ SDE2 and 3′ *ura4* (1), followed by restriction of the 5′ and 3′ PCR products with *Avr*II and *Hin*dIII respectively. Ligation of these PCR products into p*URA4* restricted with *Avr*II and *Hin*dIII formed pUM. This mutagenesis of SDE2 substituted AGTA with CTGC, abolishing the ability of SDE2 to direct 3′ end formation (unpublished data). The 300 bp *Avr*II–*Hin*dIII* fragment from pUM was then ligated, in combination with the fragment X*Bg800 described above, into pNU restricted with *Avr*II and BglII, producing pNUM. Deletion of the DSE was also achieved by PCR. A PCR fragment generated with primers 5′ *ura4* and 3′ M DSE on the pUM template was restricted with *Avr*II, combined with the fragment X*Bg800 described above, and ligated into pNU cut with *Avr*II–*Bgl*II, producing pNUM-DSE. Sequence analysis revealed the presence of a unique *Spe*I site at position 1776 on the *ura4* gene (Figure 1). pNU-DSE was produced by generation of a PCR product using the primers 5′ *ura4* and 3′ DSE *Nhe*I on the pNU template. This fragment was restricted with *Avr*II, then ligated into pNU restricted with *Avr*II–*Spe*I*. pNUM: r5′ DSE was constructed by replacement of the 5′ DSE in pNUM with the unique restriction site *Nhe*I, producing pNUM-5′ DSE *NheI*, then re-introduction of 5′ DSE at *NheI*. pNUM-5′ DSE *NheI* was constructed by generating PCR fragments with primers 5′ *ura4* and 3′ M DSE *Nhe*I and 5′ DSE and 3′ *ura4* (2) on the pNUM template. These fragments were restricted with *Avr*II and *Spe*I respectively, and ligated into pNU cut with *Avr*II and *Spe*I. Construction of pNUM: r5′ DSE involved introduction of a 49 bp *Rsa*I–*Acc*I* fragment into *Nhe*I*-restricted pNUM-5′ DSE *NheI*.

Table I. List of oligonucleotides used as PCR primers in DNA constructions

Name	Location	Sequence
5′ *ura4*	1351–1368	TTTGGTTGGTTATTGAAA
3′ *ura4* (1)	1762–1737	GCTTGTGATATATTGACGAAACTTTTTGACATC
3′ *ura4* (2)	1810–1792	ATTGTGTATTATACTCCGA
3′ *ura4* (3)	1953–1933	GTGTTTCATACTGACAATGCA
3′ SDE2	1525–1502	AGATAGCAAATTACTTTTTATTCC
5′ SDE2	1526–1539	GCATTTATTAAACATGCTCCTACA
3′ M DSE	1627–1612	TCCCATAGTTATCCCCAGGGG
5′ DSE	1675–1692	TCCGTAGACAAACAAGATAAA
3′ DSE *Nhe*I	1622–1607	ATGCTAGCATCATTACAAGTCTAA
3′ M DSE *Nhe*I	1613–1596	ATGCTAGCGGGGAAAAAAATTCACT
5′ DSE (a)	1631–1650	ACAAAGCCAATGAAAGATGT
3′ DSE (a)	1650–1631	ACATCTTTCATTGGCTTTGT
5′ DSE (b)	1648–1663	TGTATGTAGATGAATG
3′ DSE (b)	1663–1648	CATTCATCTACATACA
5′ DSE (c)	1661–1678	ATGTAAAATACCATGTAG
3′ DSE (c)	1678–1661	CTACATGGTATTTTACAT
3′:2’ *ura4*	1570–1554	GAGAAAAGATTGTGGTA
5′:3’ *ura4*	1571–1591	TTGGATTGACATTGAATAAGA
5′:7’ *ura4*	1812–1830	TCGGTGCAAATAGGTTTTA
5′:8’ *ura4*	1880–1899	AAGTATTCCCAAAATAATTC
5′ *CYC1*	352–370	TATCGATATCATGTAATTAGT
3′ *CYC1* 236	588–570	TAAGCGTCCCAAAACCTTCTC
3′ *CYC1* 83	532–511	TACGTCTGTACAGAAAAAAAAGAA

The location at which each *ura4* primer hybridizes to the *ura4* gene is given relative to the *Hin*dIII site at position (0) shown in Figure 1. The position at which the *CYC1* primers hybridize is given with respect to the *CYC1* translation start site.

PAC assay constructs

Constructs 2–5 used in the *ura4–nmt1* PAC assay (Figure 5B) were generated by restricting constructs pNU (2), pNUM-5′ DSE *NheI* (3), pNUM (4) and pNUM: r5′ DSE (5) with *Avr*II* and *Spe*I*, then ligating these fragments into the unique *Stu*I site present in the plasmid p*nmt41* (Maundrell, 1990). After removal of a second *Nhe*I site present in a pPAC:NUM-5′ DSE *NheI* construct (3), annealed oligo pairs (a), (b) and (c) were inserted into the remaining *Nhe*I* site to form constructs 6, 7 and 8 respectively.

CYC1 fragments were generated using PCR on template pGYC1 (Butler and Platt, 1988) which carries the wild-type *CYC1* 3′ end formation signals and pG200R (Butler and Platt, 1988) which contains a 38 bp deletion described previously (Zaret and Sherman, 1982). PCR products were either ligated directly or combined with 5′ DSE in the pGEM7Zf(+) polylinker before being introduced into the unique *Stu*I site in p*nmt41*. The *CYC1* 236 and *CYC1* Δ236 fragments were generated with primers 5′ *CYC1* and 3′ *CYC1* 236 on templates pGYC1 and pG200R respectively. *CYC1* 83 and *CYC1* Δ83 were generated with primers 5′ *CYC1* and 3′ *CYC1* 83 on templates pGYC1 and pG200R respectively, then restricted with *Eco*0190I*. *CYC1* 236, *CYC1* 83 and *CYC1* Δ83 were introduced into the *Hin*cII site in pGEM3. The 49 bp *Rsa*I–*Acc*I* fragment (5′ DSE) was then ligated into the *Bam*HI* site. Fragments released with *Pst*I*–*Eco*RI* were ligated into the *Stu*I site in p*nmt41*.

M13 constructs

Single-stranded probes for TRO analysis were constructed by ligating fragments released by restriction digest, or generated by PCR followed by restriction, into the *Hin*cII site of either M13mp18 (RF) or M13mp19 (RF). Probes 1–6 and probes 1’, 4’ and 5’ were constructed by appropriate restriction digest of pUL. Probe 2’ was generated by PCR using primers 5′ *ura4* and 3′:2’ *ura4* followed by restriction with *Sty*I*, probe 3’ was generated using primers 5′:3’ *ura4* and 3′ *ura4* (1) followed by restriction with *Rsa*I, probe 6’ using primers 5′:3’ *ura4* and 3′ *ura4* (2) followed by restriction with *Hin*dIII*, probe 7’ using primers [5′:7’ *ura4* and 3′ *ura4* (3) followed by restriction with *Dde*I*, probe 8’ using primers 5′:8’ *ura4* and 3′ *ura4* (3)] followed by restriction with *Dde*I*.

Transformation and RNA analysis

Schizosaccharomyces pombe transformation was performed as described elsewhere (Broker, 1987). RNA extraction followed the protocol for isolation of RNA from yeast ascospores (Kohrer and Domdey, 1991) but used LiCl (0.5 M) rather than NaAc (0.3 M) to precipitate RNA. The method of Northern blot analysis has been described previously (Humphrey *et al.*, 1994). After hybridization, filters were rinsed and washed twice in 0.2× SSC, 0.1% SDS at 42°C for 20 min, then visualized and quantitated with a Molecular Dynamics phosphorimager.

The TRO assay followed a protocol modified from those described for *S.cerevisiae* (Elion and Warner, 1986; Warner, 1991) and *S.pombe* (Maundrell, 1990). Cultures of 300 ml were grown in the presence of thiamine at a final concentration of 20 μM where appropriate, to an OD_{600} of ~0.1. Cells were harvested, washed and permeabilized for 20–30 min before being resuspended in 40 μl of 2.5× transcription buffer (50 mM Tris–HCl pH 7.7, 500 mM KCl, 80 mM $MgCl_2$; Jerome and Jaehning, 1986). Transcription was allowed to proceed for 2 min following the addition of dithiothreitol (DTT, 2 mM), rATP, rGTP, rCTP (1 mM) and 100–200 μCi of [α-^{32}P]rUTP (800 Ci /mmol, Amersham). Cells were then washed briefly with TMN (10 mM Tris–HCl pH 7.4; 5 mM $MgCl_2$; 100 mM NaCl) before total RNA was extracted as detailed above. After partial RNA hydrolysis for 5 min at 4°C (0.2 M NaOH) and neutralization (0.2 M Tris–HCl pH 7.2), samples were pre-hybridized with 15 μg of single-stranded M13mp18 (RF) for 1 h, before being hybridized to immobilized single-stranded M13 probes. Uniformly labelled transcripts were synthesized with T7 polymerase made under standard conditions. Filters were rinsed and washed twice in 0.2× SSC, 0.1% SDS for 20 min at 42°C. An additional RNase A wash step was performed on filters with short probes 1’–8’ as described previously (Tantravahi *et al.*, 1993). ‘Run-on’ signals were then visualized and quantitated with a Molecular Dynamics phosphorimager. ‘Run-on’ data presented have been confirmed in at least three independent assays.

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