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Nucleotides flanking the promoter sequence influence the transcription of the yeast mitochondrial gene coding for ATPase subunit 9

(transcription control/mitochondrial promoter/*Saccharomyces cerevisiae*)

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ABSTRACT **The conserved nonanucleotide sequence functions as a promoter in the yeast (*Saccharomyces cerevisiae*) mitochondrial genome. A mitochondrial gene, *Oli 1*, which codes for ATPase subunit 9, has two identical nonanucleotide promoter sequences separated by 78 nucleotides, but they initiate transcription with very different efficiencies *in vivo* and *in vitro*. Deletion analysis has revealed that the nucleotide at position +2 of the weak downstream promoter accounts for its poor *in vitro* transcriptional activity. This finding was confirmed with site-specific mutations at +2 and +3 positions of a consensus synthetic promoter. The nonanucleotide mitochondrial promoter with a pyrimidine at position +2 acts as a weak promoter, whereas the same sequence with a purine at the +2 position functions as a strong promoter. The nucleotide at the +3 position further contributes to the relative promoter strength. These results suggest not only that the conserved nine-nucleotide sequence is required for the correct transcriptional initiation but also that other neighboring nucleotides influence the efficiency of promoter function.**

Several features distinguish the expression of the 75-kilobase-pair yeast mitochondrial genome and the more compact mammalian mitochondrial genome. Only one-fifth of the potential information in a single strand equivalent of yeast mitochondrial DNA encodes the known mitochondrial genes, which include sequences specifying three of at least nine subunits of ATPase, three of the seven or more subunits of cytochrome oxidase, one subunit of cytochrome b–c_1 complex and var 1, a mitochondrial-ribosome-associated peptide (1–3). Mitochondrial DNA also encodes two rRNAs and 25 tRNAs. Unlike the mammalian mitochondrial genome (4, 5), the yeast mitochondrial genome contains multiple transcription units. The different transcription initiation sites have been identified by *in vitro* capping of the transcripts with guanylyltransferase (6). The distribution and multiplicity of the transcription initiation sites throughout the genome (7–9) suggest the possibility of differential gene expression. The transcripts specifying the sequences of protein, rRNA, and tRNA products are all initiated at the last nucleotide of a strongly conserved nonanucleotide consensus sequence (6, 9) that appears to be all that is absolutely required for the correct initiation of transcription (10). The presence of a common promoter element suggests that a single polymerase acts on all mitochondrial transcription units. However, some sites of the nonanucleotide consensus appear to be transcriptionally inactive (6). This presents the opportunity for investigating the factors that determine the utilization of a particular promoter sequence. A mitochondrial gene, *Oli 1*, coding for ATPase subunit 9, has two closely spaced perfectly matched nine-nucleotide sequences that function quite differently as promoters (6, 7). The upstream promoter is effectively used, while the downstream nonanucleotide sequence serves as a weak promoter. Other regulatory elements probably influence the promotion activity of these nine-nucleotide sequences. We have designated the upstream promoter of the *Oli 1* gene as Op_1 and the downstream promoter as Op_2. In this study deletion mutagenesis of the sequences downstream of Op_2 shows that the nucleotide at the +2 position is very important for the apparent promoter activity of the nonanucleotide. This observation was reinforced by the transcription results with site-specific mutations at positions +2 and +3.

MATERIALS AND METHODS

Strains and Plasmids. Yeast mitochondrial RNA polymerase was purified from *Saccharomyces cerevisiae* strain D273-10b. Plasmid $Op_1$5′-67 was derived from pK_9 (7) by 5′-deletion mutagenesis. Bacterial strain RR1ΔM15 (11) and plasmid pUR 250 (12) have been described.

Oligonucleotide Synthesis and Cloning. Oligonucleotides that correspond to both strands of the conserved nine-nucleotide sequence but with base changes at +2 and +3 positions were synthesized by the phosphoramidite method by using an Applied Biosystems (Foster City, CA) Model 380A DNA synthesizer and were purified by electrophoresis on a 12% sequencing gel.

Two complementary oligonucleotides were denatured by heating at 90°C for 5 min followed by quick chilling in ice water and allowed to hybridize overnight at 4°C. The scheme used to generate point mutations at +2 and +3 positions downstream of the mitochondrial promoter is shown later in Fig. 4. This synthetic duplex has one complementary end (*Hin*dIII) and one blunt end. Ligation was performed in a defined orientation by combining *Hin*dIII–*Hin*cII-cut plasmid vector (pUR 250) and duplex oligomers in a molar ratio of 1:15 (plasmid/oligomer) in 15 μl of ligase buffer (50 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$/20 mM dithiothreitol/1 mM ATP). After incubation overnight at 8°C with 2 units of T4 DNA ligase, the reaction mixture was used to transform *Escherichia coli* strain RR1ΔM15 (11). Transformants lacking β-galactosidase activity were selected from indicator plates and streaked onto an ampicillin plate, and a single colony formed from each transformant was grown in liquid medium for further screening. After isolation of plasmid DNA, the precise sequence of the promoter insert was determined by using the dideoxy method directly on plasmid DNA (13).

Transcription Assay. A standard 25-μl reaction mixture contained 10 mM Tris·HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM KCl, 5% (vol/vol) glycerol, 0.5 mg of rabbit serum albumin per ml, 125 μM each ATP, GTP, CTP, and UTP/50 μCi of [α-^{32}P]UTP (1 Ci = 37 GBq) and a total of 40 μg of DNA per ml comprising the two linear templates each present in equal

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concentration. These same assay conditions were used to prepare end-labeled transcripts except that 100 μM ATP containing 125 μCi of [γ-^{32}P]ATP was added in place of radioactive UTP. Reactions were started by the addition of 1–2 μl of highly purified yeast mitochondrial RNA polymerase purified as described by Levens *et al.* (14) and generously provided by B. Ticho. Under these conditions the reactions were saturated for DNA. Incubation was carried out at 28°C for 15 min, and the reaction was terminated by the addition of 25 μl of stop solution containing 0.3 M sodium acetate, 0.3% $NaDodSO_4$, 1.0 mM unlabeled nucleotide (UTP or ATP as appropriate) and 200 μg of tRNA/ml. The reaction mixture was then extracted with 50 μl of phenol. After phenol extraction unincorporated labeled nucleotides were separated from high molecular weight products on a spin column containing Bio-Gel P-6 matrix (equilibrated with 0.3 M sodium acetate and 0.3% $NaDodSO_4$) (15). The material excluded from the column was collected and precipitated with 2.5 vol of absolute ethanol. Samples were dried, resuspended in 90% (vol/vol) formamide containing 10 mM EDTA and marker dyes, and electrophoresed through an 8% (wt/vol) polyacrylamide/50% (wt/vol) urea gel. Autoradiograms were obtained by using Kodak XAR-5 film with an intensifier screen at −70°C. For quantitative assessment, the exposed bands were eluted from photographic plates with 1 M NaOH, and the reduced silver grains were measured spectrophotometrically (16).

RESULTS

Construction of 3′-Deletion Mutants. A 78-base segment separates the initiation nucleotides of the two *Oli 1* promoters. To test whether the 3′ flanking sequence of the *Oli 1* gene influences the activity of Op_1 and Op_2 promoters we have generated a series of 3′-deletion mutants. These mutants were generated from a parent plasmid $Op_1$5′-67 that contains the mitochondrial sequences 67 base pairs upstream and 240 base pairs downstream from the initiation nucleotide of the Op_1 promoter (Fig. 1). To construct the 3′-deletion mutants the parent plasmid was cleaved at the *Eco*RI site (258 base pairs downstream of Op_1) and digested with BAL-31 as

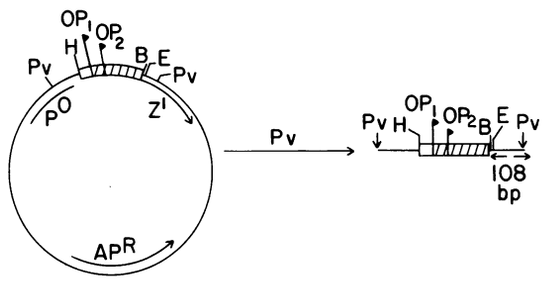

FIG. 1. Physical maps of mitochondrial *Oli 1* gene promoter-bearing plasmids. Mitochondrial DNA deletion mutants were cloned by insertion into pUR 250. The mtDNA segment was deleted to a different extent from the 3′ side by BAL-31 treatment and was inserted into *Hin*dIII/*Hin*cII sites in pUR 250. The single line represents the vector DNA with the antibiotic resistance markers and *lacZ′* genes as indicated. The wide double lines represent the mtDNA inserts. The open area in the bars represents the region upstream of the major transcriptional initiation sites, and the hatched area represents the transcribed region. Closed flags designate the promoters. The different deletion mutants have 3′-mitochondrial flanking sequences of differing lengths. To obtain a template yielding a substantial *in vitro* transcript length, advantage was taken of the downstream *Pvu* II restriction endonuclease site in the vector as shown. The undeleted *Pvu* II fragment is expected to yield two transcripts, 353 bases and 275 bases from Op_1 and Op_2 promoters, respectively, while progressive shortening of these transcript lengths is expected as 3′ sequences are deleted. Restriction site abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Pv, *Pvu* II.

described (10). The 3′-deleted mitochondrial fragments were separated from the vector by digestion with restriction endonuclease *Hin*dIII and isolated by 6% polyacrylamide gel electrophoresis. The isolated fragments were then inserted into pUR 250 that had been digested with restriction endonucleases *Hin*dIII and *Hin*cII. The precise extent of the 3′ deletions was determined by DNA sequencing (Fig. 2). The deleted mutant DNA is designated by its most 3′ mitochondrial nucleotide—e.g., Op_1+11 indicates that all mitochondrial bases downstream of the 11th nucleotide from the initiating nucleotide of Op_1 are deleted. Similarly, Op_2+33 indicates that all mitochondrial bases downstream of the 33rd nucleotide from the initiation nucleotide of the Op_2 promoter are deleted.

The recloned set of deletion mutants was cleaved to provide templates for *in vitro* run-off transcription assays. The *Pvu* II restriction endonuclease site in pUR 250, 108 base pairs downstream of the *Hin*cII site, was used. The length of the transcript expected with correct initiation of transcription is equal to the length of the mitochondrial sequence retained downstream of the initiation site plus the 108 nucleotides from the endpoint of the deletion to the *Pvu* II site in the vector. A 3′-deletion mutant of yeast 14S rRNA gene, TB3′+64 (10) was digested with *Eco*RI and used as a control template.

The transcripts produced from 3′-deletion mutants were analyzed by gel electrophoresis (Fig. 3). In each lane the 70-nucleotide run-off transcript is the product of transcription from the control plasmid. The progressive shortening of the test transcripts from the different 3′-deletion mutants reflects the differing extent of deletions. Since [γ-^{32}P]ATP was the label used in the transcription reaction, only the 5′ nucleotide is labeled, and the amount of radioactivity incorporated into each band is independent of the length of the transcript. The second through fourth lanes show the transcripts from clones deleted up to Op_2+163, +33, and +9, respectively. In each lane, transcription from both promoters (Op_1 and Op_2) is similar to that observed with the undeleted parent clone as template, indicating no effect of the deleted sequences on transcription. The transcript from Op_2 is very faint in these three lanes although more visible on the original autoradiograms. On the other hand, the fifth lane shows the transcription product from a plasmid containing only the initiation nucleotide of Op_2 promoter and all other downstream mitochondrial sequences deleted. Transcription from Op_2 in this deletion mutant is enhanced several-fold although the transcription from Op_1 is not markedly altered. Deletion to position −1 of Op_2 (lane 6) eliminates transcription from the second promoter. As expected, deletion of the second promoter eliminates the second transcript while leaving the promoter activity of Op_1 essentially unchanged (lanes 7–9). Hence the 3′ boundary of the promoter sequence absolutely required for selective initiation at Op_2 appears to be between +1 and −1.

Effect of Base Changes at Positions +2 and +3. The enhancement of the promoter activity of Op_2 revealed in the Op_2+1-deletion mutant could result from the change of the nucleotide at position +2 (thymidine to guanosine). This suggests that the nucleotide at the +2 position could be an important determinant of the extent of transcription from the nonanucleotide promoter in the yeast mitochondrial genome. A major drawback of deletion studies aimed at identifying the regulatory sequences involved in sequence-specific protein–DNA interactions is that the sequences surrounding the deletion point are often totally changed. An alternative way of studying important regulatory sequences is to make site-specific mutations at the point of interest while keeping the flanking sequence intact. To this end, plasmid inserts containing site-specific substitution at +2 and +3 positions

		TEMPLATE ACTIVITY
WILD TYPE (Op_2)	TTATATAAGT ATATATATATATTATTAATATAATGAACATCTATTAAATAA	+/-
Op_2+33	TTATATAAGT ATATATATATATTATTAATATAATGAACATgacggatccgg	+/-
Op_2+9	TTATATAAGT ATATATATAgacggatccggggaattcactggccgtcgttt	+/-
Op_2+1	TTATATAAGT Agacggatccggggaattcactggccgtcgttttacaacgt	+++
Op_2-1	TTATATAAGT gacggatccggggaattcactggccgtcgttttacaacgtc	-

FIG. 2. Sequence of selected deletion mutants. The mtDNA sequences are shown in capital letters, while sequences derived from the vector pUR 250 polylinker cloning region are in lowercase. The nine-nucleotide promoter sequence is shown in boldface type. The asterisks over nucleotides indicate the site at which initiation occurs *in vivo* and *in vitro*. The column at the right indicates the semiquantitative results of the transcription experiment shown in Fig. 3, where +/− represents weak template activity, +++ represents markedly enhanced template activity, and − represents no template activity.

have been constructed to determine if nucleotides at these positions regulate the level of transcription.

The procedure employed to construct the related site-specific mutants is outlined in Fig. 4. In brief, two partially complementary oligonucleotides (one is a 25-mer and another is a 21-mer) were synthesized chemically. After purification, the complements were hybridized. The synthetic hybrid contains an intact nonanucleotide sequence with one sticky end and one blunt end. One strand has base changes at positions +2 and +3. The synthetic hybrids were inserted into pUR 250 that had been treated with *Hin*dIII and *Hin*cII endonuclease. The mutants were identified by DNA sequencing. Out of 16 possible combinations 11 different types of mutants were obtained by screening approximately 110 clones. Plasmid DNA was digested with *Pvu* II that should yield a run-off transcript of 117 nucleotides. Examination of template activity of mutant DNAs demonstrates their different levels of activity (Fig. 5). The relative promoter strength among these synthetic templates is compared (Table 1). Nonanucleotide sequences with adenosine or guanosine at the +2 position function as strong promoters whereas the same sequence with pyrimidine at that position acts as a weak promoter. The base changes at the +3 position also have some, though less pronounced, effects on mitochondrial promoter activity. Thus, the nucleotides at the +2 and +3 positions greatly influence the relative template activity of the nonanucleotide sequence without affecting the selectivity of transcriptional initiation.

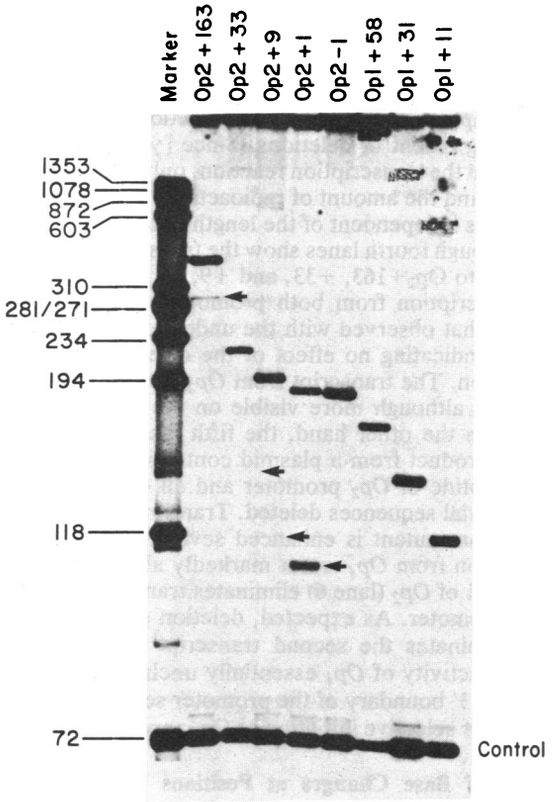

FIG. 3. *In vitro* transcription of 3′-deletion mutants. An autoradiogram of a polyacrylamide gel analysis of *in vitro* synthesized RNA is shown. RNA was labeled with [γ-^{32}P]ATP during transcription. Lane 1 shows DNA size standards of end-labeled *Hae* III fragments of phage ΦX174. Lanes 2–9 are labeled with the positions of deletion endpoints, numbered relative to the transcription initiation sites of the indicated promoter. Positive numbers indicate positions downstream from the initiation site, and negative numbers indicate the position upstream from the initiation site. The specific transcripts from the two mitochondrial promoters in undeleted insert are about 353 nucleotides and 275 nucleotides in length, decreasing with the extent of deletion. The bands corresponding to the transcripts arising from downstream promoter are indicated by arrows. The control plasmid, *Eco*RI-digested TB3′+64 yields a transcript of 70 nucleotides.

DISCUSSION

In contrast to transcription of the mammalian mitochondrial genome, the mitochondrial genome of yeast is expressed through multiple transcription units, many of which encode more than one gene product. Transcription of each of these units in yeast, *Saccharomyces cerevisiae*, is initiated at a consensus promoter made up of nine nucleotides. The uniformity of the sites of transcriptional initiation suggests that a single polymerase is involved in the transcription of rRNA, tRNA, and mRNA genes. The existence of multiple transcription units suggests the possibility of differential transcriptional regulation of mitochondrial gene expression. Indeed, evidence indicates that there are quite marked differences in the level of transcription at the various transcription units (ref. 24 and D. Mueller, personal communication). Such differences may be observed even when the same consensus nonanucleotide functions as promoter. We (10) suggested that strong promoters do not depend on specific positive effector sequences in their flanking regions, but that weak promoters may be influenced by inhibitory sequences in their neighboring regions. In this paper we provide evidence that certain nucleotides at the +2 position can in fact reduce the level of yeast mitochondrial transcription.

As a model for investigating the influences on transcriptional efficiency, the tandem promoters upstream of the *Oli 1* gene were studied. These two nonanucleotide promoters that are separated by 78 nucleotides have identical sequences, yet the upstream promoter is used much more efficiently *in vivo* (6) and *in vitro* (7). Our observations have confirmed these earlier *in vitro* findings. Also the 3′-deletion studies reported here have confirmed again that the 3′

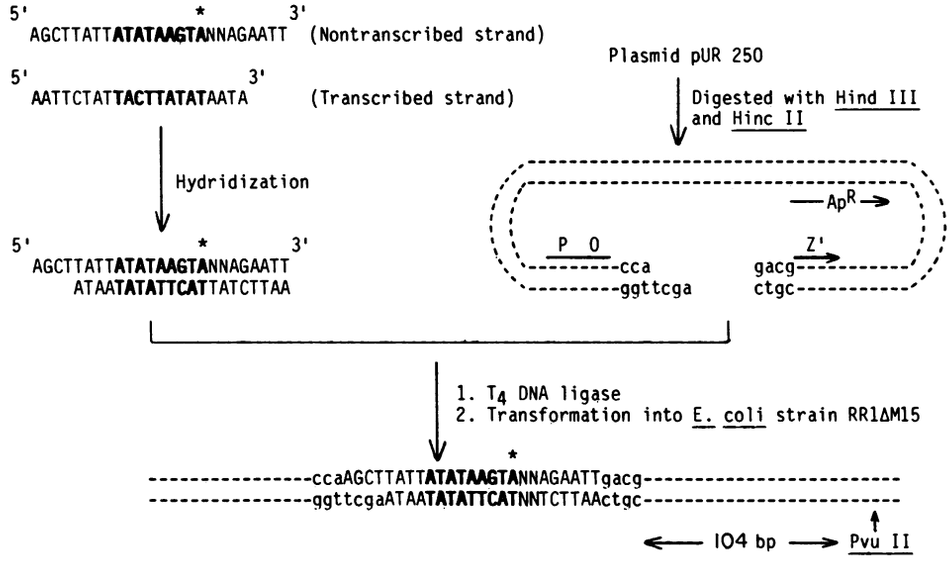

FIG. 4. Construction of a plasmid DNA with base changes at positions +2 and +3. Mutant oligonucleotides, 25 nucleotides in length and containing 16 bases of the yeast mitochondrial promoter sequence and its 21-mer complementary oligonucleotide, were synthesized and purified. These two complementary oligonucleotides were heated at 90°C for 5 min and cooled quickly in ice water. Hybridization was carried out overnight at 4°C. The synthetic duplex oligonucleotides containing one sticky end (*Hin*dIII) and one blunt end were inserted into *Hin*dIII and *Hin*cII sites of pUR 250 (with disruption of *Hin*cII sites). The positions marked NN in the nontranscribed strand are the sites of specific mutations, +2 and +3. The products of ligation were used to transform *Escherichia coli* strain RR1ΔM15.

boundary of the promoter for efficient transcription is at the +1 nucleotide (10).

The importance of the +2 position was first highlighted by the 3' deletion of sequences downstream of the second, and notably weaker, promoter Op_2. All such deletions, except one, leave the Op_2 promoter as a weak *in vitro* promoter. The one exception is the deletion of all nucleotides downstream of the weak promoter Op_2 save the +1 nucleotide. This deletion mutant had a notably increased transcription efficiency. In this mutant downstream sequences were replaced by plasmid sequences. This involved the replacement of the +2 thymidine with a plasmid guanosine. Inspection of the sequences of nucleotides surrounding promoters with a range of transcriptional efficiencies revealed that at least five weak promoters had thymidine in the +2 position, while this nucleotide did not occupy the +2 position in any of the known strong promoters (ref. 24 and D. Mueller, personal commu-

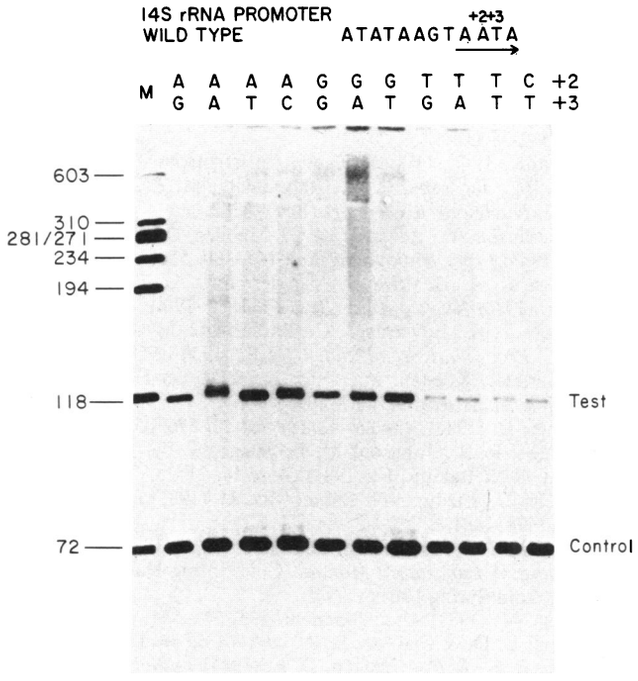

FIG. 5. Run-off transcription from synthetic mutant promoters. Lane 1 contains end-labeled molecular size markers, the sizes of which are indicated in nucleotides to the left of the autoradiogram. Transcription reactions were carried out with [α-^{32}P]UTP, as labeled precursor. The products of the transcription assays are displayed in lanes 2–12. In each case, the band of 70 nucleotides is the transcript from the control plasmid. The band of 117 nucleotides is the transcript from various synthetic templates digested with *Pvu* II. The lanes were labeled with different letters representing nucleotides present at +2 and +3 positions in the synthetic templates.

Table 1. Relative promoter strengths of site specific mutants

	Nucleotide substitution, position		Relative band intensity
	+2	+3	
Wild-type sequence	A	T	
Mutant sequence	A	G	0.56
	A	A	0.33
	A	T	1.00
	A	C	0.81
	G	G	0.52
	G	A	0.78
	G	T	1.08
	T	G	0.16
	T	A	0.14
	T	T	0.12
	C	T	0.16

The wild-type promoter sequence is TTATTATATAAGTA*AT*-TAG (nucleotides +2 and +3 are in italics). Different nucleotides were substituted into positions +2 and/or +3 and the promoter strengths were measured. The band intensities of different synthetic promoters and control plasmid were quantitated spectrophotometrically following elution of silver grain from the autoradiograms. The relative transcription shown by the nucleotide sequence was calculated as follows: [Band intensity of test plasmid (NN)/band intensity of the control plasmid in the same lane)/(Band intensity of test plasmid (AT)/band intensity of the control plasmid in that same lane]. These results are the average of three separate experiments.

nication). These observations suggested that a +2 thymidine might account for the low level of transcription observed with several weak promoters.

Since deletion mutagenesis has the defect that the deleted sequence is replaced by a totally new sequence, conclusions about a single nucleotide change derived from deletional analysis have to be interpreted with care. However, the available evidence on natural yeast mitochondrial promoters does underline the attenuating effects of the +2 thymidine. This conclusion was confirmed with site-specific mutations involving the +2 and +3 positions downstream of a synthetic strong consensus promoter. In contrast to the relatively strong transcriptional activity of the promoter with a purine at the +2 location, a pyrimidine at the +2 position resulted in a weak *in vitro* promoter. On the other hand, at the +3 position, the presence of a pyrimidine results in a more effectively utilized promoter than with a purine in this position. Thus, the relative promoter strengths of the upstream, Op_1, and downstream, Op_2, promoters seem to be explained in part by the inhibitory effects of the +2 thymidine associated with Op_2. Another explanation may be related to the influence of the upstream promoter on the downstream promoter.

We have reported (10), based on deletion mutagenesis, that the boundaries of the 14S rRNA promoter extend from the +2 to the −10 nucleotides. Replacement of the +1 adenosine by the plasmid guanosine essentially eliminates promoter activity. The present results deal with the modulating effects of the contiguous +2 and +3 nucleotides. Changes especially at the +2 position result in differences in transcriptional activity extending over a wide range (Fig. 5), with +2 thymidine being least active. These results raise the question about how the promoter is defined. Should the +2 nucleotide be regarded as part of the promoter? Since any nucleotide at this position is compatible with selective initiation of transcription, albeit at widely different levels, the +2 nucleotide should probably not be considered part of the promoter. However, two reservations are in order. The mechanism and the extent of mitochondrial RNA polymerase interaction with its promoter and neighboring nucleotides are not clearly defined. Also the precise compositional plasticity within the consensus nonanucleotide that is compatible with selective transcriptional initiation at the +1 nucleotide is yet to be fully defined. Knowledge on these two issues will help to refine the definition of this promoter.

Candidate promoters on the strands of human mitochondrial DNA have been mapped by deletion analysis and *in vitro* transcription (5, 17) and by *in vitro* capping of a primary transcript of human mitochondrial DNA (18). Transcription of the heavy strand initiates 16–19 nucleotides upstream of the start of $tRNA^{Phe}$ (17, 18). Deletion analysis has defined the outer boundaries of the putative heavy-strand promoter within −16 and +7 nucleotides of the transcriptional start site, i.e., a total of 23 nucleotides, which includes a 15-nucleotide consensus stretch that encompasses the start site and is highly conserved in the putative promoters of the human mitochondrial DNA. This consensus sequence also occurs in the conserved sequence block (CSB-3) upstream of the heavy-strand promoter. Yet no transcription occurs at this site (17), a finding of interest in the context of our results with the yeast *Oli 1* tandem promoters. Also, little transcription originated in the region between the $tRNA^{Phe}$ and the 12S rRNA genes (17), in contrast to the model proposed to account for differential ribosomal gene transcription (19). It was postulated in this latter model that a pair of tandem promoters on the heavy-strand genome of human mitochondria are differentially used in the transcription of the rRNA genes more often than the downstream genes. The putative promoter near the 5′ end of the 12S rRNA gene is thought to be used for the transcription of a polycistronic molecule covering most of the heavy strand (19). Site-specific mutagenesis of sequences in and around the human mitochondrial promoters revealed that single-nucleotide substitution upstream of the transcriptional start sites and even outside of the promoter site defined by deletion analysis (17) could abolish transcription (20).

The physiological significance of tandem promoters remains unclear. In some other systems multiple promoters appear to relate to the need for rapid expression of a gene as for the rRNA genes in *E. coli* (21) and in *Xenopus laevis* (22). Alternatively tandem promoters of a gene could be regulated differentially to serve specific cellular functions as in the case of the *gal* operon in *E. coli* (23). In the yeast mitochondrial genome no initiation site or nonanucleotide sequence has been found between the neighboring genes *Oli 1*, $tRNA^{ser}$ and *Var 1* genes suggesting that these genes are transcribed together and subsequently processed to yield the respective mature RNAs. It remains possible that attenuation may play a role under some physiological conditions in the differential expression among these three genes perhaps coupled to the differential utilization of the tandem *Oli 1* promoters. Such a coupling of differential gene expression through attenuation and differential use of tandem promoters merits consideration in the case of mitochondrial multigene transcripts in yeast.

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