Volume 11 Number 23 1983 Nucleic Acids Research

Initiation of transcription of the yeast mitochondrial gene coding for ATPase subunit 9

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Received 6 September 1983; Revised and Accepted 19 October 1983

ABSTRACT

We have determined transcriptional initiation sites for the ATPase subunit 9 gene on the yeast mitochondrial genome. Using S1 nuclease mapping, in vitro capping of primary transcripts with GTP and guanylyl transferase, and in vitro transcription analysis with purified mitochondrial RNA polymerase, we find the major site of transcriptional initiation to be at a point 630 nucleotides upstream of the coding region for the gene. In addition, we find much lower levels of initiation at a second site 78 nucleotides downstream of the first. Both initiation sites occur at the same position within a nonanucleotide sequence which we have previously found associated with initiation of rRNA synthesis. This work further supports the notion that this nonanucleotide sequence is an integral component of mitochondrial promoters and indicates that the same RNA polymerase is used for transcription of both mRNA and rRNA in yeast mitochondria.

INTRODUCTION

The 80,000 bp mitochondrial genome of baker's yeast is known to code for a limited number of proteins involved in oxidative phosphorylation and one ribosome-associated protein (1,2). In addition, the genome contains several as yet unidentified open reading frames and specifies 2 rRNAs and about 25 tRNAs. These known coding regions account for only about 25% of the entire genome. The rest of the DNA consists of long AT rich stretches puncuated by short GC clusters. Although only a fraction of the genome codes for known products, almost a full single strand equivalent is transcribed into RNA (3). Some of the genes coded by mitochondrial DNA contain one or more optional introns. This contributes to the complex array of transcripts generated during extensive splicing and processing steps (4,5).

The presence of low abundance long transcripts and extensive processing has obscured the pattern of transcriptional initiation in yeast mitochondria. In such a heavily processed system, S1 nuclease mapping is not sufficient to determine sites of transcriptional initiation because two

classes of 5' ends are present: those originating from initiation and those which are products of processing reactions.

We have recently investigated transcriptional initiation by specifically labeling RNA molecules retaining their 5' initiating nucleotide with the enzyme guanylyl transferase purified from vaccinia virions (6,7). The method relies on the specificity of the capping reaction for labeling 5' polyphosphate terminated RNA molecules whose only known source in mitochondria is the initiation of transcription. This analysis has revealed that initiation occurs at a minimum of seventeen specific sites scattered widely throughout the genome (8).

We have previously employed guanylyl transferase in the determination of the transcriptional initiation sites for the two most abundant mitochondrial transcripts, the large and small rRNAs (6,9). Furthermore, we have shown that a highly purified preparation of yeast mitochondrial RNA polymerase initiates transcription at these two sites with high fidelity in vitro (10). Comparison of the DNA sequences at the 5' ends of these two genes has revealed that the initiating nucleotide and the next eight bases immediately upstream are identical in two distantly related yeasts, Saccharomyces cerevisiae and Kluyveromyces lactis (11). The retention of this nonanucleotide sequence (5'-ATATAAGTA-3') at the initiation sites of both the large and small rRNA genes in these two species suggests that it is a conserved sequence necessary for correct initiation.

With this in mind, we searched the available yeast mitochondrial DNA sequences to see if the nonanucleotide sequence is present elsewhere in the genome associated with other genes. The nonanucleotide sequence was found in two copies upstream of the coding region for subunit 9 of the oligomycin-sensitive ATPase (genetic locus oli I, coding region identified in reference 13) as well as a number of other positions in the genome including the putative origins of replication (11). The position of the two closely spaced nonanucleotide sequences upstream of the oli I gene maps in the vicinity of the 5' end of a 12S RNA that has been identified as the major stable transcript of this gene (5,12).

In this paper, we demonstrate that the major site of transcriptional initiation for the oli I gene is at the upstream copy of the nonanucleotide sequence about 630 nucleotides upstream of the coding region for the ATPase subunit. The following lines of evidence support this view. First, we confirm that the strain we used, KL14-4A, does indeed contain two copies of the nonanucleotide sequence in front of the oli I gene as had been reported

for the strain D273-10B (13). Second, we localize the 5' end of the 12S oli I transcript to the position of the upstream copy of the nonanucleotide sequence using S1 nuclease mapping. Third, we show that this site is used as an initiation site in in vivo by sequencing guanylyl transferase-labelled primary transcripts isolated from petite yeast retaining the oli I region. Finally, we demonstrate that this site can be used as an initiation site in a homologous in vitro transcription system. In addition, we also observe transcriptional initiation at the site of the second nonanucleotide sequence 78 nucleotides downstream of the first with both in vitro capping and in vitro transcription analysis.

MATERIALS AND METHODS

The general procedures for growth of the yeast strains, purification of mtDNA, mtRNA, the replicative form of M13 clones, and of E. coli plasmids, restriction enzyme incubation, labeling DNA with T4 polynucleotide kinase, Southern blot transfer and S1 nuclease analysis (unless otherwise stated) have been described previously (9,11,14). The molecular weight markers used for calibration of the DNA fragment protected from S1 nuclease are end labeled HinfI fragment of pBR322. The markers for calibration of transcription products are end labeled HinfI-SalI-PvuI fragments of pBR322.

Yeast Strains.

The wild type yeast S. cereviaiae KL14-4A has previously been described (15). Petite mutant LH 30D3 was obtained from petite induction of the strain AMR34-17B (nuclear genotype: a, trp^-, tsp25, mitochondrial genotype: KL14-4A)(16) and retains a segment of the mitochondrial genome containing the var I and oli I genes and the 3' end of the cob gene. Petite mutant DS400/A12, derived from D273-10B, was a gift of Alexander Tzagoloff. The entire sequence of this petite has been reported (17,18). Petite mutants O_I-10 and O_I-O_{II} which do not produce the downstream transcript in vivo have been described (19).

Construction of Recombinant Clone M-12A and pK9.

After digestion of petite LH30D3 with Eco RI and HindII, fragment RT10 was isolated from low-melting agarose (cf. Fig. 1). RT10 was then digested with MspI, after which the 890 bp MspI fragment was purified from low melting agarose. This DNA, containing the beginning of the gene coding for ATPase subunit 9, was ligated to AccI cut-phage M13 mp7 (20) and transfected into E. coli strain JM101. To transfer the insert to the vector pUR222, both pUR222 and the replicative form of M-12A were cut with BamHI,

mixed and ligated. The products were used to transform $F^-Z^-\Delta$ M15recA$^-$ as described (21).

DNA Sequence Analysis.

Single-stranded M13 DNA for use in the dideoxy sequencing method of Sanger et al.(22) was prepared from recombinant M13 plaques (23) and complementary DNA synthesis started with a 18-mer synthetic primer complementary to the sequence of M13 flanking the insert.

Guanylyl Transferase Reactions.

Guanylyl transferase was isolated from cores of vaccinia virus by the procedure of Levens et al., (6). In vitro capping reactions were performed and stopped as described (6,9). The reaction mixture was extracted twice with phenol and once with chloroform prior to quick column chromatography (10,24). The excluded material was precipitated with three volumes of ethanol. T_1 digestions and RNA sequencing reactions were performed by the methods of Donis-Keller et al., (25) and Silberklang, et al., (26).

In vitro Transcription Reactions.

Mitochondrial RNA polymerase was purified as described by Levens et al., (27) except the entire purification was carried out in 0.1mM dithiothreitol and DEAE Sephacel was substituted for DEAE Sephadex A50. At the end of the purification, the polymerase was dialyzed against 20mM potassium phosphate (pH 7.6), 15% glycerol, 0.1mM dithiothreitol, 0.1% NP40, and 0.05 mM EDTA to remove KCl. To prepare end labeled transcripts for sequencing, 2 mCi of [γ^{32}P]-ATP (Amersham, 3,000 Ci/mMol) were lyophilized and resuspended in 5 µl of a 5X reaction mixture. 20 µl of RNA polymerase were added to start the reaction. Final concentrations of components of the reaction mix were as follows: 50µg/ml pK9 DNA; 10mM Tris HCl (pH 7.9); 10mM $MgCl_2$; 125µM GTP and UTP; 25µM ATP; and 0.5mg/ml rabbit serum albumin. The reactions were carried out at 30°C for 40 minutes. The reactions were stopped and products analyzed as previously described (10). RNA sequencing reactions were performed as described under "in vitro capping procedures."

RESULTS

A map illustrating relevant features of the region around the oli I gene is shown in figure 1. The coding region for the ATPase subunit 9 is indicated by the shaded area. A transcript of 12S maps in this region (5) and its approximate position as determined by electron microscopy of RNA/DNA heteroduplexes (28,12) is indicated by the interrupted line. The DNA sequence of this region in the strain D273-10B has been reported (13)

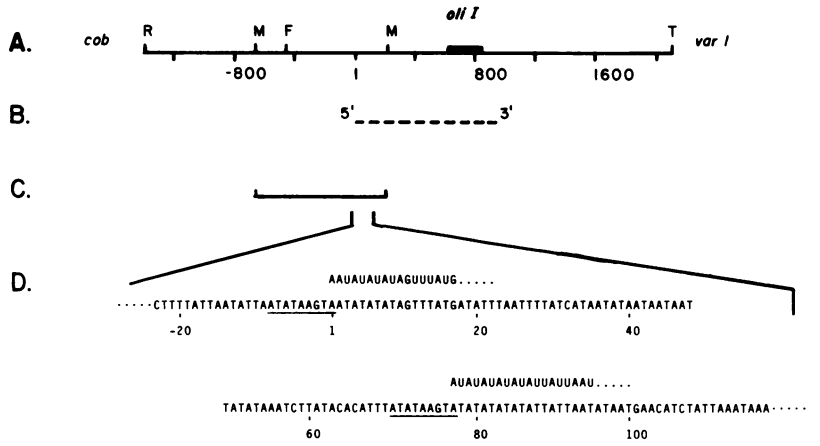

Figure 1. Diagram of the oli I coding region. A. Physical map of the oli I region (Hensgens et al., 1979; Tzagoloff et al., 1980). The shaded region indicates the coding sequence for ATPase subunit 9. The sequences are numbered from the major transcriptional initiation site as determined in this paper. The restriction sites are labelled as follows: R, Eco R1; T, HindII; M, MspI; F, HinfI. Only the relevent MspI and HinfI sites are indicated. B. Localization of the 12S RNA as determined by Hensgens et al, 1979. C. The MspI fragment inserted into M13 clone M12A and pUR222 clone pK9. D. Part of the sequence determined for the insert in clone M12A. The sequences are numbered from the major transcriptional initiation site. Copies of the conserved 9 nucleotide sequence are underlined. The nucleotde sequence of the primary transcripts detected from this region are printed above the corresponding DNA sequence.

and in the vicinity of the 5' end of the 12S RNA there are two copies of the nonanucleotide sequence which has been implicated as part of a yeast mitochondrial promoter (11).

In order to ensure that the nonanucleotide sequences are present in our strain, KL14-4A, as well as in D273-10B, we inserted the 890 bp MspI fragment upstream of the oli I coding region from KL14-4A into an M13 cloning vector and determined the sequence of the insert. The results are shown in figure 1d. Both copies of the nonanucleotide sequence are present in KL14-4A and are underlined in the figure. Comparison of this sequence with that of D273-10B reveals only two discrepancies, one of which proved to be relevant: in KL14-4A, the first cytidylyl residue in a transcript initiated at the upstream nonanucleotide sequence will be at position 32 instead of 31. The position of the nonanucleotide sequences relative to the start of the ATPase coding region is not exactly known because a short GC cluster lying between the 890 bp MspI fragment and the coding region

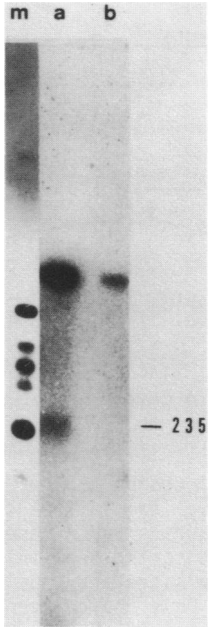

Figure 2. S1 mapping of the 5' end of the 12S mRNA. Hybrids were formed with the 650 bp HinfI-MspI fragment labeled at the MspI site and total KL14-4A mitochondrial RNA. Hybridization was carried out at 39°C for 16 hours in 0.04 M PIPES, 1 mM EDTA, 0.4 M NaCl and 80% formamide. S1 nuclease treatment was done at 37°C. The S1 nucease resistant fragments were electrophoresed through an alkaline 2% agarose gel, blotted onto nitrocellulose and visualized by autoradiography. M. Molecular weight markers (1637, 517/506, 396, 344, 298, and 221/220 base pairs). A. Fragments protected after treatment with 130 u/ml S1 nuclease. B. Undigested HinfI-MspI fragment. The apparent size of the protected fragment is indicated at the right.

remains unsequenced.

S1 Nuclease Mapping

Previous electron microsope heteroduplex studies had only roughly mapped the position of the 5' end of the 12S transcript. In order to determine its position more precisely, we used S1 nuclease mapping. The 890 bp MspI fragment from upstream of the oli I region was isolated, labeled at its 5' ends with polynucleotide kinase, and then cut with HinfI. The 650 bp HinfI-MspI fragment was isolated and hybridized with total mitochondrial RNA. After treatment with S1 nuclease, the protected DNA was analyzed by alkaline agarose gel electrophoresis and autoradiography. The results are shown in figure 2. Based on the length of the protected DNA,

the 5' end of the 12S RNA is about 235 bases removed from the MspI site or about 630 bases upstream of the coding region for the ATPase subunit. This is the position of the upstream nonanucleotide sequence. Hence, within the limits of resolution of this experiment, the 5' end of the major stable transcript of the oli I locus maps at the upstream nonanucleotide sequence.

Capping Analysis

We have recently completed an extensive study of mitochondrial transcriptional initiation which will be reported in detail elsewhere (8). In short, we isolated total RNA from a collection of petite deletion mutants whose genomes overlap and which together cover the entire genome. RNA from each petite was then labeled with guanylyl transferase and [α^{32}P]-GTP. This procedure specifically labels RNA molecules which retain their initiating nucleotide since only 5' polyphosphates serve as substrates in this reaction and the only known source of such molecules in yeast mitochondria is the initiation of transcription.

To isolate oligonucleotides suitable for sequencing from the complex mixture resulting from labeling total mitochondrial RNA, we took advantage of the low GC content of mitochondrial DNA. Capped RNA was digested to completion with the ribonuclease T_1 which cuts 3' to guanylyl residues in RNA. The resulting capped oligonucleotides were separated in a 20% acrylamide, 8.3 M urea gel. Each primary transcript is thus characterized by the number of nucleotides from the initiation site to the first guanylyl residue.

T_1 digestion products of capped RNA from two oli I-region petites are shown in figure 3a. In all the petites retaining this region, we found an eleven base T_1 oligonucleotide that had been labeled by guanylyl transferase. In instances where the T_1 digestion was not quite complete, we also found a seventeen base labeled oligonucleotide. These oligonucleotides were isolated from the gels and their sequence determined directly using base specific RNases. The results are shown in figure 3b. The sequences we determined for the eleven and seventeen base oligonucleotides match exactly the DNA sequence immediately downstream of the first nonanucleotide sequence. The entire sequence of DS400/A12, one of the petites from which this oligonucleotide was isolated, has been reported (17,18). The sequence of these oligonucleotides is not repeated anywhere else in this petite and so both oligonucleotiodes must result from T_1 digestion of a primary transcript initiated at the nonanucleotide sequence about 630 nucleotides upstream of the coding region for the ATPase subunit.

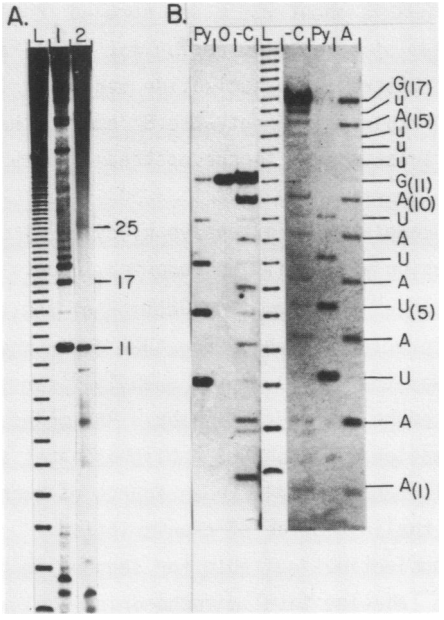

Figure 3. Capping analysis of oli I transcripts. A. Total mt RNA from the petite strains O_I-10 (lane 1) and DS400/A12 (lane 2) were labelled with guanylyltransferase and digested to completion with ribonuclease T_1. The products were separated on a 20% acrylamide, 8.3 M urea gel. L: reference ladder made by alkaline digestion of total capped RNA. The unmarked oligonucleotides in the O_I-10 lane represent transcription from other promoters present in that petite (8). B. RNA sequencing of T_1 oligonucleotides. The 11 and 17 base oligonucleotides from part A of this figure were eluted from the gel and subjected to enzymatic sequencing reactions. Leftmost three lanes: sequence determination of the 11 base oligonucleotide. Rightmost three lanes: sequence determination of the 17 base oligonucleotide. The lanes are labeled as follows. Py: products of partial digestion by the nuclease from Bacillus cereus, specific for cutting 3' to pyrimidine residues. O: undigested sample. -C: products of partial digestion by the nuclease from Physarum, specific for cutting at all sites except 3' to cytidylyl residues. A: products of partial digestion by the enzyme U2, specific for cutting 3' to adenylyl residues. L: reference ladder, produced as in part A. The deduced sequence is printed to the right of the autoradiogram.

In addition to the eleven base oligonucleotide which arises from initiation at the upstream nonanucleotide sequence, we found a low abundance oligonucleotide of 25 bases in DS400/A12, the smallest of the petites that we analyzed retaining this region (figure 3a). Because of the low amount of radioactivity in this fragment, we were unable to determine its complete sequence. However, the position of its first guanylyl residue at

position 25 and the position of pyrimidine residues at its 5' end as determined by partial digestion with ribonuclease from Bacillus cereus (data not shown) reveal that this transcript could only have originated from the downstream nonanucleotide sequence 553 nucleotides upstream of the ATPase coding region. Since this capped oligonucleotide was demonstrated in only one of the several oli I petites we investigated, transcription from the second site may be an artifact of that particular petite. Alternatively, transcripts from the second site may be normal low abundance species which are greatly amplified or stabilized in this small petite.

In Vitro Transcription

We have recently reported that purified yeast mitochondrial RNA polymerase is capable of correctly initiating transcription at the two ribosomal RNA initiation sites (10). To see if our in vitro system is competent to initiate transcription correctly at this newly discovered promoter, we used the same polymerase preparation to transcribe the plasmid pK9 which contains the 890 bp MspI fragment inserted into pUR222. To assay specificity we synthesized RNA in the absence of CTP. Under these conditions, we expect the mtRNA polymerase to initiate at the correct site and elongate the transcript until it reaches the point for insertion of the first CTP. This should yield a transcript of predictable length depending on the sequence of the particular DNA that is being transcribed. We have previously used this method to assay specificity at the rRNA promoters.

RNA was synthesized from the cloned MspI fragment using purified polymerase with [γ^{32}P]-ATP and non-radioactive GTP and UTP in the absence of CTP. The transcripts are thus uniquely labeled at the 5' initiating nucleotide, yielding a substrate suitable for sequencing. A transcript initiated from the upstream nonanucleotide sequence would have its first cytidylyl residue at position 32 while the first cytidylyl residue in a transcript initiated at the downstream nonanucleotide sequence would be at position 28. The results are shown in figure 4a. The major product is about 31 nucleotides, the expected size of a transcript from the upstream nonanucleotide sequence. In addition, two other prominent transcripts are synthesized, one of approximately 27 and one of about 57 nucleotides.

To determine the identity of these transcripts, the RNA bands were exised from the gel and subjected to enzymatic RNA sequencing reactions. The results are shown in figure 4b. The 31 nucleotide transcript has the same sequence as that determined for the major capped RNA of the oli I region and predicted by the DNA sequence for a transcript initiated at the

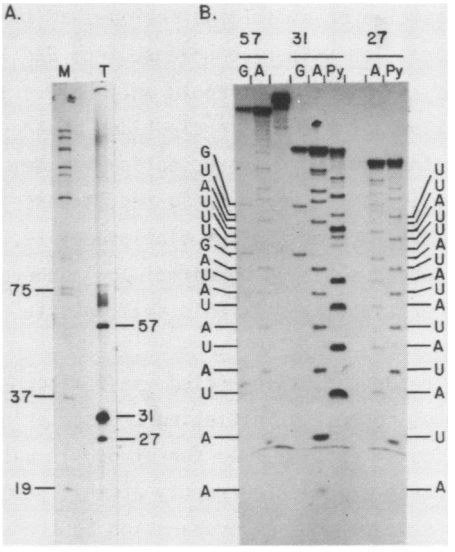

Figure 4. In vitro transcription at oli I promoters. A. The products of transcription using [γ^{32}P]-ATP in the absence of CTP from plasmid pK9 were separated on an 8% acrylamide, 8.3 M urea gel. M: molecular weight markers as indicated. The sizes of the major transcripts are indicated on the right. B. RNA sequence determination of the products of transcription. The 5' labeled transcripts shown in part A were eluted from the gel and subjected to enzymatic RNA sequencing reactions. The sequencing data for the 57 nucleotide transcript is shown in the left panel, the 31 nucleotide transcript in the center, and the 27 nucleotide transcript on the right. The lanes are labeled as in figure 3b with the additional lane G: products of partial digestion by the nuclease T_1, specific for cutting 3' to guanylyl residues. Since there are no cytidylyl residues in these transcripts, any band in the Py lanes represents a uridylyl residue. The deduced sequences for the 57 and 31 nucleotide transcripts are printed at the left, the sequence of the 27 nucleotide transcript is on the right.

first nonanucleotide sequence. The 57 nucleotide transcript was determined to have the exact same 5' sequence as the 31 nucleotide transcript. This 57 nucleotide transcript thus results from read-through of the first CTP insertion site and termination at the second which is at nucleotide 58. Readthrough could be caused by low levels of mistakes by the polymerase or by cross contamination of our nucleotide stocks. In any event, transcription is predominantly from the site of the upstream nonanucleotide sequence. The 27 nucleotide transcript's sequence was determined to be identical to the DNA sequence immediately downstream of the second nonanucleotide sequence, the same 5' terminal sequence that was found for the minor capped RNA species from petite DS400/A12. Clearly the site of the

second nonanucleotide sequence can function as a promoter *in vitro* although it is relatively weak compared to the upstream promoter.

DISCUSSION

In this paper, we show that the major transcript of the ATPase subunit 9 gene is initiated at a point approximately 630 nucleotides upstream of the coding region for this gene at a copy of the nonanucleotide sequence using S1 nuclease mapping, *in vitro* capping and *in vitro* transcription methods of analysis. In addition, capping data and *in vitro* transcription studies show that the site of a second nonanucleotide sequence 78 nucleotides downstream of the first also can function as a promoter both *in vivo* and *in vitro*. At both of these newly identified promoters, the initiating nucleotide is at the ninth position in the conserved nonanucleotide sequence as has been found to be the case for the rRNA transcripts.

These results have significant implications for our understanding of transcription of the yeast mitochondrial genome. First, there is clearly no fundamental difference in the pattern of transcription from this protein coding gene and the rRNA genes. For both types of transcripts, RNA synthesis begins at precisely defined sites. Strikingly, the four sites of transcriptional initiation that have so far been described all lie deeply imbedded in the AT rich regions of the mitochondrial genome. Clearly these regions are not only informationless spacer. On the contrary, they contain the sequences intimately involved in expression of the genome.

Second, the fact that the same highly purified RNA polymerase is capable of correct initiation at the mRNA initiation sites described in this paper as well as the rRNA initiation sites previously delineated indicates that the same transcriptional machinery is involved in the expression of these two types of genes. Since at least one tRNA has been shown to be part of the 21S rRNA primary transcript (29), there is now evidence that the same RNA polymerase is responsible for transcription of three classes of mitochondrial transcripts: mRNA, rRNA and tRNA. Recent evidence indicates that this same polymerase is also involved in priming of mtDNA replication. The nonanucleotide sequence is found in putative origins of replication (11) and in one case we have found that a nonanucleotide sequence from an origin region can serve as a promoter for transcriptional initiation (K. Osinga and H. Tabak, unpublished results). The mitochondrial RNA polymerase preparation used in these studies has been

extensively purified (27). Still, the polymerase has not been purified to homogeneity so it is not clear whether the polymerase alone is capable of specificity or whether other modulating factors are necessary.

Third, the conservation of a nonanucleotide sequence at each initiation site presented here plus the lack of any other extended homology suggests that the nonanucleotide sequence itself is an essential component of the yeast mitochondrial promoter. Recently, several other initiation sites have been identified by matching the sequence of the 5' terminal T_1 fragment of _in vitro_ capped transcripts with known DNA sequences. In each case, the initiating nucleotide lies in the ninth position of a DNA segment which is identical or strongly homologous to the nonanucleotide sequence (8). However, the nonanucleotide sequence is clearly not the only determinant of promoter function in yeast mitochondria. Both promoters upstream of the _oli I_ gene contain perfect copies of the nine nucleotide sequence and yet they show markedly different strengths _in vitro_.

Finally, our results show that there are two transcriptional initiation sites upstream of the _oli I_ region situated 78 nucleotides apart. We have shown that both promoters can function _in vitro_ and in _in vivo_, transcripts from the upstream promoter being more abundant in both cases. However, we find transcription from the downstream site in only the smallest of the several _oli I_-region petites we analyzed. In addition, S1 analysis of total grande RNA fails to reveal stable RNA molecules with 5' ends at the downstream site, but because of the high AT content of this region, S1 heteroduplex mapping is technically difficult (31) and low level transcripts might not be detected. Hence, the significance of the second promoter in _in vivo_ remains unclear.

What purpose could be served by tandem promoters Since initiation is the rate limiting step in most transcription systems, multiple promoters could simply allow more rapid expression of a gene, a rational that has been suggested for the existence of multiple promoters upstream of the rRNA genes in _E. coli_ (33) and _X. laevis_ (34). This reasoning probably does not apply to the tandem promoters in yeast mitochondria because the two most abundant transcripts, the large and small rRNAs, are transcribed from single promoters. Alternatively, the two promoters could be subject to different regulation and serve different cellular functions. This appears to be the case with the tandem promoters in front of the _E. coli gal_ operon (35) and has been suggested for the tandem initiation sites in mammalian mitochondria (32).

Acknowledgements

We thank professors P. Borst and L. A. Grivell for helpful discussions. J. C. E. is supported by a grant from the National Institutes of Health. K. A. O. was the recipient of a Short-Term Fellowship from EMBO. This work was supported by grants from the National Institutes of Health, the American Cancer Society, the Louis Block Fund of the University of Chicago, and a grant to P. Borst and L. A. Grivell from the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

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