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Characterization of a yeast mitochondrial promoter by deletion mutagenesis

(*in vitro* transcription/*Saccharomyces cerevisiae*/14S rRNA gene)

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ABSTRACT **We have generated collections of mutants of the promoter for the small rRNA gene from the mitochondria of yeast deleted from either the 3′ or 5′ end. Plasmids containing the partially deleted promoter were assayed for their ability to direct correct transcriptional initiation in a homologous *in vitro* system. We find that the region required for high-efficiency promoter function lies between positions −10 and +2. Our methods detected no effect of flanking sequences on the strength of this promoter.**

Expression of the mitochondrial genome is a complex process that requires interaction between nuclear and mitochondrially coded functions. The initiation of transcription of genes coded in the mitochondria is a central process in the coordination between nucleus and mitochondrion. In this process, the nuclear-coded, cytoplasmically synthesized mitochondrial RNA polymerase interacts with promoter sequences on mitochondrial DNA to yield the appropriate levels of transcripts of the mitochondrially encoded genes.

Recently, transcriptional initiation sites on the mitochondrial genome of the yeast *Saccharomyces cerevisiae* have been characterized extensively (1–7). By using the method of *in vitro* capping with guanylyltransferase, at least 19 unique transcriptional initiation sites have been identified. The initiation sites are spread throughout the 80,000-base-pair (bp) genome, and the locations of the initiation sites suggest a peculiar pattern of gene expression in which some genes are transcribed alone, some groups of functionally unrelated genes are transcribed as parts of a single transcript, and some transcription units are transcribed from tandem promoters (4–7). In hopes of identifying DNA sequences that define the yeast mitochondrial promoter, the sequences surrounding the transcriptional initiation sites were carefully inspected for shared elements. The initiating nucleotide and the first 8 bases upstream were found to be very strongly conserved at each of the initiation sites identified to date (4, 7). Interestingly, the same consensus sequence was found at the transcriptional initiation sites of the two rRNA genes in the mitochondria of a distantly related yeast, *Kluyveromyces lactis* (8).

We have recently developed a homologous *in vitro* transcription system for yeast mitochondrial genes in which correctly initiated transcripts are synthesized with high fidelity from a template consisting of promoter-bearing mitochondrial DNA fragments inserted into bacterial plasmids (9). This transcription system allows us the opportunity to test directly which sequences constitute a promoter for the mitochondrial RNA polymerase. We chose for our study the promoter of the gene encoding the small ribosomal RNA (14S rRNA). The exact site of transcriptional initiation *in vivo* and the ability of the mitochondrial RNA polymerase to use this promoter *in vitro* have been described previously (3, 9). Initiation of the 14S rRNA transcript occurs at an exact copy of the 9-nucleotide consensus sequence ≈80 bp upstream of the 5′ end of the mature rRNA. Levels of cappable transcripts from this site *in vivo* and incorporation studies using a transcription complex isolated from yeast mitochondria suggest that, along with the 21S rRNA gene, this is one of the most heavily transcribed genes on the yeast mitochondrial genome (4, 10).

In this paper, we report the generation of a collection of plasmids in which the 14S rRNA promoter region is progressively deleted from either the 5′ or 3′ ends using the exonuclease BAL-31. The deleted plasmids were then assayed for the ability to direct correct initiation at the 14S rRNA promoter *in vitro*. Our results indicate that the region required lies between position −10 and position +2, in excellent agreement with the conclusion based on homology among the various initiation sites.

MATERIALS AND METHODS

Strains and Plasmids. Plasmid pJE14S was derived from pYM132a (9) during a previous round of BAL-31 deletion mutagenesis. Plasmid pUR250 (11) and bacterial strain RR1ΔM15 (12) have been described previously. *S. cerevisiae* strain D273-10b (ATC no. 25647) was used for purification of yeast mitochondrial RNA polymerase.

BAL-31 Deletion. BAL-31 was purchased from New England Biolabs. Twenty micrograms of linearized DNA was incubated in a total volume of 40 μl in a solution of 300 mM NaCl/20 mM Tris, pH 8.0/12 mM $MgCl_2$/12 mM $CaCl_2$/1 mM EDTA with 4 units of BAL-31. Reactions were carried out at 30°C. Aliquots were removed at various times from 0 to 3 minutes and the reaction was stopped by mixing with equal volumes of 25 mM EDTA. The DNA was phenol extracted and precipitated with ethanol. T4 DNA polymerase (Amersham) reactions were carried out as described (13), again followed by phenol extraction and ethanol precipitation. The DNA was dissolved in a small volume of water and then brought to a final volume of 20 μl in 25 mM Tris, pH 7.4/5 mM $MgCl_2$/5 mM dithiothreitol/0.25 mM spermidine/1 mM ATP/1.25 mM hexamine cobalt chloride/10 μg of bovine serum albumin per ml/50 μg of phosphorylated *Eco*RI linkers per ml with 2 units of DNA ligase and 8 units of RNA ligase. The reaction mixture was incubated at 4°C overnight. Restriction endonuclease digestions, electrophoresis, isolation of DNA from acrylamide gels, DNA ligation, and bacterial transformations were carried out by standard methods as described (13).

DNA Sequencing. Deleted plasmids of interest were sequenced either by the chemical method of Maxam and Gilbert (14) or by the dideoxy sequencing method using a

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Abbreviation: bp, base pair(s).

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primer complementary to vector sequences bordering the mitochondrial insert (15).

Transcription Reactions. Reactions were carried out as described previously (9) in a volume of 25 μl of 10 mM Tris, pH 7.9/10 mM $MgCl_2$/40 mM KCl/5% glycerol/500 μg of rabbit serum albumin per ml/125 μM ATP, GTP, and CTP with 30 μM [α-^{32}P]UTP (20 Ci/mmol; 1 Ci = 37 GBq). Plasmid DNAs for transcription reactions were purified through cesium chloride gradients by standard procedures (13). After digestion with the appropriate restriction enzyme, the DNA was digested with RNase A, phenol extracted, purified through a spin column (13), sequentially extracted with chloroform and ether, and then precipitated with isopropanol. The DNA was dissolved in water and the concentration was determined by absorbance at 260 nm. The total DNA concentration in each transcription reaction was 40 μg/ml, each template present in equal concentration. Two microliters of polymerase, purified as in ref. 16, was used for these reactions. Under these conditions with this particular polymerase preparation, the reactions were not saturated for DNA or polymerase.

RESULTS

Generation of 5′-Deleted Plasmids. Maps of relevant DNAs and plasmids are shown in Fig. 1. The parent plasmid for our deletion mutagenesis was pJE14S (Fig. 1*A*), which contains ≈400 bp upstream and ≈120 bp downstream of the transcriptional initiation site of the 14S rRNA gene inserted into the *Eco*RI site of pBR322 (with the disruption of the *Eco*RI site). To generate 5′-deleted clones, the parent plasmid was digested within the mitochondrial DNA insert at the single *Sac* II site 235 bp upstream of the initiation site. The linearized DNA was digested with BAL-31 for varying times to yield deletions of 109–231 bp. The ends of the plasmids were made blunt with T4 DNA polymerase in the presence of all four deoxynucleotide triphosphates and then ligated to octanucleotide *Eco*RI linkers.

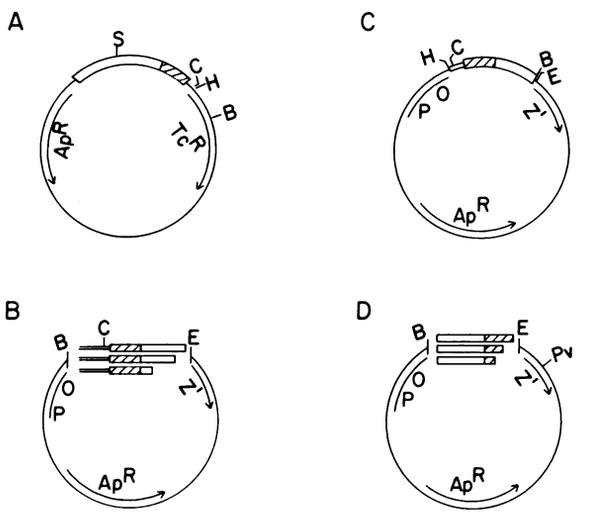

FIG. 1. Physical maps of 14S rRNA promoter-bearing plasmids. In each diagram, the single line represents the vector DNA with the antibiotic resistance markers and *lacZ′* genes as indicated. The wide double lines represent the mitochondrial DNA insertions. The clear area in the bars represents the region upstream of the 14S rRNA transcriptional initiation site and the hatched area represents the transcribed region. (*A*) pJE14S. (*B*) Maps of representatives of the 5′-deleted plasmid collection. The narrow double lines represent pBR322 sequences that were transferred from pJE14S along with the 5′-deleted mitochondrial DNA. (*C*) pTB14S. As in *B*, the narrow double lines represent pBR322 sequences that were transferred with the mitochondrial sequences. (*D*) Maps of representatives of the 3′-deleted plasmid collection. Restriction site abbreviations: B, *Bam*HI; C, *Cla* I; E, *Eco*RI; H, *Hin*dIII; S, *Sac* II; Pv, *Pvu* II.

The deleted plasmids with intact linkers attached were digested with *Eco*RI to eliminate multiple linkers and to leave an *Eco*RI complementary end at the end point of the BAL-31 deletion on each plasmid. The plasmids were then cut at the single *Bam*HI site, releasing the partially deleted mitochondrial insert with a 5′-terminal *Eco*RI linker and connected at its 3′ end to 375 bp of vector DNA. The products of the *Eco*RI/*Bam*HI double digestion were separated on a 5% acrylamide gel and DNA was isolated from the region of the gel containing fragments of 480–630 bp. This population of DNA fragments should contain the mitochondrial sequence deleted to various extents from its 5′ end, an *Eco*RI linker at the site of deletion, and a *Bam*HI complementary end at about 495 bp downstream of the initiation site. The isolated DNA fragments were then inserted into plasmid pUR250, which had been digested at the *Eco*RI and *Bam*HI sites present in the polylinker region of the plasmid. The products of ligation were used to transform *Escherichia coli* strain RR1ΔM15 and transformants were screened for the size of the insert. The precise extent of the deletion was determined by DNA sequencing. As shown in Fig. 1*B*, the resulting plasmids contain the 14S rRNA promoter deleted to various extents from the 5′ end, the deleted sequences replaced with the *lacZ′* sequences of pUR250.

Promoter Function Assay of 5′-Deleted Plasmids. To assay for the ability of the deleted plasmids to function as a template for correct initiation of transcription at the 14S rRNA promoter, we used a run-off transcription assay. In this assay, template DNA is digested with a restriction endonuclease downstream of the transcriptional initiation site. Correct initiation and elongation to the end of the template result in a transcript of characteristic length. We were interested not only in the absolute ability of the deleted plasmids to function as templates for correct initiation but also in the relative strength of the partially deleted plasmids that retained functional promoters. To avoid artifacts due to variable levels of inhibitory contaminants in the plasmid preparations or due to differential recovery of reaction products, a control plasmid was included in each reaction mix in equal concentration with the deleted plasmid to be assayed. The control promoter plasmid used for the 5′ deletions is a plasmid generated by the above procedure in which only 1 base had been deleted, retaining the mitochondrial sequences to position −234. For the promoter function assay, the control plasmid was digested at the *Eco*RV site 309 bp downstream of the initiation site and the test plasmids were cut at the *Cla* I site 145 bp downstream of the initiation site. Thus, in each reaction, two templates were present in equal concentration: an undeleted control plasmid, which would yield a run-off transcript of 309 nucleotides, and a 5′-deleted plasmid, which, if competent, would yield a transcript of 145 nucleotides. A variation in the relative intensity of the two run-off bands from lane to lane would reflect a change in the strength of the 14S rRNA promoter with deletion of 5′ sequences.

The results are shown in Fig. 2. The second through seventh lanes show the products of transcription from test plasmids deleted to positions −234, −125, −75, −32, −12, and −10 as labeled. Each of these plasmids is capable of directing correct initiation of transcription. The relative intensity of the 145-nucleotide test run-off product and the 309-nucleotide control run-off product does not vary with the extent of deletion, indicating that the deleted sequences have no effect on the strength of the promoter in the *in vitro* system. The eighth and ninth lanes display the products of transcription from plasmids deleted to positions −5 and −3. With either of these test plasmids as template, the expected

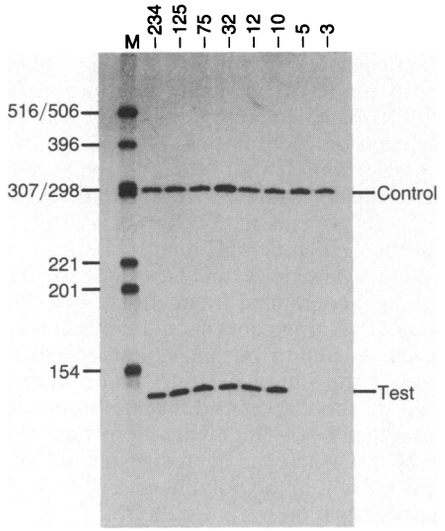

FIG. 2. Run-off transcription from 5′-deleted templates. The lane marked M contains end-labeled molecular size markers, the sizes of which are indicated in nucleotides to the left of the autoradiogram. The products of the transcription assays are displayed in the second through ninth lanes. In each case, the band of 305 nucleotides is the transcript from the undeleted control template digested with *Eco*RV. The band of 145 nucleotides is the transcript from the various deleted test templates digested with *Cla* I. The lanes are labeled with the position of the last base retained in the deleted test template as numbered from the initiation site.

product of transcription is not detectable while the transcript from the control plasmid is present at the usual level.

To determine whether the physical presence of upstream DNA is required for correct initiation, the plasmid deleted to −10 was digested with both *Cla* I and *Eco*RI and used as a template in the transcription assay. The expected transcript was synthesized from the truncated template, indicating that the presence of upstream DNA is not required for promoter function (data not shown).

The results localize the 5′ end of the mitochondrial promoter between positions −10 and −5. Furthermore, replacement of the entire region upstream of position −10 with an unrelated sequence has no detectable effect on promoter strength in the *in vitro* system.

3′-Deleted Plasmids. To facilitate the generation of 3′ deletion mutants, a different parent plasmid was constructed. The mitochondrial promoter-bearing DNA fragment of pJE14S from the *Sac* II site at position −235 to the *Hin*dIII site at position 150 was transferred to the *Hin*dIII/*Hin*cII sites of pUR250 (with disruption of the *Sac* II/*Hin*cII sites). The resulting plasmid, designated pTB14S, is diagramed in Fig. 1*C*. pTB14S was deleted with BAL-31 from the *Cla* I site at position +145, and the deleted DNA was purified by size and inserted into pUR250 in a manner entirely analogous to the procedure described above. As diagramed in Fig. 1*D*, the resulting plasmids contain the 14S rRNA promoter in which 3′ sequences have been deleted to various extents, the deleted DNA replaced with the *lacZ′* gene sequences of pUR250. As above, sequences were determined for representative deleted plasmids.

A set of relevant plasmids was selected for promoter function assay. An undeleted promoter plasmid generated during the deletion mutagenesis was used as a control in each transcription reaction. The *Pvu* II site, located within the vector sequences 95 bp downstream of the *Eco*RI site of pUR250, was used for run-off transcription. In each case, the length of the expected transcript is equal to the length of mitochondrial sequences retained downstream of the initiation site plus the 95 nucleotides from the *Eco*RI site at the end point of the deletion to the *Pvu* II site in the vector.

The results are shown in Fig. 3*A*. In each lane, the 246-nucleotide run-off transcript is the product of transcription from the control plasmid, which retains mitochondrial sequences to approximately position 120. The progressive shortening of the test transcript reflects the fact that, as the extent of deletion increases, the *Pvu* II run-off site in the vector is moved closer and closer to the transcriptional initiation site. The second through seventh lanes show the assay of transcription from clones deleted to positions +64, +49, +31, +15, +4, and +2 as labeled. In each case, the plasmid is capable of serving as a template for selective transcriptional initiation, indicating that the promoter sequences have not been disrupted in any of these plasmids. The eighth lane shows the transcription assay from a plasmid deleted to position −1 in which the initiating nucleotide is replaced with the first G of the *Eco*RI linker. Transcription from this template is drastically reduced but still detectable on long exposure. Templates deleted to position −3 (ninth lane) or beyond (data not shown) are incapable of directing correct initiation. Hence, the 3′ border of the sequences absolutely required for selective initiation appears to be between positions −1 and −3, although altering the first 2 nucleotides in the transcript clearly has a profound effect on promoter strength.

The interpretation of relative promoter strength of the 3′-deleted plasmids is more difficult than above because of the varying lengths of the test transcripts. Since [α-^{32}P]UTP was the label used in the transcription reactions, the amount of radioactivity incorporated into each band is dependent on the number of uridylyl residues incorporated per transcript. The sequence of the transcribed region is known in each case and the uridine content of the transcripts vary, with 78 uridine residues per transcript of the undeleted control

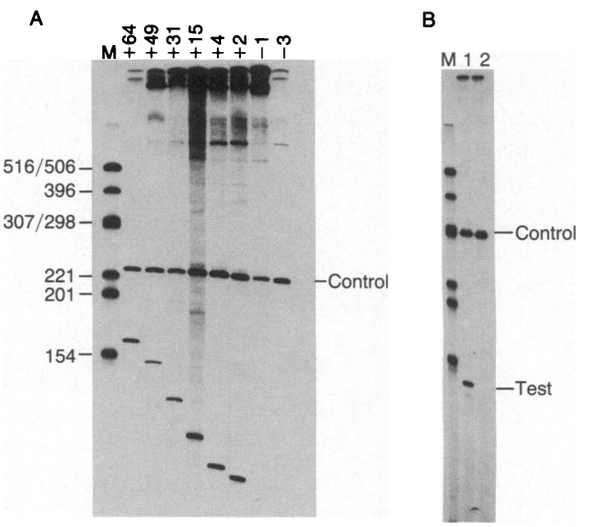

FIG. 3. (*A*) Run-off transcription from 3′-deleted templates. The lane labeled M contains molecular size markers (shown in nucleotides) as indicated. The products of transcription assays are displayed in the second through ninth lanes. The band of 225 nucleotides is the product of transcription from the undeleted control template. The sizes of the transcription products from the deleted templates vary from 160 to 100 nucleotides. The lanes are labeled with the position of the last base retained in the deleted test template as numbered from the transcriptional initiation site. (*B*) Run-off transcription from linker substitution mutant. The bands of 305 and 145 nucleotides are the control and test transcripts as in Fig. 2. Lane M, molecular size markers as labeled in *A*. Lane 1, test template is 5′ deletion pTB5′−234 (assay also shown in second lane, Fig. 2). Lane 2, test template is linker substitution mutant pTBS1 (Fig. 4*D*).

			TEMPLATE ACTIVITY
A.	WILD TYPE	TTATTTATTATT**ATATAAGT AA**TAAATAATAGTTTTATAT	+
		-20 1 20	
B.	5' DELETIONS		
	pTB5'-12	tgaattccTATTATATAAGT AATAAATAATAGTTTTATAT	+
	pTB5'-10	agtgaattccTTATATAAGT AATAAATAATAGTTTTATAT	+
	pTB5'-5	cggccagtgaattccTAAGT AATAAATAATAGTTTTATAT	-
	pTB5'-3	gacggccagtgaattccAGT AATAAATAATAGTTTTATAT	-
C.	3' DELETIONS		
	pTB3'+4	TTATTTATTATTATATAAGT AATAggaattcactggccgt	+
	pTB3'+2	TTATTTATTATTATATAAGT AAggaattcactggccgtcg	+
	pTB3'-1	TTATTTATTATTATATAAGT ggaattcactggccgtcgtt	±
	pTB3'-3	TTATTTATTATTATATAAgg aattcactggccgtcgtttt	-
D.	SUBSTITUTION MUTATION		
	pTBS1	TTATTTATTA·ggaattcc· AATAAATAATAGTTTTATAT	

FIG. 4. Sequence of selected deleted templates. Uppercase letters are bases retained from wild-type mitochondrial DNA. Lowercase letters are bases from inserted linkers and plasmid DNA. (*A*) Wild-type sequence. The region shown in this study to be required for high-efficiency promoter function is underlined. The 9-nucleotide conserved sequence is shown in boldface. (*B*) 5' deletions. (*C*) 3' deletions. (*D*) Linker substitution mutant.

plasmid, 51 uridine residues per transcript from the plasmid deleted to position +64, and 22 uridine residues per transcript from the plasmid deleted to position +2. The intensity of the bands resulting from transcription of functioning test promoters in Fig. 3 appears to be consistent with the variation in uridine content. Therefore, we conclude that our methods detect no major effect on the strength of the 14S rRNA promoter when sequences up to position +2 are deleted and replaced with unrelated sequences. In contrast, deletion and replacement of sequences up to position −1 clearly have a profound effect on promoter strength without totally disrupting promoter function. Deletion to position −3 or beyond totally destroys promoter function.

The sequences surrounding the initiation site in those plasmids deleted close to the initiation site are shown in Fig. 4. A drawback to deletion mutagenesis for investigating sequences involved in sequence-specific protein–DNA interactions is that very large sequences are removed and replaced with completely unrelated sequences. A more elegant method of probing for important sequences is to make site-specific mutations within a putative protein binding sequence, leaving the flanking regions intact. We used our set of deletions to generate one such mutant in which the 9-nucleotide promoter sequence is removed and replaced with an *Eco*RI linker without disrupting either of the flanking sequences. The plasmid, pTBS1, which was generated by linking together a 5' deletion that had been deleted to position +1 with a 3' deletion that had been deleted to position −11, is diagramed in Fig. 4*D*. This plasmid is incapable of serving as a template for correct initiation of transcription, as shown in Fig. 3*B*.

DISCUSSION

In this paper, we describe the generation of collections of promoter-bearing plasmids deleted from either the 3' or 5' direction and the use of these mutants in characterizing the borders of the 14S rRNA promoter in yeast mitochondria. Our results indicate that the sequences required for efficient selective transcriptional initiation lie between positions −10 and +2 as numbered from the initiation site. This interpretation is further verified by the analysis of a third class of mutation in which the sequences from positions −10 to −1 are replaced with an *Eco*RI linker, leaving the flanking sequences intact. This construct cannot function as a promoter.

The identity of promoter sequences on yeast mitochondrial DNA has been suggested previously by comparison of sequences surrounding known promoters. On this basis, it has been proposed that promoter sequences for the yeast mitochondrial RNA polymerase consist of a sequence from positions −8 to +1, and our results are in good agreement with that conclusion. Two preliminary reports of studies of yeast mitochondrial promoter sequences using directed mutagenesis and *in vitro* transcription speak to this same issue. In agreement with the results presented in this paper, we reported previously the use of a different set of 5' deletion mutants to demonstrate that the 5' border of sequences required for correct initiation lies within 12 nucleotides of the initiation site (17). In contrast with our present results, Tabak *et al.* (18), using oligonucleotide-directed mutagenesis, found no effect of several base substitutions within the 9-nucleotide sequence on promoter function, which was, however, disturbed by a double base substitution at positions +10 and +13. This last result is not easily reconciled with the results presented in this paper in which we show that replacement of all sequences downstream of position +2 is without any apparent effect on transcription. In our experiments, the deleted mitochondrial sequences were replaced with *lacZ'* sequences of pUR250 that show minimal homology to the mitochondrial sequences they replace (Fig. 4). Specifically, the bases at positions +10 and +13 in the mutant deleted to position +2 are not identical to bases found at the corresponding positions of the native mitochondrial sequence. However, these bases are not identical to the bases in the double mutant reported previously either. Perhaps the double mutation has generated a specific inhibitory sequence that down-regulates the adjacent promoter.

Effect of Flanking Sequences on Promoter Strength. Although these studies strongly implicate the 9-nucleotide consensus sequence as a sufficient sequence for the yeast mitochondrial promoter, other studies suggest that promoter function is probably modulated by sequences outside the 9-nucleotide sequence. All hitherto characterized yeast mitochondrial promoters share close homology to the 9-nucleotide sequence, although the apparent strengths of various promoters vary considerably. In particular, two transcriptional initiation sites are present in tandem upstream of the O_I gene, each at an exact copy of the 9-nucleotide sequence. However, the two promoters show different strengths *in vitro* and appear to be used to different

extents *in vivo* (5). Several copies of the 9-nucleotide promoter sequence are present on the mitochondrial genome that appear not to function as promoters *in vivo* at all (4). These observations suggest that the 9-nucleotide sequence may define an initiation site for mitochondrial RNA polymerase but that the strength of the promoter is modulated by flanking sequences.

In the experiments presented here, promoter strength was not affected by deletion of all of the sequences upstream of the short promoter sequence. Interpretation of the relative strengths of the 3'-deleted promoters is more difficult, as discussed above. However, our results indicate that downstream sequences also do not exert a major effect on the strength of this promoter. Since the promoter we studied is one of the strongest promoters on the yeast mitochondrial genome, the results suggest that strong promoters do not depend on specific positive effector sequences in their flanking regions but that weaker promoters are influenced by negative effector sequences in their flanking regions.

Comparative Anatomy of Promoters. Recently, the structure of mammalian mitochondrial promoters has been analyzed by deletion mutagenesis and *in vitro* transcription (19, 20). As we have found in yeast, the sequences required for promoter function in mammalian mitochondria are limited to a short region surrounding the initiation site. However, the sequence of the promoter region shows no homology to the yeast mitochondrial promoter. Perhaps, as yeast and humans evolved, the general structure of the mitochondrial promoter has been retained, although the actual sequence that interacts with mitochondrial RNA polymerase and the promoter binding site on the polymerase itself has diverged.

The rather simple structure of mitochondrial promoters is in sharp contrast to promoter structure in most other systems. Both prokaryotic and nuclear eukaryotic promoters in general consist of multiple noncontiguous blocks of sequences (21–26). The structure of the mitochondrial promoter seems more closely related to that of bacteriophage T7 in which the promoter consists of a short sequence immediately upstream of the transcriptional initiation site (27).

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