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Initiation of transcription of a mitochondrial tRNA gene cluster in *S. cerevisiae*

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ABSTRACT

In Saccharomyces cerevisiae most mitochondrial tRNA genes are clustered in a 9 kbp region between the cap and oxi1 genes. Polygenic transcripts of this region have been previously identified. A transcriptional initiation site at a TTATAAGTA box, located upstream from the $tRNA^{cys}$ gene, has now been detected by S1 mapping experiments and by the capping of primary transcripts. Results are consistent with the hypothesis that this box represents the initiation site for transcription of a cluster of tRNA genes, while the adjacent $tRNA_2^{thr}$ is cotranscribed with the 21S rRNA. Results obtained with various strains are compared, and the efficiency of this sequence as a transcriptional initiation site in different mitochondrial contexts is discussed.

INTRODUCTION

The yeast mitochondrial (mt) genome codes for two ribosomal RNAs and about twenty-five tRNAs, which represent the RNA complement required for autonomous translation in this organelle. Most tRNA genes are located between the two rRNA genes (16 of them are clustered in the cap-oxi1 region), while the genes for $tRNA^{ser}$, $tRNA^{glu}$ and $tRNA^{trp}$ are scattered in different regions of the genome (1-9). The large (21S) and the small (14S) rRNAs are independently transcribed; the processing pathways leading to the mature products have been described in detail (10-14).

Recent reports have shown the involvement of a particular oligonucleotide sequence in the initiation of transcription of many mitochondrial genes (15-17); the same sequence has also been reported to occur in ori sequences (18) and to play a role in priming the replication of mitochondrial DNA (19). Initiation of transcription has been studied in w.t. strains as well as in rho$^-$ mutants, and has been found (in the cases so far examined) to be the same in w.t. and mutant strains (17).

Several tRNA genes are cotranscribed with protein genes (20-22), while separate promoters have been identified for the transcription of the

$tRNA_1^{thr}$, $tRNA^{fmet}$ and $tRNA^{phe}$ (17,23,24) genes. However, questions still remain concerning the transcription of the cluster of 16 tRNA genes located in the 10 kbp region encompassed by the 21S rRNA and oxi1 genes. The tRNA genes from this region are transcribed as multigenic transcripts (25), but the transcriptional initiation site (or sites) has not been identified. A computer inspection (17) has revealed only two nonanucleotide sequences in this region, which has been almost completely sequenced (4-6); they are localized upstream from the $tRNA_2^{thr}$ and the $tRNA^{cys}$ genes respectively. However, no evidence for primary transcripts initiating at these two potential promoters has yet been reported; moreover, results obtained with the rho⁻ strain F11 have shown that transcripts from the 21S rRNA gene also contain the $tRNA_2^{thr}$ and possibly the $tRNA^{his}$ and $tRNA^{cys}$ sequences (13).

We have investigated the initiation of transcription for the tRNA genes located downstream from the 21S RNA gene by using rho⁻ mutants that are defective in tRNA maturation (26); such mutants might be expected to accumulate primary transcripts. The data reported here show that one of the nonanucleotides present in this region can function as an initiation site for the transcription of tRNA genes.

MATERIALS AND METHODS

Strains and growth conditions

The wild type strain D273-10B and its rho⁻ derivatives DS502 and DS504 (kindly provided by Dr. Alex Tzagoloff) were used. The deletion structures of the rho⁻ strains are shown in Fig.1.

Strains were routinely grown until the end of exponential phase on YEP medium (1% yeast extract, 1% peptone) containing 2% galactose. When noted, cells were collected at the mid-exponential phase of growth on the same medium.

Preparation and labelling of mitochondrial nucleic acids

Mitochondria were prepared using the method of Faye et al. (27). For the preparation of mtRNA, protoplasts were lysed in the presence of the ribonuclease inhibitor vanadium adenosine (28). Purified mitochondria were then lysed in 50 mM Tris containing 1% sodium dodecyl sulphate (SDS) and extracted twice with water-saturated phenol. After dialysis of the supernatant, the RNA was precipitated five times with 3 volumes of ethanol in the presence of 0.3M sodium acetate (procedure a). In some cases, mitochondria were lysed in the absence of SDS and dialysis was omitted (procedure b).

Mitochondrial DNA was prepared from purified mitochondria from strain DS504 as previously reported (29) and treated with TaqI restriction endonuclease. The TaqI fragments were electrophoresed on 1% agarose gels, photographed under ultraviolet light after staining with EtBr, and then blotted on diazobenzyloxymethyl (DBM) paper following the procedure of Wahl et al. (30).

For the preparation of 5'end-labelled, single-stranded probes, the 5' termini of the TaqI fragments were dephosphorylated with calf intestine phosphatase (Boehringer, 2U/μg of DNA) and treated with T4 polynucleotide kinase (NEN, 1U/μg DNA) in the presence of γ-^{32}P ATP (NEN, 10-20 μCi/μg of DNA). Strand separation was performed as described by Maxam and Gilbert (31), and the bands were identified by sequencing and comparing with the published sequences.

S1 nuclease mapping

S1 nuclease mapping was performed following the Weaver and Weissmann (32) modification of the Berk and Sharp method (33). About 10 nanograms of the labelled fragments of the coding strand were hybridized for 2hr 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 indicated times. Controls including the omission of S1 digestion and the treatment of the unhybridized probe with S1 nuclease were routinely performed.

A portion of each labelled probe was retained for a purine-specific DNA sequencing reaction (31). S1 resistant fragments were fractionated on 10% polyacrylamide 8M urea sequencing gels next to the corresponding DNA sequencing sample. Gels were run at about 60°C.

In vitro capping of RNA and hybridization to Southern blots of mtDNA

About 10 μg of mtRNA were treated with 5mM methyl mercury hydroxyde for 10 min at room temperature. The volume was then adjusted to 20 μl with 50 mM Tris-HCl (pH 7.9), 1 mM $MgCl_2$, 1 mM dithiothreitol and 20-40 μM α-^{32}P GTP (410 Ci/mmole) (final concentrations).

Reactions were initiated by the addition of 8-12 units of guanylyltransferase (BRL Bethesda, Md.), incubated for 15' at 37°C and stopped by adding an equal volume of a 1%SDS, 10 mM EDTA (pH 7.5), 50 ug of E.coli tRNA and 10 mM Tris-HCl (pH 7.5) solution. After extraction with an equal volume of water-saturated phenol, the supernatant was loaded on a 5 ml Sephadex G-50 column equilibrated with 10 mM Tris-HCl (pH 7.5), 2 mM EDTA (pH 7.5) and 0.2% SDS. 250μl fractions were eluted with the same buffer, and radioactivity was measured in a LKB scintillation counter. The peak

fractions were adjusted to 1M ammonium acetate and precipitated with 2.5 volumes of ethanol. Labelled RNA pellets were redissolved in H_2O and stored at -80°C.

The RNA capped *in vitro* was hybridized to Southern blots of digested mtDNA in 8xSSC, 0.1% SDS and 100 µg/ml *E.coli* tRNA for 12-16 hr at 65°C; the DBM strips were then washed twice with 200 ml of 2xSSC containing 0.1% SDS for 15' at 42°C. Prior to hybridization, RNA was treated with RNAse-free DNAseI (Miles, Elkhart, Ind.)at 37° for 15' in 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$; the mixture was then heated at 90°C for 5' in order to inactivate the DNAse and to fragment the RNA molecules.

RESULTS

To investigate the transcripts of the first cluster of tRNA genes shown in Fig.1, we used mutants that are known to be defective in tRNA maturation; these mutants might be expected to accumulate primary transcripts as well as other tRNA precursors. We had previously investigated, in S1 mapping experiments, the transcripts of the $tRNA_2^{thr}$ and $tRNA^{cys}$ genes (25); the 5' ends of these transcripts were localized at or near sites corresponding to the two sequences indicated as α and β in Fig.1. However, the 5' ends of the transcripts mapped in these experiments could have arisen either from initiation of transcription or from processing of longer transcripts. To resolve this question, we used guanylyl-transferase plus α -^{32}P GTP to label di-

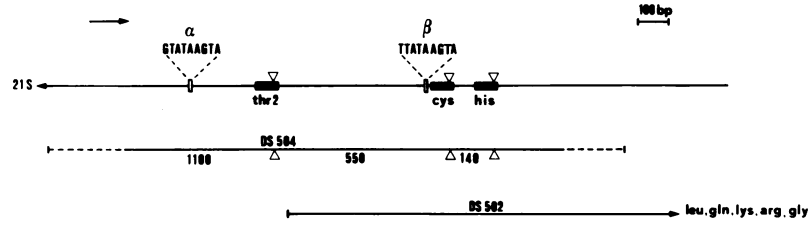

Figure 1 - The structure of the region of the genome containing the $tRNA_2^{thr}$ and $tRNA^{cys}$ genes is shown. The positions of the nonanucleotides GTATAAGTA (α) and TTATAAGTA (β) are noted, as are the regions retained in the mutants DS502 and DS504. In strain DS504 the structure of the 1100 bp junction fragment has not been precisely determined (as indicated by the dashed lines). The positions of the tRNA genes are indicated by the standard three letter code of the corresponding aminoacid. mtDNA from strain DS502 retains in addition to the region noted in the figure, the genes for an additional five tRNAs (also indicated by the corresponding symbols).
The arrow indicates the direction of transcription.
△TaqI restriction site

or triphosphate-terminated transcripts. Labelled RNAs were then hybridized to blots of TaqI-digested mtDNA from strain DS504.

Fig.2 shows the hybridization of capped mtRNA from strain DS504 with restriction fragments of mtDNA from the same strain. Hybridization was observed with the 550 bp Taq fragment that carries sequence β, but not with the 1100 bp fragment that contains sequence α. No hybridization was detected when the same experiment was performed with mtRNA from w.t. or DS502 cells (not shown).

The results of a high resolution S1 mapping experiment performed with the coding strand of the same 550 bp fragment are shown in Fig.3. Signals corresponding to the 5' end of the mature $tRNA^{cys}$ were observed when the probe was hybridized with mtRNA from w.t. cells prior to S1 treatment (lanes 2,3). These signals were not detected when the experiment was performed with mtRNA from strain DS504, which is defective in tRNA maturation; instead, a

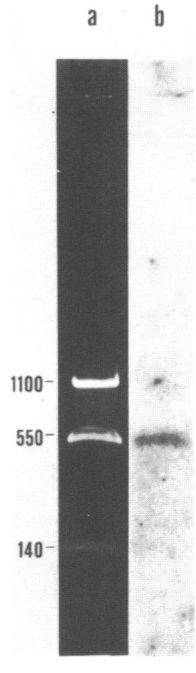

Figure 2 - Hybridization of capped transcripts from strain DS504 to DNA from the same strain (blotted on DBM paper). Lane a: the EtBr staining of TaqI digested DNA from strain DS504, after electrophoresis on a 1% agarose gel. Lane b: autoradiogram obtained after hybridization of capped RNA from strain DS504 to a Southern blot of the DNA fragments shown in lane a. The sizes of the fragments in base pairs are indicated.

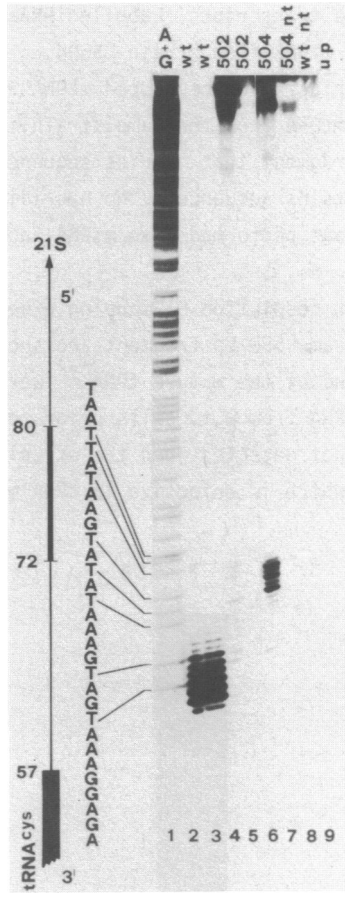

Figure 3 - Localization of the 5' ends of transcripts in the region upstream from the $tRNA^{cys}$ gene.
The 5' end-labelled coding strand of the 550 bp TaqI fragment was hybridized with mtRNA from strains D273-10B, DS502 and DS504. After S1 digestion, the protected fragments were electrophoresed on 10% acrylamide 8M urea sequencing gels; purine-specific reactions of the probe were used as reference ladders. Lanes were as follows.1) Purine-specific reaction of the probe. 2) Probe hybridized with 20µg mtRNA from strain D273-10B and treated with S1 nuclease for 2hr; 3) as in lane 2, but hybridization with 40 µg mtRNA from strain D273-10B; 4) Probe hybridized with 40 µg mtRNA from strain DS502 and treated with S1 nuclease for 2hr; 5) As in lane 4 but hybridization with 20 µg mtRNA from strain DS502; 6) Probe hybridized with 40 µg mtRNA from strain DS504 and treated with S1 nuclease for 2hr; 7) As in lane 6, but untreated; 8) As in lane 2, but untreated; 9) S1 treatment performed on unhybridized probe.
A schematic drawing shows the position of the $tRNA^{cys}$ gene and of nonanucleotide β (indicated by a bar). The sequence of the complementary noncoding strand is also reported. Numbers give the distance in nucleotides from the TaqI site at which the probe was labelled. Samples that were not treated with S1 nuclease are indicated by "n.t." and the unhybridized probe by "u.p.".

group of signals corresponding to the position of nonanucleotide β was present (lane 6). Since these signals were the only ones detectable when the probe was hybridized with mtRNA from strain DS504, we conclude that in this strain transcription initiates at nonanucleotide β and that a primary (cappable) transcript slightly longer than mature tRNA accumulates due to the lack of 5' end processing (25,29).

Surprisingly, no S1 resistant fragments were detected when the experiment was performed (lanes 4,5) with mtRNA from strain DS502 (see also ref.29). This rho⁻ strain retains part of the tRNA cluster under study, including 7 tRNA genes (from $tRNA^{cys}$ to $tRNA^{gly}$) but not $tRNA_2^{thr}$ (see Fig.1), and had previously been shown to accumulate discrete mitochondrial transcripts in amounts similar to those present in strain DS504 (25).

In w.t. cells, the absence of cappable transcripts hybridizing with the DNA fragment carrying nonanucleotide β can probably to be ascribed to the rapidity of processing and the instability of the precursor. Actually, faint signals corresponding to the position of nonanucleotide β were observed when mtRNA was prepared, using a very delicate procedure (procedure b, see Materials and Methods), immediately after the preparation of mitochondria from exponentially growing w.t. cells.

DISCUSSION

We had previously demonstrated that a group of mitochondrial tRNA genes localized in the cap-oxiI region are transcribed as polygenic precursors, which are then processed to yield the mature tRNA species (25). The results described here demonstrate that the sequence TTATAAGTA, located upstream from the $tRNA^{cys}$ gene (sequence β in Fig.1), can be used an initiation site for mitochondrial transcription.

On the contrary, no evidence for the localization of 5' ends of primary transcripts at sequence α has been obtained. This sequence might therefore be used as a site of processing, as observed by Zassenhaus and Butow (22) for a similar nonanucleotide localized in the var region. Alternatively, the S1 signal observed at a position corresponding to nonanucleotide α (25) might have been caused by the formation of secondary structures in a region that contains alternating G and C strectches.

The results are therefore consistent with the hypothesis that $tRNA_2^{thr}$ is cotranscribed with the 21S rRNA gene (as proposed by Locker and Rabinowitz ref.13), while sequence β is the initiation site for transcription of the downstream cluster of tRNAgenes. An identical sequence is used

as initiation site of transcription of the $tRNA^{glu}$, $tRNA^{fmet}$, $tRNA^{phe}$ and $tRNA_1^{thr}$ genes (17,23,24). At present, clear evidence for the involvement of sequence β in the initiation of transcription has been obtained only in strain DS504. The faint S1 signals obtained with mtRNA preparations from exponentially-grown w.t. cells might indicate that the same initiation site is used in w.t. cells, and that the difficulty in demonstrating primary transcripts initiating at sequence α might be due to rapid processing in the w.t. strain. However, we cannot completely eliminate the possibility that in w.t. cells the first cluster of tRNA is cotranscribed with the 21S rRNA, and that the nonanucleotide acts as an initiation site of transcription when the 21S rRNA promoter is lacking, as in strain DS504.

The results obtained with strain DS502 are surprising: like DS504 this strain also lacks the strong 21S rRNA promotor and contains a high level of discrete mitochondrial transcripts, but transcripts initiating at nonanucleotide β were not detected. The localization of the 5' ends of DS502 transcripts (obtained by S1 mapping, ref. 25,29) corresponds to processing sites. One might suggest that transcription in this strain initiates at another strong promotor that is localized downstream from the $tRNA^{his}$ gene and absent in strain DS504. However, no consensus nonanucleotides are present in the sequenced regions of the DS502 genome (only about 220 nucleotides out of 3500 have not been sequenced, ref.4,6) and no capped species initiating in this area have so far been detected. Alternatively, it is possible that a random initiation of transcription might be followed by precise nucleolytic cleavage events that produce discrete RNA species.

As a whole, the results suggest that sequences involved in transcriptional initiation might function differently in dissimilar mitochondrial contexts. A flexibility in the use of initiation sites has also been observed in other studies of mitochondrial transcription (22,34,35); such adaptability could be important in the regulation of mitochondrial transcription.

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