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Ordered processing of the polygenic transcripts from a mitochondrial tRNA gene cluster in *K. lactis*

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Abstract. In *Saccharomyces cerevisiae*, transcription of the mitochondrial genome starts at multiple initiation sites and is followed by the processing of multigenic transcripts at the 5′ and 3′ termini of tRNA sequences and in some intergenic regions. We have used a comparative approach to investigate the structure and function of the latter processing sites. We present here an analysis of the transcripts of a cluster of tRNA genes from the mitochondrial genome of *Kluyveromyces lactis*. The gene order of this cluster is the same as that of the cluster in *S. cerevisiae* but the sequence of the intergenic regions is different. A detailed analysis of transcripts has been performed using S1 mapping and primer extension techniques. The results can be summarized as follows: (1) transcription of the cluster very probably starts at initiation sites having the nonanucleotide sequence TTATAAGTA (which acts as a promoter in *S. cerevisiae*) and yields polygenic transcripts; (2) processing of these transcripts seems to occur through an ordered pathway of endonucleolytic events in which some tRNA sequences are preferentially excised and some endonucleolytic cuts occur more readily than others; (3) in two intergenic regions, strong signals indicate the existence of processing events. The sequences around these sites are similar in sequence and localization to *S. cerevisiae* intergenic processing sites, indicating a possible functional importance in maintaining a conserved order of tRNA genes in different species of yeasts.

Key words: Mitochondrial transcription processing – tRNA genes – *K. lactis*

Introduction

The mitochondrial genome of the yeast *Kluyveromyces lactis* is about 39 kb long and its gene content is similar to that of the mtDNA of other known budding yeasts. However, despite the similarity in gene content, genome size and, in general, gene order differ considerably from species to species. The location of the genes on the mt genome of *K. lactis* has already been determined (Ragnini and Fukuhara 1988). The genes for apocytochrome b (Brunner and Coria 1989), for subunits 1 and 2 of the cytochrome oxidase complex (Hardy and Clark-Walker 1990, 1991) and for 22 tRNAs have been sequenced (Wilson et al. 1989; Hardy and Clark-Walker 1990). Given the deduced codon usage, these tRNA genes may represent the entire set in the *K. lactis* mitochondrial genome. Most of the tRNA genes in *K. lactis* mtDNA are located in clusters near the rRNA genes. Comparison of the tRNA gene order in *K. lactis*, *S. cerevisiae*, *T. glabrata* (Hardy and Clark-Walker 1990) and *S. douglasii* mtDNA (Tian et al. 1991) have revealed a number of conserved blocks. The intergenic sequences in these blocks, however, are completely different.

In *S. cerevisiae*, the main tRNA gene cluster, which is located downstream from the LSUrRNA gene and contains 16 of the 24 tRNA genes encoded by the genome, is transcribed from three main promoters (Palleschi et al. 1984a; Bordonnè et al. 1987). Transcription from these promoters yields polygenic transcripts which undergo a number of endonucleolytic events leading to the mature form of each tRNA. Both in-vivo and in-vitro studies have shown that maturation of the 5′ end of a tRNA occurs via the endonucleolytic cutting action of mitochondrial RNase P (Martin et al. 1985; Morales et al. 1989). A different endonucleolytic activity produces the mature 3′ end of the tRNA; the enzyme responsible for this activity has been only partially purified (Chen and Martin 1988) but it appears capable of recognizing the secondary and/or tertiary structure of a tRNA (Zennaro et al. 1989). Studies on the processing of mitochondrial polygenic transcripts, have revealed, furthermore, that the extremities of many intermediate transcripts fall in intergenic regions (Francisci et al. 1987), though the nature of the processing enzyme(s) and the recognized signal(s) are still unknown. The complexity of RNA processing in the mitochondria suggests that the regulation of the steady-state level of biosynthe-

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Table 1. Recombinant plasmids used in this work

Plasmid name	Source of insert	Insert size	Insert description
111 p19	*K. lactis* mtDNA	2 340 bp	Deletion fragment starting 28 bp from the 3′ end of $tRNA^{Phe}$ and ending at the *Hin*dIII site inside $tRNA^{Asn}$
130 p18	*K. lactis* mtDNA	2 032 bp	Deletion fragment starting at 43 bp from the 5′ end of $tRNA^{Lys}$ and ending in the *Hin*dIII site inside $tRNA^{Asn}$

Table 2. S1 mapping probes

Probes	Size (bp)	Description	Labelled end
A	85	*Acc*I-*Acc*I fragment derived from 111p19. It contains 61 bp of $tRNA^{Leu}$ and 8 bp of $tRNA^{Gln}$	3′
B	188	*Hin*fI-*Taq*I fragment derived from 111p19. It contains 12 bp of $tRNA^{Gln}$ and 56 bp of $tRNA^{Lys}$	5′
C	438	*Taq*I-*Alu*I fragment derived from 130p18. It contains 15 bp of $tRNA^{Arg1}$ the entire $tRNA^{Gly}$ and 10 bp of the $tRNA^{Asp}$ as well as the intergenic regions between those tRNAs	3′
D	1 080	*Dde*I-*Hin*dIII fragment derived from 111p19. It contains 12 bp of $tRNA^{His}$ the entire $tRNA^{Met}$ and $tRNA^{Tyr}$ and 23 bp of $tRNA^{Asn}$ as well as the intergenic regions between those tRNAs	3′
E	209	*Hin*fI-*Taq*I fragment derived from 130p18. It contains 13 bp of $tRNA^{Lys}$ and 57 bp of $tRNA^{Arg1}$	5′
F	72	*Hin*fI-*Taq*I fragment derived from 130p18. It contains 2 bp of $tRNA^{Asp}$ and 65 bp of $tRNA^{Ser2}$	5′
G	391	*Acc*I-*Alu*I fragment derived from 111p19. It contains 64 bp of $tRNA^{Gln}$, the entire $tRNA^{Lys}$ and the intergenic sequence at the 5′ end of $tRNA^{Arg1}$	3′

tic compounds may also be achieved by controlling the processing apparatus. To understand the pathway by which individual tRNAs are obtained from a common precursor transcript, how the processing machinery is regulated, and if the steady-state level of each tRNA is somehow controlled, one needs to identify and characterize the intermediates between the primary transcript and the mature tRNA. Moreover, since the secondary and/or tertiary structures of precursor transcripts may act as signals for certain maturation events, comparative studies of different, evolutionarily-related, organisms may help to obtain a more precise picture of the processing pathway.

The presence of conserved blocks of tRNA genes in the mitochondrial genomes of various yeasts should prove particularly useful in this regard. The most notable of them is a group of seven tRNA genes (leu-gln-lys-arg1-gly-asp-ser2) which are a part of a large cluster which is located downstream from the LSUrRNA gene both in *S. cerevisiae* and *K. lactis*. In this paper we characterize the in-vivo transcripts of this region of the mitochondrial genome of *K. lactis*. Our results show that, in vivo, primary polygenic transcripts appear to be processed in an ordered manner. Transcripts ending in intergenic regions have been identified and compared with those of *S. cerevisiae*.

Materials and methods

Strains and growth conditions. The *K. lactis* strain 2360/7 (*alfa, lysA*, K^-), an auxotrophic subclone isolated by A. Algeri from the strain CBS 2360 (Centraalbureau voor Schimmelcultures, Delft), was used. For the preparation of mtRNA, this strain was grown until the end of the exponential phase on 1% yeast extract, 1% peptone supplemented with 2% ethanol.

Preparation of mtRNA. Mitochondria were prepared by differential centrifugation and mtRNA was extracted as previously described (Ragnini and Fukuhara 1988).

Clones and labelled probes used in S1 mapping experiments. K. lactis mtDNA fragments cloned in plasmids pTZ18R and pTZ19R (Table 1), kindly donated by Dr. C. Wilson, were used for the generation of probes used in S1 mapping experiments (Table 2). For the restriction map and sequence of the *K. lactis* mtDNA fragments retained in these clones see Wilson et al. (1989). All labelling reactions were performed as described by Maniatis et al. (1989).

S1 mapping. The S1 mapping experiments were performed as follows. The labelled mtDNA dissolved in water was denaturated for 5 min at 90° C, added to 30–60 µg of purified mtRNA and immediately dried. The pellet was dissolved in 20 µl of hybridization buffer (80% formamide, 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA pH 7.5). Nucleic acids were denaturated for 15 min at 65° C and rapidly shifted to the temperature chosen for each probe (between 43° C and 47° C depending on the probe length and G/C content), as described by Seraphin (1992) for 1 h 30 min. One-hundred microliters of ice-cold S1 buffer (250 mM NaCl, 5% glycerol, 30 mM sodium acetate, 1 mM $ZnSO_4$) was added to the hybridization reaction with 500 U of S1 enzyme (Miles Scientific) and the mixture was incubated for 30 min at 37° C. The reaction was extracted with phenol/chloroform and the nucleic acids precipitated by adding of 100 µl of 4 M ammonium acetate 0.1 M EDTA, 5 µl of 5 mg/ml *E. coli* tRNA carrier and 2.5 vol of ethanol. Nucleic acids were then resuspended in sequence loading buffer (95% formamide,

20 mM EDTA, 0.05% Bromophenol Blue, 0.05% xylene Cyanol FF) and applied to an 8% polyacrylamide-6 M urea gel for analysis. Gels were exposed with and without an intensifying screen on Cronex^R Dupont films.

Primers and primer extension reactions. The following oligonucleotides were used for primer extension reactions: 17-mer primer (5'CATGTACATAAATCTTC3') complementary to residues 33–40 of the tRNA^{Phe}; 17-mer primer (5'CTTACCTATTAGTCTAC3') complementary to residues 7–23 of the tRNA^{Gln}; 17-mer primer (5'ACCATTAAACAATATTC3') complementary to residues 7–19 of the tRNA^{Lys}; 17-mer primer (5'ACCATTAAGCTAAGAGA3') complementary to residues 3–19 of the tRNA^{Arg1}; 17-mer primer (5'GTTTGGAAGACATTCAG3') complementary to residues 24–40 of the tRNA^{Gly}; 17-mer primer (5'ACTATTAAGCTACAGAT3') complementary to residues 3–19 of the tRNA^{Asp}; 19-mer primer (5'CTCAGATTTAACGCGCCAC3') complementary to residues 34–52 of the tRNA^{His}; 17-mer primer (5'CAATTAAACTATACAAG3') complementary to residues 2–18 of the tRNA^{Met}. Sixty picomole of each primer were labelled using 20 µCi of (γ-^{32}P) ATP (3 000 Ci/mmol) by 7–10 U of T4 polynucleotide Kinase (NEN). The primer extension reactions were carried out as follows: 30–60 µg of mtRNA dissolved in 10 µl of water were mixed with 100 ng of end-labelled primer and 4 µl of reverse transcriptase 5 × buffer (375 mM KCl, 250 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 0.1 M DTT). Nucleic acids were denaturated at 90° C for 2 min and then passed at 65° C in a heating block. For the annealing the temperature was allowed to decrease to 43° C before the addition of 2 µl of 0.1 M DTT, 2 µl of 10 mM dNTP mix and 200 U of M-MLV H$^-$ Superscript^{TM} reverse transcriptase (BRL). The extension reaction was carried out for 1 h and then extracted with phenol/chlorophorm followed by precipitation with 2.5 M ammonium acetate and 2.5 vol of ethanol. The DNA was resuspended in sequence loading buffer as above and applied to an 8% polyacrylamide-urea gel for analysis.

DNA sequencing. S1 mapping and primer extension size marker sequences were performed using the dideoxy chain-termination method on single-strand DNA of the clones 111p19 and 950p18 (containing a 950-bp *TaqI* fragment of *S. cerevisiae* mtDNA of known sequence cloned in the *AccI* site of plasmid pTZ18), using (α-^{35}S) dATP as labelling nucleotide, M13 reverse primer (Pharmacia) and Sequenase^{TM} (USB) T7 DNA polymerase. The reactions were performed following the USB manufacturer's instructions.

Results

The sequence of the tRNA genes present in the cluster located downstream from the LSUrRNA gene of *K. lactis* was determined previously (Wilson et al. 1989). A schematic representation of the region being studied is shown in Fig. 1. Osinga et al. (1982) have shown that the yeast mitochondrial promoter sequence A/TTATAAGTA is also the transcriptional initiation signal for *K. lactis* mitochondrial rRNA genes. This nonanucleotide box occurs three times in the studied tRNA gene cluster (Fig. 1), upstream of tRNA^{Phe}, upstream of tRNA^{Tyr} and within the last nine nucleotides of the tRNA^{Met} coding sequence. Even if this tRNA^{Met} tail-endbox is not functional, the two other promoters can account for the transcription of the entire region. To analyze the in-vivo transcription of the conserved blocks of tRNA genes (leu-gln-lys-arg1-gly-asp-ser2), and of the other tRNA genes present in the same region in *K. lactis* mtDNA, we performed a series of S1 and primer extension analyses using 5' or 3' end-labelled restriction fragments and oligonucleotide primers complementary to wild-type *K. lactis* mitochondrial RNAs.

Analysis of the (phe-leu-gln) tRNA gene transcripts

Figure 2 shows the primer extension patterns obtained using oligonucleotides complementary to tRNA^{Phe} (Fig. 2 a) and to tRNA^{Gln} (Fig. 2 b). A signal corresponding to the last base of the consensus promoter sequence 5'TTATAAGTA3' located 10 bp upstream of the tRNA^{Phe} gene (C box in Fig. 2 a and b) was observed in both experiments. The fact that the primer complementary to tRNA^{Gln} can be elongated as far as the promoter consensus sequence clearly indicates that all the tRNAs in the group (phe-leu-gln) derive from a common precursor transcript and lends support to the hypothesis that transcription initiates at this consensus box.

A signal corresponding to the mature 5' end of tRNA^{Phe} is easily detectable in Fig. 2 a (nucleotide 49), but evident only after long exposure in the experiment shown in Fig. 2 b (nucleotide 197).

Other signals present in Fig. 2 b include bands corresponding to the 3' end of the gene for tRNA^{Phe} (nucleotide 125) and to the 5' end of the gene for tRNA^{Leu} (nucleotide 120), while no specific signal corresponding to the 3' end of the gene for tRNA^{Leu} can be observed, and that corresponding to the 5' end of tRNA^{Phe}, as noted above, is observable only after prolonged exposure. The signal corresponding to the 5' end of tRNA^{Gln} was clearly discernible

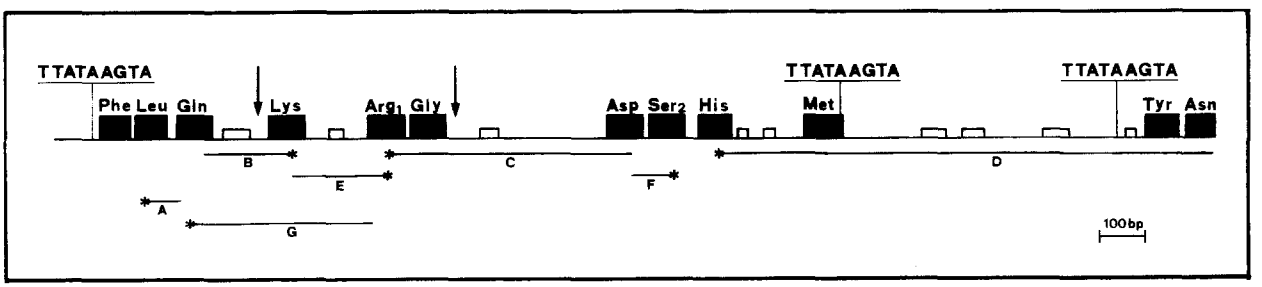

Fig. 1. Schematic representation of the studied region and probes used in S1 mapping analysis. The tRNA genes are indicated by *closed boxes. Open boxes* indicate G/C clusters. TTATAAGTA indicates the position of a promoter consensus sequence. *Straight lines* at the bottom of the figure indicate the size and localization, with respect to the upper scheme, of the probes used in S1 experiments (see also Table 2). The *asterisks* indicate the labelled end of each probe. The *arrows* in the upper scheme indicate the position of the main intergenic processing signals

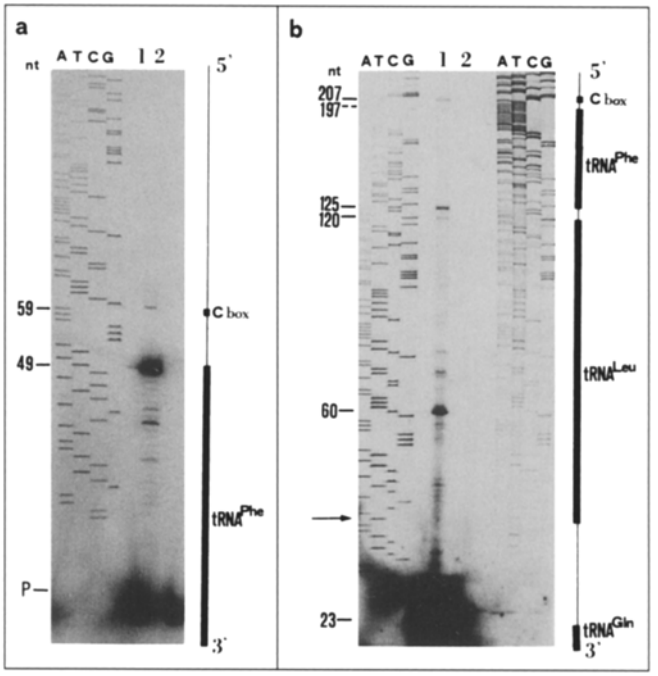

Fig. 2 a, b. Primer extension analysis of the transcripts encompassing $tRNA^{Phe}$, $tRNA^{Leu}$ and $tRNA^{Gln}$. **a** mapping of the 5′ end of transcripts encompassing $tRNA^{Phe}$. The oligonucleotide 5′CATGTACATAAATCTTC3′ complementary to $tRNA^{Phe}$ was annealed to *K. lactis* mtRNA, elongated by reverse transcriptase and the cDNA fragments obtained were separated on a polyacrylamide-urea gel as described in Materials and methods. The exposure time was 48 h. On the left of the figure: *numbers* indicate the distance in nucleotides from the first nucleotide of the primer, *P* indicates the signal corresponding to unextended primer. *Lanes ATCG*, ladder sequences (see Materials and methods). *Lane 1*, primer extension reaction of the oligonucleotide complementary to $tRNA^{Phe}$. *Lane 2*, primer extension control reaction: as per the primer extension reaction except for the omission of the reverse transcriptase. In the scheme at the right the phenylalanyl tRNA is indicated by a *closed box*; the consensus promoter sequence TTATAAGTA is indicated by "*C box*" **b** mapping of the 5′ end of the transcripts encompassing $tRNA^{Phe}$, $tRNA^{Leu}$ and $tRNA^{Gln}$. The oligonucleotide 5′CTTACCTATTAGTCTAC3′ complementary to $tRNA^{Gln}$ was annealed to *K. lactis* mtRNA as described above. The autoradiographic pattern obtained after 1 week of exposure is shown. On the left of the figure: *numbers* indicate the distance in nucleotides from the first nucleotide of the primer; the *arrow* indicates the expected position of the 3′ end of $tRNA^{Leu}$. *Lanes ATCG*, as in Fig. 2 a. *Lane 1*, primer extension reaction of the oligonucleotide complementary to $tRNA^{Gln}$. *Lane 2*, primer extension control reaction: as per the primer extension reaction except for the omission of the reverse transcriptase. In the scheme at the right $tRNA^{Phe}$, $tRNA^{Leu}$ and $tRNA^{Gln}$ are indicated by *closed boxes*; the consensus sequence is indicated by "*C box*"

after 1 day exposure (data not shown) but, due to its proximity with the primer, it appears as an overexposed area in the bottom of Fig. 2 b which shows the autoradiogram obtained after 7 days of exposure. The pattern of intensities of the above-mentioned signals does not show the decrease in intensity one would expect to occur at increasing distances from the primer, and we must conclude that transcripts including the $tRNA^{Gln}$ sequence are preferentially processed at the 3′ end of $tRNA^{Phe}$ and at the 5′ end of $tRNA^{Leu}$. If we assume that the intensity of individual signals at a certain site is a measure of the relative abundance of transcripts terminating at that site, we must conclude that the processing of $tRNA^{Phe}$ at the 3′ end occurs preferentially before the maturation of the 5′ end of $tRNA^{Leu}$. Alternatively, we could hypothesize that there is a relevant difference in stability between transcripts which, in the latter case, terminate at sites located five nucleotides apart. The signal located at position 60 in Fig. 2 b represents a transcript with a 5′ end that falls inside $tRNA^{Leu}$ (see later for an analysis of this point).

Processing at intergenic sequences

It has been previously shown that in *S. cerevisiae* some partially-processed transcripts have their extremities in intergenic sequences. Comparisons of the sequences around the processing sites have not always revealed obvious homologies, though some are located near G/C-rich sequences (G/C clusters) that might fold and give rise to stem-and-loop structures. It has been suggested that the processing enzyme(s) responsible for these events may recognize a short A/T-rich sequence located near to a secondary structure (Zassenhaus et al. 1984; Francisci et al. 1987; Smooker et al. 1988). *K. lactis* intergenic regions have the same characteristic A/T-rich stretches interrupted by G/C clusters as those of *S. cerevisiae* (Ragnini and Fukuhara 1988). To determine whether maturation of polygenic transcripts at intergenic sequences is a common feature in yeast mitochondria, we have performed a series of S1 mapping experiments using single-stranded labelled fragments complementary to the intergenic regions located between $tRNA^{Gln}$ and $tRNA^{Lys}$, $tRNA^{Lys}$ and $tRNA^{Arg1}$, $tRNA^{Gly}$ and $tRNA^{Asp}$, $tRNA^{His}$ and $tRNA^{Met}$.

The probes used in the S1 mapping experiments are shown in Fig. 1. Figure 3 shows the S1 analysis of the transcripts encompassing the intergenic region between $tRNA^{Gln}$ and $tRNA^{Lys}$ that was performed using probes G (Fig. 3 a) and B (Fig. 3 b), labelled at opposite ends. The faint signal at position 64 in Fig. 3 a corresponds to the 3′ end of $tRNA^{Gln}$. Three strong signals visible in Fig. 3 a between position 139 and position 146 (indicated by the arrow) reveal the presence of an intergenic processing site between $tRNA^{Gln}$ and $tRNA^{Lys}$. Signals corresponding to the same site (indicated by the arrow between position 84 and 90 in Fig. 3 b) were also detected in the S1 mapping experiment performed using probe B. The transcripts ending at position 146 in Fig. 3 a and at position 90 in Fig. 3 b abut upon each other in the underlined nucleotide within the sequence 5′TAGTTACGTCTCCTT3′, indicating that endonucleolytic events may be responsible for these signals. The occurrence of this intergenic processing site was confirmed by a primer extension analysis performed using an oligonucleotide complementary to the 5′ end of $tRNA^{Lys}$ (data not shown). Inspection of the sequence around this processing site has shown that five-nucleotides downstream there is a sequence, 5′AAGAAGAT3′, which is similar to the 5′AAGAAT/GAT3′ sequence often found near to processing sites in *S. cerevisiae* (Simon and Faye 1984). The faint bands visible at position 181 in Fig. 3 a and position 56 in Fig. 3 b correspond to the 5′ end of $tRNA^{Lys}$. Stronger signals, indicated by an

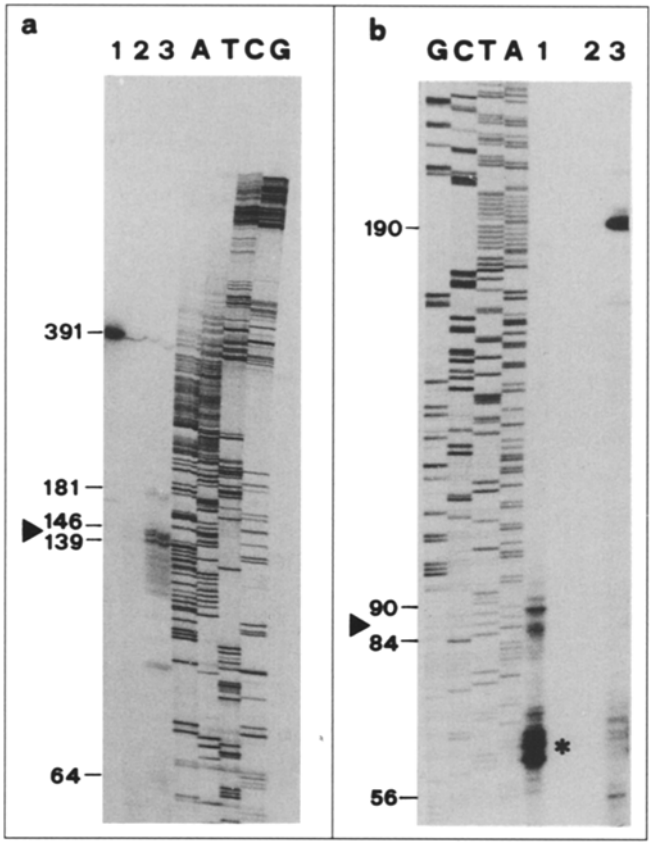

Fig. 3 a, b. S1 mapping of the transcripts with extremities in the region between $tRNA^{Gln}$ and $tRNA^{Lys}$. **a** Mapping of the 3′ end of the transcript encompassing the $tRNA^{Gln}$ and $tRNA^{Arg1}$ region. A 3′ end-labelled fragment of 391 nt (probe G in Table 2 and Fig. 1) was hybridized with mtRNA and treated with S1 nuclease as described in Materials and methods. Protected fragments were separated on buffer gradient 8% polyacrylamide-urea gels and dideoxy chain-termination sequence reactions of a known DNA fragment were used as reference ladders. After electrophoresis, gels were exposed to Dupont Cronex^R films. *Numbers* on the left of the figure indicate the distance in nucleotides from the labelled end of the probe. *Lanes ATCG*, sequence reactions used as size marker. *Lane 1*, untreated probe G. *Lane 2*, control reaction: probe G annealed with *E. coli* 5*s* ribosomal RNA instead of with mtRNA and treated with S1 nuclease. *Lane 3*, probe G annealed with mtRNA of *K. lactis* and treated with S1 nuclease. **b** S1 mapping of the 5′ end of transcripts between $tRNA^{Gln}$ and $tRNA^{Lys}$. A 5′ end-labelled fragment of 187 nt (probe B in Table 2 and Fig. 1) was annealed, S1 treated, and the protected fragments electrophorezed and exposed as described above. *Numbers* on the left and *Lanes GCTA* as in Fig. 3 a. *Lane 1*, probe B annealed with mtRNA and treated with S1 nuclease. *Lane 2*, control reaction: probe B annealed with *E. coli* 5*s* ribosomal RNA instead of mtRNA and treated with S1 nuclease. *Lane 3*, untreated probe B. The *arrows* in **a** and **b** indicate the signals corresponding to the intergenic processing sites. The *asterisk* indicates the signals corresponding to the anomalous 5′ end of $tRNA^{Lys}$

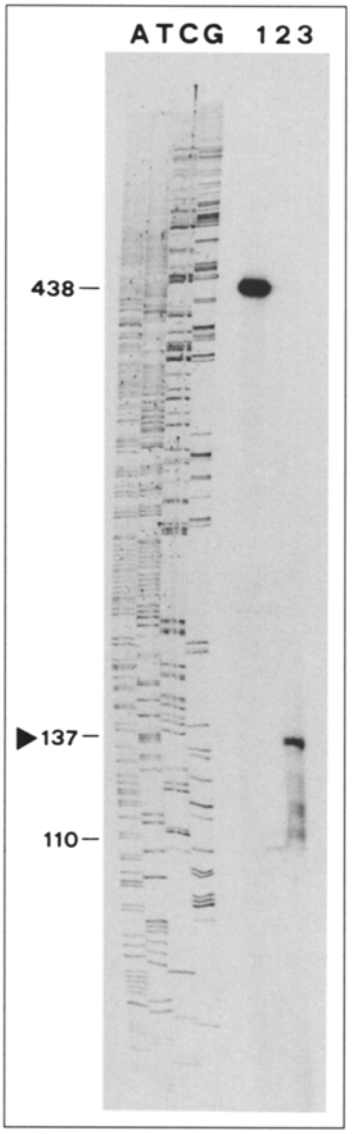

Fig. 4. S1 mapping of the 3′ end of the transcripts between $tRNA^{Arg1}$ and $tRNA^{Asp}$. A 3′ end-labelled fragment of 438 nt (probe C in Table 2 and Fig. 1) was hybridized with mtRNA and treated with S1 nuclease and protected fragments were separated as described above. After electrophoresis, gels were exposed to Dupont Cronex^R films. *Numbers* on the left of the figure indicate the distance in nucleotides from the labelled end of the probe. *Lanes ATCG*, sequence reactions used as size markers. *Lane 1*, untreated probe C. *Lane 2*, control reaction: probe C annealed with *E. coli* ribosomal RNA instead of with mtRNA and treated with S1 nuclease. *Lane 3* probe C annealed with mtRNA of *K. lactis* and treated with S1 nuclease. The *arrow* indicates the signal corresponding to the intergenic processing site

asterisk in Fig. 3 b, were present a few nucleotides upstream of the 5′ end of $tRNA^{Lys}$. This is the only case in which relevant amounts of a tRNA inaccurately processed at its 5′ end have been found in this study. This may be due to the G/C-rich nature of the sequence upstream of this tRNA, for in-vitro studies have shown that yeast mitochondrial RNase P will not efficiently cut the 5′ end of a tRNA having a G/C-rich sequence immediately upstream of the correct cutting site (Hollingsworth and Martin 1987).

Figure 4 shows the result of the S1 mapping performed using probe C. The signal at position 137 corresponds to a transcript ending in the underlined nucleotide within the sequence 5′TATAAATACATAATA3′ and indicates the presence of an intergenic processing site located 30 bp downstream from the $tRNA^{Gly}$. Position 110 in the same figure corresponds to the expected 3′ end of this gene. A series of primer extension analyses using oligonucleotides complementary to $tRNA^{Arg1}$, $tRNA^{Gly}$, $tRNA^{Asp}$,

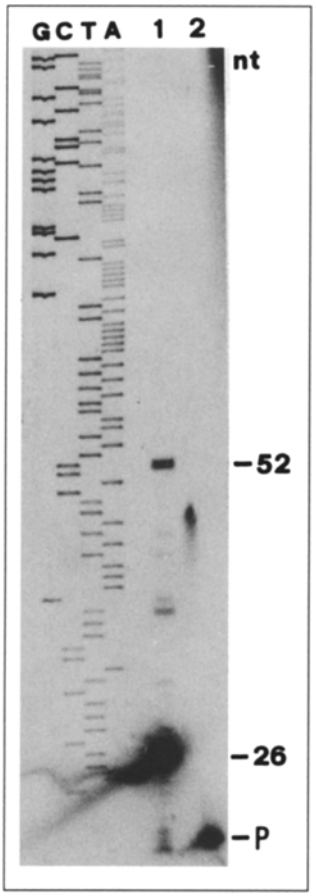

Fig. 5. Primer extension analysis of the 5′ end of $tRNA^{His}$. The oligonucleotide 5′CTCAGATTTAACGCGCCAC3′ complementary to $tRNA^{His}$ was annealed to *K. lactis* mtRNA, elongated by reverse transcriptase and the cDNA fragments obtained were separated as described in Materials and methods. The autoradiographic pattern obtained after 48 h of exposure is shown. *Lanes GCTA*, ladder sequence: dideoxy chain-termination sequence reactions performed elongating the oligonucleotide 5′CTCAGATTTAACGCGCCAC3′ on cloned mtDNA fragment carrying the $tRNA^{His}$ gene. *Lane 1*, primer extension reaction of the oligonucleotide complementary to $tRNA^{His}$. *Lane 2*, control reaction: as above except for the omission of the reverse transcriptase. On the right fo the figure: *numbers* indicate the distance in nucleotides from the first nucleotide of the primer, *P* indicates the signal corresponding with unextended primer. The band at nucleotide 26 corresponds to the intragenic processing signal. The band at nucleotide 52 corresponds to the expected 5′ end of $tRNA^{His}$

$tRNA^{His}$ and $tRNA^{Met}$, and/or S1 mapping analyses with probes A, D, E and F, failed to reveal other strong intergenic processing sites in the studied region.

Processing at intragenic sequences

As mentioned above, the strong signal at position 60 in Fig. 2b corresponds to the 60th nucleotide of $tRNA^{Leu}$ which is located inside the stem of the TΨC arm, just after a double G-C pairing. Another intragenic processing site was found by primer extension analysis using an oligonucleotide complementary to the 3′ end of $tRNA^{His}$. This intragenic processing site corresponds to the 27th nucleotide of the gene (position 26 from the primer in Fig. 5), which is located at the end of the anticodon arm, just before an unpaired nucleotide. The signal at position 52 in Fig. 5 corresponds to the 5′ end of the gene. Intragenic processing signals in tRNA genes have also been reported by other authors (Shu and Martin 1991). In principle, intragenic signals might be due to premature arrests of the reverse transcriptase which would be expected to occur in front of strong secondary structures. Therefore, we tried to overcome this problem by performing the same analysis at 45° C (the stability limit of the reverse transcriptase that we used) but, except for the disappearance of minor intragenic signals (data not shown), the result was the same. We cannot rule out the possibility that even at this temperature a secondary structure, due to the folding of the tRNA sequence into a cloverleaf structure, can be formed. But if this is the case we have to note that the positions of the signals do not correspond to those expected from premature detachment of the polymerase.

Discussion

The general order of the genes coded for by the mitochondrial genome varies in different yeasts; there are groups of tRNA genes, however, that maintain the same relative order in several yeast species, even if the intergenic regions between them differ completely in sequence. Jacobs et al. (1989) have proposed that the organization of tRNA genes in clusters, typical of lower eukaryotes, is ancestral with respect to the distribution of the tRNA genes in vertebrate mtDNA. In the latter, the dispersed distribution of the tRNA genes in the mitochondrial genome is fundamental for the correct maturation of the polygenic transcripts (which represent one of the main processing signals recognized by the processing enzymes). In organisms in which tRNA genes are grouped in clusters, it is possible to observe a different, more complex, pattern of processing which involves not only maturation at the extremities of the genes but also intergenic cuts.

In this paper we report data on the transcription and processing of the main conserved yeast tRNA gene block present in the *K. lactis* mtDNA. The results obtained from the study of the transcription of the region containing $tRNA^{Phe}$, $tRNA^{Leu}$ and $tRNA^{Gln}$ provide some information on the in-vivo process by which a precursor transcript yields mature tRNA. The precursor polygenic transcripts containing these genes are maturated at the 5′ and 3′ ends of tRNAs as a result of endonucleolytic events. The first tRNA of the polygenic transcript ($tRNA^{Phe}$) appears to be preferentially excised from the precursor before the second tRNA ($tRNA^{Leu}$) is maturated at its 5′ end, while the 3′ end of $tRNA^{Leu}$ seems to be maturated only after the maturation of the 5′ end of the third tRNA ($tRNA^{Gln}$) in this polygenic transcript. The results suggest the possibility that, for some tRNAs, a downstream-cut by RNAse P serves to separate the tRNA from its downstream partner, while for others the 3′ endonuclease plays this role: in other words in-vivo processing may follow different pathways in different regions of the same polygenic transcript. In-vitro studies performed on synthetic tRNA precursor sub-

strates using purified or partially-purified 3′ processing endonucleases from different sources (Garber and Gange 1979; Castano et al. 1985; Fredewey et al. 1985; Manam and van Tuyle 1987; Chen and Martin 1988) have shown the inability of the 3′ end of the processing enzyme to mature 5′ unprocessed precursors. Studies performed in vivo on the transcription and processing in yeast mitochondrial petite mutants lacking RNase P activity, on the other hand, have clearly shown that the 3′ end of a tRNA can be matured even if the 5′ end is not (Frontali et al. 1982; Martin et al. 1985). The difference in structure between a transcript bearing a tRNA sequence flanked by short 5′ and 3′ extensions and a complex polygenic transcript bearing several tRNA sequences might explain the difference between the in-vivo and the in-vitro results.

Our data clearly show that both intergenic sequences and the extremities of a tRNA are recognized as processing signals by endonucleolytic enzymes. In the region under investigation we were able to detect intergenic processing sites between $tRNA^{Gln}$ and $tRNA^{Lys}$ and between $tRNA^{Gly}$ and $tRNA^{Asp}$. Evidence for intergenic processing sites has previously been found in *S. cerevisiae* mitochondria (Simon and Faye 1984; Palleschi et al. 1984 b; Zassenhaus et al. 1984; Francisci et al. 1987; Smooker et al. 1988; Kang and Miller 1989). By combining the varied observations made in *S. cerevisiae* we conclude that it is possible to divide the sequences into two types: type I (5′AAGAATAT3′) was first proposed by Simon and Faye (1984), and is located at or near the processing site; type II (5′AATATAA3′) was first proposed by Zassenhaus et al. (1984), and is located near a palindromic sequence (such as a G/C cluster) which can fold into a secondary and/or tertiary structure. Inspection of the *K. lactis* sequence 5′ATTTATAAGAAGAT3′ located immediately downstream from the processing site between $tRNA^{Gln}$ and $tRNA^{Lys}$ revealed its similarity to the type-I sequence of *S. cerevisiae*. Moreover, except for one nucleotide (the third to the last nucleotide is a G instead of a T) this sequence is identical to a sequence located a few nucleotides downstream from the processing site which generates the mature 5′ end of the 15*s* rRNA (Osinga and Tabak 1982) in *S. cerevisiae* mtDNA. As for the intergenic processing site found between $tRNA^{Gly}$ and $tRNA^{Asp}$, the 5′ATAAATA3′ sequence located just upstream of the processing site is identical to a sequence which Smooker et al. (1988) found was important for the correct processing of the polygenic transcript containing the var1 coding sequence. A mutation in the central A of this box, located 109 nucleotides upstream of the var1 reading frame, results in the accumulation of var1 precursor transcripts.

We have also to note that in the mtDNA of *K. lactis* and *S. cerevisiae* the conserved block of tRNA genes (from $tRNA^{Leu}$ to $tRNA^{Ser2}$) is transcribed in a polygenic transcript that is processed in intergenic sites located between the same tRNA genes in both yeasts. Thus, not only do the tRNA genes of this block retain the same relative location in both yeasts, but a similar pathway of transcription and processing is also maintained. This common feature between different yeast species cannot simply be explained by the similarity between the nucleotide sequences located near the processing sites, because even in the same species a certain sequence motif does not by itself seem to be sufficient to cause processing; nor does it always correspond to processing sites. The difficulty in identifying the signals recognized by the intergenic processing enzyme(s) might be due to the ability of the processing enzyme(s) to recognize structures created by the folding of distant parts of the polygenic precursor transcript. A particular sequence might be only part of a more complex three-dimensional structure of the precursor polygenic transcript. Unfortunately, the difficulties in accurately predicting the possible secondary structure of large RNA molecules on the basis of their nucleotide sequence render the further elucidation of this point difficult at present.

In conclusion, our work has shown the existence of preferred processing sites at the ends of some tRNAs belonging to the same polygenic transcript, as well as the existence of intergenic (and possibly intragenic) processing cuts. We suggest that these preferred processing sites are necessary to create changes in the conformation of the precursor transcript and hence create the premise for subsequent processing at the extremities of each tRNA.

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