Endonucleolytic Cleavage of a Long 3′-Trailer Sequence in a Nuclear Yeast Suppressor tRNA†

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ABSTRACT: Transcripts of *Saccharomyces cerevisiae* nuclear tRNA genes are normally terminated within a few nucleotides of the tRNA coding region, in contrast to mitochondrially encoded tRNAs, which are contained within polycistronic transcripts and thus require 3′-processing by mitochondrial endonucleases. We show that 3′-processing activities capable of removing artificially extended 3′-trailer sequences from some tRNA substrates are also present in the yeast nucleus. Correct 3′-processing in vivo resulted in the formation of functional suppressor tRNA. The 3′-processing activities were also identified in vitro through analysis of transcription-processing products in cell-free yeast S-100 extracts. Comparison of several pre-tRNA substrates showed that the tRNA structure played a major role in determining the processability of a substrate but that the nature of the 3′-trailer sequence also modulated the rate of 3′-processing. Pre-tRNA containing mitochondrial $tRNA^{Val}$ sequence was a good substrate for in vitro processing, independent of its 3′-trailer. A 200-nt-long pre-tRNA, encoding the nuclear *SUP4* tRNA gene and a mitochondrial 3′-trailer, was processed in yeast S-100 extract in a multistep pathway into mature-sized $tRNA^{Tyr}$. Part of the 3′-processing was due to an endonuclease which cleaved near or precisely at the 3′-end of the coding region of the tRNA. A short sequence around this endonucleolytic 3′-cleavage site was crucial for the formation of active suppressor tRNA in vivo. A 9-nt-long sequence motif derived from the mitochondrial 3′-trailer allowed processing, while sequences derived from *lacZ* or pBR322 DNA were processed neither in vitro nor in vivo.

Transcription of eukaryotic nuclear tRNA genes by RNA polymerase III (pol III)[1] is directed by initiation signals within and preceding the tRNA coding region and by an oligo(T) termination sequence 3′ to the gene (Geiduschek & Tocchini-Valentini, 1988). Mutational studies have established the requirement for four or more consecutive T residues to terminate *Xenopus* pol III transcription (Bogenhagen & Brown, 1981) and for five or more consecutive T residues to terminate yeast pol III transcription (Allison & Hall, 1985). For the vast majority of yeast nuclear tRNA genes this terminator is no more than three or four base pairs downstream from the 3′-end of the tRNA coding region (Sprinzl et al., 1989). Consequently, 3′-processing of yeast nuclear pre-tRNA transcripts to remove these short trailer sequences generally requires only exonuclease trimming. By contrast, the nuclear tRNA genes of *Drosophila, Bombyx mori, Xenopus laevis,* and other higher eukaryotes frequently have distances of 10 base pairs or more between the tRNA 3′-terminus and the terminator sequence (Frendewey et al., 1985; Hagenbüchle et al., 1979; Mazabraud et al., 1987; Zasloff et al., 1982). In vitro transcripts made from these genes are substrates for 3′-endonucleolytic tRNA processing (Garber & Gage, 1979); using both the direct pol III transcription products and semisynthetic pre-tRNAs as substrates, eukaryotic endonucleases that remove the bulk of the 3′-trailer sequence have been detected (Castano et al., 1985; Solari & Deutscher, 1983). A similar activity exists in the mitochondrion of yeast, where it appears to function in the maturation of mitochondrial pre-tRNA transcripts that contain 3′-distal mRNA sequences (Chen & Martin, 1988). Higher eukaryotic nuclear processing activities that have been described require for their action the prior removal of 5′-leader sequences from their pre-tRNA substrates (Castano et al., 1985; Frendewey et al., 1985).

Since a nuclear processing activity is found in higher eukaryotes, and since at least one yeast nuclear tRNA gene contains a 27-nt-long 3′-trailer (Valenzuela et al., 1978), we undertook a combined molecular and classical genetic approach to identify similar processing activities in the yeast nucleus. A number of altered tRNA genes were constructed containing sizable insertions between the end of the tRNA and the terminator. In vivo, a yeast tyrosine suppressor tRNA gene having as 3′-trailer a sequence derived from yeast mitochondrial DNA *is* 3′-processed, as demonstrated by suppression of *ochre*-marked genes. In vitro, one endonucleolytic activity cuts within the mitochondrial trailer sequences of this template, just downstream of the tRNA coding sequence. This result is in contrast to a previous study of the expression of a yeast $tRNA^{Tyr}$ gene with 3′-trailer derived from bacterial (*lacZ*) sequences. Such a template could not be processed by the yeast nuclear system, nor did it produce functional suppressor in vivo (Allison & Hall, 1985). However, when nine nucleotides in the *lacZ* sequence of this nonprocessed pre-tRNA substrate were replaced with mitochondrial DNA, in vivo suppression was restored. Since the in vitro processing site overlaps with the nine exchanged nucleotides which permit in vivo processing, it is likely that the same activity is involved. In future experiments, one can take advantage of the in vivo suppression mediated by the semisynthetic tRNA to isolate the gene encoding the endonucleolytic activity.

MATERIALS AND METHODS

Yeast Strains and Plasmids. Yeast strain PJ17-6A (*MATα trp1-1 ura3-1 ade2-1 lys2-1 met4-1 can1-100 gal10-1 his5-2*

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[1] Abbreviations: pol III, RNA polymerase III; nt, nucleotide; mt, mitochondrial.

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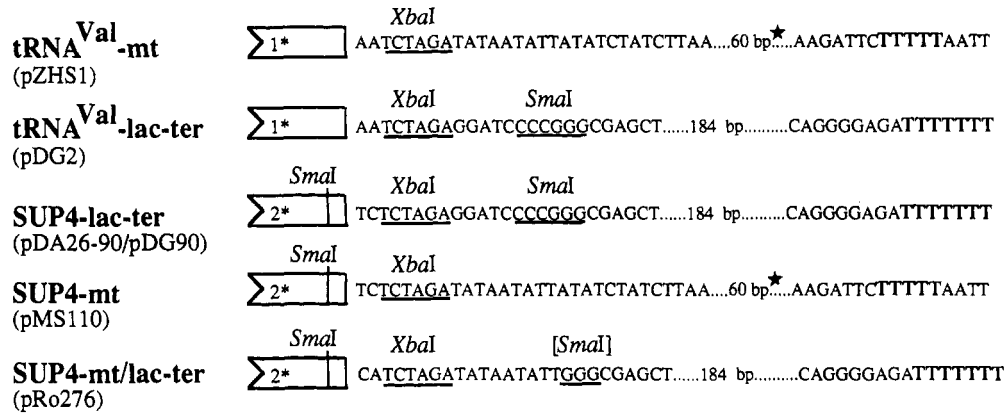

FIGURE 1: Sequences of 3′-trailers. tRNA^{Val}-mt (pZHS1) encodes a mitochondrial tRNA^{Val} gene, with its natural 3′-trailer, which contains only the two G residues shown; the 1* coding sequence is according to Miller et al. (1980). tRNA^{Val}-lac-ter has a different 3′-trailer than tRNA^{Val}-mt. SUP4-lac-ter (pDA26-90/pDG90) encodes a *SUP4* allele with *lacZ* DNA inserted between its 3′-end and its terminator. The two plasmids are based on two different centromere vectors (see Materials and Methods). The 2* *SUP4* coding sequence is described in Goodman et al. (1977). SUP4-mt and SUP4-mt/lac-ter differ from SUP4-lac-ter in their 3′- trailer. ⊃——⊂ indicates the 3′-boundaries of the tRNA genes. ☆ indicates that within these 60 nt no G residues are found. Possible pol III terminators are in boldface type.

leu2-1) is described in James and Hall (1990); the methionine, lysine, and adenine auxotrophies are *ochre*-suppressible. The *SUP4* wild-type allele (pAsup4-4, designated SUP4-ter) and allele pDA26-90 (designated SUP4-lac-ter) are described in Allison and Hall (1985). Plasmid pZHS1 (designated tRNA^{Val}-mt), carrying the mitochondrial tRNA^{Val} on a 2.9-kilobase-pair insert, is described in Müller et al. (1984) (see Figure 1). Its sequence is from Thalenfeld and Tzagoloff (1980) and Miller et al. (1980). Plasmid pCT10 contains mitochondrial promoter consensus sequences (Fangman et al., 1990).

Plasmid pMS110 (designated SUP4-mt) is a YCp50-based plasmid, carrying the *SUP4* allele of plasmid pDA26-90 in which the *lacZ*-derived 3′-trailer was replaced by the 3′-trailer of the mitochondrial tRNA^{Val} gene by using the unique *XbaI* site immediately downstream of the coding sequences of the two genes (see Figure 1). Plasmid pDG90 carries the same *SUP4* allele as plasmid pDA26-90 but is based on the vector YCp50 rather than pTC3; both plasmids are designated SUP4-lac-ter. Plasmid pRo275 (designated SUP6Δ32-mt) was derived from pDG90 by replacing the *SUP4* sequences upstream of the *SmaI* site with the corresponding sequences of the *SUP6Δ32* allele, a tRNA^{Tyr} allele lacking its intron (Johnson & Abelson, 1983). Plasmid pRo276 (designated SUP4-mt/lac-ter) is identical with plasmid pDA26-90, except that the sequence between the 3′-end of the tRNA^{Tyr} gene and the *SmaI* site in the 3′-trailer (stemming from the *lacZ* polylinker sequence) was replaced by synthetic DNA containing the sequence of the tRNA^{Val} 3′-trailer in the corresponding positions (see Figure 1).

Manipulation of Yeast Strains. Yeast strains were transformed according to Beggs (1978). Suppressor activity was tested either by replica plating on media lacking a single amino acid or, for a more quantitative determination, by spotting 5 μL of an exponentially growing yeast culture (2×10^7 cells/mL) after 1-, 10-, and 100-fold dilutions. Media are described in Sherman et al. (1983).

Yeast Transcription Extracts, in Vitro Transcription and Processing Reactions, Isolation of tRNA Precursors, and RNase P and T1 Cleavage. Yeast S-100 transcription extracts were prepared according to Baker and Hall (1984), and nuclear extracts, according to Wiederrecht et al. (1987) and Huibregtse et al. (1987). Transcription reactions were done in 20 mM HEPES, pH 7.9, 0.1 mM EDTA, 5 mM $MgCl_2$, 7% (v/v) glycerol, 4 mM 2-mercaptoethanol, 150 mM KCl, 0.5 mM ATP, CTP, and GTP, 0.05 mM UTP, 5 μCi of [α-^{32}P]UTP (Amersham; 400 Ci/mmol), and 150 ng of template DNA in 25 μL at 25 °C for 30 min. 3′-Processing reactions were done under the same conditions as transcription reactions, except that the template DNA and the labeled nucleotide were omitted from the reaction. As substrates, gel-purified precursor tRNAs were used. A 10-fold scaled-up transcription reaction was separated on an 8% polyacrylamide gel containing 8 M urea and TBE buffer; bands were excised from the gel, crushed and eluted into a buffer of 10 mM Tris, pH 8, 1 mM EDTA, and 200 mM NaCl overnight at room temperature or for 1 h at 65 °C. Several thousand counts per minute were used per processing reaction. Several S-100 extracts were initially tested for processing activity. Two out of five extracts were superior because they produced less nonspecific degradation of precursors and processing products; the reason for this behavior was not further investigated.

The RNA moiety of *Escherichia coli* RNase P was transcribed with T7 RNA polymerase from plasmid pDW25 (kindly provided by N. Pace). RNase P cleavage was done in high-salt buffer (2 M NH_4OAc, 10 mM Tris, pH 8.0, 50 mM $MgCl_2$, 0.1% SDS, and 0.05% NP-40) for 6 h at 37 °C. RNase T1 digestions were done in a buffer of 40 mM Tris, pH 7.5, and 0.5 mM EDTA at 37 °C for 15 min with 350 units of RNase T1 (BRL, Bethesda, MD). RNA size markers were made by linearizing the Bluescript vector KS+ (Stratagene, La Jolla, CA) at the *Eco*RV site (73 nt), the *ClaI* site (86 nt), and the *SalI* site (96 nt) within the polylinker and transcribing the linearized plasmids with T7 RNA polymerase in the presence of radiolabeled nucleotides.

RNase T1 Fingerprinting. Digestion with RNase T1 in the presence of 5 μg of yeast tRNA and 10 000–20 000 cpm of [α-^{32}P]GTP-labeled RNA and fingerprint analyses were performed as described by Silberklang et al. (1979).

RESULTS

Synthesis and Processing of Pre-tRNAs with Long 3′-Trailers in Yeast S-100 Extracts. As an initial test for yeast tRNA 3′-processing activity, different plasmid-borne tRNA genes were used as templates for in vitro transcription. For ease and clarity of reference they are henceforth designated SUP4-ter (a normally terminating *SUP4–o* tRNA^{Tyr} gene; Koski et al., 1980); SUP4-lac-ter (a *SUP4* gene with a 220-nt-long piece of *lacZ* DNA inserted between the coding sequence of the tRNA^{Tyr} gene and its natural terminator, equivalent to pDA26-90; Allison & Hall, 1985); tRNA^{Val}-mt

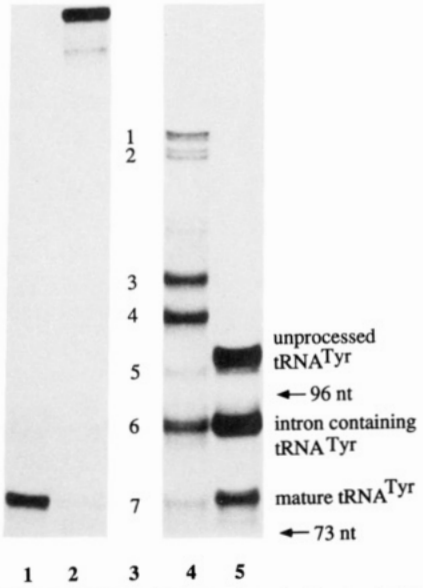

FIGURE 2: Transcription and processing of mitochondrial and nuclear yeast tRNA genes in a yeast S-100 extract. Transcription of 150 ng of template DNA with 3 μL of S-100 extract took place for 30 min at 25 °C; final KCl concentration was 115 mM. Templates used were (lane 1) tRNA^{Val}-mt, (lane 2) SUP4-lac-ter, (lane 4) SUP4-mt, and (lane 5) SUP4-ter. In lane 3 the bands of the SUP4-mt transcripts are indicated by numbers as used throughout the text. Arrows mark the position of radiolabeled size marker RNAs.

(a yeast mitochondrial tRNA^{Val} gene contained within a 2.9-kb mt DNA clone; Thalenfeld & Tzagoloff, 1980), and SUP4-mt (a recombination product made by ligation together of a 5′ *SUP4* fragment and 3′ mt DNA fragment from the tRNA^{Val}-mt gene, each obtained by cutting at an *Xba*I site homologously placed immediately downstream of the two genes) (see Figure 1). Allison and Hall (1985) previously observed that the 320-nt-long direct transcript of SUP4-lac-ter was not processed by the yeast nuclear system into shorter species. In addition, templates derived from *SUP4* constructs containing two similar inserts of pBR322 sequences did not provide any functional suppressor tRNA (Allison & Hall, 1985). Those results imply either that RNA sequences transcribed from *lacZ* and pBR322 inserts inhibit 3′ tRNA processing or, alternatively, that yeast nuclei lack the required endo- or exonuclease.

We have observed quite a different 3′-processing outcome with pol III transcripts of the tRNA^{Val}-mt and SUP4-mt templates that have as trailer the sequence downstream of the yeast mitochondrial tRNA^{Val} gene. This particular mt tRNA gene was chosen as a template because its internal sequences closely resemble the A and B box internal promoter elements of nuclear tRNA genes (Ciliberto et al., 1983). The tRNA^{Val}-mt template is actively transcribed in a yeast S-100 extract (Figure 2, lane 1). Because no mitochondrial promoter has been identified in the 2.9-kb mt DNA insert in tRNA^{Val} (N. C. Martin, personal communication), we ascribe its transcription to RNA polymerase III rather than mitochondrial RNA polymerase. Consistent with this view are the results of competition experiments (data not shown) wherein a plasmid bearing the nuclear *SUP4* tRNA gene partially inhibits tRNA^{Val}-mt transcription, while a plasmid bearing a yeast mitochondrial promoter (pCT10; Fangman et al., 1990) has no effect.

The expected size for a pol III transcript of tRNA^{Val}-mt is about 180 nucleotides since the first possible terminator, a stretch of five T residues in the 3′-flanking sequence, is located 98 bp downstream of the coding sequence of the gene (Figure 1). Transcription of tRNA^{Val}-mt in vitro produced a major RNA of about 78 nucleotides in size and small amounts of larger transcripts that appear to be precursors of the mature mt tRNA^{Val} (Figure 2, lane 1). The appearance of an abundant 78-nt transcript and the lack of substantial amounts of the hypothetical 180-nt precursor suggest that the precursor was efficiently processed at its 3′-end by the S-100 extract. Since this initial test indicated that the mitochondrial 3′-trailer sequence is a suitable substrate for a 3′-processing activity in our S-100 extract, we then tested the 3′-processability of pre-tRNA transcribed from SUP4-mt, a chimera containing a nuclear tRNA coding region and a mitochondrial tRNA^{Val} trailer sequence. The SUP4-lac-ter, SUP4-mt, and SUP4-ter DNAs were transcribed under the same conditions in the yeast S-100 extract (Figure 2). The SUP4-lac-ter template gave rise to the expected precursor of about 320 nt, with very little mature processed tRNA^{Tyr} (Figure 2, lane 2; Allison & Hall, 1985). SUP4-ter produced a precursor of 102–106 nt which is processed into a 92-nt intron-containing intermediate and then to a 78-nt mature tRNA^{Tyr} (Figure 2, lane 5). The SUP4-mt template yielded a transcript of about 200 nt (Figure 2, lane 4, band 1) consistent with termination at the first stretch of five consecutive T residues in the mitochondrial sequence. Several shorter RNAs (named bands 2–7; see Figure 2) were also observed in the transcription products of the SUP4-mt template. The same pattern of RNA bands was also observed using yeast nuclear extracts rather than S-100 extracts (not shown). Two transcripts (bands 6 and 7) comigrated with tRNA^{Tyr} transcripts from SUP4-ter, suggesting that the 200-nt precursor from SUP4-mt can be processed at its 3′-end. However, the precursor of tRNA^{Val}-mt was more efficiently processed than the SUP4-mt pre-tRNA.

Table I: Growth of Yeast Transformants of *SUP4* Alleles with Different 3′-Flanking Sequences[a]

allele expressed in PJ17-6A	–met	–lys	–ade
SUP4-ter (pAsup4-4*)	++	++	++
SUP4-lac-ter (pDG90**/pDA26-90*)	–	–	–
SUP4-mt (pMS110**)	+	–	–
SUP6Δ32-mt (pRo275**)	±	–	–
SUP4-mt/lac-ter (pRo276*)	+	–	–

[a] The listed alleles were transformed into strain PJ17-6A and tested for growth on selective plates, lacking the supplement indicated above the individual columns. (++) growth within 1 day; (+) growth within 2–3 days; (±) weak growth within 5–7 days; (–) no growth within 7 days. All plasmids transformed are described in Materials and Methods and carry the indicated *SUP4* alleles on the single-copy vector pTC3* or YCp50**.

The SUP4-mt Allele Yields Functional Suppressor tRNA. The appearance of transcripts comigrating with mature tRNA^{Tyr} after the in vitro transcription of the SUP4-mt gene suggests that correct 3′-processing occurs. Yeast suppressor tRNA genetics can be used to determine whether correct processing also occurs in vivo; correct 3′-processing of SUP4-mt will lead to a functional tyrosine suppressor tRNA, detectable in vivo through suppression of appropriate auxotrophic marker genes. To this end, yeast nuclear transformants were obtained for the chimeric *SUP4* alleles SUP4-lac-ter and SUP4-mt in yeast strain PJ17-6A, using as vector the *URA3 CEN* plasmid YCp50. Transformants were tested for the ability of the plasmid-borne *SUP4* allele to suppress chromosomal *met4*, *lys2*, and *ade2 ochre*-suppressible markers (Table I), allowing growth on medium lacking methionine, lysine, or adenine. None of the transformants with the SUP4-lac-ter could grow on any of these plates, as found earlier (Allison & Hall, 1985). However, the SUP4-mt plasmid could

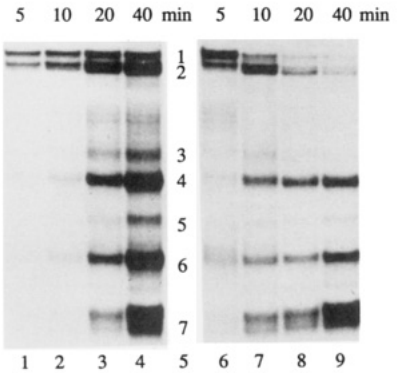

FIGURE 3: Time course of tRNA SUP4-mt synthesis and processing. The SUP4-mt template was transcribed and processed with 3 μL of S-100 extract in 130 mM KCl. Aliquots of the reaction were stopped after the reaction times indicated above the individual lanes. In lanes 1–4 transcription was in the presence of 5Ci/mmol radiolabeled GTP; in lanes 6–9 the specific activity of the radiolabeled nucleotide was reduced 20-fold after 5 min. Lane 5 shows the band numbering as introduced in Figure 2.

support slow growth on a plate lacking methionine but not on plates lacking lysine or adenine, since the *ade2-1* and *lys2-1* alleles require a higher level of suppressor activity for growth (James & Hall, 1990). The finding that SUP4-mt can yield functional suppressor tRNA in yeast transformants proves that its transcript can be correctly processed in vivo. The presence of active suppressor also argues that the appropriate processing activities must be located in the nuclear compartment, as the splicing endonuclease and ligase which are necessary for the proper function of the tRNA *SUP4* are located in the nucleus and splicing is thought to occur only after 5′- and 3′-processing has taken place (Hopper et al., 1978; Knapp et al., 1978).

Characterization of in Vitro Transcripts. The initial in vitro and in vivo tests strongly suggested the presence of a nucleolytic activity in the yeast nucleus. To elucidate the origin and the composition of the different transcripts observed in the in vitro transcription–processing reaction of the SUP4-mt template, the transcripts were analyzed by several means. First, a pulse–chase experiment showed that long transcripts from the SUP4-mt template are formed within a short time and are converted into shorter transcripts after a chase with cold nucleotides. The SUP4-mt template was transcribed for 5 min in the presence of high concentrations of labeled GTP, and then a 20-fold excess of cold GTP was added to the reaction mixture. Aliquots of the reaction were stopped after an additional 0, 5, 15, and 35 min and separated on a denaturing gel. Control samples were taken at the same time intervals without the cold GTP chase (Figure 3, lanes 1–4). In the chased samples (Figure 3, lanes 6–9), the highest molecular weight band (band 1) appeared first and rapidly diminished in intensity after the chase with cold GTP, while the bands of lower molecular weight gained in intensity during the course of the reaction, showing that band 1 is the primary transcript and that the other bands are derived directly or indirectly from band 1.

Second, RNaseT1 fingerprints of several SUP4-mt bands were compared to the fingerprinting patterns generated by $tRNA^{Tyr}$ transcripts. Bands 1, 2, 4, and 6 were isolated and digested with RNaseT1, and the products of the hydrolysis were analyzed by two-dimensional fingerprint analysis (Silberklang et al., 1979) (Figure 4; Table II). Fingerprinting allowed unambiguous identification of transcripts that have been processed at the 5′-end and still contain the intervening sequence. As expected for a primary SUP4-mt transcript,

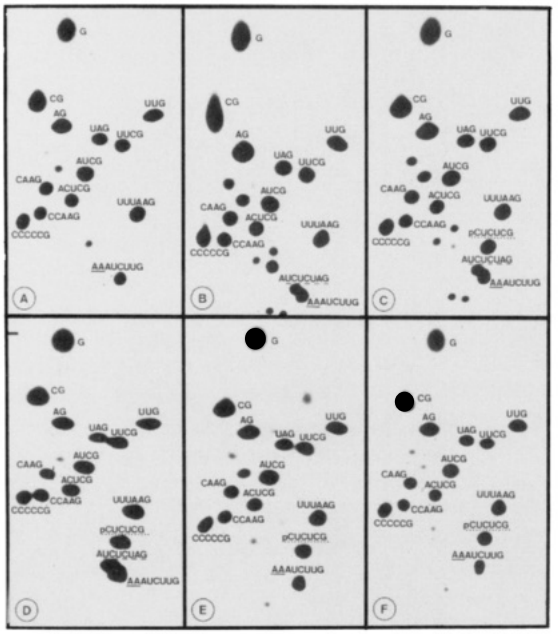

FIGURE 4: RNase T1 fingerprint analysis of [α-^{32}P]GTP-labeled pre-tRNAs. Pre-tRNAs were synthesized as described in Figure 2, and individual bands were isolated from a preparative gel and digested with RNAse T1. Oligonucleotide fractionation was by electrophoresis at pH 3.5 on cellulose acetate in the first dimension (left to right) and by homochromatography in 30 mM KOH on DEAE-cellulose thin-layer plates at 65 °C (bottom to top). The oligonucleotides were identified by comparison with published data (Koski et al., 1980). (—) Nucleotides present in the intervening sequence of pre-$tRNA^{Tyr}$. (- - -) Nucleotides present in the 5′-end of the mitochondrial 3′-trailer. (···) Oligonucleotide derived from the mature 5′-end of $tRNA^{Tyr}$. (A) Pre-$tRNA^{Tyr}$; (B) band 1; (C) band 2; (D) band 4; (E) band 6; (F) unspliced $tRNA^{Tyr}$.

Table II: Diagnostic Oligonucleotides in Two-Dimensional RNase T1 Fingerprints As Depicted in Figure 4[a]

pre-tRNA	pCUCUCG[b]	AAAUCUUG[c]	AUCUCUAG[d]
band 1 of SUP4-mt	–	+	+
band 2 of SUP4-mt	+	+	+
band 4 of SUP4-mt	+	+	+
band 6 of SUP4-mt	+	+	–
pre-$tRNA^{Tyr}$	–	+	–
unspliced $tRNA^{Tyr}$	+	+	–
mature $tRNA^{Tyr}$	+	–	–

[a] The fingerprint of mature $tRNA^{Tyr}$ is not shown. (+) Corresponding oligonucleotide present; (–) corresponding oligonucleotide absent. [b] Oligonucleotide of the mature 5′-end of the $tRNA^{Tyr}$. [c] Oligonucleotide spanning the 3′-splice site of the intervening sequence. The two underlined (—) A residues are part of the intervening sequence. [d] Oligonucleotide spanning the 3′-end of the $tRNA^{Tyr}$ and the mitochondrial 3′-trailer sequence. The mitochondrial sequence is underlined (- - -).

the pattern of band 1 (Figure 4B) shows the spots diagnostic for a pre-$tRNA^{Tyr}$ (Figure 4A); i.e., an oligonucleotide diagnostic for the intervening sequence is present, and the oligonucleotide diagnostic for the mature 5′-end is absent. Bands 2, 4, and 6 generated fingerprints very similar to that of the unspliced $tRNA^{Tyr}$; spots indicating the presence of the intervening sequence as well as the processed 5′-end were found (Figure 4C–F). Bands 3 and 5 were not fingerprinted; however, digestion of bands 1, 3, and 5 with bacterial RNase P (which specifically removes the 5′-leader) reduced their sizes to those of bands 2, 4, and 6, respectively, strongly indicating the presence of the 5′-leader in bands 1, 3, and 5 (not shown). Bands 2, 4, 6, and 7 were not reduced in size upon RNaseP digestion (not shown).

An unprocessed 3′-end is documented in the fingerprint by the presence of the oligonucleotide AUCUCUAG, which spans

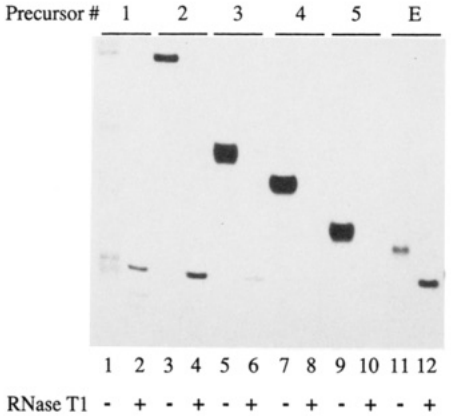

FIGURE 5: RNase T1 digestion of isolated SUP4-mt bands and the processing product band E. The isolated bands used as precursors for the reactions are indicated above the appropriate lanes. About 2000 cpm of each isolated band was digested with 350 units of RNase T1 for 15 min at 37 °C. Band E (see Figure 6, lanes 3, 4, and 6) was isolated after a preparative processing reaction of bands 1 and 2 with S-100 extract. The reaction without RNase T1 (–) contained half the amount of radiolabeled substrate.

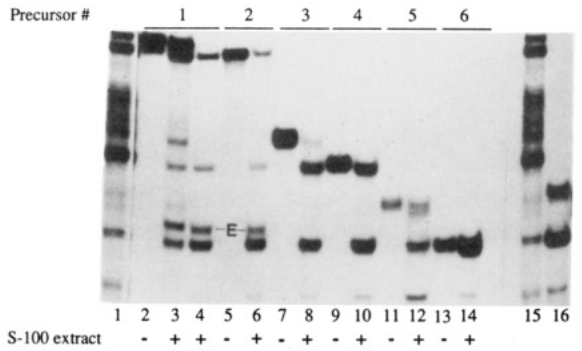

FIGURE 6: Processing of isolated SUP4-mt bands in an S-100 extract. Isolated tRNA precursors (about 3000 cpm/reaction) were processed with 3 μL of S-100 extract at 25 °C for 40 min in 115 mM KCl and 5 mM $MgCl_2$, except for the reaction in lane 3, where the KCl concentration was reduced to 75 mM and the reaction time was 20 min, and in lane 4, where the KCl concentration was 115 mM and the $MgCl_2$ concentration was 10 mM. The isolated bands used as precursors for the reactions are indicated above the appropriate lanes. The reactions without S-100 extract (–) contained half the amount of radiolabeled transcript. Lanes 1 and 15 contain an aliquot of the original transcription reaction of the SUP4-mt template from which the individual bands were isolated. Lane 16 shows the $tRNA^{Tyr}$ transcripts. The low abundance of the mature $tRNA^{Tyr}$ in the processing reactions is due to low splicing activity under the reaction conditions used. Lanes 1, 15, and 16 were exposed for a shorter period of time but run on the same gel. Band E is discussed in the text.

the 3′-end of the $tRNA^{Tyr}$ and the first seven nucleotides of the 3′-trailer. This oligonucleotide was found in bands 1, 2, and 4, indicating that these bands contain at least part of the 3′-trailer (Figure 4, panels B, C, and D, respectively). In contrast, in the fingerprint of band 6 this oligonucleotide was missing (Figure 4E). Band 6 is therefore processed at its 3′-end. For the accurate mapping of the length of the 3′-ends of bands 1, 2, and 4, the fingerprint analysis had technical limitations. The 3′-trailer of the pre-tRNA SUP4-mt does not contain any G residues for most of its length (Figure 1). Therefore, some of the expected fragments from the 3′-end are too large to be separated properly by the fingerprinting technique. To test for the presence of such fragments, complete RNase T1 digests of bands 1–5 were sized on a denaturating polyacrylamide gel. Bands 1 and 2 gave rise to an RNase T1 resistant band of 86 nt (Figure 5, lanes 1–4). Pre-tRNA terminated at the first stretch of five consecutive T residues in the mitochondrial sequence would contain such an 86-nt fragment within its intact 3′-trailer (Figure 1). Bands 3, 4, and 5, on the other hand, did not show substantial amounts of this 86-nt fragment (Figure 5, lanes 5–10), indicating that a full-length 3′-trailer in these bands is no longer present. From denaturing polyacrylamide gels the overall length of bands 3 and 4 was estimated to be around 124 and 112 nt, respectively. Since band 4 is processed at its 5′-end but band 3 is not, both bands are likely to have the same 3′-extension of about 23 nt. This suggests that bands 3 and 4 are 3′-processing intermediates which will subsequently be fully 3′-processed in an additional step that removes the last 23 nt. The estimated size of band 5 is 100 nt. Its gel mobility, slightly ahead of the pre-$tRNA^{Tyr}$, is consistent with the mobility of a pre-$tRNA^{Tyr}$ without any 3′-extension.

Taken together, these experiments show that bands 1, 3, and 5 contain the 5′-leader. Band 1 contains the intact 3′-trailer and band 3 contains a 3′-extension of about 23 nt, while band 5 is likely to be accurately 3′-processed. Bands 2, 4, and 6 are identical to bands 1, 3, and 5, respectively, except that they are processed at the 5′-end. Bands 1–6 still contain the intervening sequence.

In Vitro Processing Reactions. To determine whether a strictly ordered processing pathway was followed, the maturation of the major bands of SUP4-mt was studied in the absence of transcription. Bands 1–6 were isolated from a preparative gel, incubated either with or without S-100 extract at 25 °C for 40 min and then the resulting products were separated on a denaturing gel (Figure 6). Isolated SUP4-mt band 1 could be converted into every other band. The conversion of band 1 into band 2 was more efficient in high-salt buffer (Figure 6, lane 4) than in low-salt buffer (Figure 6, lane 3). Band 2 was converted into bands 4, 6, and 7 but not into band 3 and 5 (Figure 6, lane 6). Band 3 was produced by in vitro processing of band 1 and was further processed into bands 4, 6, and 7 (Figure 6, lane 8). Band 4 could be generated from band 1 via band 2 (Figure 6, lane 6) or from band 3 (Figure 6, lane 8). Band 4 was further processed into bands 6 and 7 (Fig. 6, lane 10). Band 5 was only seen very weakly in the processing reaction of band 1 (Figure 6, lane 3) but not in any other processing reaction. It could be processed further to bands 6 and 7 (Figure 6, lane 12). Band 6 could be processed to form band 7, which comigrated with the mature $tRNA^{Tyr}$ and was not further reduced in size by incubation with S-100 extract (not shown). Increased amounts of band 7 could be observed by using higher salt concentrations in the processing reactions and longer reaction times than used in the experiment shown in Figure 6 (not shown). The finding that isolated bands from transcription of SUP4-mt can serve as processing substrates to yield shorter species further corroborates that these bands are genuine processing intermediates of band 1 rather than prematurely terminated transcription products. It is interesting to note that band 1 can be 3′-processed to yield band 3 without prior 5′-processing, while band 2 is 3′-processed after removal of the 5′-leader. During processing reactions of bands 1 and 2, a new band of about 95 nt in length appears (Figure 6, lanes 3, 4, and 6). The nature of this band (named band E) is discussed below.

Detection of a 3′-Endonucleolytic Fragment. The results discussed above demonstrate that the SUP4-mt pre-tRNA transcript can be matured through several alternative processing pathways in vitro. Processing at the 5′-end can occur independent of whether 3′-processing has taken place. Processing at the 3′-end leads to two subsets of intermediates; one subset (bands 3 and 4) contains a 3′-extension of around 23 nucleotides, and the other (bands 5 and 6) is accurately

processed at the 3′-end. It is possible that two different exo- and/or endonucleases are responsible for the two subsets of 3′-processed intermediates or that one endonuclease makes an initial cut and the remaining 23 nucleotides are removed later. An endonucleolytic cleavage should leave behind an intact 3′-trailer. It was not possible to directly identify a band corresponding to the cleaved-off 3′-trailer in the transcription reactions because of an abundance of weak bands in the region (around 70-100 nts) where the 3′-trailers were expected to migrate. On the basis of its size, we suspected that band E, seen in processing reactions of bands 1 and 2, might be an intact 3′-trailer produced by endonucleolytic cleavage (see Figure 6, lanes 3, 4, and 6). Band E was prepared by incubating about 20 000 cpm of pooled bands 1 and 2 with yeast S-100 extract. After 20 min at 25 °C, the processing products were separated on a polyacrylamide gel and band E was isolated. An aliquot of band E was digested with RNase T1 (Figure 5, lanes 11 and 12) under the same conditions used to study the isolated bands from the transcription reactions (Figure 5, lanes 1–10). Upon RNase T1 digestion, the band E material was shortened from a 95-nt-long fragment to a 86-nt fragment, comigrating with the RNase T1 resistant fragments of bands 1 and 2 (Figure 5, lanes 1–4). This indicates that band E is the cleaved-off 3′-trailer of bands 1 and 2 and must result from an endonucleolytic, rather than exonucleolytic, 3′-processing cleavage in the in vitro reaction. On the basis of the size of band E (migrating just ahead of a 96-nt marker RNA, not shown) and an assumed 3′-trailer length of 95–100 nt, the position of the 3′-endonuclease cleavage is near or precisely at the 3′-end of the $tRNA^{Tyr}$. The accurate 3′-trimming of a few remaining residues could be done by an exonuclease.

An endonuclease that cuts band 1 or 2 very near the 3′-end of the tRNA would directly generate the subset of bands 5 and 6. Since bands 3 and 4 contain 23 nucleotides at the 3′-end, they must be generated by a second 3′-processing activity. A 3′-trailer corresponding to an endonucleolytic cut at this position was not detected under our experimental conditions.

Structural Elements of the Chimeric tRNA Genes Influencing 3′-Processing. In the in vitro transcription–processing reactions described above, we observed a wide range of 3′-processability, with the pre-$tRNA^{Val}$-mt being most readily cleaved, the SUP4-lac-ter not cleaved at all, and SUP4-mt RNA showing intermediate behavior. In order to localize the structural elements responsible for these differences, additional chimeric pre-tRNAs were tested in vitro and in vivo.

The in vitro processability of $tRNA^{Val}$-mt, SUP4-lac-ter, and SUP4-mt was compared with that of $tRNA^{Val}$-lac-ter (a recombination product made by ligation together of a 5′ mt $tRNA^{Val}$ fragment and the 3′ *lacZ* terminator fragment derived from SUP4-lac-ter, each obtained by cutting at the *XbaI* site homologously placed in the two genes; Figure 1). While the 3′-trailer derived from *lacZ* DNA in the SUP4-lac-ter pre-tRNA completely prevents its in vitro processing (Figure 7, lane 5), this 3′-trailer does not block the efficient 3′-processing when fused to the mitochondrial pre-$tRNA^{Val}$. In an in vitro transcription–processing reaction, more precursor was accumulated using the $tRNA^{Val}$-lac-ter template (Figure 7, lane 1) compared to the $tRNA^{Val}$-mt template (Figure 7, lane 2). Nevertheless, the amount of processed product from pre-$tRNA^{Val}$-lac-ter was considerably higher than obtained from pre-tRNA SUP4-mt (Figure 7, lane 4). This indicates strongly that 3′-processability is predominantly determined by the tRNA moiety of the substrate. However, the 3′-trailer sequence can have a modulating effect, as demonstrated by the difference in 3′-processing between the SUP4-lac-ter and SUP4-mt precursors and between the $tRNA^{Val}$-mt and $tRNA^{Val}$-lac-ter precursors.

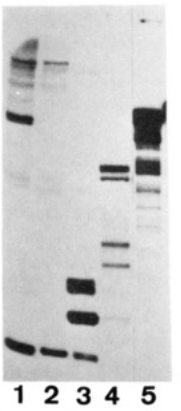

FIGURE 7: Transcription and processing of two mitochondrial $tRNA^{Val}$ alleles with different 3′-trailers. Transcription of 150 ng of template DNA took place for 40 min at 23 °C; final KCl concentration was 120 mM. Templates used were (lane 1) $tRNA^{Val}$-lac-ter, (lane 2) $tRNA^{Val}$-mt, (lane 3) SUP4-ter, (lane 4) SUP4-mt, and (lane 5) SUP4-lac-ter. The last three lanes are shown for reference.

While the chimeric SUP4-mt transcript yields mature and functional $tRNA^{Tyr}$ molecules by the dual criteria of in vitro RNA size measurements (Figure 2, lane 4) and in vivo nonsense mutant suppression (Table I), in both respects it lacks full substrate activity. On the one hand, the $tRNA^{Val}$-mt transcript is much more rapidly 3′-processed in vitro, and on the other hand, the SUP4-ter construct yields much greater in vivo suppression than does SUP4-mt. To explore the basis for this difference, a further alteration was made in the chimeric SUP4-mt gene to increase its structural similarity to the $tRNA^{Val}$-mt gene. The SUP6Δ32-mt gene contains a yeast $tRNA^{Tyr}$ gene identical in structure to the *SUP4* gene but lacking the intron (Johnson & Abelson, 1983); it has the same trailer structure as SUP4-mt. SUP6Δ32-mt gave no better in vitro 3′-processing than SUP4-mt (not shown) and provided less in vivo suppressor activity than SUP4-mt (Table I). This indicates that the intron itself has no negative effect and that more subtle differences in the tRNA structure influence processing rate.

In order to test the effect on 3′-processability of pre-tRNA sequences at or near the endonuclease cut site, the nine base pairs immediately 3′ of the *XbaI* site as well as 2 bp preceding the site were altered. In the resulting construct (SUP4-mt/lac-ter), 14 consecutive base pairs in the cut site region are identical in sequence to the $tRNA^{Val}$-mt trailer (Figure 1); further 3′, the SUP4-mt/lac-ter trailer sequence is identical to that of SUP4-lac-ter. The construct SUP4-mt/lac-ter provided the same level of in vivo suppressor activity as did SUP4-mt (Table I). Thus we have mapped the feature of the *lacZ* trailer sequence that blocks endonuclease processing in SUP4-lac-ter to the immediate vicinity of the *XbaI* site.

DISCUSSION

The yeast mitochondrial $tRNA^{Val}$ gene is actively transcribed and processed in our cell-free yeast S-100 extract. This observation indicates that the S-100 extract contains a 3′-processing activity which cleaves off the expected 100-nt-long 3′-trailer of the pre-$tRNA^{Val}$. We exploited this finding by constructing a yeast nuclear suppressor tRNA gene with an artificially long 3′-trailer, derived from the mitochondrial DNA. The transcript of this chimeric gene SUP4-mt can be

used as a substrate to detect 3′-processing activities in vivo and in vitro. In earlier experiments it was found that the transcript of a SUP4-lac-ter template, containing an insert of *lacZ* or pBR322 DNA between the *SUP4* coding region and the downstream terminator, was not processed at all (Allison & Hall, 1985). In contrast, we have shown by demonstrating in vivo suppressor activity that the transcript of the SUP4-mt template is correctly processed in vivo. In vitro experiments showed that the 200-nt-long pre-tRNA transcribed from the SUP4-mt template can be processed at both its 5′- and 3′-ends in a multistep pathway to produce mature-sized $tRNA^{Tyr}$. On the basis of the detected intermediates of this processing pathway we postulate at least two modes of 3′-processing. One activity removes endonucleolytically a 95-nt-long piece from the pre-tRNA of SUP4-mt, cutting near or precisely at the 3′-end of the coding sequence. A possible remaining short 3′-extension might be removed exonucleolytically. Whether this endonuclease requires a 5′-processed substrate cannot be concluded from our results. Another activity removed about 75 nt from the 3′-trailer of SUP4-mt RNA. While the 3′-moiety of this cleavage escaped detection, each of the 5′-moieties (bands 3 and 4) were stable enough to be accumulated to high levels. These two intermediates differ by the presence of absence of the 5′-leader, respectively, but most probably have the same 3′-extension of about 23 nt. The accumulation of these two intermediates suggests that, under the reaction conditions used, the corresponding nuclease activity is equally reactive with substrates having or lacking a 5′-extension. This behavior is distinct from other eukaryotic 3′-nucleases investigated so far, which prefer 5′-processed substrates (Frendewey et al., 1985; Castano et al., 1985).

The RNA processing substrates studied in vitro cover a wide range of 3′-processability, with the pre-$tRNA^{Val}$-mt and pre-$tRNA^{Val}$-lac-ter being most readily cleaved, the SUP4-lac-ter transcript not cleaved at all, the SUP4-mt RNA showing intermediate behavior. The structural relationships between these genes suggest that the main basis for this difference must lie inside the coding regions of the two tRNA genes. Because the *SUP4* gene has an intervening sequence, and the valine tRNA gene does not, and because we observe intron splicing only *after* 3′-processing of the SUP4-mt transcript, we tested the possibility that the intron of *SUP4* slows the processing of SUP4-mt transcript. We constructed a nuclear tyrosine suppressor allele, SUP6Δ32-mt, which has the same 3′-trailer sequence as SUP4-mt and differs from it internally only by deletion of the 14-bp intervening sequence. Rather than giving increased formation of mature $tRNA^{Tyr}$, the intronless tRNA gene is slightly *less* active than SUP4-mt in vivo, as measured by the respective suppressor activity of the two genes (Table I). A reduced suppressor activity in the absence of the intron was also observed with the native *SUP6* gene and is due to the lack of intron-directed modification of the suppressor tRNA (Johnson & Abelson, 1983). We conclude that the governing factors for substrate reactivity may be subtle structural differences. In yeast mitochondria, Zennaro et al. (1989) have observed that a single base-pair substitution within the TΨ region of an aspartate tRNA gene is sufficient to block removal of a long 3′-trailer from the precursor tRNA.

Although the tRNA coding region seems to be the major determinant for 3′-processing, an interference of the trailer sequences with the coding sequences cannot be excluded and might also influence processing. Additionally, the mitochondrial 3′-trailer sequence confers greater sensitivity to 3′-endonuclease cutting than does the *lacZ* trailer sequence when fused to the nuclear *SUP4* gene. We detected two sites for 3′-nuclease cutting in vitro, one close to the 3′-end of the $tRNA^{Tyr}$ and one around 23 nt downstream of the coding sequence. The endonuclease cut site adjacent to the 3′-end of the tRNA is likely to be relevant for in vivo processing. Sequences immediately 5′ and 3′ of the *Xba*I site, in the region where the endonuclease cut site was mapped in vitro, were altered in the SUP4-lac-ter so that they closely resembled the mitochondrial trailer sequence. Significantly, the resulting chimera, named SUP4-mt/lac-ter (Figure 1), could be processed in vivo, yielding the same *ochre* suppressor activity (Table I) as did SUP4-mt. Hence, the position of the sequences that affect in vivo suppressor activity of tRNAs with the *lacZ*-derived 3′-trailer overlap the endonucleolytic cleavage site mapped in vitro. Additionally, the rather poor activity of the SUP4-mt suppressor gene correlates with the in vitro observation that the pre-tRNA is not an ideal substrate for the endonuclease. The in vivo processability of SUP4-mt/lac-ter also indicates that some features of the sequence near the *Xba*I site are responsible for the failure of SUP4-lac-ter to be efficiently 3′-processed. The sequences that could be processed have a higher AT content than the ones that were not processed and also have decreased opportunity for stem–loop formation.

The in vitro processing site located 23 nt downstream of the coding sequence, yielding bands 3 and 4, seems less likely to be relevant for in vivo processing. This site is present within the *lacZ* sequences in the same position in relation to the tRNA in the processed SUP4-mt/lac-ter RNA as well as in the nonprocessed SUP4-lac-ter RNA. In addition, we do not observe a band 3/4-like intermediate in the in vitro transcription–processing of the SUP4-mt/lac-ter template (not shown). These two bands seem more likely to be specific intermediates of the SUP4-mt RNA and might be very abundant due to their stability.

The endonuclease described in this investigation has the ability to process long 3′-trailer sequences attached to pre-tRNA transcripts. The nuclear tRNA gene with the longest 3′-trailer found to this day ($tRNA^{Phe}$; Valenzuela et al., 1978) contains 27 additional nucleotides. It is also possible that another, yet undiscovered tRNA gene exists in yeast which encodes a long 3′-trailer and which conditionally requires a nuclear 3′-endonucleolytic activity. However, the 3′-endonuclease activity described here might have as its primary function the processing of substrates other than tRNAs; as possible candidates we suggest 5S and 37S rRNA (Piper et al., 1987). Further biochemical characterization, as well as isolation of the gene encoding the 3′-endonuclease, may lead to the discovery of the primary in vivo substrates. With the semisynthetic processing substrates and the different suppressible marker genes used in this investigation, the necessary tools are at hand.

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