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Control of mitochondrial gene expression in the yeast *Saccharomyces cerevisiae*

(transcriptional regulation/mitochondrial promoter)

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ABSTRACT **Mitochondrial promoters in *Saccharomyces cerevisiae* contain an identical octanucleotide [TATAAGTA-(+1)] sequence present just upstream of the initiation site (at the left end of the arrow). Studies have shown that the transcription rates of mitochondrial genes vary from 7- to 15-fold. The nucleotide at position +2 regulates the efficiency of mitochondrial promoters but does not affect the specificity of initiation. The data presented herein demonstrate that the variable transcription rates of mitochondrial genes are due to different levels of transcriptional initiation. The rate of first phosphodiester bond formation between a purine and a pyrimidine on a weak promoter is much lower than that of purine-purine on a strong promoter. A dinucleotide corresponding to positions +1 and +2 acts *in vitro* as a primer, bypassing the first phosphodiester bond formation at the initiation site. When these dinucleotides were used to prime transcription, the activities of the strong and weak promoters were found to be identical. In heparin-challenge experiments, there is no significant effect of dinucleotide on heparin-resistant DNA–RNA polymerase complex formation. These results indicate that the low level of transcription from the weak mitochondrial promoter is due to the slow rate of formation of the first phosphodiester bond.**

Significant progress has been made toward an understanding of the control of mitochondrial gene expression in the yeast *Saccharomyces cerevisiae* (1–12). The biogenesis of mitochondria is regulated by the coordinated expression of both mitochondrial and nuclear genes (13). The 76-kilobase mitochondrial DNA of yeast encodes a limited number of mitochondrial proteins, two rRNAs, and a set of tRNAs necessary for translation of mitochondrial mRNAs. The remaining mitochondrial proteins including factors required for mitochondrial transcription, replication, and translation are encoded by the nuclear genome and imported into mitochondrial.

Mitochondrial RNA (mtRNA) polymerase consists of a core enzyme and a specificity factor, both of which are required for specific transcription of mitochondrial genes (8–10). The mitochondrial promoter consists of an 8-nucleotide sequence [5′-TATAAGTA(+1)-3′] that has been identified in the mitochondrial rRNA, tRNA, and mRNA genes as well as at the origins of replication (2). *In vitro* transcription studies of variant promoter mutants showed that a short octanucleotide sequence, 5′-TAtAaGtN-3′, is the minimum sequence requirement to direct accurate initiation of transcription by mtRNA polymerase (6). The lowercase letters indicate positions at which nucleotide variants are permissible and N designates any nucleotide that is used as an initiating nucleotide.

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Although mitochondrial genes share an identical promoter sequence, they are transcribed at different rates (5, 12). The promoters can be grouped into two classes: strong promoters and weak promoters. The strong promoters have 7–15 times the activity of the weak promoters. Promoters are present singly or in tandem in the mitochondrial genome. Where there are tandem promoters, the upstream element is always active and strong whereas the downstream promoter is used poorly or not at all (7, 12). The strength of mitochondrial promoters is determined by the nucleotide at position +2 (2). The octanucleotide promoter with a purine at position +2 acts as a strong promoter whereas the same sequence with a pyrimidine at +2 functions as a weak promoter. To characterize further the regulation of transcriptional initiation at the mitochondrial promoter, I used various mitochondrial promoters in *in vitro* reaction mixtures. The results presented here demonstrated that transcription of yeast mitochondrial genes is mainly regulated at the level of initiation.

MATERIALS AND METHODS

Plasmids and Strains. Plasmids containing native mitochondrial *Oli1* gene promoters (5) and synthetic mitochondrial promoters (6) were described. The mtRNA polymerase was purified from *S. cerevisiae* strain D273-10B as described (8). Fractions containing selective activity were used for *in vitro* transcription.

Transcription Reaction. Plasmid containing the mitochondrial *Oli1* gene promoters was digested with the restriction endonuclease *Eco*RI and plasmids having synthetic promoters were digested with *Pvu* II endonuclease. These linear DNAs were used as a template for *in vitro* transcription assay. The standard 25-μl reaction mixture contained 10 mM Tris·HCl (pH 7.9), 10 mM $MgCl_2$, 20 mM KCl, 5% (vol/vol) glycerol, rabbit serum albumin (0.2 mg/μl), 125 μM ATP, 125 μM GTP, 125 μM CTP, 125 μM UTP, and 10–20 μCi of [α-^{32}P]UTP or [γ-^{32}P]ATP (1 Ci = 37 GBq). As specified, various concentrations of a reaction component (a rNTP, KCl, glycerol, etc.) were employed to determine its effect on promoter function. After addition of mtRNA polymerase, the reaction mixture was incubated at 28°C for 7 min. With the particular preparation of mtRNA polymerase transcription was linear for 7 min at 28°C. The reaction was terminated by the addition of 25 μl of stop solution [0.3% SDS/tRNA (200 μg/ml)] followed by phenol extraction. High molecular weight RNA products were separated from the unincorporated labeled nucleotide by precipitation with 50 μl of 5 M ammonium acetate and 250 μl of ethanol. Transcripts were separated through a 5% or 8% polyacrylamide/8 M urea gel by electrophoresis and then visualized by autoradiography. Transcription products were quantitated by scanning the intensity of transcript bands on autoradiograms using a scanning densitometer from Hoefer Scientific Instruments.

Abbreviation: mtRNA, mitochondrial RNA.

RESULTS

In vitro transcription at the tandem promoters of mitochondrial *Oli1* gene differs by 15-fold (Fig. 1). To extend these studies on the regulation of transcriptional initiation, the effects of dinucleotide on *in vitro* transcription of mitochondrial promoters were investigated using purified mtRNA polymerase and cloned mitochondrial promoters.

Stimulation of Transcription by Dinucleotide Primers. Dinucleotide priming of transcription has been found with bacterial RNA polymerases (14, 15) and with eukaryotic RNA polymerases (16, 17). The mtRNA polymerase also utilizes dinucleotides corresponding to only two positions (positions +1, +2 and positions −1, +1) as a primer (18).

The transcriptional activities of the mitochondrial strong and weak promoters increased with increasing concentrations of the first two monoribonucleotides corresponding to positions +1 and +2, up to 140 μM (Fig. 2). Half-maximal RNA synthesis occurred when these nucleotides were ≈65 μM whereas <10 μM of the subsequent nucleotides were required for transcriptional elongation. At low concentration of the initiating nucleotide (i.e., ≈5 μM), the rate of RNA synthesis was reduced by a factor of 15–20. RNA synthesis in the rNTP-limited (5 μM ATP) reaction mixtures was markedly stimulated by the addition of sequence-specific dinucleotide (50 μM) corresponding to positions +1, +2 and positions −1, +1 (Figs. 2 and 3). Dinucleotides allowed transcription to occur at 5 μM, a concentration of rNTPs that is sufficient only for elongation. This suggests that the dinucleotide acts as a primer and eliminates the requirement for a high concentration of initiating nucleotide. The effect of dinucleotides on the strong and weak promoter activities was examined in *in vitro* transcription reactions (Fig. 3). At optimal and suboptimal concentrations of initiating nucleotide, dinucleotide stimulated transcription at the weak promoter 6–8 times whereas in the presence of dinucleotide the activity of the strong promoter remained almost constant whatever the concentration (optimal or suboptimal) of the initiating nucleotide. These results confirm that high monoribonucleotide concentration is required only for the first phosphodiester bond formation rather than elongation and, furthermore, suggest that the low activity of weak promoters is a consequence of a reduced rate of the first phosphodiester bond formation.

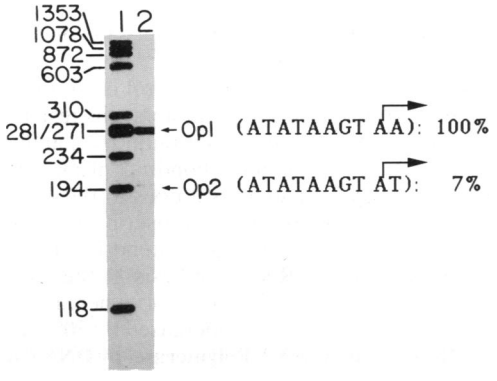

FIG. 1. *In vitro* transcriptional activities of the strong and weak promoters of mitochondrial *Oli1* gene coding for ATPase subunit 9. Run-off transcriptional products of these promoters were end-labeled with [γ-^{32}P]ATP (the initiating nucleotide of both promoters) to determine the relative amounts of transcripts. Transcripts were separated through 5% polyacrylamide/8 M urea gel by electrophoresis and then detected by autoradiography. Lanes: 1, molecular size markers (in nucleotides) of end-labeled *Hae* III-digested φX174 DNA; 2, the 266- and 188-nucleotide transcripts originated from the strong (Op_1) and weak (Op_2) promoters.

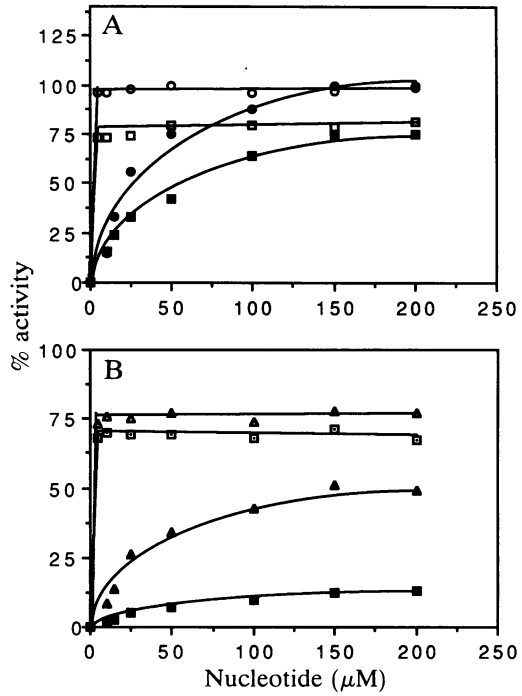

FIG. 2. Dependence of transcription on the concentrations of ribonucleotides in standard or in dinucleotide-primed reactions. Four promoters containing adenine or cytosine nucleotide at position +1 or +2 were used in this experiment. The sequences of these promoters are 5′-ATATAAGTAG-3′, 5′-ATATAAGTGA-3′, 5′-ATATAAGTCA-3′, 5′-ATATAAGTAC-3′—the initiation nucleotide at position +1 is at the left end of the arrow. Three rNTPs (each at 125 μM) and various concentrations of the fourth nucleotide (5–200 μM) were used. The K_m values for these nucleotides were determined by double reciprocal plot. Similar experiments were done in the presence of 50 μM dinucleotide primer corresponding to positions +1 and +2. The transcriptional activity was expressed as a percent activity considering the activity of a strong promoter as 100%. Solid symbols indicate transcriptional activity in the presence of monoribonucleotides and open symbols designate dinucleotide-primed transcriptional activity. (*A*) ●, ATP(+1); ○, ATP(+1) + AG dinucleotide; ■, ATP (+2); □, ATP(+2) + GA dinucleotide. (*B*) ▲, CTP(+1); △, CTP(+1) + CA dinucleotide; ■, CTP(+2); ⊡, CTP(+2) + AC dinucleotide.

Some mitochondrial genes contain a strong promoter and a weak promoter in tandem, and the presence of a strong promoter further reduces the activity of the weak promoter (7). These interactions have been further studied using tandem promoters of mitochondrial *Oli1* gene and dinucleotide primers (Fig. 4). When all four rNTPs (each at 125 μM) are present, the activity of the weak promoter was ≈15 times less than the upstream strong promoter (Fig. 4, lane 1). When the concentration of the initiating nucleotide ATP was reduced to 5 μM, transcription from both the strong and weak promoters was drastically reduced (Fig. 4, lane 2). When the dinucleotide (AA) complementary to the first two nucleotides of the upstream strong promoter and suboptimal concentrations of the initiating nucleotide were used, only the upstream strong promoter was fully expressed and the downstream weak promoter was not used at all (Fig. 4, lane 3). Similarly, when dinucleotide (AU) complementary to the first two nucleotides of the downstream weak promoter was used, transcription only from the downstream promoter occurred (Fig. 4, lane 4). When both dinucleotides were added together in the same reaction mixture both promoters were almost equally active (Fig. 4, lane 5). These results again demonstrate that the weak promoter is mainly blocked at the step of the first phosphodiester bond formation rather than from competition with the strong promoter for RNA polymerase.

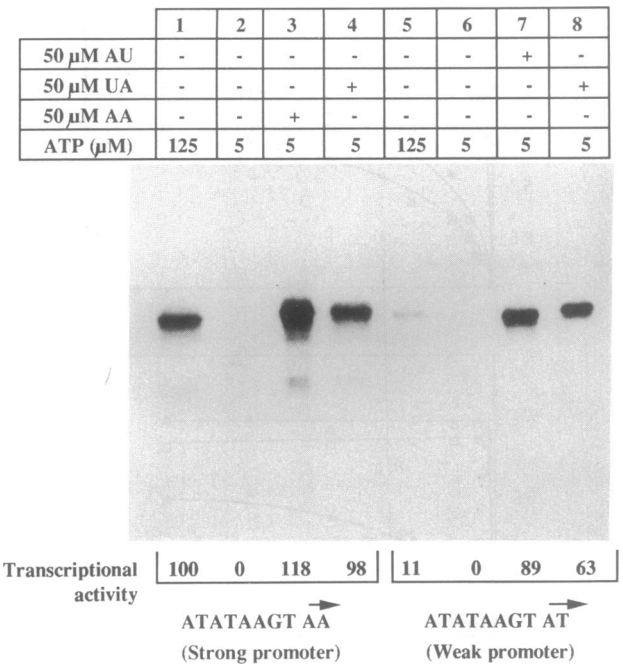

FIG. 3. Dinucleotide priming of transcription. The linear DNA containing mitochondrial promoters was used as a template in *in vitro* transcription reaction mixture that yields a 117-nucleotide transcript. Sequences of the promoters and the relative transcription are shown at the bottom. The standard transcription reaction mixtures contained all four rNTPs (each at 125 μM) (lanes 1 and 5), and the rest of the reaction mixtures contained 5 μM ATP, the other three rNTPs (each at 125 μM), and no dinucleotide (lane 2), AA dinucleotide (lane 3), UA dinucleotide (lane 4), no dinucleotide (lane 6), AU dinucleotide (lane 7), or UA dinucleotide (lane 8). Dinucleotides (50 μM) were used in these reactions.

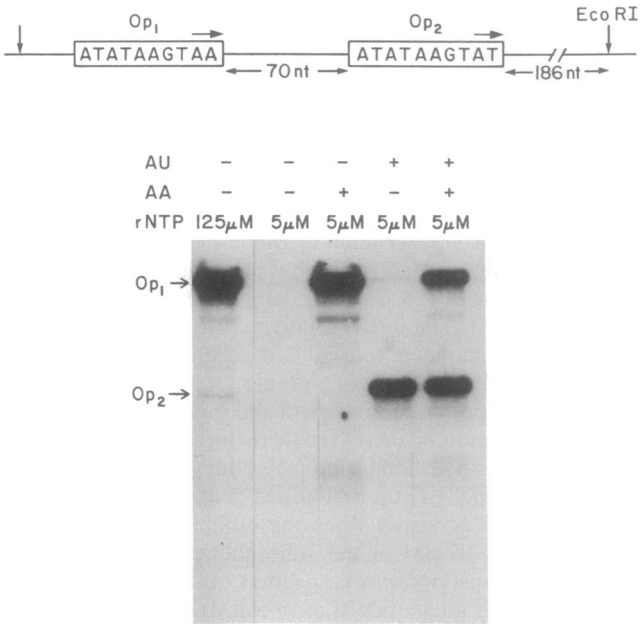

FIG. 4. Dinucleotide priming of transcription at the tandem promoters. The transcriptional activity of the *OliI* gene promoters in the presence of dinucleotides is shown. A strong promoter (Op_1) and a weak promoter (Op_2) are present in tandem and are separated by 70 nucleotides. *Eco*RI-digested template DNA gives 266-nucleotide and 188-nucleotide transcripts from the strong (Op_1) and weak (Op_2) promoters, respectively. Lane 1 shows transcripts obtained from the tandem promoters with all four rNTP (each at 125 μM). The rest of the transcription reactions were carried out with 5 μM ATP, the other three rNTPs (each at 125 μM), and no dinucleotide (lane 2), AA dinucleotide (lane 3), AU (lane 4), AA, and AU dinucleotides (lane 5).

RNA Synthesis at Various Reaction Conditions. The efficiency of transcriptional initiation depends mainly on two separate steps (19): (*i*) initial binding of polymerase to the DNA template (K_B) and (*ii*) isomerization of the closed complex into the open complex (k_2). The strong and weak activities of mitochondrial promoters could be due to differences in K_B, k_2, or both. To identify any differences in these steps, the effects of different reaction conditions on the promoter function were examined.

DNA Concentration. The efficiency of DNA–RNA polymerase interactions is determined by the binding affinity (K_B) of the polymerase for the promoter, which is defined by association constant (K_a) and dissociation constant (K_d). The weak transcriptional activity of a promoter could be due to its low K_a value. In this case, increasing concentration of template DNA or RNA polymerase will enhance transcription. Different concentrations of strong or weak promoter were used in transcription reactions to determine the optimal concentration of template DNA required for transcription (Fig. 5*A*). The maximum amount of transcripts was obtained with both templates at a concentration of 40 μg/ml, although transcription from the weak promoter was always lower than the strong promoter.

Temperature Effect. In many cases of bacterial transcription (20), isomerization of closed complex to open complex (k_2) is temperature dependent; e.g., higher temperature increases the open-complex formation. Since poor isomerization is one of the main characteristics of the weak promoters, the effect of temperature on RNA synthesis at the strong and weak promoters was tested (Fig. 5*B*). RNA synthesis at these promoters was increased with increasing temperature up to 37°C. In both cases, transcription increased 5- to 7-fold after changing the temperature from 10°C to 37°C.

Effect of Salt Concentration. The ionic environment is an important factor for DNA–protein interactions (21). The effect of salt concentrations (KCl) on transcriptional activity of the strong and weak promoters is shown in Fig. 5*C*. Transcription from the strong promoter increased as the salt concentration elevated from 5 mM to 50 mM and then was inhibited at higher salt concentration. In contrast, maximum activity of the weak promoter was obtained at low salt concentration (5 mM). In dinucleotide-primed transcription reaction, the effect of salt on weak promoter activity was different. In this case, the amount of transcripts from the weak promoter increased with increasing salt concentration up to 50 mM and then declined with higher salt concentration, as is the case with the strong promoter.

Effect of Glycerol. The effect of glycerol on transcriptional activity of the strong and weak promoters was tested *in vitro* since glycerol might activate transcription by stabilizing the structural alteration of the mitochondrial promoter during preinitiation complex formation as it does to the *Escherichia coli* RNA polymerase–*gal* promoter transcription system (22). The addition of glycerol to the mitochondrial transcription reaction mixture reduced RNA synthesis at the strong promoter (50% inhibition with 25% glycerol) whereas transcription from the weak promoter was increased by 30% (Fig. 5*D*).

Effect of Heparin on mtRNA Polymerase–mtDNA Complex. To further study whether dinucleotide is required for the formation or the stability of enzyme–promoter complex, heparin was added to the reaction mixtures since it inactivates free polymerase (23). Mitochondrial holoenzyme, but not the core polymerase, becomes partially resistant to heparin after preincubation with promoter at 30°C for 5 min (data not shown). The effects of preincubation in the presence and absence of dinucleotide and ATP on the heparin-resistant complex formation were studied. The mtRNA polymerase was almost completely inactivated when heparin (200

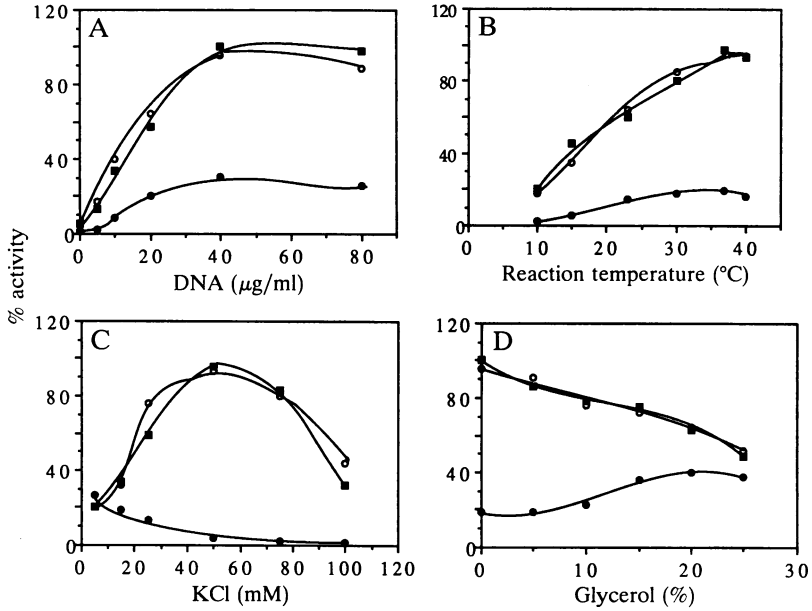

FIG. 5. Promoter activity in various reaction conditions. The transcriptional activities of a strong promoter (■), a weak promoter (●), and a weak promoter plus AU dinucleotide primer (○) are shown. Reaction conditions were similar to the standard assay conditions. Each promoter was used in a separate reaction mixture. The relative activity of these promoters was determined by considering the maximum activity of the strong promoter (Op_1) that was achieved in each reaction condition as 100%. (*A*) Saturation curve of template DNA. The linear DNA was added to the reaction mixture at various concentrations (5–80 μg/ml). (*B*) Effect of temperature on transcription. All reaction components and mtRNA polymerase were mixed together and then incubated at various temperatures (10–40°C). (*C*) Effect of salt (KCl) on transcription. KCl was added to the reaction mixture at various concentrations (5–100 mM) before the addition of RNA polymerase. (*D*) Effect of glycerol on transcription. Various concentrations of glycerol (0–25%) were added to the reaction mixture before the addition of RNA polymerase.

ng/ml) was preincubated with polymerase or when heparin was added with other reaction components (Fig. 6, lanes 2, 5, 8, and 11). When enzyme and template DNA were preincubated either with or without dinucleotide plus ATP for 5 min at 28°C before the addition of all four rNTPs to initiate transcription, 50–70% of enzymatic activity became resistant to heparin (Fig. 6, lanes 3, 6, 9, and 12). Inclusion of dinucleotide and ATP (Fig. 6, lanes 6 and 12) in the preincubation reaction mixture did not change the efficiency of heparin-resistant complex formation compared to preincubation without dinucleotide and ATP (lanes 3 and 9), indicating that dinucleotide and ATP do not have any role in heparin-resistant complex formation.

DISCUSSION

Transcriptional initiation on yeast mitochondrial strong and weak promoters has been further characterized, based on the observations that higher monoribonucleotide concentrations are required for initiation of transcription than are needed for subsequent elongation by mtRNA polymerase and that sequence-specific dinucleotides can prime transcription by mtRNA polymerase. Herein, I have explored the basis for the various strengths of mitochondrial promoters that exist in yeast mtDNA by studying the first phosphodiester bond formation of mtRNA and the role of dinucleotide in the process of transcriptional initiation.

mtRNA synthesis is sensitive to the concentrations of ribonucleoside triphosphate. At a low concentration of all four rNTPs (<10 μM), the rate of RNA synthesis is reduced >15 times. However, if the concentration of the two ribonucleoside triphosphates involved in the formation of the first phosphodiester bond is increased up to 140 μM, both the rate of chain initiation and the overall rate of RNA synthesis are maximized, as is the case with prokaryotic RNA polymerase

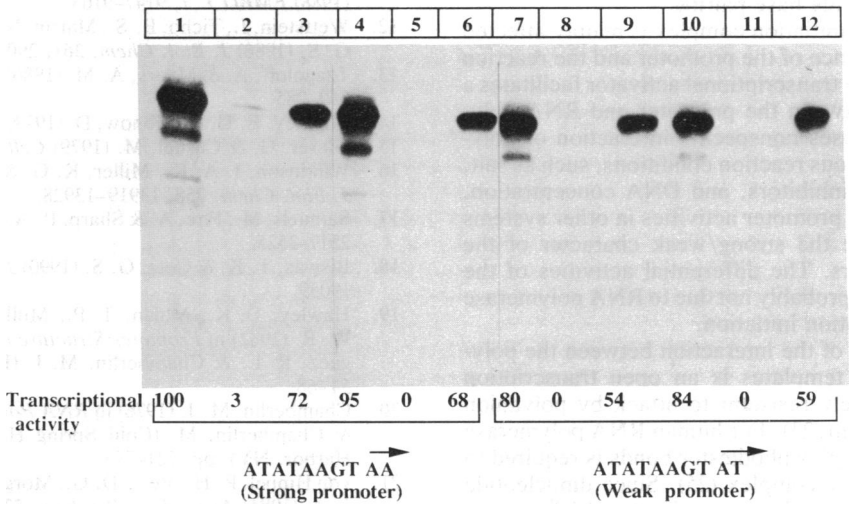

FIG. 6. Effects of preincubation in the presence or absence of dinucleotide and heparin on *in vitro* transcription of the strong and weak promoters. The relative transcriptional activity of each reaction mixture is indicated below each lane as a percent of the activity of the strong promoter shown in lane 1. Conditions for each reaction were as follows: In lane 1 (minus heparin), mtRNA polymerase and template DNA were preincubated at 30°C for 5 min before the addition of dinucleotide and all four rNTPs to initiate transcription, which was carried out for 7 min at 30°C. In lane 2 (plus heparin, no preincubation), template DNA, dinucleotides, all four rNTPs, heparin, and mtRNA polymerase were added together to initiate transcription. In lane 3 (plus heparin after preincubation), mtRNA polymerase and template DNA were preincubated, as in lane 1, and then dinucleotides, all four rNTPs, and heparin were added together to start transcription. The reactions shown in lanes 4 and 6 were identical to those shown in lanes 1 and 3, except that dinucleotide and ATP were added during the preincubation instead of after. The reaction in lane 5 was the same as in lane 2. Conditions for the reactions shown in lanes 7–12 were identical to lanes 1–6, respectively, except the reaction mixtures contained a template DNA with a weak promoter rather than a strong promoter.

(24) and eukaryotic RNA polymerase I (16). These results show that the K_m values of mtRNA polymerase for the first two nucleotides are ≈10-fold higher than for the succeeding nucleotides. The mtRNA polymerase might have two binding sites for the initiating or primer nucleotide and for the elongating nucleotide, as has been suggested for prokaryotic RNA polymerases (23). The high K_m values for the first two initiating nucleotides (positions +1 and +2) may be due to a requirement of the first two mononucleotides at the same time to form the first phosphodiester bond. Second, the high affinity (low K_m) of RNA polymerase for the elongating nucleotides might be due to the specific change of polymerase during conversion of the binary complex to ternary complex. A third explanation is that initially some of the nucleotides are used as an energy source to drive binary RNA polymerase–DNA complex into ternary conformation. Since the K_m values of mtRNA polymerase for any nucleotide at positions +1 and +2 are equal, the difference between strong and weak promoter activities are probably not due to the differences in nucleotide binding affinity of the polymerase.

The mtRNA polymerase utilizes dinucleotides to initiate transcription according to the promoter-specific base-pairing rules (Fig. 3). Since mtRNA polymerase only uses dinucleotide corresponding to positions +1 and +2 and positions −1 and +1, dinucleotide appears to be used at the promoter sites rather than to cause nonspecific initiation of transcription. Dinucleotide stimulates transcription on the weak promoter at both optimal and suboptimal concentrations of all four rNTPs. In this way it resembles the *Xenopus* transcription system (16). In contrast, dinucleotide does not change the total activity of the strong promoter under normal conditions. However, at suboptimal concentrations of the initiating nucleotide, dinucleotide does increase transcription from its low level to a normal level as is the case with dinucleotide-primed transcription of an *E. coli* rRNA gene promoter (15). These results demonstrate that dinucleotide is not very different from a mononucleotide as a precursor to the 5′ end of an RNA chain. The main differences between the two reactions are (*i*) the dinucleotide-primed reaction bypasses the rate-limiting step of the first phosphodiester bond formation and (*ii*) DNA·dinucleotide base pairing is more stable than DNA·mononucleotide base pairing.

The formation of the initiation complex is mainly affected by the nucleotide sequence of the promoter and the reaction conditions. Generally, a transcriptional activator facilitates a stronger interaction between the promoter and RNA polymerase and also decreases nonspecific interaction of polymerase with DNA. Various reaction conditions, such as salt, temperature, glycerol, inhibitors, and DNA concentration, that differentially affect promoter activities in other systems (19, 20) do not change the strong/weak character of the mitochondrial promoters. The differential activities of the two promoter types are probably not due to RNA polymerase binding but to transcription initiation.

The ultimate product of the interaction between the polymerase and the DNA templates is an open transcription complex that is relatively resistant to attack by polyanion inhibitors such as heparin (23). For human RNA polymerase II, the formation of two phosphodiester bonds is required to generate a stably ternary complex (25). Since dinucleotide enhances transcription from the weak mitochondrial promoters, the possible role of dinucleotide in ternary transcription complex formation has been tested using heparin as an inhibitor of free polymerase. Heparin at 200 ng/ml inactivates free mtRNA polymerase but does not inactivate the polymerase bound to the promoter (Fig. 6). The formation of this heparin-resistant complex does not need any mononucleotide or dinucleotide, indicating that dinucleotide does not have any role in the initial interaction of polymerase with the promoter.

The results presented in this paper demonstrate that, at the initial phase of mitochondrial transcription, the PP_i exchange reaction during the first phosphodiester bond formation is predominantly into a purine nucleotide, not into a pyrimidine nucleotide; i.e., the reaction pppN + pppR → pppNpR + PP_i (where R is a purine) on a strong promoter predominates over the reaction pppN + pppY → pppNpY + PP_i (where Y is a pyrimidine) on a weak promoter. Dinucleotide priming of transcription supports this hypothesis since the dinucleotide stimulates weak promoter activity by bypassing the rate-limiting step of the first phosphodiester bond formation between a purine and a pyrimidine but does not change the reaction rate between a purine and a purine on a strong promoter.

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