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# Expression of the clustered mitochondrial tRNA genes in *Saccharomyces cerevisiae*: transcription and processing of transcripts

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**The transcripts of a cluster of eight tRNA genes localized in the *Cap-oxiI* region of the mitochondrial genome of *Saccharomyces cerevisiae* were investigated by hybridization of gene-specific probes on Northern blots of mitochondrial RNA and by S1 mapping of the 5′ termini of the transcripts. Two *rho*⁻ mutants that lack mature tRNA species and accumulate precursors have been used to detect transcripts that are not detectable in wild-type (w.t.) mitochondria. The results have shown the existence of polygenic transcripts carrying at least 5–7 tRNA sequences, both in w.t. and in *rho*⁻ strains. The existence of several alternative processing pathways, which involve cleavage at the 3′ and 5′ ends of the tRNA sequences and in the long intergenic regions (possibly at GC clusters), is suggested. Cleavage at the 5′ ends of tRNA sequences is defective in the mutant strains. The transcripts of the genes for $tRNA^{Thr}_{ACN}$ and $tRNA^{Cys}$ (the tRNA genes immediately downstream from the 21S rRNA gene) have been analyzed; the possibility that these species represent primary transcripts is considered, and potential sites for initiation of transcription of the clustered tRNA genes are discussed.**
*Key words:* mitochondrial tRNA genes/transcription/processing of transcripts/*Saccharomyces cerevisiae*

## Introduction

In the mitochondrial genome of *Saccharomyces cerevisiae*, nearly all tRNA genes are localized in a region spanning one third of the 70-kbp genome, between the two rRNA genes. In particular, 16 tRNA genes are clustered in a 9-kbp region between the 21S rRNA and *oxiI* genes; no other genes are present in this region. This region has been almost completely sequenced (Berlani *et al.*, 1980a, 1980b; Bonitz and Tzagoloff, 1980), but little is known about its transcription. The organization of these tRNA genes is similar to that observed in the mitochondrial genomes of other lower fungi (Heckman *et al.*, 1979; Köchel *et al.*, 1981) and very different from that present in mammalian mitochondrial DNA (mtDNA), where tRNA genes are interposed between protein genes (Montoya *et al.*, 1981, 1982).

The transcription of the mitochondrial genome of *S. cerevisiae* has been investigated recently in several laboratories. A technique allowing the identification of primary transcripts by the labelling of polyphosphate-terminated molecules has allowed rapid progress in this field. Experiments have shown that the mitochondrial genome of *S. cerevisiae* is transcribed starting from several initiation sites (Levens *et al.*, 1981; Christianson *et al.*, 1983). A nonanucleotide sequence has been identified as the promoter of transcription for the two rRNA genes (Osinga and Tabak, 1982; Osinga *et al.*, 1982) and as the initiation site of transcription in *rho*⁻ strains that retain *ori* sequences (Baldacci and Bernardi, 1982). The same nonanucleotide sequence also serves as the starting point for transcription of other genes or groups of genes (Christianson *et al.*, 1983; Miller *et al.*, 1983). Transcription initiating at this sequence has also been observed *in vitro* using purified mitochondrial RNA polymerase (Edwards *et al.*, 1982).

Despite this extensive progress, many questions remain concerning the transcription of the clustered mitochondrial tRNA genes. In bacteria, clustered tRNA genes are transcribed as polygenic transcripts from external promoters, while nuclear encoded eukaryotic tRNA genes are transcribed as single gene transcripts from internal promoters (Sharp *et al.*, 1981; Galli *et al.*, 1981; Ciliberto *et al.*, 1982). Information concerning the expression of mitochondrial tRNA genes in *S. cerevisiae* has been provided by *rho*⁻ mutants, which lack large portions of the mitochondrial genome. No mature tRNA species are synthesized in *rho*⁻ mutants lacking a region adjacent to the 14S rRNA (Morimoto *et al.*, 1979). These mutants accumulate high mol. wt. transcripts that hybridize with tRNA gene probes (Martin and Underbrink-Lyon, 1981). The region that is essential for synthesis of mature tRNAs has been precisely mapped adjacent to the gene for $tRNA^{fMet}$ (Underbrink-Lyon *et al.*, 1983) and its RNA product has been identified (Miller and Martin, 1983). Some of the transcripts of the tRNA region present in *rho*⁻ strains that fail to synthesize mature tRNA have been identified and shown to contain the tRNA sequence and a 5′ extension up to the 3′ end of the preceding tRNA gene (Frontali *et al.*, 1982). Here were present the first clear evidence that, unlike other eukaryotic tRNA genes, the clustered mitochondrial tRNA genes are transcribed, both in wild-type (w.t.) and mutant cells of *S. cerevisiae*, as polygenic transcripts carrying several tRNA sequences. Several aspects of the process leading to the formation of mature tRNA species have also been elucidated and transcriptional initiation of the clustered tRNA genes is discussed.

## Results

### *Polycistronic transcripts of the tRNA region in w.t. mitochondria*

We used highly radioactive, 5′ end-labelled, single-stranded probes containing tRNA genes to analyze the transcripts of the first cluster of tRNA genes in the *Cap-oxiI* region. The organization of the studied region (~4000 bp) as well as the structure of the probes and of the mutants used are shown in Figure 1. All probes were labelled at *HinfI* or *TaqI* sites within the tRNA genes. Probes 2, 3 and 5, carrying the genes for $tRNA^{Cys}$, $tRNA^{His}$ and $tRNA^{Leu}$ + $tRNA^{Gln}$ respectively, were used for hybridization on mitochondrial RNA (mtRNA) from w.t. strain D273-10B.

Results reported in Figure 2 showed that, in addition to the strong signals corresponding to the mature tRNA species, a distribution of discrete higher mol. wt. bands was observed; their apparent size ranged between 340 and 3100 nucleotides.

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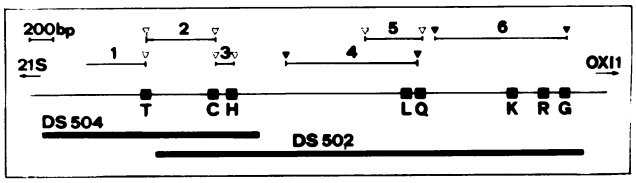


**Fig. 1.** Organization of the 4-kbp region of the mitochondrial genome under study and structure of the probes (1–6) obtained by restriction endonuclease digestion of DNA from strains DS502 and DS504. The region of the genome retained by these mutants (derived from strain D273-10B) is also noted. The one letter amino acid code (T = threonine; C = cysteine; L = leucine; Q = glutamine; K = lysine; R = arginine; G = glycine) is used to indicte the corresponding tRNA genes. △ *Taq*I; ▲ *Hinf*I site. The gene for $tRNA^{Gln}$ (Q) contains two *Hinf*I sites, one of which is very near the 5′ end of the gene.

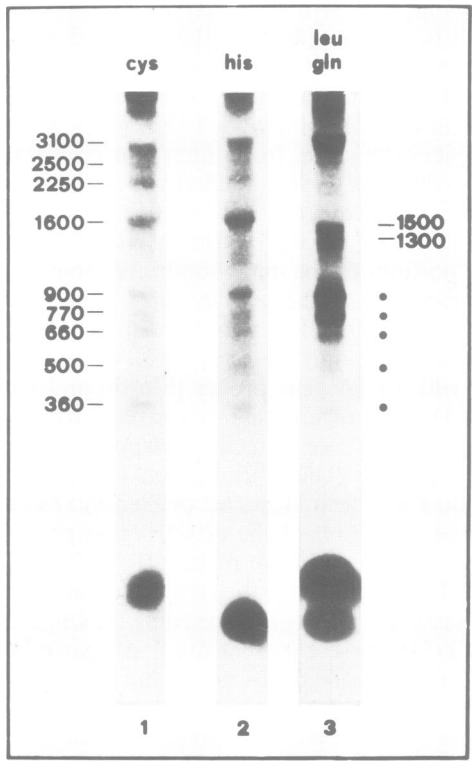


**Fig. 2.** Hybridization of tRNA gene-specific probes on Northern blots of mtRNA from the w.t. strain D273-10B. Hybridization was performed with probe 2 carrying the $tRNA^{Cys}$ gene **(slot 1)**, with probe 3 carrying the $tRNA^{His}$ gene **(slot 2)** and with probe 5 carrying the genes for $tRNA^{Leu}$ and $tRNA^{Gln}$ **(slot 3)**.

The hybridization patterns obtained with probes 2 and 3 were practically identical except for the slightly different position of the mature tRNAs, while the pattern obtained with probe 5 exhibited differences in the position of some bands. The higher mol. wt. bands hybridizing with the three probes had the same apparent size, but differences were observed in the range of 1000–2000 nucleotides. Surprisingly, five bands in the range of 300–900 nucleotides (indicated by dots in Figure 2) exhibited a very similar pattern of hybridization with the three probes; the accuracy of these measurements did not allow us to establish whether the sizes are identical or slightly different.

Since $tRNA^{Cys}$ and $tRNA^{Gln}$ genes are separated by >1500 bp, only the higher mol. wt. bands could contain all four tRNA sequences. The similarity of the band pattern in the lower mol. wt. range could suggest that a similar pattern of events might occur in the processing of polygenic transcripts in the regions surrounding the two doublets of tRNA sequences $tRNA^{Cys}$-$tRNA^{His}$ and $tRNA^{Leu}$-$tRNA^{Gln}$ that are separated by 68 and 33 nucleotides respectively. This possibility is considered further in the discussion.

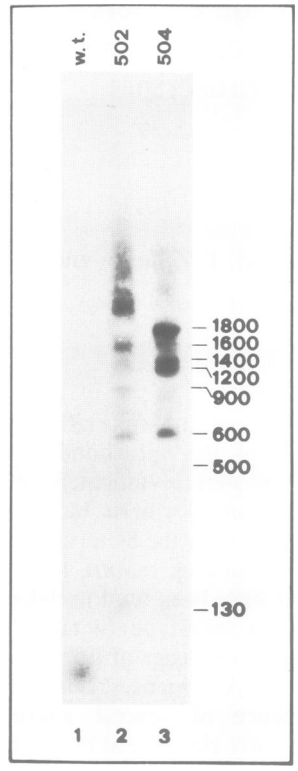


**Fig. 3.** Pattern of the transcripts of the $tRNA^{His}$ gene in w.t. and *rho*$^-$ strains. Probe 3, carrying the gene for $tRNA^{His}$ was hybridized on Northern blots of mtRNA from strain D273-10B **(slot 1)**, DS502 **(slot 2)** and DS504 **(slot 3)**.

*Use of rho*$^-$ *mutants for the analysis of processing intermediates*

As already mentioned, it is known from the work of several laboratories that *rho*$^-$ mutants lacking a mitochondrial locus localized near the gene for $tRNA^{fMet}$ do not synthesize mature mitochondrial tRNA and accumulate transcripts from tRNA genes which are probably precursors of the mature species. Since Christianson and Rabinowitz (1983) have shown that initiation of transcription is the same in w.t. and *rho*$^-$ strains, the *rho*$^-$ strains that accumulate precursors and processing intermediates constitute invaluable tools for the study of mitochondrial tRNA transcription and processing.

The hybridization pattern obtained when probe 3 (carrying the $tRNA^{His}$ gene) was hybridized on Northern blots of mtRNA from w.t. and different *rho*$^-$ strains is shown in Figure 3. A strong accumulation of high mol. wt. transcripts was observed in the mutant strains. A similar band pattern became detectable only after long exposure in Northern blots of w.t. mtRNA; in the exposure conditions of Figure 3, discrete bands could be detected only in the mutants. The lower mol. wt. bands were of the same size (600, 500 and 130 nucleotides) in the mutant strains; the different sizes of the higher mol. wt. bands reflected the different portions of the genome retained in the mutants.

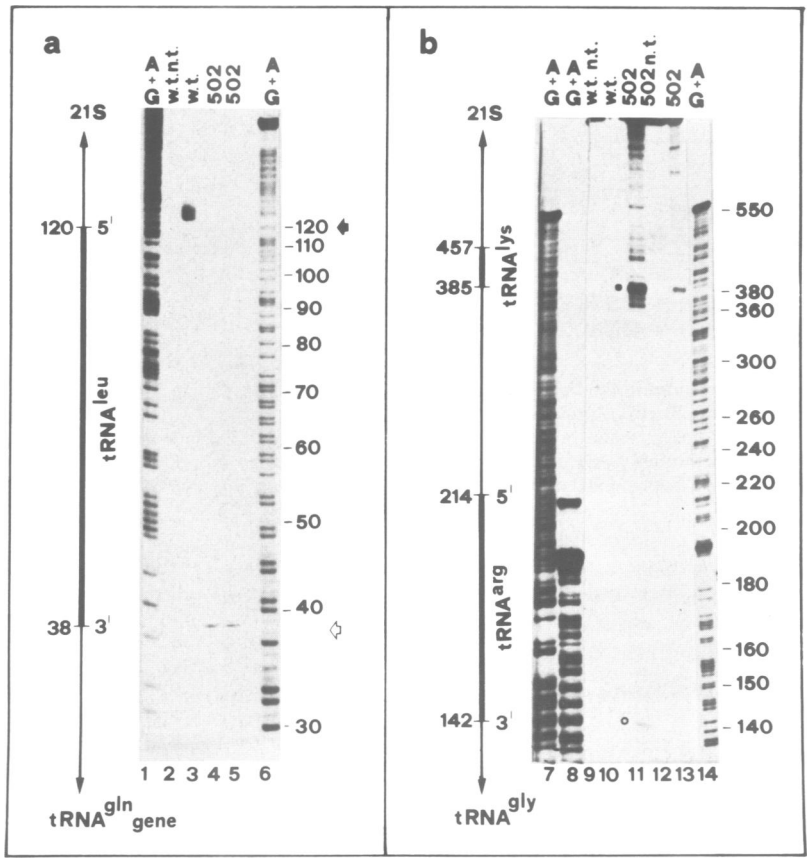


**Fig. 4.** Localization of 5′ ends of transcripts in the regions flanking tRNA genes. Probe 4 **(panel a)** and probe 6 **(panel b)** were hybridized with mtRNA from strains D273-10B an DS502. After S1 digestion, the protected fragments were run on 10% acrylamide 8 M urea sequencing gels using purine-specific reactions of DNA fragments of known sequence as reference ladders. Lanes were as follows: **(1)** probe 4 cleaved by a purine-specific reaction; **(2)** probe 4 hybridized to mtRNA from w.t. cells, untreated; **(3)** as **lane 2** but treated with S1 nuclease for 2 h; **(4)** probe 4 hybridized to mtRNA from strain DS502 and treated with S1 nuclease for 1 h; **(5)** as **lane 4** but treated with S1 nuclease for 2 h; **(6, 7, 8** and **14)** purine-specific reactions of fragments of known sequence; **(9)** probe 6 hybridized with mtRNA from w.t. cells untreated; **(10)** as **lane 9** but treated with S1 nuclease for 2 h; **(11)** probe 6 hybridized to mtRNA from strain DS502 treated with S1 nuclease for 2 h; **(12)** as **lane 11** but not treated with S1 nuclease; **(13)** as **lane 11** but lower amount of mtRNA. In both panels the distances in nucleotides from the *Hinf*I cleavage sites used to prepare the probes are reported and a schematic drawing shows the position of the genes for $tRNA^{Leu}$, $tRNA^{Arg}$ and $tRNA^{Lys}$. In **panel a**, the positions of protected fragments terminating at the 5′ ends of the $tRNA^{Leu}$ gene are indicated by heavy solid and open arrows, respectively. In **panel b**, protected fragments terminating at the 3′ ends of the $tRNA^{Lys}$ and $tRNA^{Arg}$ genes, are noted by solid and open circles, respectively. Samples that were not treated with S1 nuclease are indicated by 'n.t.'. No bands were observed when the probes were treated with S1 nuclease without prior hybridization.

The smallest transcript (130 nucleotides), which has not been detected in w.t. cells, has previously been shown to contain the sequence of $tRNA^{His}$ and the upstream intergenic sequence up to the 3′ end of the preceding $tRNA^{Cys}$ gene (Frontali *et al.*, 1982).

*Normal processing of 3′ but not 5′ ends of tRNA sequences in the mutants*

To detect those 5′ termini of transcripts that might map in the flanking regions of tRNA genes, we made use of probes 4 and 6, each carrying sequences corresponding to more than one tRNA gene. The results (Figure 4) showed that when probe 4 was annealed with mtRNA from strain DS502 and then digested with S1 nuclease, a fragment terminating at the 3′ end of the gene for $tRNA^{Leu}$ was detected. This fragment is indicated by the open arrow in Figure 4, lanes 4 and 5. When the same experiment was performed with mtRNA from w.t. cells, this signal was absent, but another one corresponding to the 5′ end of the gene for $tRNA^{Leu}$ was present (indicated by the solid arrow in Figure 4, lane 3). Since probe 4 was labelled at the *Hinf*I site within the $tRNA^{Gln}$ gene, this experiment demonstrated the existence in the mutant strains of a transcript carrying the $tRNA^{Gln}$ sequence plus a 5′ extension to the 3′ end of the preceding gene; it showed moreover that in w.t. cells processing events generated a precursor, carrying at least two RNA sequences, which had been properly processed at the 5′ terminus of the $tRNA^{Leu}$ sequence.

To investigate the transcripts of another group of tRNA genes ($tRNA^{Lys}$, $tRNA^{Arg}$ and $tRNA^{Gly}$, retained in the mt genome of strain DS502), S1 mapping experiments were performed with probe 6 (Figure 4b). After hybridization with mtRNA from w.t. cells, only the signal corresponding to mature $tRNA^{Gly}$ was detected (not shown). In contrast, hybridization of probe 6 with mtRNA from strain DS502 followed by S1 digestion, generated two protected fragments. The first (very faint) signal (indicated by an open circle in Figure 4, lane 11) corresponded to a transcript terminating at the 3′ end of the $tRNA^{Arg}$ gene, while the second (very strong one, indicated by a dot in Figure 4, slot 11 and 13) corresponded to a transcript terminating at the 3′ end of the $tRNA^{Lys}$ gene. A diagrammatic representation of the transcripts mapped in these and previous experiments (Frontali *et al.*, 1982) is shown in Figure 5. Results show that

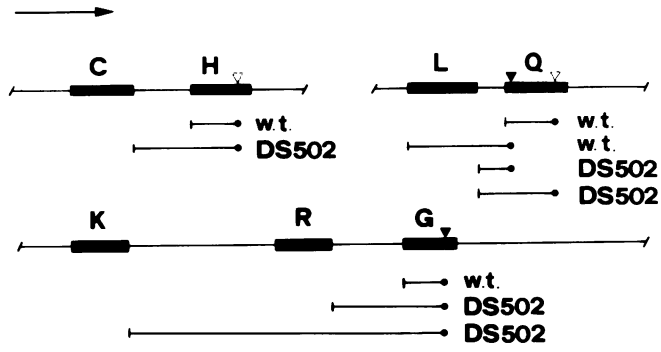


**Fig. 5.** Schematic drawing of the transcripts mapped in the flanking regions of tRNA genes. Segments (⊢—●) indicate the length of protected fragments labelled at the site indicated by the dot; △ indicates *TaqI* and ▲ *HinfI* sites. Vertical lines indicate termini identified by S1 mapping. Labelled termini are indicated by solid circles. The arrow indicates the direction of transcription.

mutants always contain transcripts carrying 5′ extensions to the 3′ ends of the preceding tRNA genes and suggest that cleavage of polygenic transcripts at 5′ ends of tRNA sequences is the defective processing step in the mutants.

*Potential role of GC clusters in processing of polygenic transcripts*

In addition to the lower mol. wt. transcripts already discussed, medium-sized (400–800 nucleotides) RNA species hybridizing with tRNA gene probes were observed in Northern blots (see Figure 2, 3 and 6a). The 5′ termini of these species can be mapped in S1 protection experiments by using long probes and performing long electrophoretic runs. When probe 4 was hybridized to mtRNA from w.t. or from DS502 cells and then treated with S1 nuclease, three signals were detected in both cell types (Figure 6b). The two shorter species (indicated by the arrows in Figure 6b, lanes 2, 3 and 4) were mapped; they represented protected fragments of 425 ± 20 and 450 ± 20 nucleotides. This could correspond to the 5′ and 3′ sides, respectively, of a GC cluster that is located between 432 and 462 bases from the *HinfI* site used to prepare the probe. The larger signal was not precisely mapped. In w.t. cells a signal was also observed at 120 nucleotides from the *HinfI* site. This signal, seen also in Figure 4a, has been discussed above.

*5′ termini of the transcripts of tRNA$^{Thr}_{ACN}$ and tRNA$^{Cys}$ genes*

The 5′ termini of transcripts of those tRNA genes localized immediately downstream from the 21S rRNA gene were mapped. Since transcription of the clustered tRNA genes might initiate within this region, the precise locations of the 5′ termini of transcripts terminating in this area are of particular interest. Results shown in Figure 7 were obtained by annealing probe 1 and 2 to mtRNA from strains D273-10B and DS504 prior to treatment with S1 nuclease. After hybridization with mtRNA from the w.t. strain D273-10B, only the signals corresponding to mature tRNAs were observed (not shown). In contrast, hybridization of probe 1 with mtRNA from strain DS504 (slot 1) yielded a very strong signal corresponding to a peculiar sequence (shown at the side). This sequence contains a nonanucleotide (GTATAAGTA, indicated by a bar) that is identical to the one known as the initiation site of mitochondrial transcription except for a G in the first position (the sequence has been checked and found to be the same as reported by Berlani *et al.*, 1980b). This sequence is flanked by a G cluster and by a pyrimidine cluster. A second,

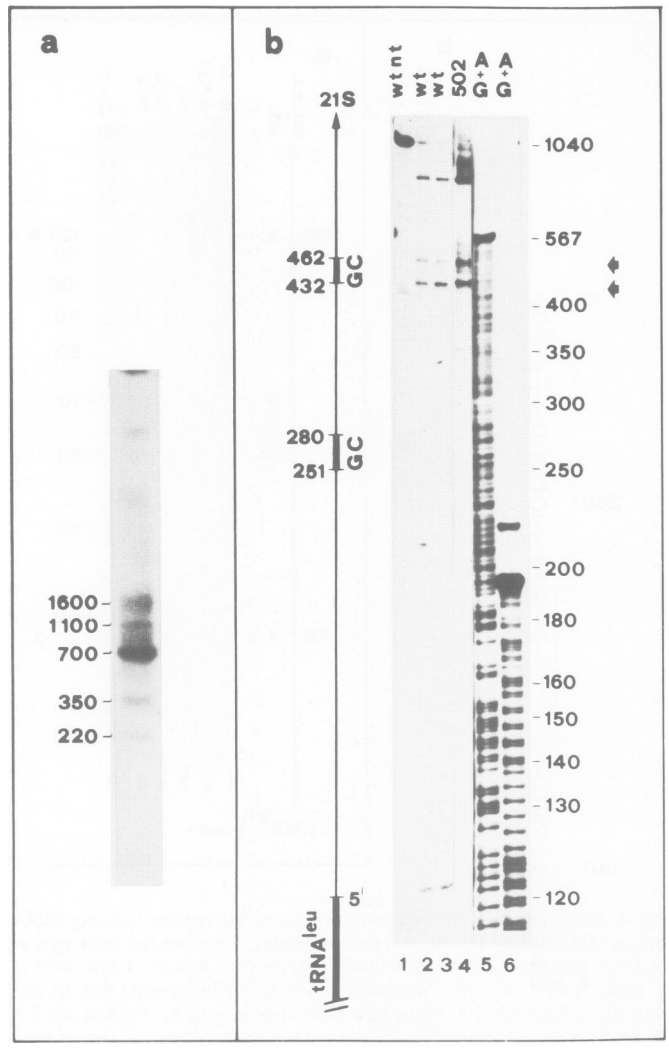


**Fig. 6.** Transcripts of the tRNA$^{Leu}$ gene and localization of 5′ termini of transcripts in the long intergenic regions. **(a)** Hybridization of probe 4 on Northern blots of mtRNA from strain DS502. **(b)** S1 mapping of 5′ ends of transcripts after hybridization of mtRNA to the same probe. Lanes were as follows: **(1)** probe 4 hybridized to mtRNA from w.t. cells untreated; **(2)** as **lane 1** but treated with S1 nuclease for 1 h; **(3)** as **lane 2** but treated with S1 nuclease for 2 h; **(4)** probe 4 hybridized to mtRNA from strain DS502 and treated with S1 nuclease for 2 h; **(5** and **6)** purine-specific reactions of known fragments used as reference ladders. The distances in nucleotides from the *HinfI* site (used to prepare the probe) are indicated. The positions of the tRNA$^{Leu}$ gene and two GC clusters are noted. The small arrow points in the direction of the neighboring 21S rRNA gene. Two heavy arrows indicate protected fragments whose endpoints lie at or near the confines of a GC cluster. Samples that were not treated with S1 nuclease are indicated by 'n.t.'. No bands were observed when the probe was treated with S1 nuclease without prior hybridization.

less intense, signal corresponded to the pyrimidine cluster.

When an equal amount of the same preparation of mtRNA from strain DS504 was annealed to probe 2, a group of faint bands was observed (Figure 7, lane 8) corresponding to the position of the nonanucleotide TTATAAGTA located immediately upstream from the tRNA$^{Cys}$ gene.

The localization of the above-mentioned signals at positions exactly corresponding to promoter-like sequences suggests that either or both of these nonanucleotides might serve as initiation site(s) for the transcription of the first cluster of tRNA genes. However, evidence for co-transcription of tRNA$^{Thr}_{ACN}$ with the 21S rRNA has been obtained by Locker and Rabinowitz (1981) in the *rho*$^-$ strain F11, which retains a

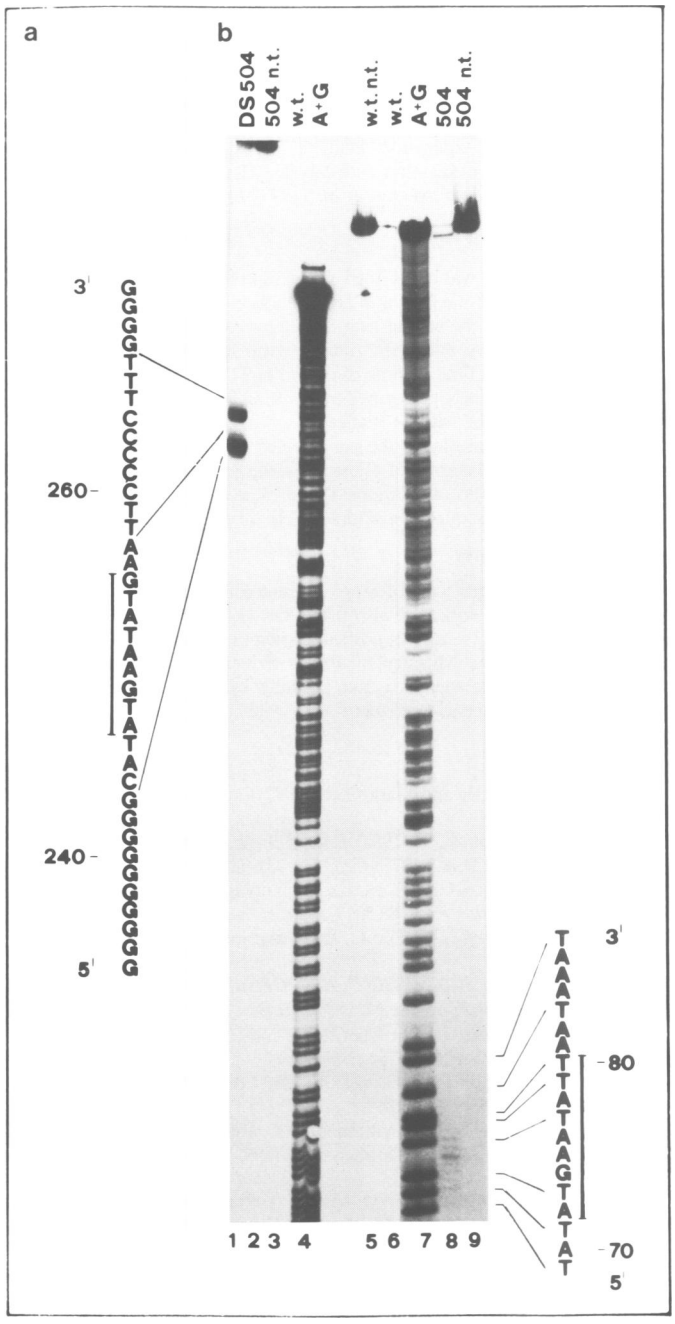


**Fig. 7.** S1 mapping of 5′ ends of transcripts of $tRNA^{Thr}_{ACN}$ **(lanes 1–3)** and $tRNA^{Cys}$ **(lanes 5–9)** genes. Lanes were as follows: **(1)** probe 1 hybridized to mtRNA from strain DS504 and treated with S1 nuclease for 2 h; **(2)** as **lane 1** but not treated with S1; **(3)** probe 1 hybridized with mtRNA from w.t. cells and treated with S1 nuclease for 2 h; **(4)** purine-specific reaction of a known fragment used as reference ladder; **(5)** probe 2 hybridized to mtRNA from w.t. cells untreated; **(6)** as **lane 5** but treated with S1 nuclease for 2 h; **(7)** probe 2 cleaved with a purine-specific reaction; **(8)** probe 2 hybridized with mtRNA from strain DS504 and treated with S1 nuclease for 2 h; **(9)** as **lane 8** but not treated with S1. The same amount of the same mtRNA preparation was used for the experiments whose results are shown in **lanes 1, 2, 8** and **9**. Samples that were not treated with S1 nuclease are indicated by 'n.t.'. No bands were observed when the probes were treated with S1 nuclease without prior hybridization. The reported sequences are those of the sense (non-transcribed) strands. The distances in nucleotides from the *TaqI* sites inside the tRNA genes at which labelling occurred are also reported. The extent of the nonanucleotides (as discussed in the text) are indicated by bars.

12-kbp region of the mitochondrial genome containing the 21S rRNA and five tRNA genes.

Possible explanations of these results include the following. (i) The 5′ termini we have mapped might have arisen from nucleolytic events occurring during the cleavage of longer transcripts. (ii) The nonanucleotide TTATAAGTA located upstream from the $tRNA^{Cys}$ gene (which has the recognized consensus sequence) might act as an initiation site for the transcription of the clustered tRNA genes, while $tRNA^{Thr}_{ACN}$ is co-transcribed with the 21S rRNA. In this case one could suggest that the nonanucleotide containing a G in the first position might act as a cleavage site of a long precursor. (iii) The nonanucleotide GTATAAGTA might be a secondary initiation site that comes into play in the *rho*$^-$ strain DS504, which lacks the 21S rRNA gene.

## Discussion

The transcription of a region spanning ~4 kbp and containing eight tRNA genes, has been examined. The results have provided the first clear demonstration of the presence of polygenic transcripts carrying several tRNA sequences in the w.t. strain D273-10B. The presence of these transcripts in w.t. strains and not only in *rho*$^-$ mutants indicates that the transcription of the clustered tRNA genes into a polygenic transcript is a normal physiological event in yeast mitochondria. The transcription of tRNA genes in mitochondria of *S. cerevisiae* should therefore utilize a transcriptional initiation site upstream from the tRNA gene cluster. The use of the same promoter for many tRNA genes is in contrast to the internal promoters characteristic of the eukaryotic nuclear tRNA genes. Also, a processing pathway involving precise endonucleolytic cleavages seems to be required for the synthesis of mature tRNA species. The length of the higher mol. wt. transcripts (~3000 nucleotides) detected in mtRNA from w.t. cells could accommodate the sequences of 5–7 tRNAs coded for by the group of tRNA genes immediately downstream from the 21S rRNA gene. The high number of processing intermediates suggests that several alternative processing pathways may function contemporarily.

The analysis of mitochondrial transcripts accumulated by the *rho*$^-$ mutants lacking mature tRNA allows some further insights into the complex process of transcription and processing of transcripts. Results obtained both from hybridization on Northern blots and from S1 mapping of the transcripts in w.t. and mutant strains indicate a process that can be summarized as follows. The transcription of the cluster of tRNA genes initiates at a sequence (or sequences) localized outside the genes. The resulting multigenic transcripts are then processed by a series of precise endonucleolytic cleavages. These cleavages occur at sites including the 5′ and 3′ termini of the tRNA sequences and some GC clusters localized in the intergenic regions. Cleavages in the long intergenic regions seem to generate smaller transcripts carrying the more closely linked tRNAs.

The nucleolytic activity involved in the processing at the 3′ ends of the tRNA sequences is present both in w.t. and *rho*$^-$ strains and should therefore be coded for by the nucleus. By contrast, the accumulation in the mutants of precursors carrying 5′ extensions shows that cleavage of multigenic precursors at 5′ ends of tRNA sequences is the defective processing step in the mutants and suggests that this endonucleolytic ac-

tivity requires the mitochondrial transcription product that has been characterized by Miller and Martin (1983). A similar requirement for an RNA moiety is exhibited by RNase P from bacteria (Stark *et al.*, 1979; Kole *et al.*, 1980).

The activity cleaving the long intergenic regions at the GC clusters appears to be present in w.t. and *rho*$^-$ strains and is therefore presumably coded for by the nucleus. GC clusters, containing several *Hpa*II/*Hae*III sites (Prunell *et al.*, 1977) are scattered in various regions of the mitochondrial genome. They often have inverted homologous sequences capable of fairly extensive base pairing. A function of these clusters in the processing of long transcripts has been suggested by Tzagoloff *et al.* (1980). However, not all GC clusters present in the studied region seem to be involved in processing of tRNA precursors, since no transcripts terminating at the GC cluster localized 250–300 nucleotides upstream from the $tRNA^{Leu}$ gene were detected. As is the case for the processing pathways of other mitochondrial transcripts, no formal proof for a precursor-product relationship has been obtained. However, the available information on the high and low mol. wt. transcripts present in w.t. and mutant mitochondria constitutes compelling evidence of such a relationship.

The mapping of the 5′ termini of the transcripts of the $tRNA^{Thr}_{ACN}$ and $tRNA^{Cys}$ genes at positions corresponding to the nonanucleotide sequences GTATAAGTA and TTATAAGTA located upstream from the $tRNA^{Thr}_{ACN}$ and $tRNA^{Cys}$ genes, respectively, suggests that these sequences may function as initiation site(s) of transcription at least in the *rho*$^-$ strain DS504, which lacks the 21S rRNA gene. The absence in w.t. cells of transcripts terminating at the same sequences might be due to rapid processing or to preferential use in w.t. cells of the promoter localized upstream from the 21S rRNA gene. This could indicate a flexibility in the use of mitochondrial promoters by the mitochondrial RNA polymerase in different mitochondrial contexts. Another interesting point concerns the presence of a limited amount of transcripts whose 5′ ends correspond to the nonanucleotide localized upstream from the $tRNA^{Cys}$ gene. Again, these transcripts could indicate that initiation occurs at various sites with different efficiency. This, in turn, could reflect significant differences in the primary (or possibly secondary) structures of the flanking sequences. Alternatively, these sequences may be involved in processing events rather than in the initiation of transcription. The hypothesis that the nonanucleotide sequences might also serve, under some conditions, as cleavage signals is interesting from an evolutionary point of view and probably deserves further investigation.

## Materials and methods

*Strains and growth conditions*

The *rho*$^+$ strain D273-10B and its *rho*$^-$ derivatives DS502 and DS504 (which were kindly provided by A.Tzagoloff) were used. The deletion structure of these strains is shown in Figure 1. All strains were grown until the end of the exponential phase on YEP medium (1% yeast extract, 1% peptone) containing 2% galactose.

*Preparation and labelling of nucleic acids*

Mitochondrial DNA and RNA were prepared from purified mitochondria as previously reported (Frontali *et al.*, 1982).

For the preparation of 5′ end-labelled single-stranded probes, mtDNA from strains DS502 or DS504 was treated with *Hinf*I (Biolab) or *Taq*I (Biolab) restriction endonuclease, respectively, followed by treatment with calf intestine phosphatase (Boehringer, 2 U/μg of DNA). Labelling was performed with T4 polynucleotide kinase (NEN, 1 U/μg of DNA) in the presence of [$^{32}$P]ATP (NEN, 10–20 μCi/μg of DNA). Strand separation was performed as described by Maxam and Gilbert (1980), and the strands were identified by sequencing and comparison with the published sequences.

*Fractionation of RNA, transfer and hybridization*

Electrophoresis of mtRNA was performed in 1.5% agarose 6 M urea gels at 4°C in Tris-phosphate buffer as described by Locker (1979). EtBr stained gels were photographed under u.v. light. Transfer to diazobenzyloxymethyl (DBM) paper and hybridization with labelled DNA probes were performed following the method of Alwine *et al.* (1977) as modified by Wahl *et al.* (1979).

*S1 nuclease mapping*

S1 nuclease mapping was performed following the Weaver and Weissmann (1979) modification of the Berk and Sharp method (1977). About 10 ng of the labelled fragments of the coding strand were hybridized for 2 h at 45°C with 20–50 μg of mtRNA and then treated with 3000 units of S1 nuclease (Seikaguki Kojo) in a final volume of 0.1 ml at 37°C for the times indicated.

Controls were routinely performed by omitting S1 digestion and by treating the unhybridized probe with S1 nuclease.

A portion of each labelled probe was retained for a purine-specific DNA sequencing reaction (Maxam and Gilbert, 1980), S1-resistant fragments were fractionated on 10% polyacrylamide 8 M urea sequencing gels alongside the corresponding DNA sequencing sample. Gels were run at ~60°C.

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