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Nascent transcription from the *nmt1* and *nmt2* genes of *Schizosaccharomyces pombe* overlaps neighbouring genes

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We have determined the extent of the primary transcription unit for the two highly expressed genes *nmt1* and *nmt2* of *Schizosaccharomyces pombe*. Transcription run-on analysis in permeabilized yeast cells was employed to map polymerase density across the 3′-flanking region of these two genes. Surprisingly, polymerases were detected 4.3 kb beyond the *nmt1* polyadenylation [poly(A)] site and 2.4 kb beyond the *nmt2* poly(A) site, which in each case have transcribed through an entire convergent downstream transcription unit. However, the steady-state levels of both downstream genes were unaffected by the high level of *nmt1* or *nmt2* nascent transcription. Analysis of *nmt1* and *nmt2* RNA 3′ end formation signals indicates that efficient termination of transcription requires not only a poly(A) signal but also additional pause elements. The absence of such pause elements close to the poly(A) sites of these genes may account for their extended nascent transcripts.

Keywords: 3′ end formation/*nmt1*/polyadenylation/*Schizosaccharomyces pombe*/transcription termination

Introduction

The 3′ end of most eukaryotic mRNAs is formed by coupled endonucleolytic cleavage and polyadenylation of the nascent transcript synthesized by RNA polymerase II (pol II). Transcription termination, defined as the dissociation of the ternary complex into its constituent parts, occurs further downstream and is required to release the polymerase for subsequent rounds of transcription, as well as to prevent transcriptional interference. Transcriptional interference arises when an elongating polymerase fails to terminate transcription and reads into a co-transcribed downstream transcription unit resulting in reduced expression of the downstream gene through disruption of interactions at the promoter (Cullen *et al.*, 1984; Bateman and Paule, 1988; Henderson *et al.*, 1989; Irniger *et al.*, 1992). Interference can be relieved by cloning 3′ end formation signals between the two tandem promoters (Irniger *et al.*, 1992; Eggermont and Proudfoot, 1993; Greger *et al.*, 1998). For convergent transcription units, the potential problem of polymerases collision or hybridization between nascent transcripts that could target the transcripts for degradation may also arise. In yeast, transcriptional interference is believed to be of particular significance due to the compact nature of the yeast genome (Oliver *et al.*, 1992). Furthermore, yeast autonomously replicating sequences (ARSs) and centromere (*CEN*) elements, which are both required for chromosome maintenance, as well as sites of meiotic gene conversion, are also sensitive to transcriptional interference (Hill and Bloom, 1987; Snyder *et al.*, 1988; Rocco *et al.*, 1992). Since transcription units have been identified in the *Saccharomyces cerevisiae* genome which direct transcription into ARSs or *CEN* elements without apparently affecting their function (Snyder *et al.*, 1988; Hegemann and Fleig, 1993; Tanaka *et al.*, 1994; Chen *et al.*, 1996), efficient transcription termination signals must exist to prevent such interference.

The signals directing cleavage/polyadenylation have been studied extensively in both mammals and the yeast *S.cerevisiae*, and are now well-characterized (Guo and Sherman, 1996; Wahle and Keller, 1996). In mammals, the almost invariant AAUAAA hexanucleotide is located 15–20 nucleotides upstream of the cleavage site, and a less well-defined GU or U-rich element is located downstream of the cleavage site (for review see Wahle and Keller, 1996). In *S.cerevisiae*, the polyadenylation [poly(A)] signals are more degenerate and comprise an upstream efficiency element (EE; consensus UAUAUA) which promotes 3′ end formation at downstream sites, and a positioning element (consensus AAAAAA or AAUAAA) which directs cleavage at preferred sites [characterized by $Y(A)_n$] 15–30 nucleotides downstream (Irniger and Braus, 1994; Guo and Sherman, 1995, 1996). The development of *in vitro* processing extracts has also led to the identification and cloning of many of the *trans*-acting factors involved in cleavage/polyadenylation (Keller and Minvielle-Sebastia, 1997). In contrast, far less is known about the process of pol II transcription termination, partly due to the instability of the primary transcript and lower transcript levels compared with genes transcribed by RNA polymerases I and III.

In higher eukaryotes, transcription run-on (TRO) assays have been employed to map sites of nascent transcription in isolated nuclei or whole cells (Citron *et al.*, 1984; Hagenbüchle *et al.*, 1984; Connelly and Manley, 1988; Maa *et al.*, 1990). Generally, transcription termination occurs in broad, poorly defined regions up to 4 kb beyond the poly(A) signal. Two signals are believed to be necessary for pol II transcription termination: a functional poly(A) signal and a downstream element (DSE) at the site of termination (Logan *et al.*, 1987). Mutation of the poly(A) signal results in continued transcription beyond the normal termination site (Whitelaw and Proudfoot, 1986; Logan *et al.*, 1987; Connelly and Manley, 1988), and the *in vitro* processing efficiency of the poly(A) signal

correlates with termination efficiency (Edwalds-Gilbert *et al.*, 1993). The nature of the DSE appears variable, but a common feature may be the pausing of the polymerase by perturbing the normal mode of elongation. The ability to induce polymerase pausing may be intrinsic to the DNA sequence being transcribed (Kerppola and Kane, 1990; Enriquez-Harris *et al.*, 1991) or be induced by a bound protein (Connelly and Manley, 1989a,b; Ashfield *et al.*, 1991, 1994) which may bend the DNA (Ashfield *et al.*, 1994).

In *S.cerevisiae*, most studies have employed indirect assays to identify putative transcription termination signals. Such assays rely on monitoring transcription-induced changes in plasmid topology (Osborne and Guarente, 1988) or increased plasmid stability conferred by cloning sequences that prevent transcriptional interference between the highly expressed *GAL1* promoter and a *CEN* element (Russo and Sherman, 1989; Russo, 1995). These studies indicate that sequences required for 3′ end formation of the *CYC1* gene are also required for transcription termination. A more direct study using a TRO assay (Osborne and Guarente, 1989) showed that an 83 bp fragment from the 3′-flanking region of the *CYC1* gene confers efficient transcription termination. Also, *in vitro* transcription studies have detected transcription up to 150 nucleotides beyond the *GAL7* and *ADH2* poly(A) sites and are consistent with polymerase pausing in this region (Hyman and Moore, 1993).

In the fission yeast, *Schizosaccharomyces pombe*, the signals required for 3′ end formation have only been defined for the *ura4* gene and comprise two site-determining elements (SDEs) which position the major and minor cleavage sites and a downstream EE required for efficient 3′ end formation at the upstream SDEs (Humphrey *et al.*, 1994). The same 3′ end formation signals are also required for transcription termination, in association with a DSE which acts by pausing the polymerases (Birse *et al.*, 1997). In this latter study, termination of *ura4* transcription was shown to occur 180–380 bp downstream of the poly(A) site using a TRO assay in permeabilized yeast cells. The aim of this study was to extend these observations on *ura4* to other *S.pombe* genes by mapping the polymerase density across the 3′-flanking region of the co-ordinately regulated *S.pombe* genes, *nmt1* and *nmt2*, and so identify where transcription termination occurs and which signals are responsible. The *nmt1* and *nmt2* genes were selected because they are highly transcribed and repressed when thiamine is included in the growth medium (*nmt*: no message thiamine) (Maundrell, 1990; Manetti *et al.*, 1994). Also, the *nmt1* promoter and 3′-flanking sequences are present in a widely used series of vectors for the expression of genes in *S.pombe* (Maundrell, 1990, 1993). Information regarding the signals required for efficient *nmt1* 3′ end formation and transcription termination are therefore of general interest.

Results

Identification of genes positioned downstream from nmt1 and nmt2

Previous analysis of the *nmt1* gene (Maundrell, 1990) provided a sequence (DDBJ/EMBL/GenBank databases accession No. J05493) extending 1.25 kb beyond the stop codon, but no information was available as to the nature, location and direction of any downstream transcription units. In contrast, available sequence extended 2.45 kb beyond the *nmt2* stop codon (DDBJ/EMBL/GenBank accession No. X82363; additional sequence kindly provided by K.Maundrell) and included a convergently transcribed open reading frame (ORF), named *avn2*, of unknown function (Manetti *et al.*, 1994). To extend the *nmt1* 3′-flanking sequence and identify any downstream transcription unit, cosmid clones were obtained from the Reference Library Data Base (RLDB; Maier *et al.*, 1992; Hoheisel *et al.*, 1993). Restriction fragments identified by Southern analysis were subcloned and sequenced. In total, 3.24 kb of novel sequence was obtained (DDBJ/EMBL/GenBank accession No. Y14993). A convergent ORF was identified (called *gut2* for glycerol utilization) which is predicted to encode a mitochondrial glycerol-3-phosphate dehydrogenase based upon the observed 56.3% identity to the *S.cerevisiae GUT2* gene (Rønnow and Kielland-Brandt, 1993).

Mapping of nmt1 and nmt2 poly(A) sites

The major *nmt1* poly(A) site was originally mapped by S1 nuclease protection analysis to 142 bp downstream of the *nmt1* stop codon (Maundrell, 1990). To confirm this result and to map the previously uncharacterized poly(A) site of *nmt2*, RT–PCR analysis was employed. Total RNA was isolated from the wild-type *S.pombe* strain 972h$^-$ and subjected to RT–PCR using a gene-specific primer and a phased oligo(dT) primer. DNA products were then subcloned and sequenced. Specific products were only obtained from yeast grown in the absence of thiamine, when *nmt1* and *nmt2* are expressed (not shown). As indicated in Figure 1A, the RT–PCR analysis confirms that *nmt1* possesses a single major poly(A) site at a position which matches that obtained using S1 nuclease protection analysis (data not shown; Maundrell, 1990). Four cDNAs also mapped to different, minor sites nearby.

For *nmt2*, two clusters of cleavage sites were identified (Figure 2A), which were again confirmed by S1 nuclease mapping (data not shown). The cleavage sites revealed by the RT–PCR mapping are consistent with the existence of poly(A) signals specifying a region in which cleavage occurs at preferred sites, usually characterized by the sequence $Y(A)_n$, although less favourable sites nearby may also be utilized. The situation observed in *S.pombe* thus appears similar to that in *S.cerevisiae* where multiple positioning elements are found directing cleavage at several clustered sites 15–30 nucleotides downstream (Heidmann *et al.*, 1992; Russo *et al.*, 1993; Guo and Sherman, 1995). An inspection of the sequence surrounding the mapped poly(A) sites for *nmt1* and *nmt2* (also *avn2*, data not shown) reveals the presence of an A-rich sequence located ~15 nucleotides upstream. A comparison with the proposed consensus positioning element (AAAAAA or AAUAAA) in *S.cerevisiae* (Guo and Sherman, 1995) and the similar positioning with respect to the cleavage site suggests an A-rich element is a candidate SDE in *S.pombe* which may act to define the cleavage site. Indeed, the 16 nucleotide minimal element defined for the *S.pombe ura4* SDE2 also contains an A-rich sequence (Humphrey *et al.*, 1994).

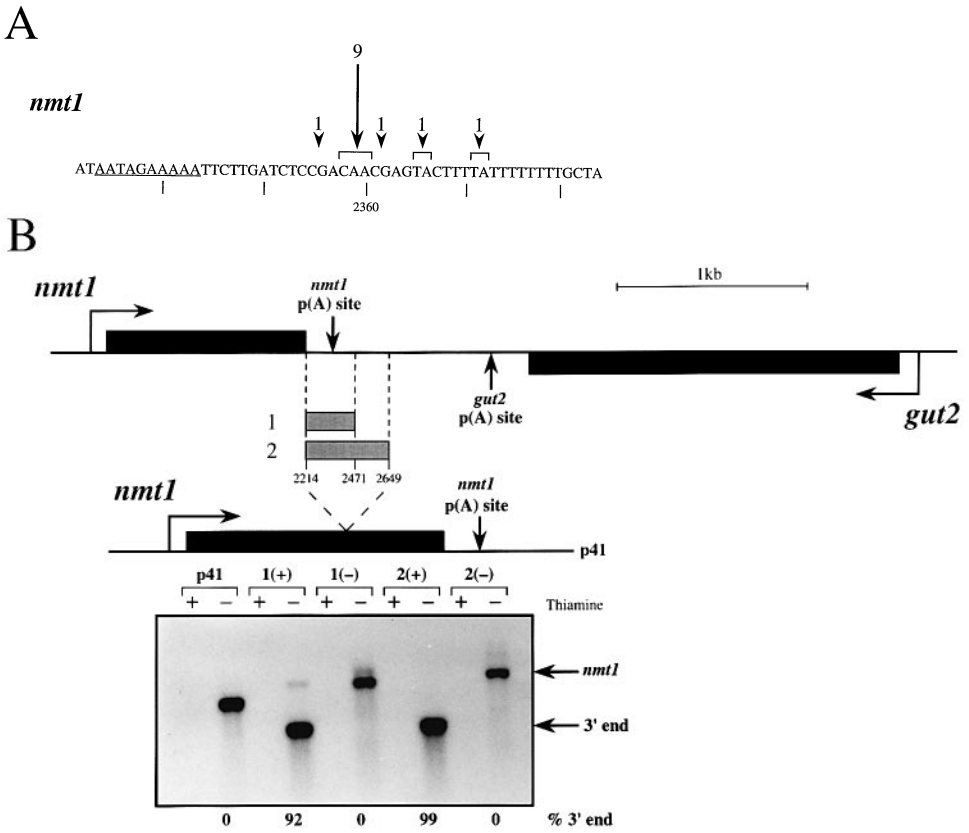

Fig. 1. *nmt1* poly(A) signals. (**A**) RT–PCR mapping of the *nmt1* poly(A) sites. The RT–PCR products were subcloned and 13 clones sequenced. The vertical arrows indicate the cleavage site positions. Where the cDNA ended with an adenosine, it was not possible to assign the cleavage site unambiguously since the adenosine may be template derived or added by poly(A) polymerase, indicated by brackets. The numbers above each arrow and the arrow length reflect the frequency with which each site was obtained. An A-rich sequence upstream of the poly(A) site is underlined. (**B**) A schematic diagram of the convergently transcribed *nmt1* and *gut2* genes is shown. The major poly(A) sites mapped by RT–PCR are indicated. The test fragments indicated were subcloned (in both orientations) into the *Stu*I site of plasmid p41, to assess their ability to direct 3′ end formation in competition with the downstream wild-type *nmt1* poly(A) site. The constructs were used to transform *nmt1* disruption strain Sp204, and total RNA was prepared, which was resolved on a 1.6% formaldehyde–agarose gel overnight, transferred to a nylon membrane and probed with a random-primed probe of the *Xho*I–*Nco*I *nmt1*-specific fragment. The *Xho*I–*Nco*I fragment has been replaced by *ura4* sequences in the host strain Sp204, so no background from the chromosome is detected. Truncated transcripts (labelled 3′ end) are due to 3′ end formation directed by the test fragment inserted at the *Stu*I site in the *nmt1* coding region. Readthrough transcripts (labelled *nmt1*) are due to 3′ end formation directed by the wild-type *nmt1* poly(A) signals located downstream. The '% 3′ end' indicates the percentage truncated transcripts of the total detected using the *nmt1*-specific probe. The inclusion of thiamine in the growth medium is indicated. In the presence of thiamine, transcription from the *nmt1* promoter is repressed.

Analysis of nmt1 and nmt2 poly(A) signals

The signals required for efficient 3′ end formation of *S.pombe* mRNA have only been characterized extensively for the *ura4* gene (Humphrey *et al.*, 1994). To begin analysis of the poly(A) signals for *nmt1* and *nmt2*, test fragments from the 3′-flanking region were cloned into the unique *Stu*I site within the *nmt1* coding region located in plasmid p41. Constructs were used to transform *S.pombe nmt1* disruption strain Sp204, total RNA isolated and subjected to Northern analysis. The ability of the test fragment to direct 3′ end formation in competition with the downstream wild-type *nmt1* poly(A) site is assessed by measuring the ratio of truncated transcripts, due to 3′ end formation directed by the test fragment, compared with readthrough transcripts, due to 3′ end formation at the *nmt1* poly(A) site (labelled *nmt1*). Typical Northern blots are presented in Figures 1B (*nmt1*) and 2B (*nmt2*). For *nmt1*, most of the signals required for efficient 3′ end formation appear to be contained within a 256 bp fragment. This fragment contains the mapped poly(A) sites and directs 3′ end formation with 92% efficiency in this context, in an orientation-specific manner. The greater efficiency observed for the larger 434 bp fragment (99%) may simply reflect increased spacing. All Northern blot signals were sensitive to the presence of thiamine.

For *nmt2* (Figure 2B), fragment 2 (259 bp) containing the mapped *nmt2* poly(A) sites was sufficient to direct 100% 3′ end formation in competition with the downstream *nmt1* poly(A) site. However, when fragment 2 is subdivided, the 5′ fragment 1 (155 bp) which still contains the mapped *nmt2* poly(A) sites showed reduced efficiency (21%), while the 3′ fragment 3 (104 bp) had no RNA 3′ end-forming activity. Therefore, sequences downstream of the *nmt2* poly(A) site contribute to efficient 3′ end formation, as for the *S.pombe ura4* gene (Humphrey *et al.*, 1994). This contrasts to the situation in the majority of *S.cerevisiae* genes examined where no role for downstream sequences has been observed (Abe *et al.*, 1990; Egli *et al.*, 1997). It should also be noted that both fragments 1 and 2 function in the reverse orientation as efficient poly(A) signals. The ability of poly(A) signals to function in both orientations has also been observed in a number of *S.cerevisiae* poly(A) signals (Irniger *et al.*, 1991). No cDNAs with 3′ ends mapping within fragment 4 were

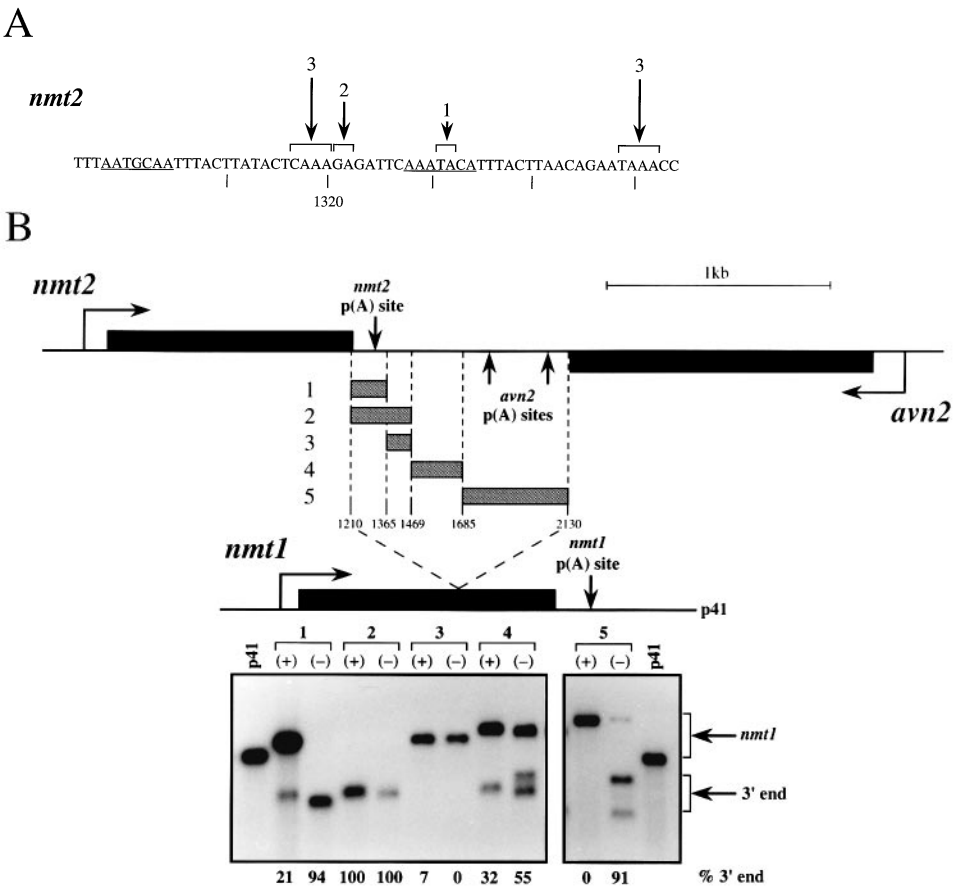

Fig. 2. *nmt2* poly(A) signals. (**A**) RT–PCR mapping of the *nmt2* poly(A) sites. The RT–PCR products were subcloned and nine clones sequenced. The vertical arrows indicate the cleavage site positions as in Figure 1. An A-rich sequence upstream of each major poly(A) site is underlined. (**B**) The upper part shows a schematic diagram of the convergently transcribed *nmt2* and *avn2* genes. The poly(A) sites mapped by RT–PCR are indicated. As in Figure 1, *nmt2* test fragments were subcloned into the *Stu*I site of plasmid p41 to assess their ability to direct 3′ end formation by Northern blotting.

identified, suggesting that the ability of this fragment to direct 3′ end formation is due to cryptic signals within the fragment, not normally utilized in the natural context. A 220 bp fragment [Figure 2B, compare 5(+) with 5(–)] in the sense orientation with respect to *nmt2* did not direct formation of a truncated transcript but in the reverse orientation directed 3′ end formation at two major sites (and one minor) which correspond to the two major 3′ ends mapped by RT–PCR analysis for *avn2* (data not shown).

Extended nascent transcription at the 3′ end of the nmt1 and nmt2 genes

A TRO assay was employed to map pol II density across the 3′-flanking region of the *nmt1* and *nmt2* genes. Briefly, detergent-permeabilized yeast cells are incubated in a moderately high salt transcription buffer containing [α-^{32}P]UTP for a short period (2–5 min). The conditions permit transcriptionally engaged polymerases to elongate a short distance and incorporate radioactive label (Birse *et al.*, 1997). Total RNA is then isolated, partially hydrolysed (to sub-probe length fragments) and hybridized to immobilized single-stranded M13 probes to localize the active polymerase complexes. The signal for each probe is proportional to the average polymerase density across the DNA fragment, within the yeast population. If the pulse-labelled RNA is hybridized to contiguous single-stranded DNA probes spanning the 3′-flanking region, a profile of polymerase density is obtained.

TRO analysis was performed on the wild-type strain 972h$^-$ grown in the presence or absence of thiamine. Figure 3 shows the results of a representative experiment when the pulse-labelled RNA is hybridized to probes spanning the 3′-flanking region of *nmt1*. High signals are observed for the *nmt1* probes when the yeast are grown in the absence of thiamine (–T), but the signals fall to background levels when the yeast are grown in the presence of thiamine (+T), confirming that the signals are due to transcription from the *nmt1* promoter. A signal was detected for the H2A probe both in the presence and absence of thiamine. The signals (–T) were quantitated, corrected for the M13 background (M13mp18 without insert) and for the U content of the nascent transcript that hybridizes to each probe, and were expressed relative to the 5′ *nmt1* probe (1-1). The polymerase profile obtained reveals that polymerases transcribe a distance of 4.3 kb beyond the poly(A) site of the endogenous *nmt1* gene, reading entirely through the convergent *gut2* transcription unit. This is surprising since all previous studies in yeast have indicated that transcription termination occurs close to the poly(A) site. Indeed, termination of transcription close to the poly(A) site is intuitively expected, given the compact nature of yeast genomes and the need to avoid transcriptional interference. A build-up in polymerase

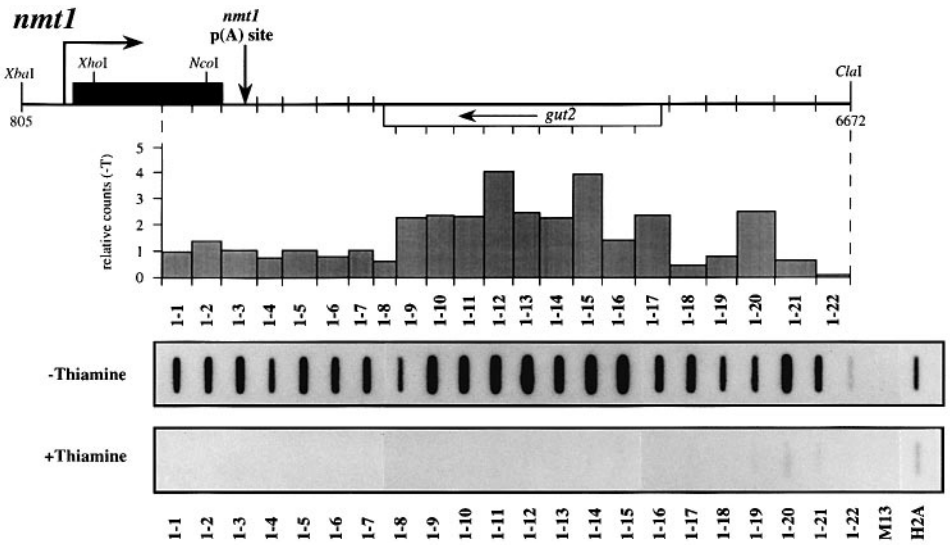

Fig. 3. Transcription run-on analysis of *nmt1*. The wild-type strain 972h$^-$ was grown overnight in minimal medium in the presence or absence of thiamine, and TRO analysis was performed. The signals obtained after the high-stringency wash are shown and are displayed graphically after quantitation. The signals were corrected for the M13 background (M13mp18 without an insert) and for the U content of the nascent RNA hybridizing to each probe, and are expressed relative to probe 1-1. The graph is drawn to scale such that the width of each bar reflects the length of the probe and the height indicates the relative average signal across the probe. The H2A probe is included as an internal control. The profile obtained was similar after an additional RNase A wash (data not shown).

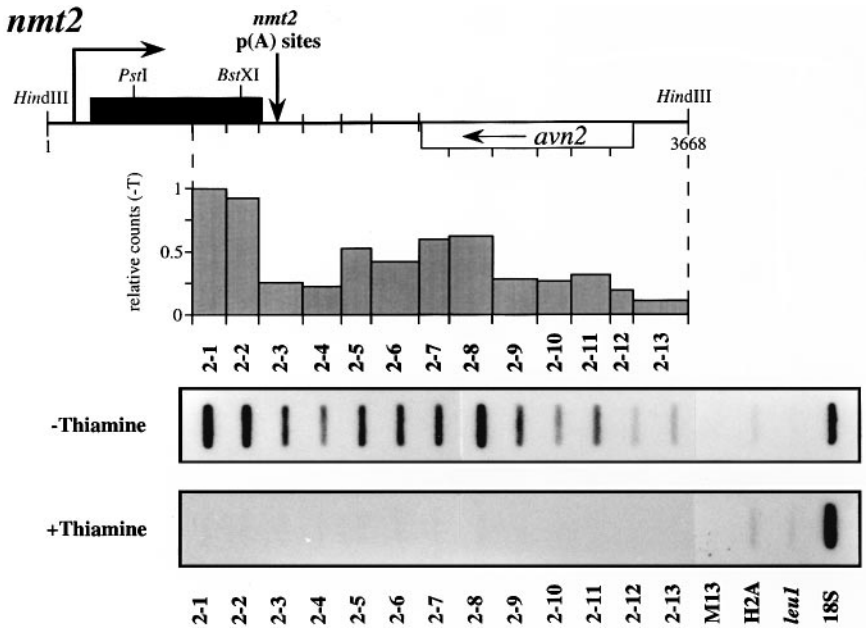

Fig. 4. Transcription run-on analysis of *nmt2*. Conditions were as for Figure 3. The signals obtained after the high-stringency wash are shown and displayed graphically after quantitation. The signals were corrected as in Figure 3 and expressed relative to probe 2-1. The H2A, *leu1* and 18S probes are included as internal controls. The profile obtained was similar after an additional RNase A wash (data not shown).

density is observed over the probes spanning the *gut2* ORF which may in part reflect a hybridization effect due to the greater GC content within the coding region. Also, a second build-up is observed over probe 1-20, just before polymerase density falls to the lowest levels detected over probe 1-22, suggesting that transcription termination may be occurring at this position.

A similar analysis for *nmt2* is presented in Figure 4. The signals obtained for *nmt2* are also sensitive to the presence of thiamine, confirming that the nascent transcription detected is due to polymerases initiating at the *nmt2* promoter. For *nmt2*, a drop in polymerase density is observed consistently over the probe containing the mapped poly(A) sites, suggesting that some polymerases may terminate transcription at this point. The polymerases that continue appear to give an increased signal over probe 2-5 and those immediately downstream, before a gradual decline in signal is observed. As for *nmt1*, it is notable that polymerases transcribe at least 2.4 kb beyond the poly(A) site and read through the downstream convergent *avn2* transcription unit.

A comparison of the level of signals obtained for *nmt1* and *nmt2* with that obtained for the other pol II-transcribed genes, *leu1* and *H2A.1*, and the RNA polymerase I-

transcribed 18S rRNA gene (Figures 3 and 4), reveals that high levels of nascent transcription are observed for *nmt1* and *nmt2* which facilitates their analysis. No signal was detected for the sense M13 probes (±T) which would be expected to detect polymerases transcribing *gut2* or *avn2* (data not shown). Presumably, *avn2* and *gut2* nascent transcription signals lie below the limits of detection using this TRO assay under the growth conditions employed.

Although representative polymerase profiles are shown in Figures 3 and 4, some variation is observed between different experiments (data not shown) in the apparent efficiency of transcription termination [distance beyond the poly(A) site at which polymerase density falls to background levels]. However, the trend in polymerase density remains consistent. For *nmt2*, polymerase density always falls over the poly(A) signals and increases over probe 2-5, but in some experiments a more pronounced build-up in signal over probe 2-5 appears to be associated with more efficient transcription termination, closer to the poly(A) site. A similar apparent increase in termination efficiency is also observed in some experiments for *nmt1*, performed in parallel, suggesting that the variation may reflect differences in the growth of the yeast. However, even in these experiments, polymerases still continue for 2.0 kb (*nmt2*) and 2.6 kb (*nmt1*) beyond the poly(A) site reading into the downstream transcription unit (data not shown).

gut2 and avn2 expression is unaffected by nmt1 or nmt2 nascent transcription

Since nascent transcription from both the *nmt1* and *nmt2* genes extends into the convergent downstream transcription unit, it was of interest to examine whether the high level of *nmt1* or *nmt2* transcription influenced the expression of the adjacent *gut2* or *avn2* genes. Therefore, the wild-type strain 972h⁻ was grown on minimal media in the presence or absence of thiamine, with 3% glucose as the sole carbon source or 3% glycerol/0.1% glucose (0.1% glucose was included to facilitate growth; Hoffman and Winston, 1989). These alternative carbon sources were used to test whether *gut2* expression was subject to glucose repression as has been found for the *S.cerevisiae GUT2* gene (Sprague and Cronan, 1977; Rønnow and Kielland-Brandt, 1993). Total RNA was prepared and subjected to Northern analysis (Figure 5A). The low levels of *gut2* expression prevent accurate quantitation. However, based on the *leu1* loading control, it is apparent that *gut2* expression is repressed by glucose, but not significantly affected by *nmt1* transcription. The size of the *gut2* transcript was estimated to be 2.6 kb based on RNA markers run alongside.

A similar steady-state Northern analysis of *avn2* expression reveals that a doublet (1.56 and 1.8 kb bands) is detected in RNA isolated from the wild-type strain 972h⁻ and the *nmt2* disruption strain Sp92, grown both in the presence and absence of thiamine (Figure 5B). The doublet corresponds to 3′ end formation at the two major poly(A) sites mapped by RT–PCR analysis (not shown) as seen in clone 5(–) in Figure 2B. Equal amounts of total RNA were loaded in each lane, therefore expression of *avn2* does not appear to be influenced by *nmt2* transcription. The lower band visible in the lane containing RNA isolated from 972h⁻ grown in the absence of thiamine represents

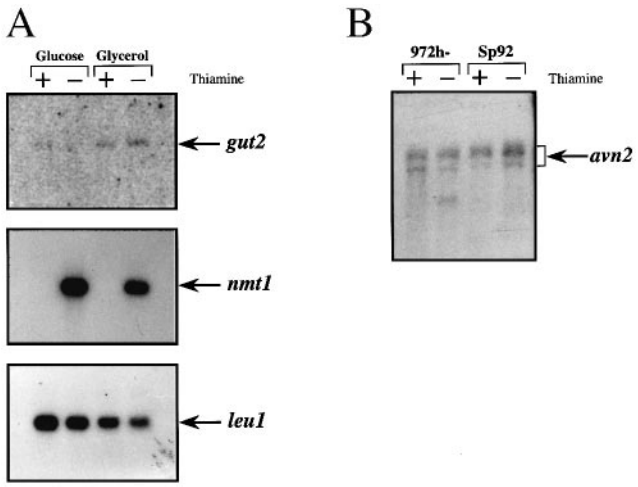

Fig. 5. Steady-state analysis of *gut2* and *avn2* expression. (**A**) The wild-type strain 972h⁻ was grown overnight in minimal medium containing 3% glucose or 3% glycerol/0.1% glucose as a carbon source, in the presence or absence of thiamine. Total RNA was prepared which was resolved on a 1.6% formaldehyde–agarose gel overnight, transferred to a nylon membrane and sequentially stripped and probed with a random-primed probe of the *Sty*I–*Nru*I *leu1*-specific fragment, an *Xho*I–*Nco*I *nmt1*-specific fragment and a *Hin*dIII–*Eco*RV *gut2*-specific fragment. The *leu1* signal acts as a loading control. (**B**) The wild-type strain 972h⁻ and the *nmt2* disruption strain Sp92 were grown overnight in minimal medium in the presence and absence of thiamine. Total RNA was prepared and equal amounts resolved on a 1.6% formaldehyde–agarose gel overnight, transferred to a nylon membrane and probed with a random-primed probe of the *Nde*I–*Sty*I fragment from the *avn2* coding region.

cross-hybridization to the *nmt2*-derived transcripts since it is absent in the *nmt2* disruption strain Sp92.

These data indicate that both the *gut2* and *avn2* genes are subjected to extensive readthrough transcription from *nmt1* and *nmt2*. However, neither this high level of transcriptional activity on the opposite strand nor the accumulation of antisense transcript appear to affect their steady-state mRNA levels.

nmt2 poly(A) signal is required for efficient transcription termination

To identify the sequences responsible for controlling the pattern of polymerase density observed at the 3′ end of a gene, it is necessary to reproduce the endogenous gene profile in a plasmid context. Therefore, a 3.67 kb *Hin*dIII–*Hin*dIII fragment encompassing the *nmt2* and *avn2* ORFs was cloned into the *Bam*HI site of pIRT2 to create plasmid p2V. Northern analysis confirmed that a thiamine-sensitive transcript was transcribed from plasmid p2V (Figure 6B). When plasmid p2V was used to transform *S.pombe nmt2* disruption strain Sp92 and TRO analysis performed, a similar polymerase profile was observed for the plasmid and endogenous genes (compare panels in Figures 6A, and 4), such that the polymerase density fell over the poly(A) site and increased over probe 2-5 before decreasing gradually over subsequent probes to background levels. However, the plasmid-derived TRO profile gave lower signals over the downstream probes covering the 3′ end of *avn2* than was observed for the endogenous gene. This may reflect differences in chromatin structure between the chromosomal and plasmid genes which might be expected to influence elongation. The build-up in signal observed

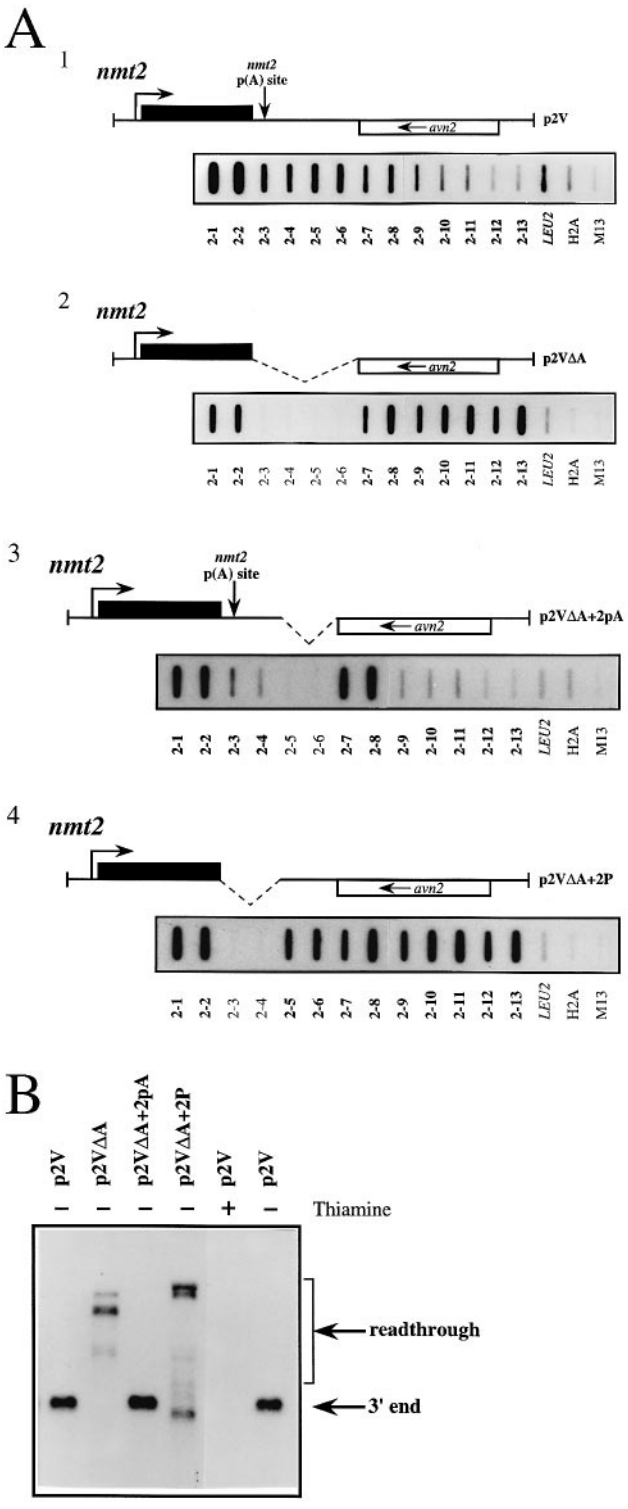

Fig. 6. Manipulation of the *nmt2* polymerase profile. (**A**) A schematic diagram of each of the constructs used to transform *nmt2* disruption strain Sp92; the location of probes used for TRO analysis is shown. The dotted lines indicate sequences deleted from a particular construct with respect to plasmid p2V. The TRO hybridization signals after an RNase A wash are shown, approximately aligned. The *LEU2* and H2A probes were included as internal controls. (**B**) Northern analysis of total RNA isolated from Sp92 transformed with the constructs indicated and grown in the presence or absence of thiamine as indicated. The RNA was resolved on a 1.6% formaldehyde–agarose gel overnight, transferred to a nylon membrane and probed with a random-primed probe of the *Pst*I–*Bst*XI *nmt2*-specific fragment.

over probe 2-5 in both the chromosomal and plasmid *nmt2* genes may represent a polymerase pause site, as has been described previously for the *ura4* gene (Birse *et al.*, 1997).

In an attempt to dissect the relative contribution of the *nmt2* poly(A) signals and the putative pause element in producing the observed polymerase profile, the intergenic region between *nmt2* and *avn2* was deleted from plasmid p2V to give plasmid p2VΔA, and a unique *Avr*II site introduced. Into p2VΔA, either a fragment containing all the sequences shown previously to direct 3′ end formation (2pA), or the putative pause element (2P), were re-introduced between the two genes. The constructs were used to transform strain Sp92, and both TRO and Northern analyses were performed (Figure 6). The results clearly reveal that when the intergenic region is deleted the polymerase density remains at a high level throughout the region probed (Figure 6A, p2VΔA), suggesting that no transcription termination is occurring. Northern analysis reveals that 3′ end formation at the normal site is abolished, with cryptic signals 0.5–1.5 kb downstream used (Figure 6B, compare p2V with p2VΔA). Insertion of the *nmt2* 3′ end formation signals restores 3′ end formation (Figure 6B, p2VΔA+2pA) and the polymerase profile resembles that of the wild-type gene, although more efficient termination is observed (Figure 6A, compare p2V with p2VΔA+2pA). Thus the TRO signals decreased over the poly(A) site and increased substantially over the two probes downstream of 2-4, which in p2VΔA+2pA are 2-7 and 2-8, before falling to background levels. The high level of signals observed for probes 2-7 and 2-8 may reflect the presence of additional pause elements within the *avn2* gene. This result implies that a poly(A) signal is required for transcription termination. Re-introduction of the putative pause element into p2VΔA produced little change in the polymerase profile; polymerases continued to transcribe the region probed at high levels (Figure 6A, p2VΔA+2P). Steady-state analysis revealed that although 3′ end formation was abolished at the normal position in p2VΔA+2P, as expected in the absence of the *nmt2* 3′ end formation signals, a number of minor sites were utilized and 36% of polymerases read through to a site 1.5–2.0 kb downstream (Figure 6B, p2VΔA+2P). This pattern of minor 3′ ends could be explained by the selection of a number of weak signals in the absence of an obvious consensus signal, enhanced by polymerase pausing in the vicinity of the end of the *nmt2* coding region, directed by fragment 2P. However, it is evident that while the DNA sequences spanned by probes 2-5 and 2-6 may be able to pause polymerases, transcription termination does not occur in the absence of an efficient poly(A) signal.

Efficient nmt1 transcription termination can be achieved by insertion of ura4 RNA 3′ end formation signals

To examine whether the polymerase profile of the endogenous *nmt1* gene can also be reproduced on a plasmid, p43B was constructed containing less 5′-flanking sequence, but additional 3′-flanking sequence compared with the parental p41 plasmid, and a unique *Bgl*II site was introduced 231 bp downstream from the major *nmt1* poly(A) site. Northern analysis confirms that p43B gives a thiamine-

sensitive transcript of the expected size when used to transform *nmt1* disruption strain Sp204 (Figure 7B). Comparison of the TRO signals of *nmt1* disruption strain Sp204 transformed with p43B (Figure 7A, panel 1) with the endogenous gene in the wild-type strain 972h$^-$ (Figure 3), reveals that similar overall polymerase profiles are observed when the *nmt1* gene is located on a plasmid compared with the endogenous gene. The main differences include a higher signal for probe 1-2. This partly reflects a background contribution from polymerases reading through the *ura4* sequences used to disrupt the chromosomal *nmt1* gene in strain Sp204 (Figure 7A, panel 5) and terminating shortly afterwards, as expected given previous results (Birse *et al.*, 1997). Also, the signals observed over probes 1-9 to 1-12 are not as high relative to 5′ probes as in the endogenous gene. As with *nmt2*, this may reflect differences in chromatin structure between the chromosomal and plasmid genes. The thiamine-sensitive TRO signals derived from the plasmid are lost when the *nmt1* promoter is deleted (data not shown). This indicates that the observed polymerase density is due to polymerases that initiated at the *nmt1* promoter and transcribed throughout the region probed, although transcript release and re-initiation cannot be excluded using this assay.

The surprising observation that polymerases initiating at the *nmt1* promoter transcribe through the *nmt1* 3′-flanking region, into and beyond the adjacent *gut2* gene, may reflect the absence of termination signals as previously defined for the *ura4* gene (Birse *et al.*, 1997). The *ura4* DSE (Birse *et al.*, 1997) or *ura4* poly(A) signals (Humphrey *et al.*, 1994) were therefore inserted downstream of the *nmt1* poly(A) site, in an attempt to alter the TRO profile. The constructs were used to transform Sp204, and both TRO and Northern analyses were performed. As shown in Figure 7A (panel 2), when the DSE is placed close to the *nmt1* poly(A) site (112 bp downstream) the TRO signals fell to low levels within 1.23 kb of the poly(A) site. However when the DSE is located further downstream (231 bp), the observed effect was greatly reduced and polymerases continued transcription throughout the 1.93 kb region probed (Figure 7A, panel 3). This indicates that the *ura4* DSE is able to increase the efficiency of transcription termination at a heterologous location, but is sensitive to distance or context effects. When efficient transcription termination was observed, this was associated with increased signal over the probes immediately downstream of the DSE. This is consistent with the DSE acting as a polymerase pause element (Birse *et al.*, 1997) and may increase termination efficiency by pausing the polymerases in the vicinity of the poly(A) signal so that the off-loading of processing factors from the C-terminal domain (CTD) of pol II onto the RNA is enhanced (McCracken *et al.*, 1997). Insertion of a fragment encompassing all the previously defined *ura4* 3′ end formation signals (Humphrey *et al.*, 1994) 231 bp downstream of the poly(A) site produced more efficient transcription termination (Figure 7A, panel 4) than the DSE inserted at either position. It seemed plausible, therefore, that the inserted *ura4* poly(A) signals are more efficient than those of *nmt1*, resulting in the more efficient termination observed. However, as shown in Figure 7B, the p43B+pA plasmid still produces transcripts that exclus-

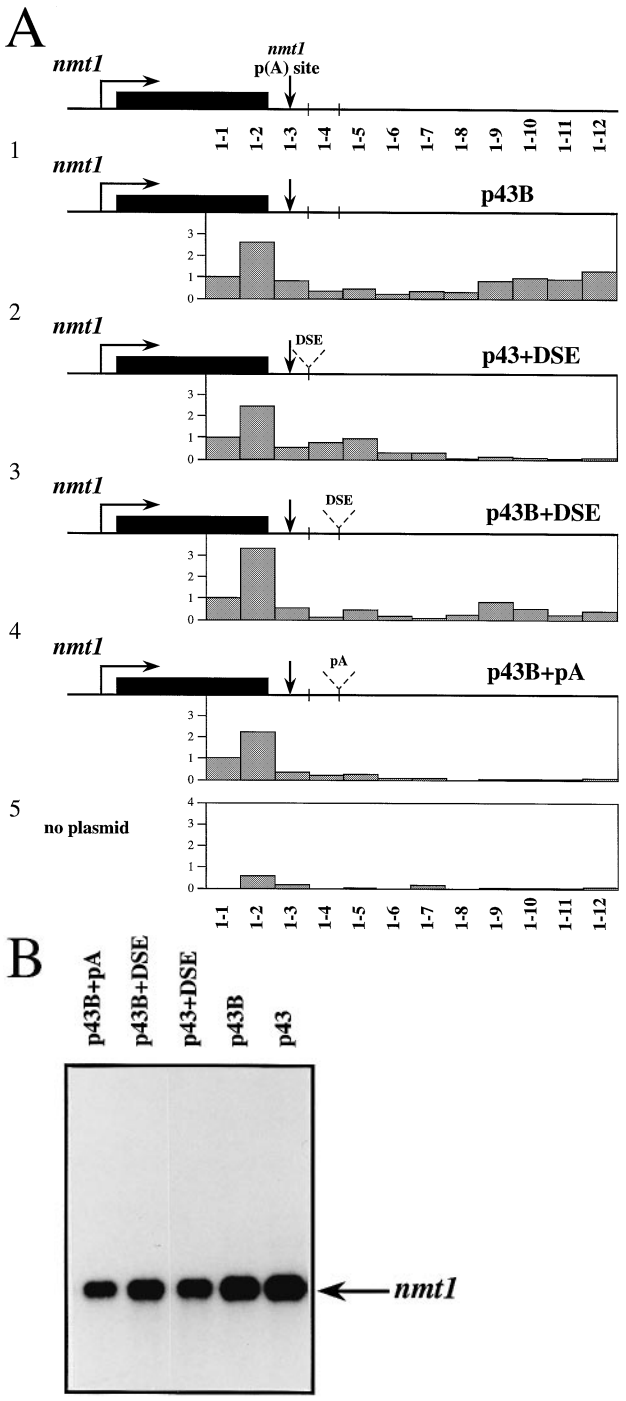

Fig. 7. Manipulation of the *nmt1* polymerase profile. (**A**) The constructs indicated were used to transform *nmt1* disruption strain Sp204, and TRO analysis was performed. The signals were quantitated after RNase A treatment, corrected for the M13 background (M13mp18 without an insert) and for the U content of the nascent RNA hybridizing to each probe, and expressed relative to the signal for probe 1-1, except for the host strain Sp204 (panel 5, no plasmid) where the signal for the H2A probe was used as an internal control to express the signals on the same scale as Sp204 transformed with p43B (panel 1). The vertical arrow indicates the major *nmt1* poly(A) site. The *ura4* DSE or *ura4* poly(A) signals were cloned either into the *Pml*I site 112 bp downstream of the *nmt1* poly(A) site or into the *Bgl*II site 231 bp downstream of the *nmt1* poly(A) site. (**B**) Northern analysis of total RNA isolated from Sp204 transformed with the constructs indicated and grown in the absence of thiamine. The RNA was resolved on a 1.6% formaldehyde–agarose gel overnight, transferred to a nylon membrane and probed with a random-primed probe of the *Xho*I–*Nco*I-specific fragment.

Table I. List of oligonucleotides used as primers

Oligo	Location	Sequence
4783	oligo(dT)	CCT GAA TTC AGT TTT TTT TTT TTT TTG
4784	oligo(dT)	CCT GAA TTC AGT TTT TTT TTT TTT TTA
4785	oligo(dT)	CCT GAA TTC AGT TTT TTT TTT TTT TTC
5906	*nmt1*: 2163–2180	CGG GAT CCC GAG GGA GGT GTC TTC AGC G
5907	*nmt2*: 1077–1096	CGG GAT CCC GGT ATG GAG CTT TCT GAA T
6331	*ura4*: 1622–1607	ATG CTA GCA TCA TTA CAA GTC TAA
8174	H2A.1: 26–45	CGG GAT CCC GGC TGC TGT TGC CAA ATC TG
8175	H2A.1: 368–390	CGG GAT CCC TGA CTA GGC TTT CCA GTG
8176	18S: 395–416	CGG GAT CCC TGA GAA ACG GCT ACC ACA TC
8177	18S: 629–650	CGG GAT CCC AGG CTC AAA GTT CAA CTA CG
8759	*nmt2*: 1656–1676	AGC CTA GGT CAT ATT GAT TGT ATT AAT TGC
8760	*nmt2*: 1219–1195	AGC CTA GGT CTT TAC AAG TAC TTT TCA TTA ACA G
8834	*nmt1*: 2590–2569	TGA AGT CAA GCT CAT AGA CTC G
8835	*nmt1*: 2591–2612	GAT CTA GAA GGC ATT AAA TCG T
9074	*nmt2*: 2142–2162	AGC CTA GGT TCA ATG GAT ATG GCG AAG TCG
9094	*nmt2*: 2134–2110	TAA ACC ACC AAT CGT CTG TAT TAG C
9271	*nmt1*: 5865–5845	TGC CTG ATA AAT TCA TTT CGT
9314	*nmt2*: 1218–1239	AGA AAA TAG TGT ACT AGC AAC C

ively utilize the *nmt1* poly(A) signal. We conclude that the *nmt1* poly(A) signals are efficient and effectively outcompete the *ura4* poly(A) signals. However, these latter signals may also act as transcriptional pause sites, accounting for their dominant effect in promoting efficient termination of *nmt1* primary transcription. These results demonstrate, therefore, that the failure of *nmt1* transcription to terminate may be ascribed to the absence of poly(A)-proximal pause sites rather than to the lack of efficient poly(A) signals.

Discussion

The aim of this study was to map polymerase density in the 3′-flanking region of two highly expressed *S.pombe* genes *nmt1* and *nmt2* using TRO analysis, identify sites of transcription termination and investigate the signals required. Surprisingly, it was found that polymerases transcribe for distances in excess of 2 kb beyond the poly(A) site, in both cases reading through a convergent downstream transcription unit, without apparently affecting the steady-state level of expression of either downstream gene. These data contrast with all previous studies of yeast pol II genes, which have suggested that transcription termination occurs within 400 bp of the poly(A) site (Osborne and Guarente, 1989; Russo and Sherman, 1989; Hyman and Moore, 1993; Birse *et al.*, 1997). Transcription interference between pol II transcription units has been demonstrated in both yeast and mammals for genes transcribed in the same direction. Although convergent transcription may also pose problems, it is apparent from these studies that expression of the *avn2* or *gut2* genes is not significantly affected by such transcription. It is possible, therefore, that precise termination of transcription following a poly(A) signal may not be required for the expression of the many convergent genes in yeast.

Our analysis of the RNA 3′ end formation signals of *nmt1* and *nmt2* emphasizes the importance of both poly(A) signals and transcriptional pause sites for efficient termination. For *nmt2*, the poly(A) signals are required for efficient termination, which is enhanced by redundant pause signals at the 3′ end of the convergent *avn2* gene. In contrast, although the *nmt1* gene possesses an efficient poly(A) signal, termination of transcription requires additional pause signals which can be provided by inserting *ura4* RNA 3′ end signals downstream from the *nmt1* poly(A) site. The absence of such signals in the wild-type *nmt1* 3′-flanking region accounts for the extensive nascent transcription that extends throughout the convergent *gut2* gene. Therefore, it is apparent that both poly(A) signals and pause sites are required for pol II termination and that their relative strengths and positions will determine the extent of nascent transcription.

The mechanism by which transcriptional pause sites enhance termination is thought to reflect the close connection between polyadenylation and transcription. Thus, pausing of polymerases in the vicinity of the poly(A) signals may facilitate the off-loading of processing factors from the CTD of pol II onto the RNA (McCracken *et al.*, 1997). Consistent with this, the efficient transcription termination observed when the *ura4* DSE pause sequence was cloned 112 bp downstream of the *nmt1* poly(A) site was associated with a build-up in polymerase density over probes immediately downstream. Indeed, a reduced termination effect was observed when the DSE was cloned further downstream, and no increase in polymerase density was observed over 3′ probes. Perhaps polymerase pausing is enhanced by a temporary association between the processing factors during transfer to the RNA from the CTD. For *nmt2*, an increased signal has been observed over probe 2-5 in the normal context, and over probe 2-7 (Figure 6A, p2VDA+2pA) or probe 2-10 (data not shown) where deletions have brought novel sequences to a position downstream of the *nmt2* poly(A) signal. This may indicate that many sequences possess an intrinsic ability to pause polymerases and/or a connection with poly(A) signal recognition as suggested above.

The results presented here are consistent with either of two models previously suggested to account for the coupling of transcription termination and polyadenylation. Transfer of processing factors from the CTD of the polymerase onto the nascent RNA (McCracken *et al.*, 1997) may modify the elongation complex, making it

Table II. Single-stranded M13 probes

Probe	Fragment	Location	Length	Us(+)	Us(–)
nmt1					
1-1	*Eco*RI*–*Bst*EII*	1797–2013	216	64	62
1-2	*Bst*EII*–*Dra*I	2009–2216	207	59	49
1-3	*Dra*I–*Bsa*AI	2217–2473	256	75	89
1-4	*Bsa*AI–*Dra*I	2474–2651	177	64	55
1-5	*Dra*I–*Xmn*I	2652–2904	252	94	75
1-6	*Xmn*I–*Dde*I*	2905–3123	218	65	83
1-7	*Dde*I*–*Afl*II*	3121–3305	184	59	69
1-8	*Afl*II*–*Hin*dIII*	3302–3465	163	55	55
1-9	*Hin*dIII*–*Xho*I*	3462–3685	223	65	64
1-10	*Xho*I*–*Hph*I*	3682–3868	186	58	52
1-11	*Hph*I*–*Bst*NI*	3868–4088	220	55	66
1-12	*Bst*NI*–*Nco*I*	4088–4290	202	56	49
1-13	*Nco*I*–*Ava*II*	4287–4479	192	59	43
1-14	*Ava*II*–*Hin*fI*	4477–4710	233	63	78
1-15	*Hin*fI*–*Dra*I	4708–4913	205	64	52
1-16	*Dra*I–*Dra*I	4914–5153	239	74	68
1-17	*Dra*I–*Ssp*I	5154–5394	240	89	46
1-18	*Ssp*I–*Dra*I	5395–5655	260	90	88
1-19	*Dra*I–oligo 9271	5656–5865	209	84	56
1-20	*Sau*3AI*–*Rsa*I	5866–6138	272	99	56
1-21	*Rsa*I–*Bgl*II*	6139–6436	297	89	92
1-22	*Bgl*II*–*Cla*I*	6433–6675	242	71	91
nmt2					
2-1	*Hpa*II*–*Dde*I*	820–1024	204	40	71
2-2	*Dde*I*–*Sca*I	1022–1209	186	49	59
2-3	*Sca*I–*Bgl*II*	1210–1469	259	84	100
2-4	*Bgl*II*–*Hpa*II*	1466–1686	220	79	83
2-5	*Hpa*II*–*Cla*I*	1685–1857	172	64	53
2-6	*Cla*I*–*Dra*I	1855–2133	278	115	69
2-7	*Dra*I–*Ava*II*	2134–2303	169	52	52
2-8	*Ava*II*–*Sty*I*	2301–2563	262	57	90
2-9	*Sty*I*–*Hae*III	2560–2812	252	58	94
2-10	*Hae*III–*Sph*I*	2813–2996	183	40	63
2-11	*Sph*I*–*Nde*I*	2997–3230	233	59	72
2-12	*Nde*I*–*Afl*II*	3229–3360	131	33	44
2-13	*Afl*II*–*Hin*dIII*	3357–3668	311	107	95
Other					
leu1	*Sty*I*–*Dpn*I	65–452	387	91	114
LEU2	*Afl*II*–*Eco*RV	315–479	435	133	122
18S	oligo 8146–8177	395–650	255	79	52
H2A	oligo 8174–8175	26–390	364	74	108

The Us(–) indicates the number of Us in the sense transcript hybridizing to the (–) orientation M13 probe, and Us(+) indicates the number of Us in a transcript hybridizing to the (+) orientation M13 probe. An * indicates a restriction site was filled-in using Klenow before the fragment was subcloned.

competent for transcription termination (Logan *et al.*, 1987). Alternatively, actual cleavage of the RNA may be required to generate an uncapped 5′ end susceptible to exonucleolytic degradation. Transcription termination then occurs when the exonuclease reaches and disrupts the ternary elongation complex (Connelly and Manley, 1988; Proudfoot, 1989). The true situation may include elements of both models. Thus, a polymerase pause site positioned close to the poly(A) site could facilitate the transfer of processing factors to the RNA, which may be of greater importance in yeast where poly(A) signals are less highly conserved. A pause element located further downstream could also play a role in slowing down polymerase elongation, so allowing an exonuclease to catch the polymerase. In both models, it is the poly(A) signal which is absolutely required and marks the end of the gene. A final solution of the mechanism of transcriptional termination by pol II will require a full biochemical dissection of this elusive process.

Materials and methods

Schizosaccharomyces pombe strains

The stains used were, wild-type strain 972h$^-$; Sp204 (*nmt1::ura4 leu1.32 ade6-704 ura4-D18 h*$^+$) (gift of K.Maundrell) and Sp92 (*nmt2:ura4 leu1.32 ade6-704 ura4-D18 h*$^+$) (gift of K.Maundrell). The *nmt1* and *nmt2* disruption strains contain a 1.76 kb fragment encompassing the *ura4* coding region inserted in place of an *Xho*I–*Nco*I fragment of the *nmt1* coding region or a *Pst*I–*Bst*XI fragment of the *nmt2* coding region, respectively.

Growth and transformation of S.pombe

Techniques for the growth and maintenance of *S.pombe* are described in Moreno *et al.* (1991). The *S.pombe* were transformed using the LiCl method (Bröker, 1987).

Numbering

For *nmt1/gut2*, +1 is the start of the *Bcl*I site upstream of the *nmt1* ORF. For *nmt2/avn2* and *ura4*, +1 is the start of the *Hin*dIII site upstream of the *nmt2/ura4* ORFs, respectively. For other genes, the translation start site is +1 except 18s rRNA where +1 is the transcription start site.

PCR primers

A list of oligonucleotides used as primers is given in Table I.

p41 constructs

The fragments indicated were produced by restriction of an appropriate plasmid or generated by PCR with or without restriction of the PCR product, blunted and cloned into the *Stu*I site within the coding region of *nmt1* in plasmid p41 (p*nmt41*; Maundrell, 1990). All constructs were confirmed by sequencing. All fragments were inserted in both the forward (+) and reverse (–) orientations.

M13 constructs

Single-stranded DNA probes for TRO analysis were constructed by subcloning appropriate fragments into the *Hin*cII site of M13mp18 (RF) and confirmed by sequencing the M13 single-stranded DNA (probes are listed in Table II). An asterisk indicates that a restriction site was filled-in using Klenow before the fragment was subcloned. The H2A probe fragment was generated by PCR from *S.pombe* genomic DNA using primers 8174/8175, restricted with *Bam*HI and cloned into the *Bam*HI site of M13mp18 (RF). The 18S probe fragment was generated by PCR as above, using primers 8176/8177, restricted with *Bam*HI and cloned into the *Bam*HI site of M13mp18 (RF).

nmt1 3′-flanking sequence

Cosmid clones were received from the RLDB (Maier *et al*., 1992; Hoheisel *et al*., 1993) and contained fragments subcloned into the *Bam*HI site of LAWRIST4. Restriction fragments identified by Southern analysis were subcloned and sequenced on both strands using an Applied Biosystems 310 sequencer. An *Nco*I*–*Nco*I* fragment encompassing 2.17 kb of the *nmt1* 3′-flanking region and a *Ban*II*–*Ban*II* fragment from cosmid clone ICRFc60B119D were cloned into the *Bam*HI* site of pGEM4 to give plasmids pGNc and pGBn, respectively. Plasmid pGBm contains a *Bam*HI–*Bam*HI fragment from cosmid clone ICRFc60B0125D, cloned into the *Bam*HI site of pGEM4.

TRO constructs

Plasmid p41Δ was derived from p41 by cloning the 0.99 kb *Bcl*I–*Hin*dIII fragment from p41 into the vector backbone of p41 restricted with *Bam*HI and *Hin*dIII. Plasmid pGHd contains the *Hin*dIII–*Hin*dIII fragment encompassing the *nmt1* ORF from p41, cloned into the *Hin*dIII site of pGEM4 (Promega). Plasmid p43 contains the *Hin*dIII–*Hin*dIII fragments from pGHd and pGNc subcloned into the unique *Hin*dIII site of p41D to give 4.3 kb of contiguous *nmt1* sequence from the *Bcl*I site (+1) to the *Nco*I site (+4286). A unique *Bgl*II site was introduced at position 2590 in p43 by PCR around the vector sequences using primers 8835/8834, and the ends were ligated to create plasmid p43B. The 132 bp *Rsa*I–*Hin*dIII* DSE fragment was inserted into the unique *Pml*I site of p43 or the *Bgl*II site of p43B. The *Avr*II*–primer 6331 (pA) fragment was subcloned into the *Bgl*II site of p43B to give p43B+pA.

Plasmid p2V was constructed by subcloning the 3.67 kb *Hin*dIII*–*Hin*dIII* fragment from p*nmt2* (Manetti *et al*., 1994) containing the *nmt2* and *avn2* ORFs, into the *Bam*HI* site pIRT2 **(**Hindley *et al*., 1987). The intergenic region (1221–2142) was deleted from p2V using the primers 8760 and 9074 to PCR around the vector sequences, followed by restriction with *Avr*II and ligation of the plasmid ends to introduce a unique *Avr*II site and create p2VΔA. The 2pA and 2P fragments were generated by PCR using primers 9314/9094 or 8759/9094, respectively, followed by restriction with *Hpa*II, blunted and cloned into the *Avr*II* site of p2VΔA to give clones p2VΔA+2pA and p2VΔA+2P.

RNA analysis

Total RNA was prepared using a protocol for the isolation of RNA from yeast ascospores (Köhrer and Domdey, 1991). Northern blot analysis was as described previously (Humphrey *et al*., 1994). RT–PCR mapping was based upon the method described by Sadhale *et al*. (1991), except that an anchored oligo(dT) primer, degenerate in the last nucleotide, was employed in a reverse transcription reaction using 1–10 μg of total RNA (strain 972h$^-$) and in a separate PCR with a gene-specific primer 5906 (*nmt1*) and 5907 (*nmt2*) to amplify specific cDNA products, which were subcloned into pGEM4 and sequenced.

TRO analysis was essentially as described previously (Birse *et al*., 1997). Briefly, 300 ml cultures were grown overnight in EMM (Moreno *et al*., 1991), supplemented as required, to an OD_{595} of 0.1. The cultures were cooled quickly, and the cells were harvested and washed in 5 ml of ice-cold TMN (10 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$, 100 mM NaCl). The pellet was re-suspended in 0.9 ml of ice-cold water and 0.5% *N*-lauryl sarcosine sulfate was added and incubated on ice for 20 min. The cells were pelleted and the supernatant removed, before re-suspension in 1× transcription buffer (20 mM Tris–HCl pH 7.7, 200 mM KCl, 32 mM $MgCl_2$), containing 2 mM dithiothreitol (DTT), 666 mM each ATP, CTP and GTP and 100–200 μCi of [α^{32}P]UTP. The reaction was allowed to proceed for 2–5 min at 20°C. Cells were washed with TMN and total RNA was extracted. The labelled RNA was partially hydrolysed in 0.2 M NaOH for 6 min on ice. After neutralization (addition of 0.2 M Tris–HCl, pH 7.2), samples were hybridized directly to excess single-stranded M13mp18 probes and immobilized on a nylon filter (Hybond-N, Amersham) using a Bio-Rad Bio-Dot SF slot-blot (5 μg of single-stranded DNA per slot), in 50% formamide/10× Denhardts/5× SSPE/0.2% SDS containing 100 μg/ml tRNA (*Escherichia coli*, Boehringer Mannheim) at 42°C. Partial hydrolysis was confirmed by running an aliquot of RNA before and after hydrolysis on a polyacrylamide gel. Filters were rinsed and washed in 2× SSC/0.1% SDS for 20 min and in 0.2× SSC/0.1% SDS heated to 65°C, for 20 min at 20°C. Filters were also treated with RNase A by washing for 30 min at 20°C in 2× SSC containing 1 mg/ml RNase A and rinsed three times, for 2 min each, in 2× SSC. Filters were covered in Saran wrap and exposed to film/PhosphorImager screen for quantitation after each wash.

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