Polymorphism in the structure of the yeast mitochondrial tRNA synthesis locus

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ABSTRACT

Yeast mitochondrial DNA contains a genetic locus, called the tRNA synthesis locus, which codes for information necessary for mitochondrial tRNA biosynthesis. A 9S RNA molecule coded by this locus is thought to be the trans-acting element required for the removal of 5' extensions from tRNA precursors. The DNA coding for this RNA maps to a region of mitochondrial DNA known to contain strain specific restriction site polymorphisms. Comparison of the tRNA synthesis locus in two such strains by sequence analysis demonstrates that the restriction enzyme polymorphisms are due to the deletion/insertion of a 50 base pair GC-rich element in the 5' flanking sequence of the 9S RNA coding region. There are also several differences between the 9S RNA coding region of these two strains which do not interfere with the tRNA synthesis function.

INTRODUCTION

The mitochondrial DNA (mtDNA) of the yeast, *Saccharomyces cerevisiae*, codes for all of the transfer RNAs (tRNAs) necessary for mitochondrial protein synthesis (1-3), and twenty-four of the mitochondrial tRNA genes of *S. cerevisiae* have been mapped and sequenced (4). Expression of these genes requires a number of nuclearly encoded functions as well as one mitochondrially encoded function. Morimoto et al. (5) and Martin and Underbrink-Lyon (6) have shown that a region of mtDNA is necessary for the production of mature tRNA. This region acts in the absence of mitochondrial protein synthesis (6). The tRNA synthesis function has been mapped to a 780 base pair region located between the tRNA^{Met}_f and tRNA^{Pro} genes (7), and a 9S RNA is produced from this locus (8). Petite deletion mutants unable to make mature tRNAs because they lack the locus, accumulate tRNA precursors with 5' extensions (9-11). We have previously proposed that this RNA functions by complexing with a nuclearly encoded protein to produce a ribonucleoprotein similar to RNAase P in *E. coli* which has been shown to remove 5' extensions from tRNA precursors (12) with tRNA processing activity. Recent evidence showing that the RNA component of RNAaseP is, under some conditions, alone

able to mature the 5'ends of tRNAs (13) raises the interesting question of whether the yeast mitochondrial 9S RNA will also be capable of tRNA transcript processing in the absence of protein.

The 9S RNA produced from the tRNA synthesis locus is extremely AU-rich (87%) but does contain a single very GC-rich region (8). In this respect the DNA coding for 9S RNA is typical of the noncoding sequences between mitochondrial genes in *S. cerevisiae* (4). In general, the mitochondrial genome of this yeast is very AT-rich and consists of three types of sequence elements. About 50% of the genome consists of very (>95%) AT-rich sequence. Another 10% consists of a number of very (50-90%) GC-rich sequence elements which are about 50 nucleotides in length. The remaining 40% is intermediately GC-rich and include the majority of mitochondrial genes. While all strains of *S. cerevisiae* have this same basic mtDNA organization, certain variations between the mtDNA of different wild-type strains exist (14-15). These variations may be due to large insertion/deletions such as the presence or absence of introns (4), rather simple sequence variation in the AT-rich regions (16,17) or variations in the presence or absence of GC-rich sequence elements (27). One such variation has been observed near the region shown to be necessary for mitochondrial tRNA synthesis (14,15,18). A Hha I restriction enzyme site upstream of the 9S RNA coding region in MH41-7B is absent in D273-10B. Since previous characterization of the tRNA synthesis locus has been done exclusively using MH41-7B and its derivative petites (8), it was of interest to compare the structure of this wild-type gene and its surrounding sequences with that of another strain.

The sequence of the tRNA synthesis locus region from a different wild-type strain, D273-10B, is reported here. A 50 base pair GC-rich sequence element upstream of the 9S RNA gene is precisely deleted in D273-10B relative to MH41-7B. Also, several examples of simple sequence variation in the AT rich 9S RNA coding region are seen. As expected from the fact that the locus is essential for tRNA biosynthesis, this region is transcribed. Since the tRNA synthesis locus functions in both wild-type strains, there must be flexibility in the allowable structure of its gene product.

MATERIALS AND METHODS

Isolation of Mitochondrial Nucleic Acids

Wild-type yeast strains D273-10B and MH41-7B, and petite strain ND157 were grown on 2% galactose, 0.2% glucose, 1% yeast extract and 1% bactopectone to late log phase and mitochondria were isolated from zymolyase-treated cells

(Miles Laboratories). mtDNA was isolated by CsCl isopycnic gradient centrifugation (19). RNA was prepared by multiple phenol extractions of mitochondria lysed in 10 mM Tris-HCl (pH 8.0), 2 mM EDTA, 0.2% SDS, 0.1% makaloid. The aqueous layer from such extractions was precipitated with ethanol and applied to 1.5% agarose, 6M urea gels for Northern blots. RNA for primer extension experiments was precipitated with ethanol, treated with RNAase-free DNAase I (Millipore, Inc.), extracted with phenol to destroy DNAase activity and precipitated with ethanol.

Nitrocellulose Filter Hybridizations

Plasmid pYm644 was identified as containing the mtDNA of interest by the in situ colony hybridization technique of Grunstein and Hogness (20) using uniformly labeled mtDNA from the petite strain ND157 (7), a strain which retains the tRNA synthesis locus. The homologous portion of this petite for the region of interest extends from position 37 to position 730 in figure 3.

Restriction enzyme digestions of plasmid DNA were resolved on 2% agarose gels and transferred in situ to nitrocellulose filters by a modification of the method of Southern (21). These filters were hybridized with the ND157 probe described above in 5XSSC, 0.1% SDS, 50 mM NaPO₄ buffer (pH 7.0), 10 µg/ml calf thymus DNA and 50% formamide at 42°C.

DNA Sequence Analysis

DNA fragments from plasmid pYm644 were labeled at the 3' end with α -³²P-NTP and *E. coli* DNA polymerase I or at the 5' end with γ -³²P-ATP and polynucleotide kinase as described by Miller *et al.* (22). Labeled fragments were sequenced by the method of Gilbert and Maxam (23).

Northern Hybridization

Mitochondrial RNA was separated by electrophoresis on 1.5% agarose, 6M urea gels, transferred to DBM paper (24) and hybridized with the radioactive probe. The radioactive probe was prepared by nick translation of the cloned Hpa II fragment located from position 501 to 730 in figure 3. These filters were hybridized under the conditions described above.

Primer Extension Sequence Analysis

The Sst II (510)-Hinf I (531) fragment was isolated from plasmid pYm644 and labeled at the 5' end of the Hinf I site with polynucleotide kinase and γ -³²P-ATP. The labeled fragment was mixed with mtRNA from D273-10B in 45 µl of 90% formamide and incubated at 68°C for 5 min. Hybridization was initiated by the addition of 5 µl of 4M NaCl, 10 mM EDTA, 1M PIPES (pH 6.4), continued at 50°C for 60 min. and terminated by precipitation with ethanol. cDNA was synthesized by incubating the annealed primer in 20 mM Tris-HCl (pH 8.4),

Figure 1. Demonstration that the mitochondrial insert in pYm644 contains the tRNA synthesis locus. Restriction enzyme digests of pBR322, pYm644 and ND157 (a petite which retains the tRNA synthesis locus) were separated on a 2% agarose gel, transferred to nitrocellulose paper and hybridized with nick translated ND157 mtDNA. The first four lanes on the left are ethidium bromide stained DNA and the next four lanes are the autoradiogram of the hybridization experiment. The last two lanes are the autoradiogram obtained when ND157 DNA is hybridized to itself as well as to pBR322.

10 mM NaCl, 6 mM $MgCl_2$, 1 mM NTPs, 20 mM dithiothreitol, and 60 U avian RNA-dependent DNA polymerase (Life Sciences, Inc.) at 42°C for 60 min. Extension products were separated on 6% acrylamide gels. Specific extension products of approximately 330 and 470 were isolated from the acrylamide gel by elution from ground up gel at 37°C overnight. cDNA fragments were concentrated by DEAE chromatography and were sized by performing the C and G sequencing reactions of Maxam and Gilbert (23).

Figure 2. Restriction enzyme map of the tRNA_f^{Met} synthesis locus region in D273-10B as cloned in pYm644. The tRNA_f^{Met} gene is indicated by the box. Restriction enzyme sites are indicated by hatches and letters as follows: AhaI (Ah) AvaII (Av), HpaII (Hp), HincII (Hc) Hinf I (Hf) MboI (Mb). Arrows under the map indicate direction and the extent of DNA sequence analysis. C and P indicate the directions of the chloramphenicol and paromomycin loci to allow orientation on the wild-type genome.

RESULTS

Identification and Characterization of pYm644.

We have previously prepared a bank of mtDNA from strain D273-10B cloned in the bacterial plasmid, pBR322 (25). To identify those colonies in the bank which contain the region analogous to the 9S RNA coding region in the wild-type strain MH41-7B, the bank was screened by the method of Grunstein and Hogness (20) using a uniformly labeled portion of mtDNA from the tRNA synthesis region of MH41-7B as a probe. A number of colonies from the bank hybridized with this probe. Several of these were analyzed by restriction enzyme digestion and one such plasmid, (pYm644), containing the HincII site within the tRNA_f^{Met} gene was further characterized by restriction analysis and by DNA sequence analysis. To demonstrate that pYm644 contains the tRNA synthesis locus, plasmid DNA was digested with HinfI and HpaII, and the electrophoretically separated fragments were transferred to nitrocellulose for hybridization with a tRNA synthesis locus probe. As can be seen in Fig. 1, DNA fragments which do not correspond to vector bands hybridize with this probe. The mtDNA fragment cloned in plasmid pYm644 includes the entire region analogous to the tRNA synthesis locus in MH41-7B as well as the tRNA_f^{Met} gene. Restriction mapping revealed that several sites known to be present in this region of MH41-7B are missing in pYm644, including the Hha I site reported previously (14,15,18). These restriction enzyme sites all fall in a GC-rich element which is completely missing in D273-10B (see below).

Sequence analysis of the tRNA synthesis locus region in D273-10B.

DNA was sequenced using the procedure of Maxam and Gilbert (23). The restriction enzyme sites used and the extent and direction of the sequence obtained is indicated by the arrows in Fig. 2.

Comparison of the sequence of this region of D273-10B (Fig. 3) with that previously obtained for MH41-7B (9) shows several regions of nonhomology. The entire GC-rich sequence element that contains the HhaI site in MH41-7B is missing in D273-10B. This GC-rich sequence element (Fig. 3, number 1) is located just upstream of the 5' end of the 9S RNA as determined by analysis of the most prominent cDNA extension product previously identified in MH41-7B (8). The sequence TA is found at the position of the deleted GC rich sequence element in D273-10B (underlined in Fig. 3). In the 9S RNA coding region there is an insertion of 17 base pairs (positions 332 to 348) in D273-10B relative to MH41-7B (underlined in Fig. 3). Further downstream the sequence TATATATATATATA in MH41-7B is reduced to TATATATA in D273-10B (Fig. 3, number 3). A single base transition is observed at position 377 (Fig. 3). There is a single base insertion in the GC rich sequence element in D273-10B (underlined in Fig. 3). A run of 3As is also not present in D273-10B so the 8 As found in MH41-7B in this location (Fig. 3, number 6) are reduced to 5 As in D273-10B.

Primer extension sequence analysis of transcripts from the tRNA synthesis region of D273-10B.

Some of the sequence differences of D273-10B relative to MH41-7B were within the region that was found to code for a 9S RNA in MH41-7B. To determine if this region in D273-10B is also transcribed, a primer extension sequence analysis was performed. MtRNA was isolated from D273-10B and annealed to a 5' end-labeled, 22 nucleotide Sst II-Hinf I restriction fragment (nucleotides 510 to 531 in Fig. 3 which acted as a primer for avian RNA dependent DNA polymerase (8). The cDNAs synthesized were separated by polyacrylamide-urea gel electrophoresis and autoradiographed. Two major cDNA extension products of about 330 and 470 nucleotides and several minor products were observed (Fig. 4A). The two major cDNAs were isolated and G and C DNA sequencing reactions performed (23). The DNA sequencing reaction products were electrophoresed on a 6% polyacrylamide 8M urea gel and autoradiographed (Fig. 4B). This gel showed that the 330 nucleotide cDNA extension product was a mixture of two fragments of approximately 321 and 325 nucleotides. The 470 nucleotide cDNA was also a mixture of fragments approximately 465 and 510 nucleotides long. The general location of the 5' ends of these cDNAs is shown in Figure 3 by asterisks.

GC Site Cluster ~ 37 Base Pairs.....AAAA *AGTATAATAA AATATATTAT 60
 template strand → TTTT TCATATTATT TTATATAATA

AAGAA*GAATA TATTATATAA TATAATAATT ATATTAATAA TATTAATAAA TAATATATAA 120
 TTCTTCTTAT ATAATATATT ATATTATTAA TATAATTATT ATAATTATTT ATTATATATT

ATAATTATAA AAAAGTATAT AATATTAATC AATTAATTAA TTAATAAATA TAAATAATAT 180
 TATTAATATT TTTTCATATA TTATAATTAG TTAATTAATT AATTATTTAT ATTTATTATA

ATTAATTTTT AATTAATTT* AATAAGATAT TTATATTATT AATAGGAAAG TCATAAATAT 240
 TAATTAAGAAA TTAATTAAC TTATTCTATA AATATAATAA TTATCCTTTC AGTATTTATA

ATAAATTATA TTATATAATT AATATAATAA TAAAAATAAT TATATTTTT ATTTATAATA 300
 TATTTAATAT AATATATTAA TTATATTATT ATTTTATTTA ATATATAAAA TAAATATTAT

TTATTTCTTT ATAAGATAAA TATTATCTAA TTAATAATTA GATTGAATAA TATTTATAAA 360
 AATAAGAAA TATTCATATT ATAATAGATT AATTATTAAT CTAACTTATT ATAAATATTT

GAAATATATA TAAAAAATCA TTATATAAAT TTAATTATAA TTAAATAAAA TTTTATATAA 420
 CTTTATATAT ATTTTTTAGT AATATATTTA AATTAATATT AAATTTATTT AAAATATATT

ATTAATATAA TATTAATAAA GTAATTAGTA TAAATAAATA ATATGAAAT AAAACTTAAT 480
 TAATTATATT ATAATTATT CATTAATCAT ATTTATTTAT TATACTTTTA TTTTGAATTA

AAATATATAA ATATAGTCCG GCCCGCCCGC CGGGCGGGAC CCGCAGGAG TCCATATATA 540
 TTTATATATT TATATCAGGC CGGGCGGGGC GCGCGCCTG GGGCGTCTC AGGTATATAT

TATATTAATA AAAAGTAATA TATATATATA TATTGGAATA GTTATATTAT TATACAGAAA 600
 ATATAATTAT TTTTCATTAT ATATATATAT ATAACCTTAT CAATATAATA ATATGTCTTT

TATGCTTAAT TATAATATAA TATCCATATT CATAATTAAT TTTTATATA TATATTATAT 660
 ATACGAATTA ATATTATATT ATAGGTATAA GTATTAATTA AAAAAATATAT ATATAATATA

TATAATATTA ATTTATATTA TAAAAATAAT ATTTATTATT AAAATATTTA TTCTCCTTTA 720
 ATATTATAAT TAAATATAAT ATTTTATTA TAAATAATAA TTTTATAAAT AAGAGGAAAT

GGGGTTCCCG
 CCCCAAGGCC

1. CCCCCGCGGGCGCCCAACCCGTTGCTCACGGGTTGGTCCACGGGGT
 GGGGGCGCCCGCGGTTGGGCAACGAGTGGCCCAACGAGGTGCCCCCA

3. TATATA
 ATATAT

4. A → G
 T C

6. AAA
 TTT

Figure 3. DNA sequence of the tRNA synthesis locus in D273-10B and a comparison with the analogous region in MH41-7B. The DNA sequence as shown was determined from pYm644 and the bottom line is the template strand. The positions of nucleotides which are present in MH41-7B but absent in D273-10B are indicated by the numbers 1,3,4,6 above the sequence. The bases missing at these sites are printed next to the corresponding numeral at the bottom of the figure. Sequences present in D273-10B but absent in MH41-7B (numbers 2 and 5) are underlined. The single base transition at position 377 (number 4) is also underlined and the base change printed at the bottom of the figure. The 5' ends of the primer extension products (see Figure 4) are indicated by asterisks (*) above the line.

Figure 4. Analysis of D273-10B tRNA synthesis locus transcripts by primer extension. A) An autoradiogram of a 6% polyacrylamide gel following separation of cDNA extension products produced by copying D273-10B mtRNA from a primer spanning nucleotides 510 to 531 (Fig. 3). B) Autoradiogram of G and C DNA sequencing reactions of the cDNA extension products. The two lanes on the left are the digestion products of the 470 base cDNA and the two to the right are of the 330 base cDNA. The numbers at the right correspond to the appropriate positions of Gs and Cs as numbered in Fig. 3.

Figure 5. Northern hybridization to detect tRNA synthesis locus gene products. RNA obtained from isolated mitochondria from MH41-7B and D273-10B was run separately and mixed on 1.5% agarose-urea gels, transferred to DBM paper and hybridized with the 230 base pair HpaII fragment from nucleotides 501 to 730 in Fig. 3. The first three lanes on the left are ethidium bromide stained RNAs and the next three lanes are the corresponding autoradiograms.

Hybridization of a 9S RNA probe with mtRNA isolated from D273-10B and MH41-7B.

We compared the RNA transcripts produced from the tRNA synthesis locus in these two strains by isolating mtRNA, separating it on an agarose-urea gel, transferring it in situ to DBM paper, and hybridizing with a radiolabeled DNA fragment containing the sequence from the tRNA synthesis locus (Fig. 5). Both MH41-7B and D273-10B have RNAs of about 9S which hybridize to the probe and at

Figure 6. Possible secondary structure of the 9S RNA from D273-10B and MH41-7B. The arrows show the location of polymorphisms and the number next to each arrow corresponds to the numbers above the sequence in figure 3.

this level of resolution, appear to be the same size. We presume this 9S RNA is the mature product of the tRNA synthesis locus. Several faint RNAs of higher molecular weight can be seen in the original autoradiogram and MH41-7B shows prominent hybridization to a larger RNA as well. This larger RNA varies in abundance between different strains and from RNA to RNA preparation. To date, the low abundance of these RNAs has prevented us from characterizing them by direct sequence analysis. The precursor-product relationship of these RNAs, if any, has not yet been shown.

RNA Secondary Structure Comparisons Between Two Wild Type Strains

We have previously computer modeled a possible secondary structure of the 9S RNA from the tRNA synthesis locus of MH41-7B (8). Figure 6 shows this structure and a similar structure constructed from the DNA sequence information for D273-10B obtained during this study. Although the overall structures of the two RNAs are similar, certain aspects of secondary structure are altered by the sequence differences. The most obvious are the extended

stem which accommodates the 17 base AT-rich insertion, a shortening of stem structure at the base of the large loop and a shortening of a smaller loop. We do not, of course, know how this computer modeled RNA resembles the RNA in vivo. Regardless, such a sequence comparison is a compelling argument that the sequences absent in one gene or the other are not essential for the locus function.

DISCUSSION

We had two specific aims in undertaking the experiments reported here. First, we wished to determine the sequence of a region of the mtDNA from the yeast strain, D273-10B, which corresponds to the tRNA synthesis locus previously characterized only for the mtDNA of the yeast strain MH41-7B. Second, we wished to ascertain that this region in D273-10B was transcribed. The results of our sequence analysis of the tRNA synthesis locus in D273-10B reveal six differences in sequence all of which are in regions which are transcribed as RNA.

The GC rich sequence element insertion/deletion at position 170 (Fig. 3) constitutes the longest sequence difference in the tRNA synthesis locus between the two strains studied. The fact that the GC-rich sequence element is absent in one strain demonstrates that this element is not essential for the expression or function of the tRNA synthesis locus. At least three other instances of GC-rich sequence element insertion/deletion not affecting gene expression have been observed. In each of these cases the GC-rich sequence element was present in the coding region of the gene in some strains and absent in others so it is clear that GC rich sequence elements can be transcribed as part of stable RNAs. Such differences are found in the 21S rRNA gene (26), the 15S rRNA gene (27), and the gene coding for the mitochondrial ribosomal protein at the var1 locus (17). The GC-rich sequence element located in the coding region of the mature 9S RNA (position 494 to 533) differs in the two strains by a single nucleotide. Further comparisons with other wild-type strains would be required to assess the possibility that this GC rich sequence element is optional. In general, the function of GC-rich elements is not understood. Gourset et al. (28) and Dujon et al. (29) have shown that some of these sequences may be involved in mtDNA replication. Tzagoloff (30) has proposed that these sequences may be involved in RNA processing. Further, several groups (8,31-33) have observed that the recombination involved in petite formation can occur between homologous portions of these elements. Bernardi (34) has proposed that these elements

may constitute a type of selfish DNA which has no function except its own propagation. These elements may be able to move to any acceptable site, e.g. an intergenic region or a non-functional portion of an RNA coding region. The fact that some of these sequences are involved with DNA replication would provide a further selective advantage for their propagation.

Besides the GC rich sequence element insertion/deletion there are five other sequence differences in the tRNA synthesis region of these strains. One is a single base insertion/deletion in the remaining GC-rich sequence element. Three of the differences are insertion/deletions of small AT rich sequences 3-17 nucleotides in length. There is one transition base substitution. Of these five, all are in the region believed to code for the 9S RNA (8).

The exact structure and relationship of the RNA molecules coded by this region of mtDNA is still unclear. S1 nuclease mapping of transcripts isolated from MH41-7B gave a unique 3' end at nucleotide 639 (Figure 3). The largest primer extension product produced from MH41-7B RNA templates had its 5' end downstream from the variable GC-rich sequence element as do the most prominent extension products from D273-10B RNA templates (C200, C206 Fig. 4). This data is consistent with the assignment of the 5' end of the 9S RNA to the region just downstream of the polymorphic GC-rich element. The size predicted by S1 mapping (8) and primer extension is consistent with the 9S RNA detected by Northern analysis.

We should point out that longer extension products are observed from D273-10B RNA templates (Fig. 4) and RNAs larger than 9S are clearly present (albeit in variable amounts) in RNA blots from both strains (8, Fig. 5). It seems likely that, as previously suggested, premature termination of cDNA synthesis due to secondary structure of the GC rich sequence in MH41-7B template RNA accounts for the lack of larger extension products in the earlier experiments (8). Future studies will focus on the origin and possible function of the RNAs larger than the prominent 9S RNA coded by this locus. We do not yet know if only the 9S RNA is active in tRNA processing or if, as is the case with the precursor to *E. coli* M1 RNA (35), larger RNAs are also capable of supporting the catalytic function.

We also do not know if these larger RNAs are precursor RNAs. The multiple transcripts arising in this region could come from RNA processing events or from different sites of initiation of transcription. The nonanucleotide consensus sequence proposed to form part of the promoter (36) in yeast mtDNA is not present in the DNA sequences reported here. The closest consensus sequence is upstream of the tRNA^{Met}_f gene (9), so the 9S RNA is presumably

co-transcribed with that tRNA in a primary transcript. If the 5' end of the active RNA in D273-10B falls around nucleotide 200 (Fig. 3), then the sequence alteration involving the GC-rich element would be in a precursor transcript, but changes between nucleotides 200 and 640 would be represented in the smallest product from the region, the 9S RNA.

There are clearly open questions concerning the nature of the transcripts from this locus even though a 9S RNA is the most abundant RNA found in wild-type strains. Nevertheless, the insertions and deletions observed when two wild type strains are compared do have some implication concerning the structure of the 9S RNA and its gene. One can clearly argue that sequences absent in one or the other strains are not essential for gene expression or function. Further, the gene product of this locus is able to tolerate structural alterations and still carry out its tRNA synthetic functions.

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