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Use of Yeast Nuclear DNA Sequences To Define the Mitochondrial RNA Polymerase Promoter In Vitro

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We have extended an earlier observation that the TATA box for the nuclear *GAL10* gene serves as a promoter for the mitochondrial RNA polymerase in in vitro transcription reactions (C. S. Winkley, M. J. Keller, and J. A. Jaehning, J. Biol. Chem. 260:14214–14223, 1985). In this work, we demonstrate that other nuclear genes also have upstream sequences that function in vitro as mitochondrial RNA polymerase promoters. These genes include the *GAL7* and *MEL1* genes, which are regulated in concert with the *GAL10* gene, the sigma repetitive element, and the 2μm plasmid origin of replication. We used in vitro transcription reactions to test a large number of nuclear DNA sequences that contain critical mitochondrial promoter sequences as defined by Biswas et al. (T. K. Biswas, J. C. Edwards, M. Rabinowitz, and G. S. Getz, J. Biol. Chem. 262:13690–13696, 1987). The results of these experiments allowed us to extend the definition of essential promoter elements. This extended sequence, -$^{A}_{C}$TA$^{T}_{A}$A$^{A}_{C}$G$^{a}_{c}$TG^{AA}-, was frequently found in the upstream regulatory regions of nuclear genes. On the basis of these observations, we hypothesized that either (i) a catalytic RNA polymerase related to the mitochondrial enzyme functions in the nucleus of the yeast cell or (ii) a DNA sequence recognition factor is shared by the two genetic compartments. By using cells deficient in the catalytic core of the mitochondrial RNA polymerase (*rpo41*$^-$) and sensitive assays for transcripts initiating from the nuclear promoter sequences, we have conclusively ruled out a role for the catalytic RNA polymerase in synthesizing transcripts from all of the nuclear sequences analyzed. The possibility that a DNA sequence recognition factor functions in both the nucleus and the mitochondria remains to be tested.

The nuclear and mitochondrial compartments must communicate and coordinate their respective biosynthetic reactions to ensure balanced cell growth. Mitochondria are themselves composed primarily of proteins encoded in the nucleus (14); the regulated expression of these nuclear gene products involves many diverse elements, but in many cases the genes are subject to repression by glucose (17, 22). Transcription of the genes encoded in the mitochondrial DNA is dependent on the mitochondrial RNA polymerase, a multicomponent enzyme which is itself encoded in the nucleus (47, 48). The regulation of this enzyme by glucose may control the rate of mitochondrial transcript synthesis in a simple way (47).

The catalytic core of the mitochondrial RNA polymerase is encoded by the *RPO41* gene (24, 47), which bears striking similarity to RNA polymerases from bacteriophages T3 and T7 (29). Although the core is transcriptionally active in vitro on nonspecific templates such as poly[d(AT)], it requires the addition of a specificity factor to initiate selectively at mitochondrial promoter sequences (37, 44, 48). The promoter for the mitochondrial RNA polymerase was initially described as a simple nonanucleotide sequence, -ATATAAGTA- (12, 34). Transcripts isolated from mitochondria and transcripts synthesized in vitro by preparations of the RNA polymerase initiate within 1 or 2 nucleotides of the end of this promoter consensus (8, 34). Analysis of templates bearing deletions and single-base mutations has refined the critical elements of the promoter in in vitro transcription reactions (6–9, 36).

A consensus mitochondrial promoter sequence is present in the upstream region of the nuclear *GAL10* gene (23, 48). This sequence, which serves as a functional promoter for mitochondrial RNA polymerase in vitro (48), is also the TATA box for transcription of the *GAL10* gene by RNA polymerase II (38). As described herein, we have also found mitochondrial promoter sequences in the upstream regions of several nuclear genes, including the *GAL7* and *MEL1* genes, which are regulated by the same metabolic and genetic factors as is the *GAL10* gene (22). These nuclear DNA sequences are efficient promoters for the mitochondrial RNA polymerase in vitro. To determine whether the presence of these sequences in the nuclear DNA reflects a role for shared regulatory components in the two genetic compartments of the cell, we have rigorously tested the possibility that a catalytic form of the mitochondrial RNA polymerase functions in the yeast cell nucleus. Our results indicate that it is very unlikely that the catalytic RNA polymerase functions in the nucleus but leave open the possibility that a sequence-specific DNA-binding factor is shared by the two genetic compartments of the yeast cell.

MATERIALS AND METHODS

Media, yeast strains, and genetic methods. The methods for media preparation, cell growth, and plasmid maintenance have been described elsewhere (3, 28, 39). The strains used are presented in Table 1. All strain constructions were performed by standard yeast mating, sporulation, and tetrad analysis techniques (39). Isonuclear [*rho*^{0}] strains were prepared by growing the corresponding [*rho*$^+$] strains to saturation in yeast extract-peptone-glucose (YPD) containing 10 μg of ethidium bromide per ml (1). The *imp*$^-$ mutation denotes a single nuclear mutation (i.e., a 2:2 segregant) that is probably allelic to the reported *imp1*$^-$ mutant gene (1). The *imp*$^-$ [*rho*^{0}] spores never grew on plates containing galactose as the sole carbon source. Plasmids were introduced into yeast strains by the lithium acetate technique of Ito et al. (21). For all experiments, plasmids were isolated from *Esch-*

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TABLE 1. Yeast strains

Strain	Genotype	Source
YJJ65	*MATa gal3 trp1 ade* [*rho*$^+$]	Y. Oshima
YJJ165	*MATa rpo41*::*URA3 imp ade5 his3Δ200 lys1 lys2-801 trp1* [*rho*^{0}]	This work
YJJ166	*MATα rpo41*::*URA3 IMP*$^+$ *his3 lys trp1* [*rho*^{0}]	This work
YJJ167	*MATa rpo41*::*URA3 imp trp1* [*rho*^{0}]	This work
YJJ170	*MATa RPO41 imp his3 trp1* [*rho*$^+$]	This work
YJJ171	YJJ170 [*rho*^{0}]	This work
YJJ172	*MATα RPO41 IMP*$^+$ *ade5 his3* [*rho*$^+$]	This work
YJJ173	YJJ172 [*rho*^{0}]	This work
YJJ174	*MATa rpo41*::*URA3 IMP*$^+$ *his3Δ200* [*rho*^{0}]	This work
YJJ179	*MATα gal4*::*LEU2 leu2 trp1-289* [*rho*$^+$]	K. Hagen
YJJ181	*MATa RPO41*$^+$ *trp1Δ1 his3Δ200 ade2-101 IMP*$^+$ [*rho*$^+$]	This work
YJJ182	YJJ181 [*rho*^{0}]	This work
YJJ189	*MATa rpo41*::*URA3 trp1Δ1 his3Δ200 ade2-101 IMP*$^+$ [*rho*^{0}]	This work
YNN13	*MATa lys1* [*rho*$^+$]	G. Fink
YNN22	*MATα trp1 pep4-3* [*rho*$^+$]	E. Jones
YNN22^0	YNN22 [*rho*^{0}]	This work

erichia coli by the alkaline lysis technique (27). Special methods for using the YARp plasmids were detailed by Fagan and Scott (16).

Plasmid constructions. Commonly used procedures for DNA manipulations were followed (27). Plasmid YRp17-Sc3121 was constructed by transferring the *Bam*HI fragment containing the *HIS3* gene from plasmid YRp14-Sc3121 (41) into the *Bam*HI site of YRp17 (40). The promoterless *HIS3* test plasmid pJJ155 was constructed by placing the *Eco*RI-to-*Bam*HI *HIS3* DNA fragment from plasmid YRp14-Sc2890 (41) between the *Eco*RI and *Bam*HI sites of YRp17 (39). Plasmids YCp19-*HIS3*$^+$ and YCp19-*his3*$^-$ have been described elsewhere (28). The synthetic mitochondrial promoter centromere plasmids (MCp; diagrammed in Fig. 5), containing the wild-type mitochondrial promoter (MCp WT), base-pair substitution (MCp −2T, MCp −2T,−5A, and MCp −5C), and promoter deletion (MCp Δ), were constructed by the following series of steps. The wild-type synthetic mitochondrial promoter and its variants were prepared by the degenerate oligonucleotide synthesis method of Oliphant et al. (33). The double-stranded cassettes were cut with *Eco*RI and fused to the *HIS3* gene by ligation into the unique *Eco*RI site in the promoterless *HIS3* test plasmid pJJ155 (described above). Individual promoter variants were isolated in *E. coli* JM83 (30) and characterized by restriction endonuclease analysis and double-stranded DNA sequencing (described below). MCp plasmids were constructed by transferring the *Bam*HI fragment (containing the synthetic promoter and *HIS3* gene fusions) from these plasmids into the *Bam*HI site of YCp19 (40). Mitochondrial promoters oriented away from the *HIS3* gene are deleted in this subcloning step (MCp Δ; see Fig. 5B). MCp plasmids containing the *HIS3* gene variants in both the clockwise and counterclockwise orientations (see Fig. 5A) were isolated.

Double-stranded-DNA sequencing. The cloned promoters were sequenced by the genomic sequencing protocol of Huibregtse et al. (20) except that 0.2 μg of plasmid DNA (cut with *Xho*I), 1.0 μg of plasmid YRp17 (cut with *Bam*HI), and 50,000 cpm of the ^{32}P-5′-end-labeled *HIS3* oligonucleotide primer were used in the dideoxy-chain sequencing reactions.

In vitro transcription reactions. Nuclear genes and synthetic constructs containing potential mitochondrial RNA polymerase promoters were assayed as described by Winkley et al. (48), with the following modifications: 0.1 to 1.0 μg of plasmid DNA, digested with the appropriate restriction endonuclease, was incubated with the mitochondrial RNA polymerase for 10 min at 30°C in a 10-μl final volume. The mitochondrial RNA polymerase was prepared from strain YNN22 grown on glucose medium as described by Winkley et al. (48). The RNA polymerase preparations (peak activity fractions from the phosphocellulose column step) contained 0.5 U of *GAL10* promoter-selective activity per ml, 0.75 U of poly[d(AT)]-nonselective activity per ml, 1.6 mg of protein per ml, and 200 mM KCl. These samples were generously provided by C. Peterson (Indiana University).

Oligonucleotide primers and primer extension assays. The oligonucleotide primers used were synthesized by L. Washington (Institute for Molecular and Cellular Biology, Indiana University). *GAL10* primer A, 5′-AGAAGTACTTTCACTT-3′ (5′ at position 107 [23]), *GAL10* primer B, 5′-GGATG GACGCAAAGAAGTTT-3′ (5′ at position 176 [23]), and the universal sequencing primer U (30) are diagrammed in Fig. 1. Primer U hybridizes to the M13 sequences bordering the *GAL10* gene in the M13 phage P43 (see below). The *HIS3* primer, 5′-TACTAGGGCTTTCTGCTC-3′, is complementary to the N terminus of the *HIS3* coding strand (42). When these primers were used for primer extension assays or for DNA-sequencing experiments, the oligonucleotides were 5′ end labeled with ^{32}P, using a fivefold molar excess of [γ-^{32}P]ATP (>6,000 Ci/mmol; Dupont, NEN Research Products) and T4 polynucleotide kinase (Bethesda Research Laboratories, Inc.) as described by Maniatis et al. (27). To analyze the 5′ ends of yeast *GAL10* RNA, approximately 50,000 cpm of the ^{32}P-end-labeled oligonucleotides (primer A or primer B) was mixed with the indicated amounts of RNA (e.g., 5 to 50 μg of total yeast RNA) in a 12-μl volume and elongated with avian myeloblastosis virus reverse transcriptase (Seikagaku) under the reaction conditions described by Huibregtse et al. (20).

RNA preparation and quantitation. Total yeast RNA was prepared by the glass bead method (28). To quantitate the sensitivity of our hybridization assays in terms of the number of RNA molecules per cell, we determined that approximately 1 mg of total yeast RNA corresponds to 1 fmol of exponentially growing (YPD or synthetic complete medium) haploid yeast cells, as determined from our RNA extraction yields and the biochemical analysis data reported by Waldron and Lacroute (46).

Northern (RNA) blot assays. RNA samples were electrophoresed, transferred to a membrane support, hybridized with the ^{32}P-labeled DNA, and washed as previously described (28). To detect *GAL10* transcripts, hybridization was performed by the single-step protocol with 50×10^6 to 100×10^6 cpm of single-stranded ^{32}P-labeled *GAL10* upstream (primer B) or *GAL10* downstream (primer U) DNA probes as described previously (28). These probes were prepared by the hybridization of 1 pmol of single-stranded M13 phage P43 with 5 pmol of oligonucleotide primer B or U, [α-^{32}P]dATP incorporation with Klenow polymerase, and polyacrylamide gel isolation as described by Church and Gilbert (13). Phage P43 is M13mp10 (29) with the *GAL1-GAL10* Sc4816 fragment (23) cloned into the polylinker. To detect *HIS3* and actin transcripts, hybridization was performed by the two-step protocol involving hybridization of unlabeled single-stranded M13 phage clones, followed by a second hybridization with ^{32}P-labeled single-stranded M13 vector sequences (28). The M13 clones consisted of the *HIS3* gene (the *Xho*I-to-*Eco*RI fragment from plasmid YRp14-Sc3121 [41])

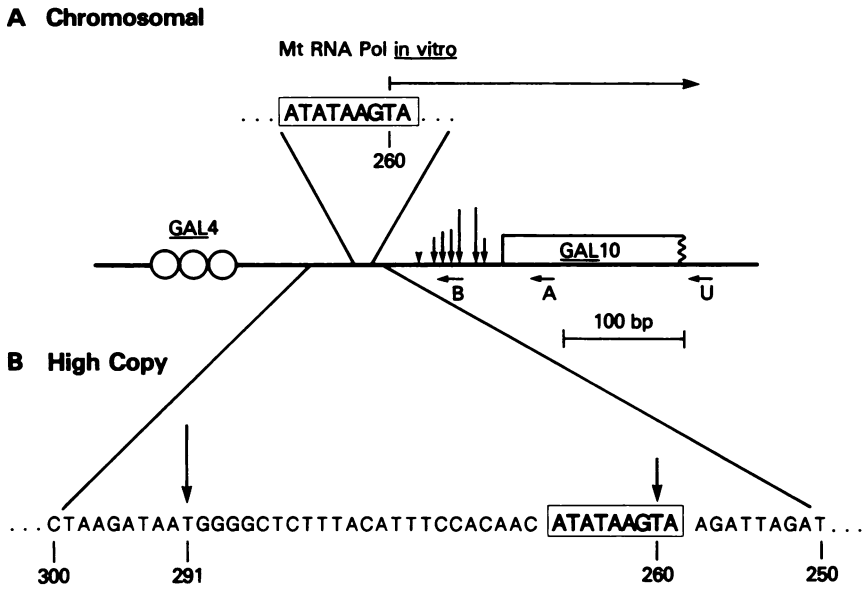


FIG. 1. (A) Transcription-landmark map of the *GAL10* gene 5′ region. Positions of the *GAL10* transcripts are presented in relation to the significant DNA sequences according to the nucleotide numbering system of Johnston and Davis (22). The in vitro mitochondrial RNA polymerase transcript start site is at position 260 (Mt RNA Pol; →). The nonanucleotide sequence is underlined. Symbols: ↓, positions of the galactose-induced RNA polymerase II transcripts, as determined by the primer extension assays discussed in the text; ▼, position of the farthest-upstream galactose-induced transcript at position 232; ○, positions of three *GAL4* protein-binding sites (21). The *GAL10* open reading frame AUG codon begins at position 139. Symbol: ←, positions of the DNA oligonucleotide primers used for sequencing and primer extension assays. bp, Base pairs. (B) Constitutive *GAL10* upstream transcripts on high-copy-number YARp plasmids. ↓, Initiating nucleotides for the two *GAL10* upstream transcripts that initiate in vivo from high-copy-number plasmids YARp16 and YARp17.

and the 5′ yeast actin gene exon (*Bam*HI-to-*Xho*I fragment from plasmid AHX-2 [32]) cloned into M13mp10 (30).

RESULTS

The *GAL10* TATA box is a functional mitochondrial promoter. We have previously demonstrated that a sequence in the 5′ region of the *GAL10* gene is an efficient promoter for the mitochondrial RNA polymerase in vitro (48). The mitochondrial nonanucleotide consensus promoter -ATATAA GTA- (12, 34) is located at position 260 in the upstream region of the *GAL10* gene, approximately 100 base pairs upstream of the major mRNA start site as defined by Johnston and Davis (23; Fig. 1A). On the basis of the photo-cross-linking experiments of Selleck and Majors (38), this sequence element has also been identified as the TATA box for the *GAL10* gene. The promoter in the *GAL10* gene is unusual in that transcription initiates with a U corresponding to the −1 T in the promoter sequence (48). Selective initiation from this sequence was demonstrated by a reverse transcriptase primer extension assay (Fig. 2). The experimental results were consistent with those of Winkley et al. (48) and clearly demonstrated that the transcript initiates at the −1 T within the nonanucleotide sequence rather than at the +1, 2, or 3 position reported for other mitochondrial promoters (6–9).

Two other *GAL*-regulated genes contain functional mitochondrial promoters. Using the mitochondrial RNA polymerase in vitro runoff assay, we observed that, in addition to the *GAL10* transcript described above, the coordinately regulated *GAL7* (41) and *MEL1* (26) genes were also selectively transcribed. Both S1 nuclease protection and transcription runoff experiments (data not shown) demonstrated that the *GAL7* transcripts initiated either at or within a few nucleotides of the +1 A from the promoter sequence presented in Fig. 3. This sequence overlaps the RNA polymerase II TATA box promoter element reported by Tajima et al. (43). This sequence, -ATAAAAGCA-, is also present in the upstream region of the *MEL1* gene, and transcription runoff experiments demonstrated that the mitochondrial RNA polymerase selectively initiates transcription from the *MEL1* promoter sequence.

Search for critical mitochondrial promoter sequences in nuclear DNA. The observations described above led us to search for additional examples of mitochondrial promoters in nuclear genes. Since there is clearly some latitude in the sequence requirements for a functional promoter, we used the elements defined by Biswas et al. as "critical" (6–9) to initiate our search. Figure 3B depicts the critical promoter, whose features include the facts that neither position −8 nor position +1 is essential for in vitro promoter function (9) and a purine is required at position +2 for maximum efficiency (8).

Using this degenerate sequence, we searched the GenBank data base and found many examples of potential mitochondrial promoter sequences in yeast nuclear DNA. A list of 28 potential mitochondrial promoter sequences, and the yeast nuclear DNA sequences in which they are found, is presented in Fig. 3A (sources for the templates are described in Table 2). The sequences are aligned with the nonanucleotide and critical mitochondrial promoter sequences. Each of the promoter sequences was assayed with purified mitochondrial RNA polymerase in a transcription runoff assay. An example of such an in vitro assay performed on one active and two inactive promoters from nuclear yeast genes is presented in Fig. 4. For quantitation, transcripts from a plasmid containing the *GAL10* promoter were prepared, and serial dilutions of the 260-base-pair *GAL10* runoff transcript were included with the other runoff assays on the same gels.

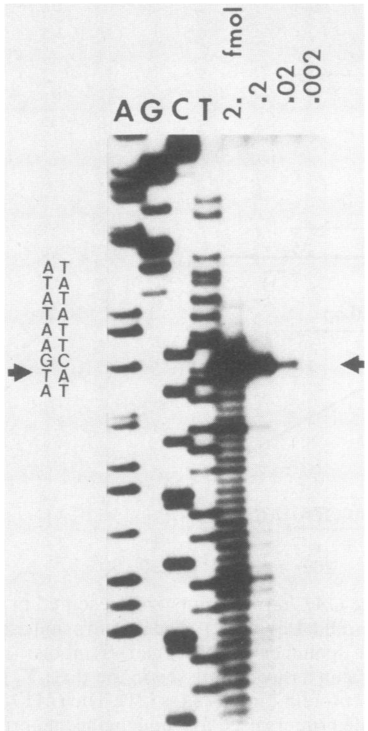


FIG. 2. Primer extension analysis of in vitro transcripts from *GAL10*. In vitro *GAL10* RNA molecules (2.0 to 0.002 fmol) were hybridized with 5 fmol (approximately 50,000 cpm) of oligonucleotide primer B (see Materials and Methods) labeled at the 5′ end with ^{32}P and incubated with reverse transcriptase under conditions that permitted the primer to be fully extended to the 5′ ends of the RNA molecules. The primer extension products were electrophoresed alongside a dideoxynucleotide-sequencing ladder generated by sequencing the *GAL10* M13 phage P43 with the same ^{32}P-end-labeled primer.

A

	-8 +1 ••• ATA TAA GTA •••	Run-Off Transcript	Location	
1	acc ATA TAA GTA aga	++	GAL10 5′	259
2	acc ATA AAA GCT agt	−	GAL10 5′	508
3	gat ATA AAA GCA ggt	+	GAL7 5′	858
4	gta ATA AAA GCA acg	+	MEL1 5′	579
5	tca CTA TAA GAA aat	−	GAL80 5′	414
6	cta CTA TAA GTG gcc	−	CYC7 5′	152
7	atc ATA TAA GTA att	++	CYC7 3′	665
8	ata ATA AAA GTA ata	++	CYC7 3′	719
9	gtg CTA TAT GGA aac	−	PUT2 5′	13
10	gct ATA TAA GGG tga	−	PUT2 5′	428
11	gga AAA TAT GTA ata	−	PUT2 5′	478
12	aca CTA AAG GAA aaa	−	ADHI 5′	435
13	aat ATA TAA GAT aaa	+	Sigma	679
14	aag ATA AAC GAA ggc	−	HIS3 5′	178
15	tga GTA TAC GTG att	−	TRP1 5′	75
16	tta TTA AAA GAT aac	−	2μ	72
17	tcg TTA AAG GAC aag	−	2μ	2457
18	ttc CTA TAC GTA gta	+	2μ	3606
19	taa GTA TAA GCA att	−	18S rRNA	71
20	aga GTA TAG GTA aca	−	25S rRNA	1437
21	gct ATA AAG GTA gtg	−	25S rRNA	2605
22	gta ATA TAC GAT gag	−	5S rRNA	151
23	ttc CTA TAG GTA aca	−	5S rRNA	416
24	caa CTA AAC GAG ggt	−	5S rRNA	844
25	agt CTA AAG GTT gtt	−	5S rRNA	1744
26	cag ATA TAG GGA aac	−	5S rRNA	2077
27	aaa ATA TAC GCT aag	−	5S rRNA	2339
28	aaa ATA AAA GTA aga	−	5S rRNA	2434

B

••• •TA [T/A]A[a/g/c] G[t/a/c]• [A/G]•• Critical Promoter (6-9)

••• [A/C]TA [T/A]A[A/C] G[t/a/c][A/T] [A/G]•• Extended Critical Promoter

FIG. 3. (A) In vitro transcription of nuclear DNA containing critical yeast promoter sequences. Three categories of promoter activity were observed: promoters comparable in strength to the nuclear *GAL10* 5′ and mitochondrial 14S rRNA promoters (++), promoters with a 10- to 50-fold-lower transcriptional activity (+), and inactive promoters (−). The location of the nonanucleotide +1 position is given according to the numbering system presented in the GenBank data base. (B) Features of the critical promoter and the extended critical promoter.

All of the mitochondrial promoters designated as being active in Fig. 3A produced runoff transcripts whose lengths agreed with those of the predicted runoff products (±5 bases). Positive transcription runoff results were confirmed by using templates digested with at least two separate restriction endonucleases.

All of the potential promoter sequences listed in Fig. 3A except the three sequences found in the 5′ region of the *PUT2* gene (24) strictly conform to the critical promoter and +2 purine sequence rules. Therefore, on the basis of the published reports of Biswas et al. (6–9), we expected all of these promoters to be active. However, as described below, only a few of these potential promoters were in fact utilized by the mitochondrial RNA polymerase.

GAL80. Our search also revealed that six critical mitochondrial promoters were associated with the *GAL80* galactose negative regulatory gene (49). Figure 3A (line 5) lists the one potential *GAL80* promoter located in the 5′ noncoding region, which also contains the +2 purine proposed to be required for efficient transcription. However, none of the six *GAL80* promoters were observed to be transcribed in vitro under conditions in which the other *GAL* genes were clearly transcribed (data not shown).

Glucose-regulated genes. The *GAL* genes and the *CYC7* gene described below are examples of yeast genes whose transcription is repressed by glucose (17, 22). In contrast, the yeast *ADHI* gene, encoding the cytoplasmic alcohol dehydrogenase, is transcriptionally activated by glucose and repressed when cells pass from fermentative to aerobic growth (5). *ADHI* also contains a potential mitochondrial promoter in its 5′ noncoding region (Fig. 3). However, this

TABLE 2. Plasmid templates

Plasmid	Relevant yeast gene	Source (reference)
pUC9-Sc4816	*GAL10*	J. Jerome (22)
YRp17-Sc731	*GAL7*	S. M. Baker (2)
pMP550	*MEL1*	J. E. Hopper (25)
pRYGAL80 (YEp13-GAL80)	*GAL80* and 2μm	M. Johnston (47)
pYeCYC7(2.2)	*CYC7*	R. S. Zitomer (30)
pSB4	*PUT2*	M. C. Brandriss (24)
pIA	*ADHI*	S. A. Johnston (5)
pSV20	Sigma	S. Van Arsdell (44)
YRp17-Sc3121	*HIS3* and *TRP1*	This work
pBD4	rDNA	J. A. Wise (4)

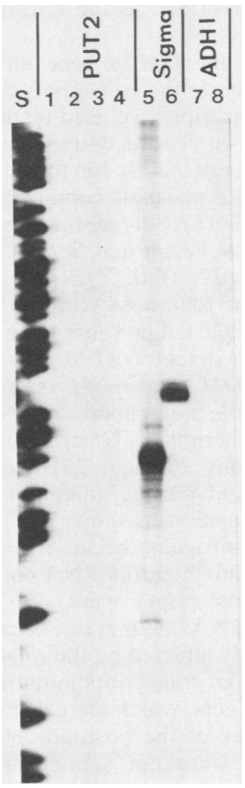


FIG. 4. In vitro transcription of nuclear genes containing critical promoters. Cut plasmid DNA (0.25 μg) was used as a template, and the RNA products were processed as described in Materials and Methods. Lanes: S, single-stranded DNA size standards produced by a dideoxy-cytosine-sequencing reaction of single-stranded M13 phage P43; 1 to 4, *PUT2* (plasmid pSB4) cut with *Taq*I, *Alu*I, *Bgl*II, and *Dde*I; 5 and 6, sigma element (plasmid pSV20) cut with *Pst*I and *Hin*dIII; 7 and 8, *ADHI* (plasmid pIA) cut with *Hae*III and *Alu*I.

promoter was inactive in the mitochondrial transcription runoff assay (Fig. 4, lanes 7 and 8).

Genes that function in the mitochondria. We used the transcription runoff assay to analyze the *CYC7* and *PUT2* genes, which both encode proteins that function in the mitochondria and which both contain potential promoters within their 5′ noncoding regions (Fig. 3). Analysis of the *CYC7* gene, which encodes the minor isoform of cytochrome *c* (31), revealed three potential mitochondrial promoters: one in the 5′ noncoding region and two in the 3′ noncoding region (Fig. 3A). No transcripts were detected from the 5′ promoter (Fig. 3A, line 6), but both 3′ promoters (lines 7 and 8) were transcribed with efficiency comparable to that of the strong *GAL10* promoter (line 1). These 3′ promoters direct in vitro transcription of the antisense strand of the *CYC7* gene.

PUT2 encodes pyrroline-5-carboxylate dehydrogenase, one of the mitochondrial proteins involved in converting proline to glutamate (25). Three potential mitochondrial RNA polymerase promoters were found in the 5′ noncoding region of the *PUT2* gene (Fig. 3A, lines 9 to 11). However, all three promoters were inactive in our assay (Fig. 4, lanes 1 to 4).

Sigma repeated element. Our search for mitochondrial promoters revealed a potential promoter within the sigma repeated element (Fig. 3A). It has been reported that the in vivo hormone (α-factor)-induced sigma transcripts initiate precisely at the +1 T nucleotide of this potential promoter (45). The sigma-element plasmid (pSV20) was subjected to the in vitro transcription runoff assay. If transcription initiates at the +1 T of this promoter, then pSV20 cut with *Pst*I and *Hin*dIII would be predicted to direct 106 and 123-nucleotide runoff transcripts, respectively. The results shown in Fig. 4 (lanes 5 and 6) are in perfect agreement with these predictions.

Transcripts regulated by mitochondrial genotype. We also searched for mitochondrial RNA polymerase promoters in the DNA sequences of the yeast 2μm plasmid and the yeast nuclear ribosomal DNA (rDNA) sequences. This search was motivated by the results of Parikh et al. (35), who observed nuclear transcripts whose abundance was affected by the DNA sequences present in the mitochondrial genome. Parikh et al. (35) presented evidence that 0.7- and 1.95-kilobase transcripts from 2μm plasmid DNA are 10 to 15 times more abundant in an isonuclear mitochondrial deletion strain ([*rho*$^-$] hypersuppressive petite strain HS40). These transcripts span the D region of the yeast 2μm plasmid (18). The sequences of three potential mitochondrial promoters from this 2μm circle region are presented in Fig. 3A. The first two promoters listed (lines 16 and 17) are located at positions within the DNA sequences spanned by both the 0.7- and 1.95-kilobase transcripts. These two promoters were inactive in the in vitro transcription runoff assay. The third promoter sequence, which was active in vitro (Fig. 3A, line 18), lies upstream of the transcripts described by Parikh et al. (35) and is nearly coincident with the 2μm origin of DNA replication (19).

Parikh et al. (35) also identified nuclear transcripts from the rDNA spacer region whose abundance is dramatically elevated in isonuclear [*rho*^{0}] (total deletion of mitochondrial DNA) strains. A computer search through the GenBank data base revealed the 10 potential mitochondrial promoters listed in Fig. 3A. An rDNA plasmid (pBD4), containing the entire rDNA repeat region (4), was used as the template in the in vitro transcription runoff assay. Although three runoff transcripts were detected with use of the rDNA templates, none of the sizes corresponded to the location of the promoter elements. We therefore concluded that all 10 potential rRNA promoters were inactive in vitro. Since the sequences in the GenBank data base do not account for all of the rDNA present on pBD4, it is probable that other mitochondrial promoters, not listed in Fig. 3A, are present within the rDNA repeat.

Amino acid biosynthesis genes. The *HIS3* and *TRP1* amino acid biosynthesis genes have no obvious connection with the mitochondria or with carbon metabolism, but they both contain critical mitochondrial RNA polymerase promoter sequences located immediately upstream of their protein-coding sequences (Fig. 3A, lines 14 and 15). However, both of these promoters were found to be inactive in the in vitro transcription runoff assay (data not shown).

Extended consensus for the functional mitochondrial promoter. On the basis of a comparison of the active and inactive promoter sequences in Fig. 3A, we propose to extend the critical promoter sequence to that shown in Fig. 3B. Since the definition of the critical promoter was based on single-base changes (6–9), the extended consensus reflects the additional information that must be present if multiple changes are made within the promoter or in the flanking nucleotides. In particular, the bases at positions −8 and +1 are essential for recognition when other bases are altered. In addition, G is not functional at the −3 position. In general, the extended consensus resembles the original nonanucleotide more closely than does the critical promoter.

Tests for nuclear transcripts dependent on mitochondrial RNA polymerase activity. We have demonstrated that several nuclear genes include mitochondrial promoter sequences in their 5′ regions, in some cases overlapping an RNA polymerase II TATA box or associated with the 5′ end of a transcript. Two theories can be proposed to account for the presence of these sequences. First, if a catalytic form of the enzyme exists in the nucleus of the yeast cell, then functional RNAs might be synthesized from these promoters. Alternatively, it is possible that a related DNA-binding protein is found in both cellular compartments, where it interacts with two very different transcription mechanisms (see Discussion). If the first theory is correct and such transcripts exist, they should (i) initiate precisely at the nonanucleotide mitochondrial promoter element, (ii) be dependent on a functional form of the *RPO41* gene, and (iii) be dependent on a sequence shown to be a functional promoter in vitro, as described above. We attempted to detect such transcripts by using the three sensitive experimental protocols described below.

Transcripts from the chromosomal *GAL10* gene. Initial attempts to detect in vivo mitochondrial RNA polymerase transcripts focused on the nuclear *GAL10* gene because of its promoter strength in vitro. To determine the in vivo pattern of transcription initiation sites at the *GAL10* gene, a primer extension experiment was performed with oligonucleotide primers A and B (Fig. 1A) and RNA prepared from cells grown on glucose or galactose. The strain that we analyzed (YNN13; Table 1) contains wild-type alleles of all of the *GAL* genes. This experiment was expected to reveal all of the major transcripts that might initiate upstream of the *GAL10* protein-coding region. The positions of the major and minor 5′ galactose-induced transcripts are indicated in Fig. 1A. Transcription initiation at the *GAL10* gene (23) and at many other yeast genes (17) is very heterogeneous. The positions of the major galactose-induced transcripts that we observe are in good agreement with previously published results (23). The farthest-upstream transcript was detected at position 232, far short of the expected mitochondrial RNA polymerase transcription initiation point at position 260.

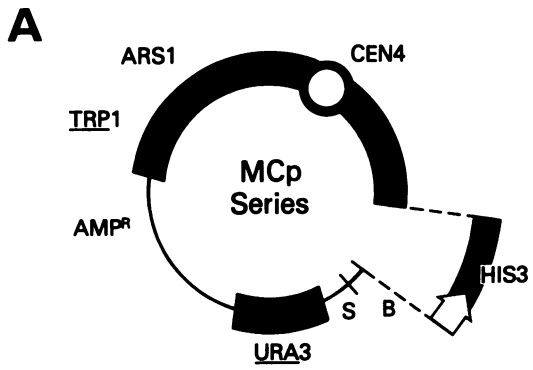
We also used a Northern blot assay to search for upstream *GAL10* transcripts; we have found this technique (see Materials and Methods), which can detect RNA molecules that are present in less than one copy per 50 cells, to be more sensitive than the primer extension assay. We analyzed *GAL10* transcripts in several genetic backgrounds (Table 1) that might influence the *GAL10* mitochondrial promoter. As discussed above, the state of the mitochondrial genome can influence nuclear transcription (35). We therefore included three pairs of isonuclear [*rho*$^+$] and [*rho*^{0}] strains (YJJ170 and -171, YJJ172 and -173, and YJJ181 and -182). We tested all four possible combinations of the *RPO41* gene with the *IMP* (1) gene previously described to couple expression of the *GAL* genes to mitochondrial function (strains YJJ166, -167, -170, and -172). A *gal4*$^-$::*LEU2*$^+$ disruption strain (YJJ179) was included to eliminate any *GAL4*-activated RNA polymerase II transcription (2). A null *gal3*$^-$ mutant strain (YJJ65) was included on the basis of the hypothesis that basal *GAL10* levels may be needed to compensate for the lack of *GAL3* function (22).

RNA was prepared from each of these strains grown under a variety of conditions. Cells were grown on defined synthetic complete medium containing either glucose, raffinose, or glycerol plus lactate as the sole carbon sources, and RNA was isolated from cultures harvested in both mid-log and stationary phases. Despite the sensitivity of the assays, no transcripts that conformed to the criteria described above were detected.

Transcription of the *GAL10* gene on high-copy-number plasmids. To increase the sensitivity of our assays for low-abundance transcripts, we used recombinant constructs of the *GAL10* gene on yeast acentric-ring plasmids (YARp), which are stably maintained at 100 to 200 copies per haploid yeast cell (50). YARp plasmids containing both orientations of the *GAL1* and *GAL10* divergent promoter region have been constructed by Fagan and Scott (16) and designated YARp16 and YARp17. Both YARp16 and YARp17 were placed into each of four host strains (YNN22, YNN22^0, YJJ166, and YJJ167) listed in Table 1. The primer extension assay was used to detect *GAL10* transcription from the plasmids. Both *RPO41*$^+$ and *rpo41*$^-$ strains were chosen to test the effect of the mitochondrial RNA polymerase on YARp *GAL10* transcription. Upstream *GAL10* transcripts initiating at positions 260 and 291 were observed from plasmids YARp16 and YARp17 (data not shown). The DNA sequence at these positions is shown in Fig. 1B. These initial observations were intriguing because position 260 is the in vitro start site for mitochondrial RNA polymerase (Fig. 1A). However, these transcripts were not produced by the *RPO41*-dependent RNA polymerase, because the transcripts were not significantly affected by the *rpo41*$^-$::*URA3*$^+$ mutation. Unlike the major transcripts initiating from the single-copy chromosomal gene which are galactose inducible (Fig. 1A), the abundances of the position 260 and position 291 *GAL10* transcripts were not significantly altered by the carbon source in the growth medium. These upstream transcripts may therefore have been artifacts of the high copy number of the plasmids (3).

Transcription of synthetic mitochondrial promoters on nuclear plasmids. The final assay for a functional form of the mitochondrial RNA polymerase in the nucleus involved constructing the mitochondrial promoter centromere plasmids (MCp, Fig. 5A) containing synthetic DNA oligonucleotides encoding variants of the mitochondrial promoter sequence inserted upstream of a copy of the *HIS3* gene lacking all promoter elements (41; see Materials and Methods). The MCp WT plasmids contain the wild-type mitochondrial promoter (Fig. 5B). A transcription runoff experiment (Fig. 6) demonstrated that both orientations (clockwise and counterclockwise) of this promoter fusion were efficiently transcribed in vitro by the mitochondrial RNA polymerase (compare lanes 1 and 2 with lanes 3 and 4 in Fig. 6). Transcription efficiency from the MCp WT promoter was comparable to that of the *GAL10* mitochondrial promoter, which served as an internal control in these transcription reactions (Fig. 6, even-numbered lanes). The MCp −2T plasmids are identical to the MCp WT plasmids except for a G-to-T change at the −2 position within the nonanucleotide sequence (Fig. 5B). The in vitro transcription results (Fig. 6) supported the essential nature of the −2 G, since MCp −2T plasmids had no activity in the in vitro transcription reaction. The MCp −2T, −5A (double point mutation) and Δ (promoter deletion) plasmids also failed to program runoff transcription.

To test these mitochondrial promoters in vivo, we introduced the MCp plasmids into *RPO41*$^+$ strains (YJJ181 [*rho*$^+$] and YJJ182 [*rho*^{0}]) and an *rpo41*$^-$::*URA3* strain (YJJ189). *HIS3* gene expression was tested by plate growth assays and by Northern blot analysis. Two growth assays were used to test for *HIS3* gene expression from the MCp plasmids: (i) growth in the absence of histidine and (ii) resistance to 3-amino 1,2,4-triazole (3AT), which requires



B

Synthetic Mitochondrial Promoters

WT	...ggatccACAACATATAAGTAATGATtagaattcc
-2T	.....ATATAATTA.....
-2T, -5A	.....ATAAAATTA.....
-5C	.....ATACAAGTA.....
Δ	.....TTTxxxxxxxxxxxxxxxAAA.....

FIG. 5. (A) Structures of mitochondrial promoter centromere plasmids. These plasmids consist of the vector YCp19 and the *HIS3* gene fused to a synthetic mitochondrial promoter sequence. Symbols: ——, bacterial pBR322 sequences; ▬, yeast chromosomal DNA sequences; ○, centromere (*CEN4*); ⇨, synthetic mitochondrial promoter. The orientation shown is counterclockwise. Abbreviations for restriction enzyme sites: S, *Sal*I; B, *Bam*HI. (B) Synthetic mitochondrial promoter sequences. Plasmid MCp WT contains the wild-type promoter sequence shown in uppercase letters. This promoter sequence is identical to the *GAL10* sequence from position 251 to 275 except for the marked T insert (compare with the sequence in Fig. 1). The *Bam*HI and *Eco*RI recognition sequences are shown in lowercase letters; the nonanucleotide promoter sequence is underlined. Four variants of the MCp WT sequence are also shown. These sequences were analyzed in the experiments shown in Fig. 6 and 7 in Table 3.

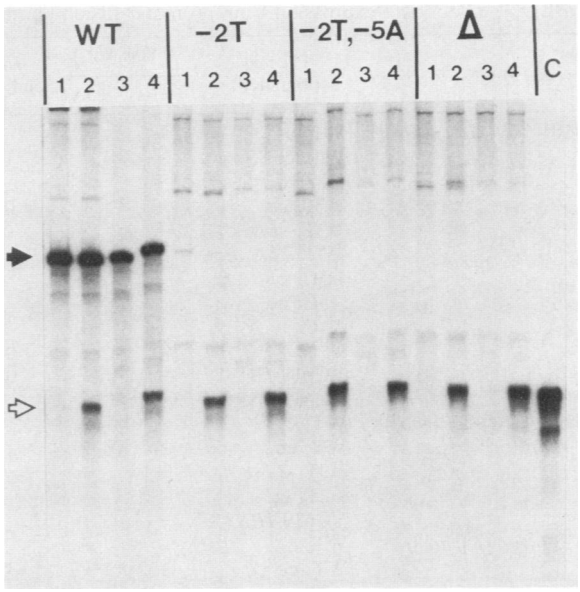


FIG. 6. Transcription of the synthetic promoters by the mitochondrial RNA polymerase. In vitro transcription runoff assays were performed as described in Materials and Methods, but the DNA template concentrations were lowered to approximately 0.15 pmol of promoter per reaction. For all numbered lanes, the indicated MCp plasmids were cut with *Bgl*II. The predicted runoff transcripts (464 nucleotides) from the MCp templates (➧) and the expected runoff transcripts (260 nucleotides) from the *GAL10* Sc4816 DNA fragment (⇨) are indicated. Lanes: 1 and 2, MCp clockwise promoter templates; 3 and 4, MCp counterclockwise promoter templates; C, control Sc4816 reaction without MCp plasmid templates. Reaction mixtures in the even-numbered lanes contained equimolar amounts (0.15 pmol) of both MCp and Sc4816 promoters.

higher levels of *HIS3* gene expression (41). To test the effectiveness of these plate assays, the strains were also transformed with a YCp19 plasmid bearing the *HIS3* gene with a fully functional RNA polymerase II promoter (YCp19-*HIS3*$^+$) and a YCp19 plasmid bearing the *HIS3* gene lacking all promoter elements (YCp19-*his3*$^-$) (30, 43). Previous Northern blot experiments have demonstrated that YCp19-*HIS3*$^+$ produces a strong *HIS3* band corresponding to an estimated 5 to 10 mRNA molecules per cell, whereas YCp19-*his3*$^-$ produces very low levels of *HIS3* mRNA (28). The Northern blot results were confirmed by the results of plate assays (Table 3). YCp19-*HIS3*$^+$ supported growth both in the absence of histidine and in the presence of 3AT, whereas YCp19-*his3*$^-$ supported growth only in the absence of histidine; 3AT completely inhibited growth.

The results of the plate assays (Table 3) demonstrated that all of the mitochondrial promoter plasmids bearing the counterclockwise *HIS3* gene fusion (as drawn in Fig. 5A) showed strong *HIS3* expression (i.e., supported 3AT-resistant growth). In contrast, none of the clockwise *HIS3* gene fusions grew in the absence of histidine. The counterclockwise *HIS3* expression was clearly not due to the catalytic subunit of the mitochondrial RNA polymerase, because the same pattern of *HIS3* expression was seen in both the *RPO41*$^+$ and *rpo41*$^-$ genetic backgrounds.

The results of these biological assays were confirmed by analyzing the *HIS3* RNA produced by these cells in a Northern blot experiment (Fig. 7). The His$^-$ MCp clockwise constructs produced low levels of nonfunctional *HIS3* transcripts that were long enough to initiate in the upstream *CEN4* DNA sequences and terminate at the 3′ end of the *HIS3* gene. Although all of the His$^+$ MCp counterclockwise constructs produced *HIS3* transcripts in both the *RPO41*$^+$ and *rpo41*$^-$ genetic backgrounds, the wild-type construct supported the highest level of expression. In addition, the *HIS3* transcripts from the plasmids were three- to fourfold more abundant in the *rpo41*$^-$ [*rho*^{0}] strain (Fig. 7 and Table 3). These transcripts comigrated with the transcripts produced by the wild-type promoter on the YCp19-*HIS3*$^+$ plasmid. Therefore, the synthetic constructs containing the mitochondrial promoter variants probably reflect the reconstruction of functional RNA polymerase II promoter elements rather than the presence of a catalytic equivalent to the mitochondrial RNA polymerase in the nucleus.

DISCUSSION

The nonanucleotide promoter (ATATAAGTA) for the yeast mitochondrial RNA polymerase was originally defined by comparison of the DNA sequences found upstream of the in vivo transcription initiation sites (12, 34). The availability of partially purified mitochondrial RNA polymerase that selectively initiates transcription from DNA templates in

TABLE 3. *HIS3* gene expression from promoter fusion plasmids

Strain	Plasmid promoter[a]	Growth[b]		*HIS3* mRNA[c]
		−His	+3AT	
YJJ181 (*RPO41*$^+$ [*rho*$^+$])	WT, c	−	−	
	WT, cc	+	+	1.0
	−2T, c	−	−	
	−2T, cc	+	+	0.5
	−2T,−5A, c	−	−	
	−2T,−5A, cc	+	+	0.4
	−5C, c	−	−	
	−5C, cc	+	+	
	Δ, c	−	−	
	Δ, cc	+	+	0.2
	YCp19-*HIS3*$^+$	+	+	0.6
	YCp19-*his3*$^-$	+	−	
YJJ182 (*RPO41*$^+$ [*rho*^{0}])	WT, c	−	−	
	WT, cc	+	+	
	−2T, c	−	−	
	−2T, cc	+	+	
	YCp19-*HIS3*$^+$	+	+	
YJJ189 (*rpo41* [*rho*^{0}])	WT, c	−	−	
	WT, cc	+	+	3.4
	−2T, c	−	−	
	−2T, cc	+	+	2.1
	YCp19-*HIS3*$^+$	+	+	1.6

[a] c, Clockwise; cc, counterclockwise.

[b] The yeast strains used contain the *his3Δ200* allele, which is a complete deletion of the *HIS3* structural gene (28). This allows a clear assay of the expression of the plasmid-borne *HIS3* gene (see Fig. 6). For each transformed cell line, four independent transformants were grown on tryptophan omission medium to ensure plasmid maintenance and tested by replica plating onto histidine omission plates (−His) with or without 20 mM 3AT.

[c] Quantitated by densitometry of the counterclockwise *HIS3* bands from Fig. 7. The actin bands in each lane served as an internal standard, and the counterclockwise *HIS3* band in lane 2 was arbitrarily normalized to 1.0 to allow comparison between lanes.

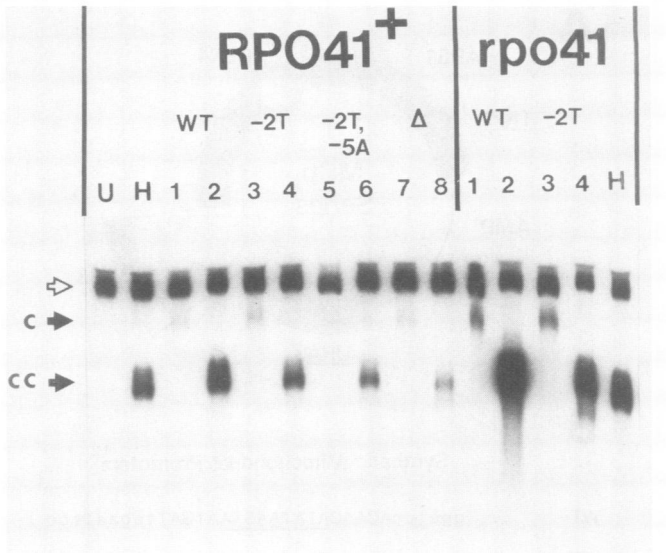


FIG. 7. In vivo *HIS3* gene transcription from mitochondrial promoter plasmids. Northern blot analysis was performed by the two-step hybridization protocol described in Materials and Methods, using 20 μg of total yeast RNA per lane. The membrane was hybridized simultaneously with single-stranded M13 recombinant DNA bearing sequences homologous to the entire *HIS3* gene and to the first exon of the yeast actin gene. ➡, Plasmid-borne *HIS3* transcripts produced by the MCp clockwise (c; odd-numbered lanes) or counterclockwise (cc; even-numbered lanes) plasmids. Lane H, RNA from cells bearing YCp19 containing the wild-type *HIS3* gene; lane U, RNA prepared from untransformed cells. ⇨, Chromosomal yeast actin band. The *RPO41*$^+$ strain is YJJ181; the *rpo41*$^-$ strain is YJJ189.

vitro has allowed detailed analysis of the sequence requirements of the promoter. The experiments have included deletion and single-base-change substitution of the promoter DNA (6–9, 36). In general, these experiments have supported the importance of the original consensus nonanucleotide, which extends from +1 to −8 (Fig. 3). It is clear that strong promoters require additional information in the form of a purine at position +2 (8). When individual bases are altered in the consensus, positions −8 and +1 are no longer required for recognition by the RNA polymerase, and the nucleotide at position −3 can be anything but T (9; the critical promoter in Fig. 3B). There has been some disagreement about the importance of the G at position −2. Biswas et al. (7, 9) found an absolute requirement for this base, whereas Schinkel et al. (36) observed only a 5- to 10-fold reduction in promoter activity when this base was altered.

Extension of the rules for a functional promoter. In this work, we tested many additional DNA sequences as templates for the mitochondrial RNA polymerase. These templates were derived from synthetic sequences and from yeast nuclear DNA fragments containing the promoter elements previously defined to be critical for recognition. In many cases, these sequences represent multiple changes from the original consensus and significant changes in the flanking DNA. We have found that most sequences which contain a critical promoter element (9; Fig. 3B) are not recognized by the RNA polymerase in vitro. The elements required for recognition define an extended critical promoter, $-\overset{A}{C}TA\overset{T}{A}A\overset{A}{C}G\overset{a}{t}\overset{A}{c}AA\overset{}{T}G-$; nucleotides at positions +1 and −8 are important, and an A is the preferred base at position −3, although a C can be tolerated if the promoter is otherwise close to consensus. We also found a complete dependence on the G at position −2, as described by Biswas et al. (7, 9; Fig. 6). The extended critical promoter clearly reflects the additional sequence information required for recognition when the promoter has multiple changes from the consensus and when the flanking DNA sequences are not optimal. The discrepancy in the results from different laboratories regarding the −2 position may reflect additional contributions of flanking nucleotides or differences in the preparations of RNA polymerase used for the in vitro reactions (see below).

The consensus mitochondrial promoter can serve as a nuclear TATA box. The screen of nuclear DNA sequences was originally motivated by our observation that some nuclear genes contain mitochondrial RNA polymerase promoters functional in in vitro assays (48). These nuclear DNA sequences were useful as a collection of critical promoters but were also interesting because, in some cases, the mitochondrial promoter sequence was coincident with the TATA box for the nuclear RNA polymerase II transcription apparatus (38, 43). In particular, the coordinately regulated *GAL7*, *GAL10*, and *MEL1* genes all contain sequences that function as mitochondrial promoters in vitro (Fig. 2 and 3). Since these genes are also sensitive to the phenotype of the mitochondria through the action of the *IMP* gene (1), we were intrigued by the possibility that these sequence elements play a role in nuclear-mitochondrial interactions. We therefore focused our initial search on genes containing critical promoter sequences that either shared regulatory elements with the *GAL* genes or were involved in mitochondrial function. Although no clear pattern emerged from this first round of screening, we did observe functional promot-

ers in the sigma repeated element coincident with the 5′ end of a hormone-regulated transcript (45) and at the 2μm plasmid origin of DNA replication (19).

When we introduced into yeast cells synthetic constructs containing sequences related to the mitochondrial promoter, replacing the natural TATA box (and upstream promoter elements) of the *HIS3* gene, we found that we had created a functional promoter (Fig. 7 and Table 3). This promoter was dependent on sequences in the flanking vector that are normally not sufficient for transcription (27). The transcripts from these synthetic constructs were elevated three- to fourfold in cells lacking functional mitochondrial RNA polymerase and mitochondrial DNA (Fig. 7 and Table 3). We have found that this increase is not a consequence of the defective *RPO41* gene but also occurs in *RPO41*$^+$ [*rho*^{0}] strains. In these strains, the differences between the wild-type and mutant consensus sequences are more striking than the twofold change shown in Fig. 7. Single-base changes from the extended consensus reduced in vivo expression from 3- to 10-fold. We have tested several additional single-base variants for the ability to serve as a TATA box on the plasmid constructs and have found a perfect correlation between the ability to serve as an in vitro promoter and the ability to function as a strong in vivo TATA box (C. Stipp, Z. Du, and J. A. Jaehning, unpublished observations). In addition, each strong TATA box responds to changes in the mitochondrial genome (Fig. 7).

To begin to determine whether these sequences have functional roles in vivo, we used the newly defined extended critical promoter sequences to search a recent data base. We have identified more than 20 examples of genes that contain sequences in their 5′ regions which we predict will function as in vitro promoters. Several of these genes are responsive to changes in mitochondrial genotype. We are currently screening these genes and additional synthetic constructs to determine whether the presence of sequences related to the mitochondrial promoter correlates with transcriptional regulation.

Does the mitochondrial RNA polymerase function in the nucleus? We have rigorously tested the possibility that this sequence in nuclear DNA actually serves as a promoter for a nuclear form of the mitochondrial RNA polymerase. Although there is precedent for gene products functioning in both the nucleus and the mitochondria (15), we used several very sensitive assays to essentially rule out the possibility that an *RPO41*-dependent RNA polymerase functions in the nucleus (Fig. 5 to 7 and Table 3). It is possible that other mitochondrial promoter sequences in the nucleus are templates for the enzyme, but it is more likely that the core polymerase is not found in the nuclear compartment.

We have not, however, ruled out the possibility that a sequence-specific DNA-binding factor interacts with this sequence in both genetic compartments. A possible candidate for such a shared binding factor would be the specificity factor required for promoter recognition by the mitochondrial RNA polymerase. Unfortunately, the nature of this factor has not been unambiguously elucidated. Schinkel and co-workers have reported that the factor isolated from whole yeast cells is a 43-kilodalton polypeptide that does not bind DNA on its own but binds it only as a part of the holoenzyme complex (37). In contrast, Ticho and Getz have described a 70-kilodalton specificity factor purified from isolated mitochondria that does form stable complexes with promoter DNA in the absence of the core polymerase (44). It is possible that these different enzyme preparations are the explanation for the discrepancies in the definition of the promoter as described above.

Yeast cells therefore may contain either a protein that binds to mitochondrial promoter sequences (44) or a protein that binds to these sequences when complexed with other transcription factors (37). We have demonstrated that some nuclear gene TATA boxes contain this sequence element rather than the canonical sequences TATAAA or TATATA (11). Although Buratowski et al. have recently described a yeast protein factor that binds to the canonical TATA boxes of the *CYC1* gene (10), it is possible that there are other TATA-box factors which interact with the noncanonical sequences found in many yeast genes. The presence of such a factor in both the nucleus and the mitochondria could be part of communication system-coordinating gene expression between the two compartments. Further characterizations of the binding proteins and target sequences are required to test this hypothesis.

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