Structure and transcription of the mitochondrial genome in heteroplasmic strains of *Saccharomyces cerevisiae*

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ABSTRACT

*Saccharomyces cerevisiae* strain FF1210-6C/170 is respiratory deficient due to a mutation of the penultimate base of the mitochondrial tRNA Asp gene. We have identified a number of progeny from this strain which have reverted to respiratory sufficiency by the excision and tandem amplification of a small region of the mitochondrial (mt) DNA carrying the tRNA Asp gene, while also maintaining the full-length mtDNA. We have studied the structure of the mtDNA and mitochondrial transcription in a number of these heteroplasmic strains. The exact site of the recombination involved in the excision of the repeating unit of the amplified mtDNA has been determined for five of the revertants. Recombination occurs between identical sequences 4–13 base pairs in length. Each of the different repeating units of the amplified DNA retains an active promoter which has been moved to a site just upstream of the tRNA Asp gene by the excision/amplification. Transcripts from the heteroplasmic strains have been characterized to determine the sites of mitochondrial RNA termini. We find that in addition to the 5' and 3' processing of the tRNAs, many of the transcripts terminate at a position about 300 base pairs downstream of the gene for tRNA Asp. We also find that 3' processing of tRNA Asp precursors is absent in petite strains which lack 5' processing indicating that 5' processing of tRNA Asp may be a prerequisite for 3' processing in this mutant.

INTRODUCTION

The mtDNA of the yeast *Saccharomyces cerevisiae* encodes a number of the protein components directly involved in respiration, as well as two rRNAs, twenty-four tRNAs, a 9S RNA and a mitochondrial ribosomal protein necessary for mitochondrial protein synthesis (1). Of the twenty-four tRNA genes, sixteen are clustered in a 6kb region between the large ribosomal RNA gene and *ort1*, defined as tRNA cluster 1 by Morimoto & Rabinowitz (2), and the remainder are distributed throughout the mtDNA.

In general the mtDNA is very AT-rich (about 82% overall). Most genes have a composition of about 60–70% A+T, although some genes including those encoding the *var1* polypeptide (3) and the tRNA synthesis locus RNA (4) have compositions as high as 85–90% A+T. Genes are separated by vast regions of approximately 95% A+T and these spacer regions are punctuated by small regions of high GC-content (45–89%) called GC clusters (5). These regions are usually 33–50 bp in length and the approximately 200 GC clusters found in yeast mitochondria are all apparently related in sequence (6).

Respiration deficient mutants of *Saccharomyces cerevisiae* arise at a relatively high frequency from the random excision of a small portion of the mtDNA by intramolecular recombination with subsequent amplification and segregation of the excised region. These 'petite' strains which retain only a small portion of the mtDNA, lack respiration due to the loss of mitochondrial protein synthesis but retain mitochondrial transcription.
In yeast, mitochondrial transcription is initiated at the conserved nonanucleotide sequence, 5'-ATATAAGTA-3'. About 20 promoter sequences have been identified and mapped on the mtDNA using an in vitro capping technique with guanylyl transferase (7). Mitochondrial RNAs containing tRNA sequences occur as polycistronic transcripts together with other tRNA (8,9), rRNA (10) or mRNA (11,12) sequences.

Production of mature tRNA from precursor RNAs requires nucleolytic cleavage at both the 5' and 3' ends of the tRNA, modification of nucleotides, and the addition of -CCA to the 3' end. In yeast the removal of 5' leader sequences from mitochondrial tRNA precursors requires a 9S RNA encoded on mtDNA (4). Therefore, mtDNA mutants which delete the gene encoding the 9S RNA cannot remove 5' leader sequences from the tRNA transcripts and tRNA precursors accumulate which are processed normally at the 3' end but which retain 5' leader sequences (8,13).

The expression of tRNA genes has been studied in both petite and wild-type strains by several groups. In tRNA cluster I, only three promoter sequences have been identified. They are located upstream of tRNA^Cys (14), tRNA^Leu (15), and tRNA^Asp (9). In each case, transcription presumably continues through two to eight tRNA genes producing multigene transcripts which must be processed to mature tRNAs. In some petite strains, intermediate RNA products have been identified which extend to possible processing signals other than the 5' and 3' ends of the tRNAs. Francisci et al. (14) reported that some termini of intermediate transcripts are located in the sequences AATATAA or AATATATTTT located immediately adjacent to GC clusters.

Most of the studies of yeast mitochondrial tRNA processing have utilized petite strains, since in wild-type strains the processing of tRNA precursors is so rapid that the steady-state levels of tRNA precursors are very low. Transfer RNA precursors do accumulate in those petite strains which lack the tRNA synthesis locus (8,9,14,16), although, these precursors are usually 5' extensions with mature 3' ends (8,13). Some transcripts in petite strains show unusual 5' processing or initiation of transcription which is not seen in wild-type strains (9,14, 16-19).

We have recently characterized a number of respiring revertants from a respiratory deficient strain harboring a mutation in the mitochondrial tRNA^Asp gene. In these revertants a small region in the tRNA gene cluster is amplified and maintained along with the full-length mtDNA (20). Maintenance of the amplified region containing the mutated tRNA^Asp gene results in an overproduction of the mutated tRNA^Asp which allows respiration in the otherwise respiratory deficient strain. It is unclear whether overproduction of tRNA^Asp is due solely to gene amplification or if enhanced transcription of the gene also contributes to its overproduction. In either case these heteroplasmic strains are useful for the mapping of processing sites since they apparently accumulate all types of processing intermediates, due to gene amplification and to the full-length mtDNA which provides 5' processing of tRNA precursors.

We report here the structure of the mtDNA and the mitochondrial transcription in a number of the heteroplasmic revertant strains. We have precisely defined the amplified region of the mtDNA in some of these strains by sequencing across the recombination site of the tandemly repeated unit. All of the amplified regions retain a promoter consensus sequence indicating that transcription may contribute to the overproduction of tRNA^Asp. RNA mapping techniques and comparison of the transcripts produced in strains with different amplified regions has allowed us to identify and precisely map several tRNA
processing sites and to detect a probable requirement for 5' processing of RNA from the mutated tRNA\(^{\text{Asp}}\) gene to precede 3' processing.

**MATERIALS AND METHODS**

**Yeast strains and media**

The revertant strains used in these experiments are respiratory competent due to the tandem amplification of a segment of the mtDNA carrying the mutant tRNA\(^{\text{Asp}}\) gene. These revertants were derived by MnCl\(_2\) mutagenesis from the respiratory-deficient *S. cerevisiae* strain, FF1210-6C/170 (*MAT\(\alpha\) ura1 ura2 rho\(^+\) syn\(^-\) [21]*), which in turn was derived from the respiratory-sufficient strain FF1210-6C (*MAT\(\alpha\) ura1 ura2 rho\(^-\)*). The revertants carry two types of mtDNA, a full-length genome and a deleted and amplified 'petite' genome (20). The strain designation and the extent of the repeating units of the amplified genome are shown in Fig. 1. A mitotic segregant from R7, which retains only the 'petite' genome, is designated R7p.

All revertants were maintained on YEPG (1% yeast extract, 1% peptone, 2% glycerol in 50 mM sodium phosphate buffer, pH 6.5) and petite strains were maintained on YEPD (1% yeast extract, 1% peptone, 2% glucose). For mtDNA and mtRNA isolation, YEPGal (the same as YEPD except it contains 0.2% glucose and 2% galactose instead of 2% glucose) was used (22).

**Mitochondrial DNA and RNA preparation**

Mitochondria were isolated from yeast grown to late log phase in YEPGal medium. The

![Fig. 1. Organization of the mtDNA in the region of the tRNA\(^{\text{Asp}}\) gene. The thick line represents a segment of mtDNA showing selected restriction enzyme sites (F, *Hin*; H, *Hpa*; N, *Nae*; R, *Rsa*; S, *Sau3AI*; T, *Taq*). Open boxes under the thick line represent tRNA genes which are labeled with their cognate amino acid. GC clusters are depicted as filled boxes above the thick line. The bars below the thick line show the extent of the repeating units of the amplified genomes of the heteroplasmic revertants. Where the exact recombination site is not known (R17, R18), the open boxes indicate the minimum extent of the repeating unit, and the thin lines indicate the maximum extent of the repeating unit. The arrow in front of tRNA\(^{\text{Ala}}\) gene shows the location of the promoter sequence described in the text.](image)
cells were treated with Novozym 123 (Novo Biolabs; 1 mg/g of wet weight cells) at 37°C for one hour to remove their cell walls and were then homogenized with a blender to release mitochondria. Mitochondria were isolated and purified by several rounds of differential centrifugation (23), and CsCl-bisbenzimide gradients as described by Hudspeth et al. (24) after lysing the mitochondria with n-Lauroylsarcosine (Sigma). Total mitochondrial RNA was prepared by a series of phenol, phenol/chloroform and chloroform extractions of SDS lysates of isolated mitochondria (25).

**Northern hybridization**

RNA separated by electrophoresis on 1.5% agarose-urea gels was transferred in situ to DBM (diazobenzyloxymethyl) paper using the method of Alwine et al. (26). Hybridization probes were labeled by nick translation in the presence of [α-32P]dATP. The blotted DBM paper was hybridized with the labeled probe at 42°C in 50% formamide overnight and washed twice for 15 min at room temperature in 2× SSC and twice for 15 min at 42°C in 0.1× SSC (4).

**Determination of the extent of tRNA^Asp transcripts using SI nuclease**

The location of the ends of tRNA^Asp transcripts produced in heteroplasmic strain R7 or in petite strain R7p was determined using SI nuclease protection as described by Berk & Sharp (27) and modified by Weaver & Weissman (28). Double-stranded DNA fragments radiolabeled at the 5’ end with T4 polynucleotide kinase (BRL) and [γ-32P]-ATP (29) or at the 3’ end with Klenow fragment and [α-32P]dCTP were used as probes. MtRNA from R7 and R7p and the labeled probe were coprecipitated and resuspended in 30 μl of 0.4M NaCl, 40mM PIPES, pH 6.4, 1mM EDTA, 80% formamide. Nucleic acids were denatured by heating at 75°C for 15 min, then annealed at 50°C for 30 min. The temperature was then decreased in 5°C increments to room temperature over a period of more than three hours. Ten volumes (300 μl) of ice-cold SI buffer (0.28 M NaCl, 50 mM sodium acetate, pH 5.2, 4.2 mM ZnSO4) containing 50 or 100 units of SI nuclease (BRL) was added to the hybridization reaction, and the mixture was incubated at room temperature for an additional hour. The reaction was stopped by adding 50 μl of 4M ammonium acetate, 0.1M EDTA. After phenol/chloroform extraction, SI nuclease-protected fragments were precipitated with an equal volume of isopropanol. The fragments were resuspended in 80% formamide and applied to a 6% polyacrylamide-urea gel for analysis.

**DNA sequencing**

DNA sequence determination was accomplished by digesting plasmids carrying mtDNA with restriction enzymes which would produce staggered ends with extended 5’ ends. These ends were labeled using the large (Klenow) fragment of *E. coli* DNA polymerase I and the appropriate [α-32P]-dNTP. Labeled fragments were sequenced by the method of Maxam and Gilbert (29).

**RESULTS**

**Organization of the mtDNA in amplification revertants**

To determine the extent of the region of mtDNA amplified in the heteroplasmic revertants, total mtDNA of seven heteroplasmic revertants was analyzed by restriction enzyme mapping and Southern hybridization. All seven revertants have a common 1.1 kb fragment which extends from the Sau3A1 site of the tRNA^Asp gene to the Hinfl site of the tRNA^Asp2 gene (Fig. 1). The mtDNA of R15 has an additional Hinfl site indicating that the repeating unit of R15 retains tRNA^Asp. The mtDNA of R21 and R16 has an additional RsaI site indicating that their repeating unit retains tRNA^Asp. The only promoter consensus sequence

8598
in this region is located 133 bp upstream of the tRNA^{Ala} gene (9). Except for R7 and R15, all the repeating units retain the NsiI site located in the middle of the tRNA^{Ala} gene, indicating that they also retain the promoter sequence. We hypothesized (20) that positioning of the tRNA^{Asp} gene downstream of this strong promoter compensates in part for the mutation in the tRNA^{Asp} gene.

To determine the relative positions of the promoter consensus sequence and the tRNA^{Asp} gene, the boundaries of the repeating unit of five revertants (R7, R15, R16, R20 and R21) were sequenced to determine the exact site of recombination. The sequences at the recombination sites are shown in Fig. 2. In each case the recombination site was located within identical sequences located upstream and downstream of tRNA^{Asp}. These identical sequences were often flanked by regions of imperfect homology. In four of the strains the recombination site was located within AT-rich sequences and the sizes of the identical AT-rich sequences were 8—13 bp in length. In the fifth revertant, recombination occurred between GC-rich sequences within a GC cluster. Here recombination must have occurred within the sequence GGGG. Thus, all the repeating units of the amplification revertants retain the promoter consensus sequence (ATATAAGTA) normally located upstream of the tRNA^{Ala} gene.

Fig. 2. Sequences flanking the site of recombination in the amplified genome of five heteroplasmic strains. The middle line in each example is the DNA sequence across the site of recombination which produced the repeating unit of the amplified mtDNA in heteroplasmic strains R7, R15, R21, R20 and R16. Wild-type sequences contributing to the 5' end of the recombinant sequence are shown above the line; wild-type sequences contributing to the 3' end of the recombinant sequence are shown below the line. Small vertical lines indicate sequence homology between the wild type and recombinant sequences. Sequences which are identical in 3', 5', and recombinant sequences are enclosed in a box. The boxed sequences define the small direct repeat (4 to 13 bp) in which recombination occurred.
Polymorphisms in strain FF1210-6C relative to strain D273-10B

The sequence of the region of the mtDNA which generated the repeating units of the heteroplasmic strains of FF1210-6C is shown in Fig. 3. The sequence of the mtDNA in this region differs from the sequence of the analogous region of strains D273-10B and SM202 at two sites (9,30-32). First, FF1210-6C does not have the short GC cluster (#16) located from 6645 to 6695 on the map of de Zamaroczy & Bernardi (5), and second, it lacks three Ts in the T-rich region located about 30 bp upstream of tRNA^Ala in strain SM202 ([9] see Fig. 3., positions 7004 to 7017 [5]).

SI nuclease mapping of tRNA^asp precursors

To test whether the promoter normally located just upstream of the tRNA^Asp gene is used for transcription initiation on the amplified segment of mtDNA, mtRNAs from R7, a heteroplasmic strain, and R7p, the rho^- segregant of R7 containing only the amplified repeating unit mtDNA, were hybridized with a plasmid containing the 632 bp mtDNA segment extending from the Sau3Al site at the 5' end of the tRNA^Asp gene through the recombination site to the TaqI site near the 3' end of the tRNA^52 gene (Fig. 1). This plasmid was linearized at the Sau3Al site of the tRNA^Asp gene by BamHI digestion and labeled at the 5' end. After SI nuclease digestion, two distinct protection fragments were observed using RNA isolated from both strains (Fig. 4). The lower band, which is about 240 bp in length, corresponds to the predicted transcription initiation site at the promoter sequence. The second band, which is about 630 bp in length corresponds to the end of the homology between the probe and mtRNA, indicating that transcripts carrying the tRNA^Asp sequence exist which are longer than those initiated at the promoter. These transcripts may result from initiation at the promoter and progression of the polymerase completely across the repeating unit. Since this promoter is retained in the repeating unit of all the amplification revertants, we feel that it is likely that this is the site of transcription initiation on the repeating units of all the amplification revertants.

Transcription in the amplification revertants

RNAs produced from the repeating unit of the amplified DNAs are selectively enriched and high molecular weight transcripts with homology to tRNAs accumulate (20). These transcripts are useful for identifying the processing sites of mitochondrial tRNA precursors. To understand the processing of tRNA^Asp-containing transcripts produced from the repeating unit of revertant R7, RNA was isolated from R7 mitochondria, separated on agarose-urea gels, transferred in situ to DBM paper, and then hybridized with nick-translated plasmids carrying mtDNA.

When mtRNAs were hybridized with a probe carrying the tRNA^Asp gene and its 3' flanking sequences, three major transcripts (Bands A, B, and D in Fig. 5) were detected.

Fig. 3. Sequence of the mtDNA around the tRNA^Asp gene. The DNA sequence presented is the 4 Kb region of S. cerevisiae mtDNA from coordinates 4001 to 8000 (5). The sequence of this region in strain FF1210-6C (wild type, parental strain of FF1210-170 and its revertants) is identical with that of strain D273-10B or SM202, except for the absence of a GC cluster located between the tRNA^Asp and tRNA^Ala genes (#16, coordinates 6646 to 6695, shown in brackets) and the absence of three Ts (indicated by dots above the sequence) from a short T-rich sequence located 30 bp upstream of tRNA^Ala (coordinates 7004 to 7017, TTTTTATTTTTTT, in SM202 correspond to TTTTTATTTTTTT in FF1210-6C). tRNA genes are underlined and their cognate amino acids are shown to the right of the sequence. The C residue (position 5538) substituted by a T in the mutated tRNA^Asp gene is marked with an asterisk. The promoter sequence is boxed with an arrow to indicate the direction of transcription and is indicated by a P to the right of the sequence. The direct repeats which are the sites of the recombination which generated the heteroplasmic strains are boxed and labeled with the heteroplasmic revertant strain number.
Fig. 4. Localization of the 5'-end of tRNA<sub>Asp</sub> precursor transcripts in strain R7. A 5'-end labeled mtDNA fragment extending from the labeled Sau3A site of the tRNA<sub>Asp</sub> gene to the TaqI site in tRNA<sub>Ser</sub> and ending in vector DNA was hybridized with mitochondrial RNA from revertant R7 (lanes 3 and 4), or from petite strain R7p (lanes 1 and 2) or with no RNA (lanes 5 to 7). After S1 nuclease treatment (50 units, lanes 1, 3 and 5; 100 units, lanes 2, 4 and 6; no S1 nuclease lane 7), the protected DNA fragments were separated by 6% polyacrylamide-urea gel electrophoresis. The vertical line to the right of the autoradiograph shows the extent of the mtDNA homology in the probe relative to a portion of the R7 mtDNA (shown at the far right). The arrow indicates the mitochondrial promoter, the filled circle indicates the 5'-labeled Sau3AI site of the probe, boxes indicate the location of tRNA genes and the x indicates the site of recombination in strain R7. Two protected transcripts of about 240 and 630 nucleotides were detected. The 240 nucleotide fragment is the size expected for an RNA extending to the promoter sequence. A 630 nucleotide fragment is the size expected for an RNA extending through the promoter sequence to the 3' end of the mtDNA homology.

Band A corresponds to the mature tRNA<sub>Asp</sub> (4S, 75 nucleotides) found in the wild-type strain. This mature tRNA is the result of both 5' and 3' processing and the addition of the CCA terminus at the 3' end of the 72 nucleotide transcript. In addition to the mature tRNA<sub>Asp</sub> transcript, a prominent RNA about 600 nucleotides in length (Band D) is seen in the RNA isolated from R7. This RNA is consistent with an RNA initiated at the promoter 240 nucleotides upstream of the 5' end of the tRNA<sub>Asp</sub> gene and terminating about 300 nucleotides downstream of the 3' end of tRNA<sub>Asp</sub> (Fig. 6). This is consistent with the observation of Francisci et al. (14) that some RNA termini correspond to a location near the GC cluster downstream of tRNA<sub>Asp</sub>. Cleavage of the 600 nucleotide transcript at the 3' end of the tRNA would then be expected to yield two RNAs each about 300 nucleotides in length (one extending from the 3' end of the tRNA to the 3' terminus of the transcript; the other extending from the 5' end of the transcript to the 3' end of the tRNA) and this is consistent with the size of band B.
Fig. 5. Northern hybridization analysis of tRNA$^{A_{mp}}$ transcripts. Total mtRNAs from the heteroplasmic revertants, R7 and R15, and the petite strain, R7p, which is a mitotic segregant of R7, were separated on 1.5% polyacrylamide-urea gels, transferred to DBM paper and hybridized with the radiolabeled Sau3A1-Hpall fragment (coordinates 5470 to 5880, see Fig. 6). Four transcripts labeled A, B, C, and D are discussed in the text. The size of each RNA in nucleotides is shown on the right.

Cleavage of the 600 nucleotide transcript at the 5' end of the tRNA gene would be expected to produce two RNAs, one about 240 nucleotides in length and the other about 375 nucleotides (Fig. 6). While the 240 nucleotide fragment would not be detected with the probe used in this experiment, the 375 nucleotide fragment would be detected. The 375 nucleotide band is clearly seen in most of the heteroplasmic revertants (e.g. see band C in R15, Fig. 5) since this region (from the 5' end of the tRNA$^{A_{mp}}$ gene to the 3' terminus of the primary transcript) is present in all of the repeating units. However, in RNA isolated from R7 this transcript is reproducibly light or below the level of detection indicating that 5' processing in R7 must be slow relative to 3' processing.

No 75 nucleotide RNA (band A) is detected in the RNA isolated from the petite strain R7p (right lane of fig. 5). This is expected since the repeating unit lacks the tRNA synthesis locus required for 5' processing of tRNAs (33). However, only one major transcript is
Fig. 6. Location of the RNA transcripts detected in the heteroplasmic revertant R7. The heavy line represents the portion of the mtDNA carrying the trNA<sup>Asp</sup> gene. The open box labeled 'Asp' indicates the trNA<sup>Asp</sup> gene; 5' and 3' represent the portion of the gene coding for the 5' and 3' ends of the tRNA, respectively. X indicates the site of recombination in strain R7 as determined by sequence analysis. The arrow labeled with P indicates a nonanucleotide promoter sequence. E indicates the site on the mtDNA corresponding to the common end of the RNAs from the trNA<sup>Asp</sup> region. The extent of the Sau3AI-HpaII probe used to detect the RNAs is shown above the line representing the mtDNA. The lines below the mtDNA line show the predicted location of six RNAs five of which were detected by the Sau3AI-HpaII probe. Each RNA is labeled with its size in nucleotides.

detected in the RNA isolated from the petite strain R7p and this transcript has the same mobility as band D (600 nucleotides), consistent with the absence in the petite strain of both 5' and 3' processing.

S1 nuclease mapping of transcripts extending 3' of trNA<sup>Asp</sup>
To determine if an RNA terminus exists which corresponds to a site about 300 bp downstream of the 3' end of the trNA<sup>Asp</sup> gene, RNA isolated from mitochondria of R7 and R7p was hybridized with the 410 bp Sau3AI-HpaII fragment (coordinates 5470–5880, Fig. 3) radiolabeled on the 3' end at the Sau3AI site. These heteroduplexes were then digested with S1 nuclease. A number of protection fragments are seen in RNA isolated from R7 mitochondria (Fig. 7). The smallest fragment is about 70 nucleotides in length and corresponds to the 3' end of trNA<sup>Asp</sup>. This fragment is missing from the RNA isolated from R7p mitochondria indicating that 3' processing is absent in the petite strain which also lacks 5' tRNA processing. The next largest protection fragment is about 300 nucleotides in length and corresponds to a site (between positions 5830 and 5855) within the GC cluster downstream of trNA<sup>Asp</sup>.

DISCUSSION
Suppression of a mitochondrial mutation by the amplification of mtDNA has been reported by Mueller et al. (34), Dieckmann et al. (35) and Kang & Miller (20). Mutations in PET494 or CBP1 (nuclear genes necessary for mitochondrial biogenesis) resulted in the inability of the gene product to recognize target sequences on mitochondrial mRNA. In both cases, respiratory-competent revertants were obtained due to the formation of heteroplasmic cells, i.e. cells with mitochondria having two different mtDNAs, one being the wild type or rho<sup>+</sup> version and the other being an tandemly amplified segment of the wild type mtDNA, the rho<sup>-</sup> version. Suppression in these revertants resulted from the replacement of the
Fig. 7. S1 nuclease protection of the R7-3' probe by the tRNA^{Atp} precursor. The 3'-end labeled, double stranded probe extending from the labeled SalI site at position 5469 to the HpaII site at 5878 was used as a probe in S1 nuclease protection studies. The probe was incubated in the presence (lanes 2, 4 and 6) or absence (lanes 1, 3, and 5) of S1 nuclease after hybridization in the absence of RNA (lanes 1 and 2), in the presence of mitochondrial RNA isolated from heteroplasmic strain R7 (lanes 3 and 4) or in the presence of mitochondrial RNA isolated from petite strain, R7p (lanes 5 and 6). Labeled fragments produced from G-specific cleavages of the radiolabeled probe used in the S1 experiments are seen in lane 7. The coordinates of the G residue cleaved in the production of each fragment in lane 7 is indicated to the right. GC indicates the location of the bands corresponding to GC cluster #14 (Fig. 3). The vertical line to the right indicates the extent of mtDNA homology in the probe relative to the region surrounding the tRNA^{Atp} gene in the amplified portion of the mtDNA of heteroplasmic strain R7 (shown as a vertical line on the far right). The open and filled boxes indicate the location of the tRNA^{Atp} gene and GC cluster, respectively. The arrow indicates the location of the mitochondrial promoter. X indicates the site of recombination which generated the amplified DNA.

unrecognized target sequences with sequences from another mitochondrial gene which does not require the PET494 or CBP1 gene products. This replacement was the result of recombination of mtDNA during petite formation. Kang & Miller (20) have reported a
similar phenomenon for the suppression of a mutation in the mitochondrial tRNA^{Asp} gene. However, in this case it was not the replacement of faulty target sequences but the amplification of the mutant gene which gave suppression, indicating that the tRNA produced from the mutated gene retains a low level of activity.

The formation of the mtDNAs in petite strains occurs in two steps. First, homologous recombination occurs between two sites on the mtDNA and then the excised region is tandemly amplified (36). Recombination occurs between direct repeats located in the A+T-rich sequences or in GC clusters. (36,37). The amplification revertants used in this study were isolated after MnCl$_2$ mutagenesis of a mitochondrial tRNA^{Asp} mutant. We have sequenced the region around the site of recombination in some of these amplification revertants in order to determine the exact site of recombination and the exact length of the repeating unit. We find that the mtDNA from four of the strains sequenced, had recombined at homologous AT-rich sequences 8 to 13 base pairs in length. The mtDNA from a fifth strain had recombined within the sequence GGGG located in two different GC clusters. This is similar to the result observed by de Zamaroczy et al. (36) for spontaneous petite formation.

In addition to amplification of the mutant gene, a second factor which probably contributes to the overproduction of the tRNA is an increase in the transcription of the gene. We have shown that all of the amplified segments of mtDNA mapped and/or sequenced so far, retain the strong promoter sequence, normally located upstream of the tRNA^{Ala} gene (9), at a new site upstream of the tRNA^{Asp} gene so that the tRNA^{Asp} gene could be transcribed directly from the promoter (in strain R7) or along with tRNA^{Ala} (in strains R17, R18, and R20) or with tRNA^{Ala} and tRNA^{Ile} (in strain R21). In the heteroplasmic strain R7, transcripts extending to the site of initiation have been detected (Fig. 4) and we feel that it is likely that the proximity of a strong promoter (233 bp upstream as opposed to 3.8 kb in the wild type strain) contributes, along with the physical amplification of the gene, to the overproduction of tRNA^{Asp}.

Mitochondrial RNA processing was studied in a number of the heteroplasmic strains. The predominant processing sites were, as reported by others (8,9,14), at the 3' end and 5' end of tRNAs even though the spacer sequences between the two tRNA genes were rearranged by recombination. One possible exception was an RNA terminus corresponding to a site about 300 nucleotides downstream of tRNA^{Asp}. This terminus maps at or near a GC cluster. This site was originally identified by Francisci et al. (14) who proposed that it was a site of RNA processing.

While the raison d'être for the GC clusters is still not known, three different features are demonstrated by these studies. First, some of the GC clusters are optional and are present in some Saccharomyces cerevisiae strains and not others (6) and the GC cluster (#16) located downstream of tRNA^{Ser} and tRNA^{Arg} in Saccharomyces cerevisiae strains D273-10B and SM202 (9,30—32) is missing in FF1210-6C. Second, the similarity of the sequences of GC clusters can provide sites for the homologous recombination that is involved in the excision of the repeating units of the amplified DNA in heteroplasmic and petite strains. This is seen in strain R16 where recombination between similar sequences within two GC clusters (#12 and #18) produced the repeating unit. Finally, we have added to the evidence that some RNA transcripts terminate at GC clusters.

That GC clusters might be RNA processing sites was first proposed by Tzagoloff et al. (38). They proposed that secondary structure conserved within the GC cluster might serve as signals for processing. However, most RNA processing sites have been located...
Fig.8. A potential hairpin structure in GC cluster # 14. Panel A: The site corresponding to the terminus of the 3' extension on the RNAs containing tRNA\textsuperscript{A\textsubscript{sp}}, is located between the dots shown next to the potential hairpin structure in the G+C-rich sequences of GC cluster # 14. The underlined sequence is the processing site proposed by Francisci et al. (14). Panel B: Two other potential hairpin structures located in GC clusters which are proposed to be RNA processing sites in yeast mitochondria (11,41) are shown. The structure on the left is located upstream of the ol2 gene (coordinate 45,431); the structure on the right is located upstream of the tRNA\textsuperscript{Thr2} gene (coordinate 1,091).

in AT-rich sequences unassociated with GC clusters (11,12,14,39–41). Francisci et al. (14) proposed that the RNA processing site downstream of the tRNA\textsuperscript{A\textsubscript{sp}} gene maps to the sequence AATATAA just proximal to GC cluster # 14 (Fig. 3). However, S1 nuclease protection of our probe by RNA isolated from the mitochondria of heteroplasmic strain R7 and petite strain R7p locates the terminus of the 615 nucleotide transcript within GC cluster # 14, 30–50 nucleotides downstream of the AATATAA site (Fig. 7). GC cluster # 14, like many other GC clusters has the potential to form a hairpin structure (Fig. 8). The location of the terminus as determined by S1 mapping is located within the hairpin. This hairpin is similar to two other hairpin loops in GC clusters which have been proposed to be RNA processing sites (11,41). All three hairpins have A+T-rich loops with stems in which one strand has 10 consecutive purines and the other strand has 9–10 consecutive pyrimidines. Our data do not distinguish whether the RNA terminus located within the GC cluster is the result of RNA processing or transcriptional termination and the potential for secondary structure at the RNA terminus is reminiscent of bacterial terminators.

However, if these termini are sites of transcriptional termination, termination can not be completely efficient since no promoter sequences are normally found between the GC cluster and the tRNA\textsuperscript{Arg2} and tRNA\textsuperscript{Ser2} genes, and since transcripts produced from the R7 repeating unit appear to be efficiently extended through this GC cluster (see fig. 4). Whether this terminus is the result of RNA processing or transcriptional termination, this evidence argues that some GC clusters correspond to RNA termini. However, it is clear that not
all GC clusters in this region correspond to RNA termini (data not shown) and it is likely that only a subset of GC clusters have this feature.

An unexpected result of the S1 nuclease protection experiments is the observation that in petite strains processing at the 3' end of the tRNA is absent although this processing site is used in heteroplasmic strains. It is likely that 3' processing in heteroplasmic strains as evidenced by the 300 nucleotide fragment results from the additional capacity of these strains to process the tRNA at its 5' end. This requirement for 5' processing is surprising since transcripts from petite strains have been identified which consist of tRNAs which are unprocessed at the 5' end and yet are mature at the 3' end (8, 13). Chen and Martin (42) have observed that the 3' processing endonuclease has a preference for 5'-mature precursors in vitro using a mitochondrial tRNA\textsubscript{Glu} precursor. Ordered processing of tRNAs (5'-processing preceding 3' processing) is well documented in eukaryotes for nuclear tRNA genes (43–45), chloroplastic tRNA genes (46–47), and rat mitochondrial tRNA genes (48). It may be that tRNA processing is ordered for certain of the yeast mitochondrial tRNAs (e.g. tRNA\textsubscript{Glu}, tRNA\textsubscript{Asp}) but not others (e.g. tRNA\textsubscript{Met}). Alternatively, the requirement for 5' processing of tRNA\textsubscript{Asp} may be the result of the mutation in the tRNA\textsubscript{Asp} gene which eliminates Watson-Crick base pairing in the last two base pairs of the acceptor stem. This alteration of base pairing may result in an altered structure which affects 3' processing in such a way as to make it dependent on 5' processing. Consistent with this idea is the recent identification of another mutation in the tRNA\textsubscript{Asp} gene (at position 61 in the T pseudouridine stem) which essentially eliminates 3'-processing of tRNA\textsubscript{Asp} even in the presence of 5'-processing (49). Further studies using mitochondrial RNAs isolated from mutant, wild type, heteroplasmic and petite strains may reveal whether altered 3'-processing is a cause of respiratory deficiency in the mutant strain.

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