Nucleotide Sequences Surrounding the Nonanucleotide Promoter Motif Influence the Activity of Yeast Mitochondrial Promoter†

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ABSTRACT: The highly conserved nonanucleotide (5′-TATAAGTAA[+2]) promoter sequence dictates initiation of gene-specific transcription by the mitochondrial (mt) RNA polymerase in yeast mitochondria. However, transcriptional efficiency of the nonanucleotide promoter in different mt genes varies severalfold. To explore the regulatory role of the promoter-proximal template sequence in mt transcription, different deletion, nucleotide (nt) substitution, and tandem promoter constructs were analyzed under in vitro transcription reaction conditions. It has been found that the conserved nonanucleotide promoter plus more than 9 nt of nonconserved sequence 3′ to the promoter were absolutely essential for mt gene-specific transcription. In addition, approximately 300 nt of nonspecific DNA sequence 5′ to the promoter was also important for efficient transcription. Interestingly, introduction of consecutive T residues in the early transcribed sequence of the template strongly inhibited mt transcription at low nt concentrations (i.e., 5 μM UTP). In contrast, neither other nt clusters nor a bacterial terminator-like sequences at that location inhibited mt transcription. Under the nonproductive reaction conditions, the full-length transcript from the mt polyT template was drastically reduced with the formation of several short abortive oligoribonucleotides. These results suggest that the transcriptional efficacy of the yeast mt promoter is influenced by sequence 3′ to the promoter.

The biogenesis of mitochondria depends on the expression of both the mt and the nuclear genomes (*1*, *2*). The 78 000 base pairs of yeast mt[1] DNA carry 35 genes that code for two ribosomal RNAs (21S and 14S), 24 transfer RNAs, an RNA subunit of mt RNase P, one ribosomal protein, and seven other proteins functioning in the respiratory chain (*3*, *4*). The rest of the mt proteins including those involved in mt DNA, RNA, and protein synthesis are encoded by nuclear genes and imported into mitochondria.

The mt genes in *Saccharomyces cerevisiae* are transcribed singly or in clusters from conserved nonanucleotide (TATAAGTAA[+2]) promoter sequences (*5*−*10*). A single mt RNA polymerase, which consists of two nuclear DNA-encoded protein subunits (a 145 kDa core polymerase and a 43 kDa specificity factor) (*11*−*16*), is responsible for transcription of all classes of mt genes. The same mt RNA polymerase might be also involved in priming of mt DNA replication (*17*, *18*). It has been shown by in vitro transcription studies that the mt RNA polymerase, like *Escherichia coli* (*19*) and bacteriophage T3/T7 RNA polymerases (*20*), fully commits to transcription only after making an RNA of 11 nt or longer (*21*, *22*). In addition to this functional similarity, the mt RNA polymerase also exhibits structural homology with the T3/T7 RNA polymerases (*16*).

Interestingly, the transcriptional rate of different mt genes varies as much as 15−20-fold even though they are transcribed from the canonical nonanucleotide promoter sequences by the same RNA polymerase (*23*−*26*). More surprisingly, some of the conserved promoter sequences are transcriptionally silent in vivo (*6*, *10*) as well as in vitro (*26*), suggesting that the transcriptional efficiency of these nonanucleotide promoter motifs varies due to the influence of their flanking sequences. Recently, it has been reported that productive initiation and processivity of T7 RNA polymerase transcription are influenced by an early transcribed sequence (*27*). To examine a similar possibility with the mt RNA polymerase, we have investigated the regulatory role of template sequences(s) surrounding the nonanucleotide promoter. Previously, we noticed that the promoter-proximal polyT sequence inhibits mt transcription under certain reaction conditions (*28*). To gain further information about the regulation of mt promoter function, we have prepared a series of 3′ and 5′ deletion mutants and analyzed them in the in vitro transcription reaction. Furthermore, different synthetic mt promoters carrying variable 3′ flanking sequence proximal to the promoter were also generated and characterized to explore the structural feature(s) of the early transcribed sequence influencing mt promoter function. The results presented here reveal three additional pieces of information about the regulation of mt transcription. (i) The nonanucleotide conserved motif and more than 9 nt 3′ to the promoter are absolutely essential for promoter-specific transcription. (ii) A 300 nt of nonspecific sequence 5′ to the promoter is also required for optimal mt transcription. (iii)

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[1] Abbreviations: mt, mitochondrial; nt, nucleotide(s); polyT, consecutive thymidine nucleotide cluster; wt, wild-type.

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A homopolymeric thymidine nt sequence in the early transcribed sequence, but no other nt clusters or bacterial terminator-like sequences tested, inhibits mt run off transcription but still allows abortive initiation of transcription.

MATERIALS AND METHODS

Chemicals and Enzymes. Nucleoside triphosphates were obtained from Pharmacia. Radioactive nt were purchased from Amersham Life Science, Inc. Restriction enzymes were from New England Biolabs. The mt RNA polymerase was isolated from yeast cells (strain D273−10B) grown to late-log phase as described previously (*8*, *13*).

Generation of mt Promoter Constructs. Deletion mutagenesis of the mt DNA template was carried out by *Bal*31 digestion. The synthetic mt promoter sequences were made by an oligonucleotide synthesizer. The oligonucleotide duplex containing the mt promoter sequence was ligated into the *Eco*RI/*Hin*dIII cloning sites of pUC19 and then transformed into *E. coli*. Plasmid DNA was isolated and the promoter sequence was confirmed by DNA sequencing.

In Vitro Transcription Assay. In vitro transcription was carried out using the following reaction conditions unless otherwise indicated. In a 25 μL reaction, 12.5 μL of 2× transcription buffer (20 mM Tris-HCl, pH 7.9, 40 mM KCl, 20 mM $MgCl_2$, 10% glycerol, 0.4 mg/mL rabbit serum albumin, 250 μM each of ATP, GTP, and CTP, and 250 μM or 10 μM of UTP), 10−50 μCi of [^{32}P]UTP or -ATP, and 1 μg of linear DNA template were added together on ice, and then the reaction volume was brought up to 24 μL with sterile distilled water. The transcription reaction was started by the addition of 1 μL of mt RNA polymerase and incubated at 30 °C for 10 min. The reaction was terminated by 25 μL of stop solution (0.3% SDS, 200 μg/mL tRNA) followed by phenol extraction. The RNA products were precipitated with 0.25 vol of 8 M ammonium acetate and 2 vol of ethanol and collected by centrifugation. The RNA pellets were suspended in 5 μL of sample buffer (90% formamide, 50 mM Tris-borate, pH 8.3, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue) and then loaded onto a 6% polyacrylamide-8 M urea gel. After electrophoresis, the RNA bands were displayed by autoradiography on Kodak XAR-5 film. The transcript levels were measured by scanning the intensity of transcript bands on the autoradiograms using a computing densitometer (Molecular Dynamics). Different exposures of the film were employed to keep the transcript band intensity in the linear range, and the gel background intensity was also subtracted from the transcript band intensity in the final quantitative measures. In one set of experiments, 5 μL of the whole transcription mixture was loaded onto a 22.5% polyacrylamide-8 M urea gel to detect short transcripts (e.g., oligoribonucleotides).

RESULTS

The highly A + T-rich yeast mt genome carries many "TATAAGTA"-like sequence motifs but only a fraction of them are used as promoters by the mt RNA polymerase (*6*, *10*). At least three of these active promoters (i.e., $tRNA^{Thr-ACN}$, $tRNA^{Cys}$, Oli1−2; see Figure 1) exhibit weak transcriptional activity due to the negative influence of their +2T nt (*23*−*25*, *29*). The other promoter-like sequences in the mt genome are apparently silent. Some of these inactive promoter-like sequences carry a nt substitution in the conserved promoter motif (Figure 1); however, our prior in vitro promoter sequence analysis (*8*, *10*) demonstrated that these nt changes do not inactivate the mt promoter function. Moreover, the TAAAAGTA, TATAAGAA, and TATAAGTAT promoter variants are used in vivo for transcription of the mt *COX*2 (30), *RPM*1(31) and $tRNA^{Cys}$ genes (*32*), respectively. These observations suggested that the transcriptional activity of a few nonanucleotide promoter sequences is negatively regulated by template sequence(s). Interestingly, our earlier work demonstrated that the thymidine nucleotides 3′ to the promoter inhibit mt transcription in vitro (*24*, *26*, *28*). To further explore the regulatory role of different template sequences in mt transcription, mt promoter activity was assayed in the context of different flanking sequences.

Transcriptional Activity of mt Promoter with Different Flanking Sequence. In the prior deletion analysis of the yeast mt promoter sequence, the activity of mt promoter mutants was assayed in the context of flanking plasmid sequences (*7*). To determine any influence of these plasmid sequences in mt transcription, we have examined the activity of different mt promoter constructs with or without the plasmid sequence. Four different 3′ deletion mutants (i.e., pTB3′ + 64, pTB3′ + 31, pTB3′ + 15, and pTB3′ + 4) of the 14S rRNA gene promoter were used for the 3′ sequence analysis (Figure 2A). These 3′ deletion mutants have an identical promoter and the same 5′-flanking sequence but carry the indicated lengths of 3′ mt DNA sequence (i.e., in the pTB3′ + 64 plasmid, all mt DNA sequence downstream of the 64th nt from the initiating site was deleted and replaced by the plasmid sequence). For runoff transcription, these templates were cleaved with *Eco*RI to keep only the mt DNA sequence or with *Pvu*II to provide an extra 95 nt plasmid sequence downstream of the mt DNA insert. *Pvu*II digestion produced blunt-ended DNA fragments whereas *Eco*RI generated linear templates with 4 nt 5′ over-hangs. A *Pvu*II-digested pTB3′ + 64 template carrying 165 nt of transcribed sequence (64 nt mt DNA sequence plus 101 nt plasmid sequence) or an *Eco*RI-digested pTB3′ + 64 template carrying 69 nt of transcribed sequence (64 nt mt DNA sequence plus 5 nt *Eco*RI linker sequence) was used as an internal control in the right or left panel of Figure 2A. Since the size of these transcripts was expected to be different, the transcription reactions were carried out with γ[^{32}P]ATP to label the 5′ end of RNA products. Under these reaction conditions, the radioactivity incorporated into each band would be directly proportion to the amount rather than the length of transcript. Interestingly, all four *Pvu*II-digested templates exhibited equal activity (Figure 2A, left panel), suggesting that no specific mt DNA sequence downstream of position +4 is important for mt promoter function. However, when the 3′ plasmid sequence was removed by *Eco*RI digestion, their transcriptional activities differed significantly (Figure 2A, right panel). The *Eco*RI-digested pTB3′ + 64, pTB3′ + 31, and pTB3′ + 15 templates behaved like their parent templates (Figure 2A right panel, lanes 1−3), whereas the *Eco*RI-cleaved pTB3′ + 4 template carrying 9 nt downstream sequence (4 nt mt DNA sequence plus 5 nt *Eco*RI linker sequence), sustained <10% of its original activity (lane 4). This result suggested that more than 9 nt nonspecific template sequence is necessary for effective mt promoter function.

Similarly, the role of 5′ flanking sequence was also examined using three different 5′ deletion mutants (pTB5′

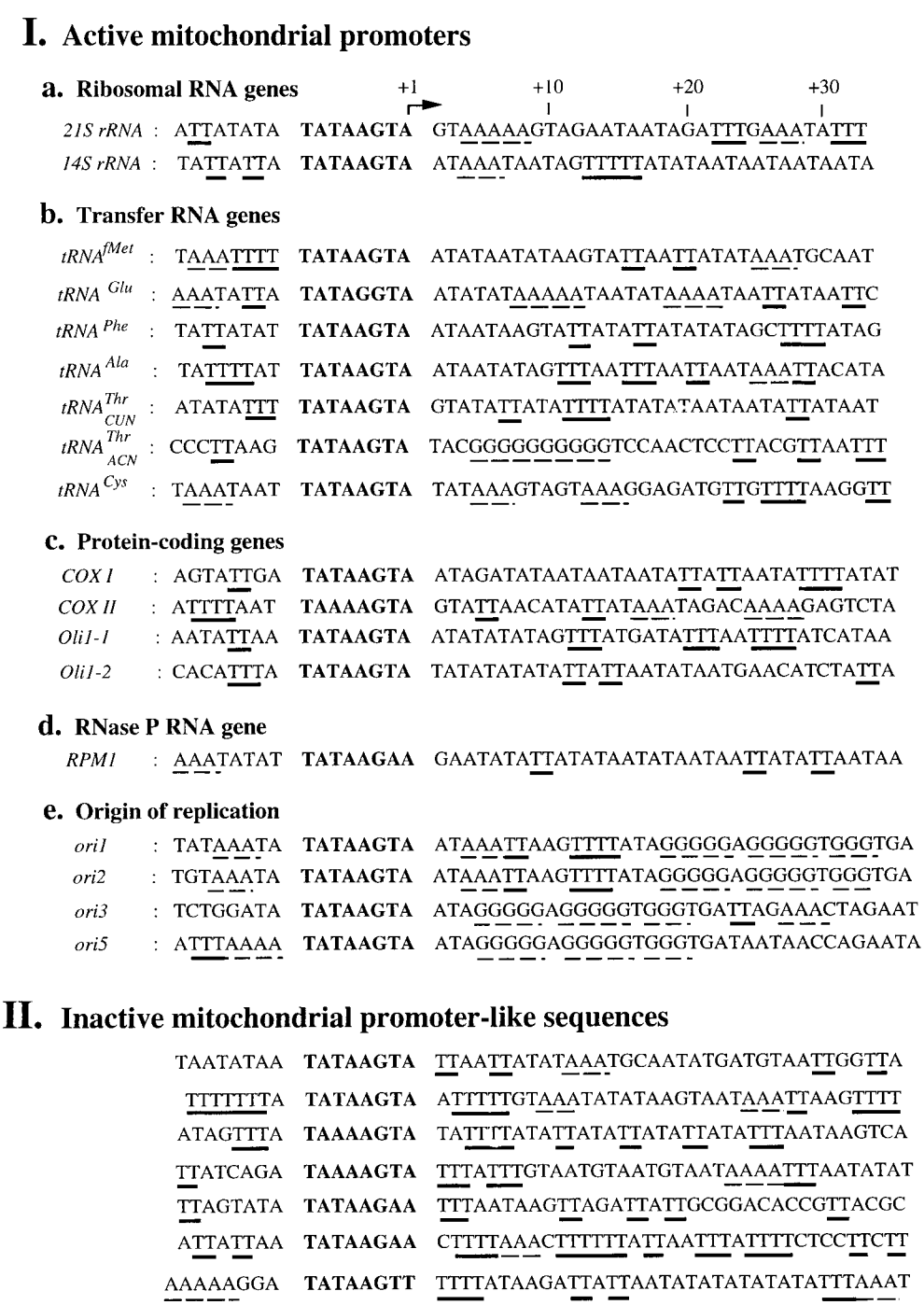


FIGURE 1: The active promoters and the inactive promoter-like sequences in the yeast mt genome. The conserved promoter sequence is highlighted by bold letters and the transcriptional start site is shown by a bent arrow line. The polyT clusters (≥2 nt) are underlined and the other homopolymeric sequences (i.e., polyA or polyG) are shown by the dashed underlines.

− 234, pTB5′ − 125 and pTB5′ − 10) of the 14S rRNA gene promoter. These mt DNA templates were almost identical except that they have different sizes of the upstream mt DNA sequence (Figure 2B). For in vitro run off transcription, these 5′ deletion templates were linearized with *Cla*I 145 nt downstream from the initiating site whereas the control 14S rRNA template was cleaved with *Eco*RI 69 nt downstream from the transcriptional start site. The *Cla* I and *Eco*RI-digested templates carried 2 nt and 4 nt 5′ overhangs, respectively. Interestingly, these *Pvu*II-digested templates exhibited similar transcriptional activity (Figure 2B, lanes 1, 3, and 5), suggesting that the mt DNA sequence upstream of −10 position is not required for mt promoter function. However, when the upstream plasmid sequence of these templates was removed by *Eco*RI digestion, their transcriptional activity dropped significantly (Figure 2B, lanes 2, 4, and 6). For example, the *Cla*I/*Eco*RI double-digested templates containing 234, 125, or 10 nt upstream mt DNA sequence plus 5 nt *Eco*RI-linker sequence, exhibited 70, 30, or 18% of the transcriptional activity seen with the *Eco*RI-digested control template. This result suggested that a 10 nt upstream mt DNA sequence is sufficient for mt promoter activity but an additional 300 nt nonspecific upstream sequence is required for optimal mt transcription.

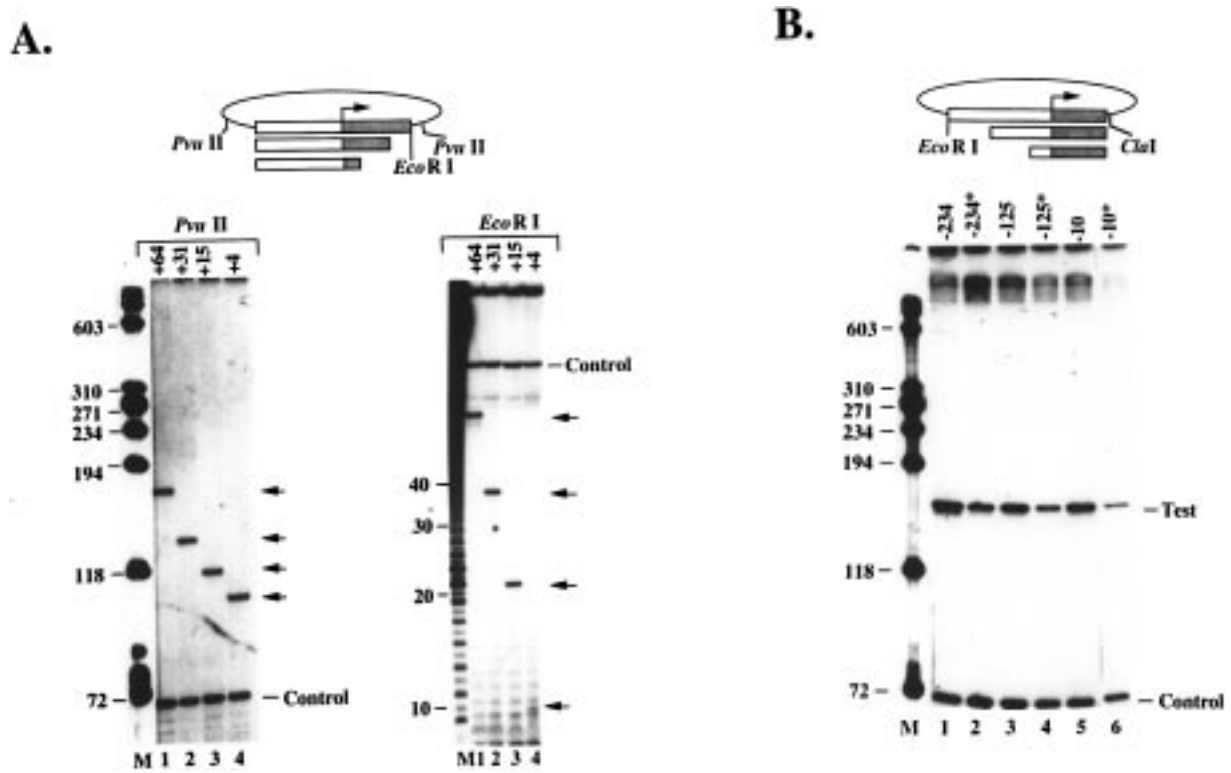


FIGURE 2: (A) The transcriptional activity of the 3′ deletion mutants of the mt 14S rRNA gene promoter. The DNA templates were linearized with *Pvu*II (left panel) or *Eco*RI (right panel) digestion which produced linear templates with blunt ends or 4 nt 5′-over-hangs. The positive number on the top of each lane represents the downstream mt DNA sequence retained in the 3′ deletion clones. The *Pvu*II-digested templates carried 159, 126, 110, and 99 nt transcribed sequence (3′ mt DNA sequence plus 95 nt plasmid sequence), respectively. The *Eco*RI-digestion of the same templates retained 69, 36, 20, and 9 nt of transcribed sequence (mt DNA sequence plus 5 nt *Eco*RI linker sequence), respectively. The transcript from the control TB3′Δ + 64 template was 69 nt long when digested with *Eco*RI (left panel) or 159 nt long when digested with *Pvu*II (right panel). The transcripts were labeled at the 5′-end with the initiating nt $\gamma[^{32}P]$ATP. (B) The transcriptional activity of the 5′ deletion mutants of the mt 14S rRNA gene promoter. The negative number on the top of each lane represents the upstream mt DNA sequence retained in the 5′ deletion clone. The templates used in lanes 1, 3, and 5 were linearized with *Cla*I whereas the templates used in lanes 2, 4 and 6 (marked by asterisk) were digested with both *Cla*I and *Eco*RI. The *Cla*I and *Eco*RI-digested templates carried 4 nt and 2 nt 5′-over-hangs, respectively. The *Eco*RI-digested TB3′Δ + 64 template of the mt 14S rRNA gene promoter was used as an internal standard. The top band (145 nt) and the bottom band (69 nt) were from the test and control templates, respectively.

Effect of Promoter-Proximal 3′ Sequence in mt Transcription. The afore-described deletion analysis indicated that the mt RNA polymerase requires the nonanucleotide promoter and more than 9 nt of 3′ sequence for effective transcription. Moreover, a sequence comparison between the strong, weak, and inactive mt promoter elements revealed that the inactive promoter-like sequences carry a thymidine nt cluster in the early transcribed sequence, the weak promoters carry a T residue at position +2 whereas strong promoters contain none of these T residues (Figure 1). This observation suggested that the mt promoter activity might be negatively influenced by an early transcribed sequence. Interestingly, the presence of another homopolymeric nt cluster in the early transcribed sequence does not inactivate the mt promoter activity (i.e., the polyA in the *21S rRNA*, *14S rRNA*, $tRNA^{Glu}$, $tRNA^{Cys}$ genes or the polyG in the $tRNA^{Thr-ACN}$ gene) (Figure 1), further indicating that the promoter-proximal polyT clusters are uniquely responsible for the inactivation of mt promoter function.

To explore the effect of the early transcribed sequence in mt promoter function synthetic mt promoters carrying different 3′ flanking sequences (i.e., homopolymeric nt cluster or bacterial terminator-like sequence) were constructed and analyzed in vitro. These nt sequences were introduced at position +6 of the synthetic mt promoter constructs (Figure 3) to approximate the location of the polynucleotide sequences shown in Figure 1. In each promoter construct, the +5 position was occupied by a T residue. Although the homopolymeric nt templates were assayed previously at high or low concentrations of the respective nt (e.g., different concentrations of ATP for the polyA but not for the polyG, polyC or polyT templates), their activities have been reexamined here at high or low concentrations (i.e., 125 or 5 μM) of each of the four rNTPs. Under these reaction conditions, the activity of most of these templates did not change, whereas the polyT template (i.e., $6T_5$) was poorly active particularly at a low UTP concentration (Figure 3). This result confirmed our earlier observation (*28*) that the polyT-dependent inhibition of mt transcription is a unique rather than the general effect of a homopolymeric nt cluster. It is worth noting that the bacteriophage T7 RNA polymerase also terminates transcription when synthesizing U-rich but not C− or A-rich transcripts, especially at low UTP concentrations (*33*). Since the mt RNA polymerase from different batches of enzyme preparation responded similarly to the polyT inhibition, it is likely that the mt RNA polymerase itself is sensitive to the polyT sequence.

In many cases of prokaryotic and eukaryotic gene transcription, a hairpin secondary structure in the transcript results in transcriptional pause or termination (*34, 35*). To explore whether a secondary structure in the early transcribed sequence of the mt template influences the mt RNA

	Nucleotide conc. (μM)				
ATP	125	5	125	125	125
GTP	125	125	5	125	125
CTP	125	125	125	5	125
UTP	125	125	125	125	5

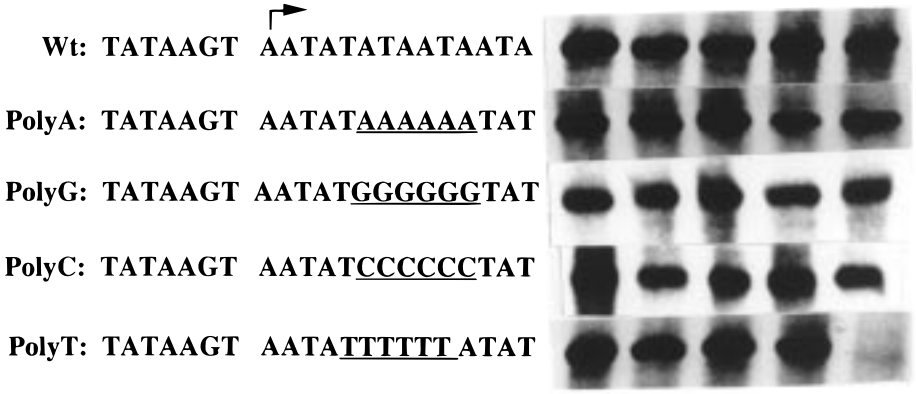


FIGURE 3: Transcriptional activity of mt promoters carrying different 3′ homopolymeric nt sequences. The activity of synthetic templates carrying six consecutive adenine, guanine, cytidine or thymidine nt at position +6 was measured in the presence of 125 μM each of 4 rNTPs or 125 μM each of three rNTPs plus 5 μM of the fourth rNTP. At low ATP concentration ApA dinucleotide was also included in the reaction to bypass the first phosphodiester bond formation of transcription requiring a high concentration of the first two nt (i.e., ATP).

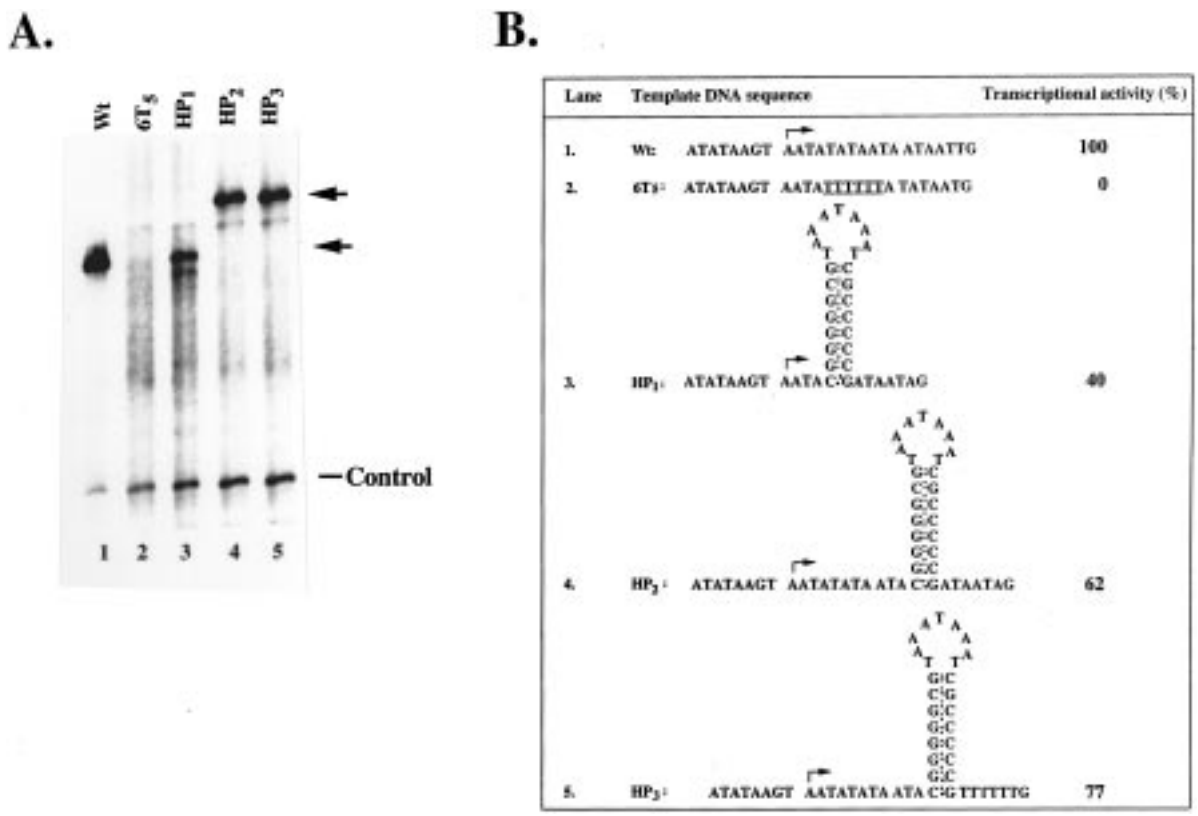
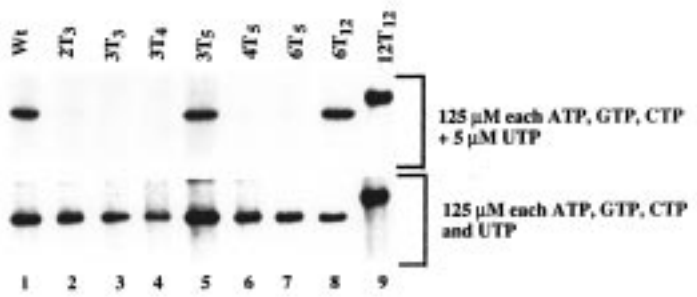


FIGURE 4: Transcriptional activity of the mt templates carrying a 3′ hairpin secondary structure. Transcription assays were carried as above except that 5 μM of UTP was used. (A) autoradiogram of the transcription gel; (B) the sequence of synthetic templates and their relative activities.

polymerase transcription, a GC-rich secondary structure was introduced at position +5 or +12 (i.e., HP1, HP2, and HP3 in Figure 4). These secondary structures were designed by comparing several prokaryotic ρ-independent terminator sequences (*36*). Transcription assays were performed as above using the $6T_5$ template as a negative control. Interestingly, the HP1 and HP2 templates containing a hairpin structure at positions +5 or +12 exhibited 40 and 60% of the wt activity, respectively (Figure 4). This result suggests that these secondary structures exhibit some position-specific inhibitory effect on mt transcription, albeit not as strong as the polyT inhibition. Since a hairpin structure followed by a

A.



B.

Lane	Template sequence	Transcriptional activity (%)	
		at 5 μM UTP	at 125 μM UTP
1	Wt : TTATATAAGTA ATATATAATATATATAGAATTC	82	100
2	$2T_3$: TTATATAAGTA ATTAATAATATATATAGAATTC	0 *	61
3	$3T_3$: TTATATAAGTA ATTTATATATATATAAGAATTC	0 *	48
4	$3T_4$: TTATATAAGTA AATTTAATATATATAAGAATTC	0 *	45
5	$3T_5$: TTATATAAGTA ATATTTAATATATAATGAATTC	98	125
6	$4T_5$: TTATATAAGTA ATATTTTATATATAATGAATTC	0 *	70
7	$6T_5$: TTATATAAGTA ATATTTTTTATATAATGAATTC	0 *	90
8	$6T_{12}$: TTATATAAGTA ATATATAATATTTTTTGAATTC	80	95
9	$12T_{12}$: TTATATAAGTA ATATATAATATTTTTTTTTTTTATGAATTC	83	112

FIGURE 5: Transcriptional activity of different polyT templates. (A) Autoradiogram of transcripts from the polyT templates carrying two to twelve successive T residues at different 3′ locations. Transcription reactions were carried out at either 5 or 125 μM UTP. The $12T_{12}$ template produced a 115 nt long transcript whereas the other templates generated a 107 nt long transcript. (B) Sequences of the polyT templates and their relative activities. The transcriptional activities of these promoters were calculated considering the wt activity at 125 μM rNTP concentrations as 100%. The reported transcript measures represent an average of two to three experiments. The asterisk (*) indicates that the transcriptional activity of these templates vary between <1 and 5% of the wt activity.

polyT cluster functions as a strong ρ-independent terminator in prokaryotes (*34*), we have also tested the effect of this sequence on mt transcription. The HP3 template carrying a ρ-independent terminator-like sequence at position +12 had 77% of the wt activity (Figure 4). This suggests that the prokaryotic terminator-like sequences that we have tested did not serve as a terminator for the mt RNA polymerase.

Inhibition of mt Transcription by a polyT Sequence. For better understanding of the inhibitory effect of the polyT sequence in mt transcription, 2−12 consecutive T residues were introduced between positions +3 and +12 of the mt DNA template. These templates were named according to the number of T residues in a cluster as well as the location of the polyT cluster with respect to the transcriptional start site (i.e., the $2T_3$ template carries a pair of T residues starting at position +3 in the nontranscribed strand). The transcriptional activity of the wt and the polyT templates was measured in the presence of 125 μM each of ATP, GTP, CTP plus 5 μM of UTP (Figure 5A, top panel) or 125 μM each of all four rNTPs (bottom panel). Under these reaction conditions, the wt promoter activity was comparable (Figure 5A, lane 1) whereas the transcriptional activity of other templates varied significantly depending on the length and position of the polyT sequence as well as the UTP concentration. The mt promoters with two to three consecutive T residues starting at position +3, or three T residues starting at position +4, were poorly active at 5 μM UTP but exhibited moderate activity at higher UTP concentrations (lanes 2−4). Another mt promoter with three T residues starting at position +5 was active at both UTP concentrations (Figure 5A, lane 5) whereas a similar promoter sequence carrying four or more T residues at that location was functional at 125 μM UTP, but inactive at 5 μM UTP (lanes 6 and 7). Interestingly, when this polyT cluster was moved further downstream, transcriptional inhibition was almost abolished. For example, the presence of 6−12 successive T residues at +12 position did not inhibit mt transcription (Figure 5A, lane 8 and 9); presumably by this point in transcription, the mt RNA polymerase fully committed to transcription (*21*). These data corroborate the in vivo scenario that the progress of mt RNA polymerase is blocked by a promoter-proximal polyT sequence whereas mt transcription apparently proceeds through many polyT clusters in other areas of the mt genome. Together, the polyT-dependent inhibition of yeast mt transcription depends on several factors: (i) the number of T residues in the polyT cluster, (ii) the distance between the polyT cluster and the transcriptional start site, and (iii) the UTP concentration in the transcription reaction.

Reduction of polyT Inhibition under Certain Reaction Conditions. It has been found that the poly[rU]:poly[dA] duplex is very unstable compared to DNA:DNA or RNA:RNA cognates (*37*, *38*). So, incorporation of multiple uridine nt in the nascent transcript might weaken the interaction between the transcript and the DNA template which, in turn, destabilizes the early transcription complex. If this was the case with the mt polyT template, one would expect that the polyT-dependent inhibition of mt transcription could be minimized by strengthening the RNA:DNA duplex interaction. To explore this possibility, three different templates (i.e., wt, polyT, and GGpolyT) were used in this experiment. The polyT and GGpolyT templates both contained six consecutive T residues beginning at position +6 but differing at the +4

DNA Template	Transcriptionl activity (%)							
	UTP (μM)				Br-UTP (μM)			
	5 μM	25 μM	50 μM	100 μM	5 μM	25 μM	50 μM	100 μM
TTA **TATAAGTAA** TAGAATTCACT	82.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
TTA **TATAAGTAA** TATT TTTTATA	0	40.0	70.0	84.0	16.0	80.0	90.0	100.0
TTA **TATAAGTAA** TGGT TTTTTAT	18.0	67.0	84.0	93.0	50.0	90.0	100.0	100.0

FIGURE 6: Transcriptional activity of the wt, $6T_5$ or $6T_6$ containing two G residues at positions +4 and +5. The reactions were carried out at different concentrations of UTP or Br−UTP.

and +5 positions, where the polyT promoter had A, T and the GGpolyT had G, G residues (Figure 6). These two G residues of the GGpolyT template were introduced just upstream of the polyT cluster to strengthen the nascent RNA:DNA duplex interaction. The Tm/Δ*G* (free-energy change) values of the predicted 12 nt nascent RNA:DNA duplex were calculated to be 78 °C/−12.4 kcal/mol, 65 °C/−7.9 kcal/mol or 73 °C/−11 kcal/mol for the wt, polyT and GGpolyT template, respectively. According to these destabilization energy values, the stability of the initial RNA:DNA duplex would be the strongest with the wt template and weakest with the polyT template. Transcriptional activity was measured at different UTP concentrations. As expected, the wt promoter was very active at any of the nt concentrations tested, whereas the activity of the polyT template was drastically reduced at low UTP concentrations (i.e., 5 μM) but exhibited moderate activity at higher UTP concentrations ($\geq$25 μM). Interestingly, the activity level of the GGpolyT template was between the levels of the wt and polyT templates. This observation suggests that the presence of two G residues upstream of the polyT cluster enhanced transcription, most probably by strengthening the interactions between the nascent transcript and the DNA template. This was further supported by Br−UTP incorporation into the transcript which also strengthened the RNA:DNA interaction (*38*) and produced more mt transcripts than when UTP used at equivalent concentrations (Figure 6).

Premature Termination (Abortive Initiation) of mt Transcription at the polyT Sequence. It was found with *E. coli* RNA polymerase that the formation of productive initiation complex is affected by a promoter-proximal polyT sequence (*39, 40*). Since yeast mt transcription was inhibited by an early transcribed polyT sequence, it is conceivable that the mt RNA polymerase might also form an unstable initiation complex with the polyT template. If this is correct, the short RNA products should be detected from such nonproductive transcription complexes. To test this possibility, the RNA products from the in vitro transcription of the wt or $3T_3$ template at 125 or 5 μM of UTP concentration were analyzed by a high-resolution polyacrylamide (22.5% polyacrylamide-8 M urea) gel electrophoresis. The $3T_3$ template carrying three consecutive T residues starting at position +3 represents the inactive promoter sequences delineated in Figure 1. Transcription assays were performed at two different UTP concentrations (i.e., 125 or 5 μM). With the wt template full-length transcript was obtained regardless of the UTP concentrations (Figure 7A, lanes 4 and 5). In contrast, with the $3T_3$ template full-length transcript was seen

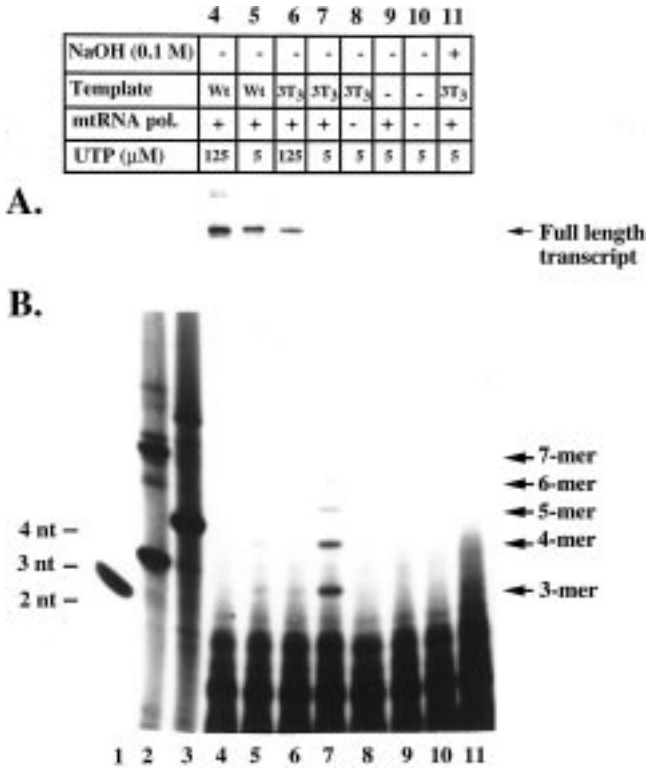


FIGURE 7: Generation of short RNA products from the polyT template. In vitro transcription reactions were performed with either the wt or $3T_3$ template as described before except that two different UTP concentrations (125 or 5 μM) were used. Transcripts from the same experiment were separated by electrophoresis either on a 6% polyacrylamide-8 M urea gel to detect the 107 nt long full-length transcript (A) or on a 22.5% polyacrylamide-8 M urea gel to detect the short transcripts (B). The lanes 1−3 represent end-labeled di, tri, and tetra nt RNA markers, respectively. Transcriptional products from the wt and $3T_3$ templates are shown in lanes 4, 5, 6, and 7, respectively. In the negative control experiments the transcriptional products in the absence of either the mt RNA polymerase, the DNA template or both are shown in lanes 8−10. The lane 11 represents an experiment identical to the lane 7 except that the RNA products were pretreated with NaOH before gel electrophoresis.

only at a high (Figure 7A, lane 6), but not at a low UTP concentration (Figure 7A, lane 7). Interestingly, the $3T_3$ template at 5 μM of UTP concentration yielded a large amount of oligoribonucleotides in the size range 2−7 nt (Figure 7B, lane 7). The 6- and 7-mer bands in lane 7 are faint although more visible on the original autoradiogram. To demonstrate whether these short bands were indeed ribonucleic acids generated by the mt RNA polymerase on the polyT template, several control experiments were also

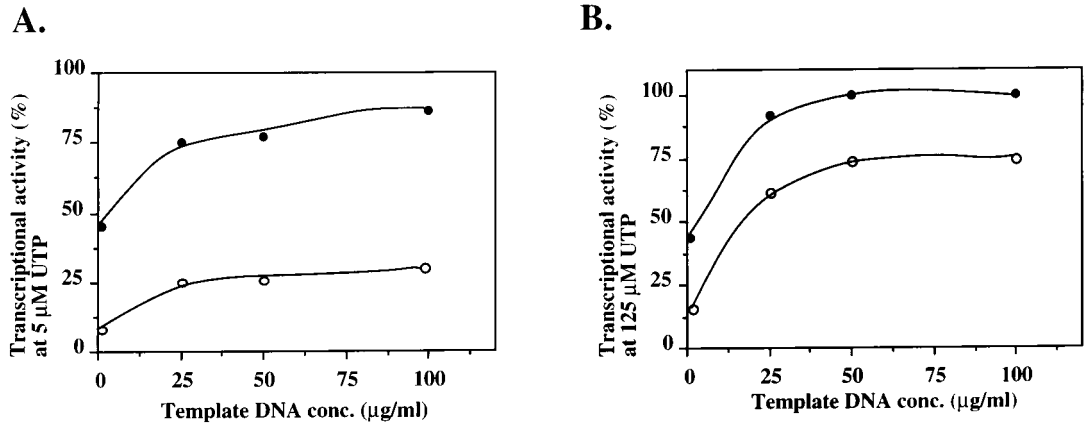


FIGURE 8: Transcriptional activity of synthetic mt tandem promoters. A tandem promoter construct (i.e., CTATATAAGTAATGGTTTTT-TATTATTGTCGACATTATATAAGTATAGAATTC) containing the GGpolyT promoter in the upstream and the wt promoter in the downstream sequence synthesized by an oligonucleotide synthesizer. The transcriptional assays were performed at different template concentrations in the presence of 5 or 125 μM of UTP concentrations. The GGpolyT and wt promoters produced 137 and 103 nt transcripts, respectively, from a *Pvu*II-digested template. The transcriptional activity was calculated considering the wt promoter activity at 125 μM rNTP concentrations as 100%, and plotted against the template DNA concentrations. (●) Wt promoter; (○) GGpolyT promoter.

performed. It has been found that neither long nor short transcripts were formed in the absence of either mt RNA polymerase (lane 8), mt DNA template (lane 9), or both (lane 10). As anticipated, these RNA products were sensitive to alkaline degradation (lane 11). These results, together with the previous observations, suggested that the disappearance of the full-length transcript in concert with the accumulation of the short-sized transcripts on the polyT template was due to the premature termination (or abortive initiation) of transcription. Scanning the autoradiogram to measure the amounts of different oligoribonucleotide transcripts revealed that the major transcription blockage (~80%) occurred between positions +3 and +4.

Transcriptional Activity of the mt Tandem Promoters. Tandem mt promoters were also generated and characterized to obtain some specific information, particularly on the affinity of mt RNA polymerase for the polyT template, as well as the potential interactions between the tandem promoters. The interaction between the tandem promoters of the mt *Oli1* gene was previously described (*41*). To address these issues, we have generated a tandem promoter construct with the GGpolyT promoter in the upstream and the wt promoter in the downstream location, and assayed at a high or low UTP concentration. Since the efficiency of productive initiation of transcription depends on the polymerase affinity for the promoter, the weak activity of the GGpolyT template could be due to the polymerase low affinity for this promoter. If this is the case, then increasing the concentration of DNA template or the RNA polymerase will enhance transcription. To examine the activity of both promoters in tandem under different polymerase-DNA ratios, the polymerase or the DNA template was used in excess by holding polymerase concentration constant and adjusting template concentration.

At 5 μM UTP, the downstream wt promoter exhibited severalfold higher activity than the upstream GGpolyT promoter (Figure 8). However, transcription on these promoters was enhanced with increasing template concentrations. The maximum amount of transcripts was obtained with both promoters at around 40 μg/mL template, although transcription from the GGpolyT promoter was always lower than the wt promoter. Interestingly, the relative activities of both promoters at different polymerase:template ratios were comparable, suggesting that these promoters might be recognized similarly by the mt RNA polymerase. This was further supported by transcription assays at high rNTP concentrations. At 125 μM UTP, the GGpolyT promoter activity was enhanced 3−4-fold relative to its activity at 5 μM UTP, whereas the wt promoter activity remained almost unchanged under these reaction conditions. The optimal template concentration for both promoter activities at high rNTP concentrations was also found to be 40 μg/mL. Interestingly, the enhanced GGpolyT promoter activity at high nt concentrations did not affect the activity of the wt promoter even when the template DNA was in excess and thus the polymerase was limited (Figure 8). Apparently, both promoters function independently under these reaction conditions; however, total transcript production was greater at high nt concentrations than at low nt concentrations particularly with the GGpolyT promoter. These results indicate that the GGpolyT and wt promoters might be recognized similarly by the mt RNA polymerase, but the variation in these promoter activities at low or high nt concentrations seems to be due to different rates of productive initiation of transcription.

DISCUSSION

The process of transcriptional initiation, elongation, and termination are relatively well-defined in prokaryotes (*42*−*46*) but only partly understood in eukaryotes (*47*−*49*). The ternary complexes of DNA-dependent RNA polymerase with its DNA template and the nascent transcript are central intermediates in transcription. Multiple factors including promoter sequence, the early transcribed sequence, and the reaction conditions have been implicated to be important for productive initiation of transcription and promoter clearance. Promoter clearance, which is defined as the switching of RNA polymerase from its initial transcribing step into a stable elongation phase, is well characterized in *E. coli* by three different biochemical changes of the transcription complexes. These include the release of specificity factor (σ), an initial translocation of RNA polymerase displacing it from the promoter, and the formation of a stable ternary complex by tight binding of RNA polymerase to both the

nascent transcript and the DNA template. However, if RNA polymerase releases the nascent RNA from the transcription complex, it cannot continue RNA synthesis, resulting in a premature termination of transcription. So, RNA polymerase at the initial stage of transcription must choose between these alternative productive-nonproductive transcriptional pathways.

The mt RNA polymerase, like other RNA polymerases, probably follows a similar initiation−elongation switching mechanism. Since our interest is in the elucidation of nucleic acid sequences that control yeast mt gene expression, we have extended our investigation of the template sequences that influence mt promoter function. Deletion analysis of the mt DNA template revealed that the nonanucleotide promoter and more than 9 nt of downstream sequence from the promoter are absolutely required for promoter-specific initiation of transcription. Moreover, the mt RNA polymerase also requires about 300 nt of sequence upstream of the promoter for optimal transcription. Since there was no apparent nt specificity to these flanking sequences, they might be used as a platform rather than for specific interactions between the mt RNA polymerase and the template. It is noteworthy that the recognition of a promoter or other signaling DNA sequences by *E. coli* RNA polymerase (*50*, *51*), the "TATA"-binding protein (*52*) or by the bacteriophage T4 protein gp45 (53) is carried out by a mechanism of protein binding and sliding along a long stretch of DNA template.

The influence of promoter-proximal 3′ sequence in mt transcription was also documented by position specific polyT inhibition of mt transcription. At low UTP concentrations, mt transcription is inhibited by a 3′ polyT cluster when located within the first 10−12 nt of the transcribed sequence. However, an identical polyT cluster further downstream did not interfere with mt transcription, suggesting that the polyT sequence is only involved in the abortive initiation of mt transcription but does not participate in the traditional terminator event. The prokaryotic terminator-like secondary structure in this early transcribed sequence of the template does not have any strong negative impact on mt transcription, suggesting that the mt RNA polymerase and the prokaryotic RNA polymerase do not respond to a similar transcriptional termination signal. This result also corroborates our earlier finding (*21*) that the mt RNA polymerase undergoes a transition from an unstable initiation complex into a stable elongation complex after making an RNA product 10−12 nt long. A similar transcriptional transition was also noticed with *E. coli* RNA polymerase (*42*, *54*, *55*), bacteriophage T7 RNA polymerase (*20*, *33*), human RNA polymerase II (*56*, *57*) and plant retrovirus polymerase (*58*). At the initial stages of transcription, these polymerases experience abortive initiations of transcription and release 2−10 nt long RNA products. The relative levels of abortive initiation vary among promoters, but the mechanism is not well understood. However, a number of recent studies suggested that the strength of the transcript-template hybrid is a major parameter for the stability of a ternary transcription complex (*38*, *59*−*61*). This RNA:DNA duplex stability has been also emphasized as an important factor for partitioning initiations between abortive and productive pathways (*28*, *38*, *57*). The bacteriophage T3 and T7 RNA polymerases undergo abortive initiation of transcription with limiting pyrimidine nt. This abortive initiation is most severe if the limiting substrate occurs within the first 8−12 nt of transcribed sequence and is particularly evident when the UTP concentration is low (*20*). The T7 RNA polymerase also terminates transcription at extended runs of T nt, especially at low UTP concentrations (*20*, *62*). The abortive initiation of transcription at low rNTP concentrations was also noticed with the bacteriophage SP6 RNA polymerase (*63*). Limiting the concentration of a ribonucleotide causes the SP6 RNA polymerase to stall only at the positions of the limited nt long enough to dissociate the polymerase from the elongation complex. This produces an oligoribonucleotide ladder including a large quantity of 6-mer or shorter transcripts generated from multiple rounds of abortive initiation (*63*).

Transcriptional blockage at the polyT sequence has also been documented with other RNA polymerases. As already mentioned, the bacteriophage T7 RNA polymerase terminates transcription when synthesizing a U-rich transcript especially at low UTP concentrations (*20*, *62*). Transcriptional termination in human mitochondria occurs at a "TTT" nt cluster preceding a tridecamer template sequence (*64*). In many cases *E. coli* RNA polymerase stops transcription at a terminator sequence consisting of several consecutive T residues (*34*, *65*). Signaling of transcriptional termination by T residues was also reported for eukaryotic RNA polymerase I (*48*, *66*, *67*), RNA polymerase II (*35*, *68*−*70*) and RNA polymerase III (*71*−*73*). For example, transcriptional termination with both yeast and mouse RNA polymerase I requires a trans-acting DNA-binding protein factor (i.e., Reb1p in yeast) and a T-rich flanking sequence in the nontemplate strand (*48*, *66*). Human RNA polymerase II terminates transcription at the TTTTTTTC−CCTTTTTT sequence of the histone H3.3 gene (*70*). Generally, the *Xenopus* RNA polymerase III requires a minimum of four adjacent T residues in the nontemplate strand for transcriptional termination (*71*). Yeast RNA polymerase III also terminates transcription in vivo at a polyT cluster of the $tRNA^{Tyr}$ gene (*72*). Since the polyT-dependent blockage of transcription is observed with both prokaryotic and eukaryotic RNA polymerases, it is likely that polyT inhibition of transcription perhaps evolved in an early form of life, but persisted in evolution due to its favorable thermodynamic mechanism. It is also worth noting that the polyT inhibition (i.e., abortive initiation) of mt transcription and the above-described polyT-dependent termination of transcription do not appear to be the same event although there are some mechanistic similarities between them.

How does the polyT sequence serve as a part of an intrinsic termination signal for RNA polymerase? There are already suggestions that T-runs weaken the interaction between the RNA and DNA (*37*, *38*) and also alter the conformation of the DNA template (*74*, *75*). The general mechanism for transcriptional termination consists of two separate events: polymerase pausing at the termination site and the release of transcript from the transcription complex. For example, pausing of RNA polymerase I at a terminator sequence is primarily regulated by a DNA-binding terminator protein, whereas a T-rich upstream sequence coding for the last 12 nt of the transcript in both yeast and mouse is mainly responsible for transcript release from the template (*48*). In the case of prokaryotic ρ-independent terminator, a highly stable hairpin structure of the G + C-rich RNA sequence impedes the progress of bacterial RNA polymerase, whereas

the polyT sequence leads to the release of RNA from the template due to the instability of the dA:rU bonds in the RNA−DNA duplex (*34*). In contrast, *ρ*-dependent terminators in *E. coli* do not contain a stable hairpin structure but may have a weak secondary structure without a polyT sequence. Since these *ρ*-dependent terminator sequences do not share the destabilizing features of other intrinsic terminators, the required destabilization of the transcription complex at the rho-dependent terminator is provided by the RNA:DNA helicase activity of the rho protein.

Inhibition of mt transcription at the polyT cluster might occur by a similar mechanism. Protein factors other than the mt holo RNA polymerase may not be necessary for this polyT inhibition of transcription since different polymerase preparations with variable purity exhibited similar sensitivity to the polyT sequence (*28*). The experimental evidence suggests that the mt RNA polymerase undergoes abortive initiation of transcription due to polymerase pausing and transcript release at the polyT cluster. For example, the incorporation of consecutive uridine residues in the nascent mt transcript perhaps weakens the interaction between the RNA and polyT template since a run of [rU]:[dA] base pairs at the 3′ end of the RNA:DNA duplex is highly unstable (*37*, *38*). Furthermore, lowering the concentration of nt (i.e., UTP) in the reaction decreases the catalytic event of polymerization (*76*), particularly at the position of the limited nt (i.e., polyT), and reduces the elongation speed of the transcription complex (i.e., polymerase pausing). Duration of polymerase pausing and the degree of instability of RNA:DNA duplex at the polyT cluster depend on the number of consecutive T residues and the distance between the polyT sequence and the transcriptional initiating site. The sum of these two independent destabilization events would exacerbate the instability of early transcription complexes and accelerate the premature termination of transcription. As predicted, several oligoribonucleotides but no full-length transcripts were obtained from the polyT template under the limiting nt conditions (Figure 7). However, in the presence of high UTP concentrations the mt RNA polymerase accelerates the polymerization reaction, suppresses the abortive initiation, and thus enhances the promoter clearance. Similarly, the presence of two G residues upstream of the polyT cluster strengthens the RNA:DNA duplex interactions, and thus increases transcription on the mt polyT template.

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