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Pleiotropic effect of a point mutation in the yeast SUP4-o tRNA gene: *in vivo* pre-tRNA processing in *S.cerevisiae*

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ABSTRACT

The expression of mutant tyrosine-inserting ochre suppressor SUP4-o tRNA genes in vivo in *S.cerevisiae* was examined as a basis for further studies of tRNA transcription and processing. In vivo yeast precursor tRNAs have been identified by filter hybridization and primer extension analysis. We have previously shown that a mutant SUP4-o tRNA gene with a C52→A52 transversion at position 52 (C52→A52(+IVS) allele) was transcribed but that the primary transcript was not processed correctly. We show here that 5′ and 3′ end processing as well as splicing are defective for this mutant but that the 5′ end processing is restored when the intron is removed from the gene by oligonucleotide directed mutagenesis (C52→A52(-IVS) allele). Our results imply that the C52→A52 transversion by itself cannot account for the lack of susceptibility to RNase P cleavage but that the overall tertiary structure of the mutant tRNA precusor is destabilized by the intron/anticodon stem. A second consequence of the C52→A52 transversion is to prevent complete maturation of the tRNA precursor at its 3′ end since intermediates containing incompletely processed 3′ trailers accumulate in the yeast cells transformed with the C52→A52(-IVS) allele. A correct structure of the T stem might therefore define a structural feature required for the recognition of the 3′ processing activity.

INTRODUCTION

Many studies on eukaryotic tRNA gene transcription and processing have been conducted in vitro (1–6) or after microinjection of cloned tRNA genes into Xenopus oocytes (7–11). Surprisingly little is known of the tRNA processing reactions in vivo. Since the in vitro or microinjection studies may not be representative of the tRNA processing in vivo it is desirable to obtain more results from in vivo studies as a further step in the determination of tRNA transcription and processing. The early work of Fradin and coworkers (12) has allowed the characterization of many precursors to tRNA by two dimensional gel electrophoresis after a brief radiolabeling of yeast *S.cerevisiae* cells. In vivo yeast precursors of $tRNA^{Tyr}$ and $tRNA^{Ser}$ have also been identified using a modification of the Northern hybridization procedure (13). Two species of pre-$tRNA^{Tyr}$ and pre-$tRNA^{Ser}$ were detected in all yeast strains examined. One of the species was the end-matured intron-containing pre-tRNA while the other one was presumed to be the primary transcript. More recently O'Connor and Peebles (14) have studied in vivo pre-tRNA processing in *S.cerevisiae* by using differential oligonucleotide hybridization, size estimation and primer extension analysis. The tRNA processing reactions of several intron-containing pre-tRNAs could be ordered and two alternative pre-tRNA processing pathways operating in vivo for *S.cerevisiae* were proposed. In one pathway, designated end processing first, the 5′ leader and 3′ extension were removed before splicing whereas in the second pathway, designated splicing first, the intron was removed before 5′ and 3′ end trimming. In both pathways the processing reactions used to remove the 5′ leader and 3′ extension were ordered 5′ end trimming before 3′ end trimming.

Genetic methods to identify trans-acting products involved in pre-tRNA processing have also been used (for a review see 15). Mutants affecting the removal of intervening sequence from pre-tRNA, tRNA base modifications or tRNA-nucleotidyltransferase have been described. However little insight into the in vivo pre-tRNA processing pathway has been gained by this genetic approach since the biochemical role of many of the identified genes remains unknown. In this report we analyse the effect of point mutations in the yeast SUP4-o tRNA gene which cause processing alteration of the pre-tRNA. The SUP4-o system has been widely used to detect loss of suppressor tRNA function in vivo by making use of yeast strains containing alleles of genes whose activity can be screened (16–18). It has been shown that many mutations in the tRNA gene inactivate or decrease the level of nonsense suppression. In most cases the nature of the defects caused in vivo by these mutations have not been identified. By using an abortive primer extension assay (19) we have previously observed that several mutants accumulate non processed tRNA in vivo. A C → A transversion at position 52 in the T arm seemed particularly interesting since large amounts of precursor tRNA could be detected. Here we have characterized in more details the effect of mutations at this position on the processing of the transcripts. Our results reveal some features of the tRNA processing pathways operating in vivo in *S.cerevisiae*.

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MATERIALS AND METHODS

Strains and plasmids

The recipient *S.cerevisiae* strain used in this work was strain AB1380 (MAT α, Ψ^+, ura3, trp 1, ade 2-1, can 1-100, lys 2-1,his 5). Ade2-1,can 1-100 and lys2-1 are ochre alleles suppressibles by SUP4-o. The SUP4-o tRNA gene was cloned into the multicopy plasmid pFL1 (20 and figure 1) and propagated in *E.coli* strain 1046 (21). Bacteriophage M13mp19 was grown in *E.coli* strain JM103.

For mutagenesis the SUP4-o gene was subcloned into the M13mp19 vector. The phages were then mutagenized using an Amersham oligonucleotide directed mutagenesis kit; the double stranded thio-DNA was used to transform *E.coli* strain TG1. Mutant clones were identified by DNA sequencing.

Transformation in *S.cerevisiae*

The procedure used for yeast transformation was essentialy as decribed by Hinnen et al. (22) using zymolyase for spheroplast formation. Transformants were selected for a ura3$^+$ phenotype. Cells were grown under selective conditions in S.D medium (containing 6.7 g yeast nitrogen base without aminoacids and 20 g glucose per liter) supplemented with 2 mg/ml casamino acid, 40 μg/ml tryptophan, and 7.5 μg/ml adenine.Two or three days after transformation the colonies which appeared on selective medium were inoculated into liquid selective medium and grown for 20h at 28°C.

Preparation of bulk tRNA from Yeast Cells

Bulk tRNA was isolated from yeast cells as described by Dingermann and Nerke (23) : after growing the transformed cells to logarithmic phase, the cells were collected by centrifugation at 4°C. The pellet was resuspended in TMS buffer (10mM Tris/HCl, pH7.5, 10mM $MgCl_2$, 2mM $Na_2S_2O_3$) and SDS was added to a final concentration of 0.5%. The RNA was extracted with 2 volumes of TMS-saturated phenol for 5min with vigorous vortexing. Bulk RNA was precipitated with 3 volumes of ethanol at −20°C in the presence of 0.3 M sodium acetate. The RNA was recovered by centrifugation, dried, dissolved in TMS buffer, and passed through a DEAE-cellulose (DE 52 Whatmann) column equilibrated with TMS buffer. The column was washed with TMS buffer containing 0.2M NaCl and the tRNA was eluted with TMS buffer containing 0.7 M NaCl. The tRNA was finaly recovered by precipitation with 3 volumes of ethanol at −20°C.

Primer extension analysis

To analyse the 5′ end of tRNA, primer extension was done with AMV reverse transcriptase. A primer complementary to the nucleotides 3 to 20 of the tRNA (primer 1 in figure 1) was 5′ labelled with γ^{32}P ATP using T4 polynucleotide kinase. The primer and total yeast tRNA were precipitated together, resuspended in 5μl reverse transcriptase buffer (50mM Tris/HCl pH 7.4, 100mM KCl, 5mM DTT and 7mM $MgCl_2$), heated 2min at 90°C and allowed to cool down slowly to room temperature. The hybridization mixture was supplemented with 5 μl of reverse transcriptase buffer containing 0.5mM each dATP, dCTP, dGTP and dTTP and 5 units AMV reverse transcriptase. Reverse transcription was performed at 37°C for 1h. 7 μl formamide dye mix were added to the reaction mixture and the extension products were analysed on a 8% polyacrylamide/7M urea gel (40cm long, 0.4mm thick) made up in TBE buffer. Electrophoresis was carried out until the bromophenol blue marker has moved to 5cm from the bottom of the gel.

The abortive primer extension assay was done by using the Klenow fragment of DNA polymeraseI instead of reverse transcriptase as described previously (19).

Polyacrylamide gel electrophoresis and Northern blot analysis

One dimensional denaturing gels were 8% polyacrylamide/7M urea made up in TBE. The gels were run at room temperature using 0.5×TBE as the running buffer. Two dimensional gels were partially denaturing 10% polyacrylamide/4M urea in the first dimension and 20% polyacrylamide/4M urea in the second dimension. The gels were run at 4°C using TBE as the running buffer. The gels were stained with methylene blue (0.2% methylene blue (w/v) in 0.2M sodium acetate pH 4.6) and destained by soaking for several hours in tap water.

For electrophoretic transfer, the gel and the membrane (Hybond N) were equilibrated in 50mM sodium phosphate pH6.5 for 10−20min. Transfer of the RNA from the gel onto the membrane was done in 50mM sodium phosphate pH6.5 at 0.1A/7V for 16h at room temperature. RNA was covalently attached to Hybond by UV cross-linking. The blot was then prehybridized in 0.1% SDS, 6×SSPE, 10×Denhardt solution, 50 μg/ml denatured salmon sperm DNA for 2h at 37°C. A ^{32}P end labelled oligonucleotide complementary to bases 42 to 55 of the tRNA (primer 2 in figure 1) was used as a hybridization probe. Blots were washed two times 10min in 0.1% SDS, 2×SSPE at room temperature. Kodak XAR films were used for autoradiography. Exposures were made at room temperature without intensifying screen.

RESULTS

Mutagenesis of the SUP4-o tRNA gene and in vivo expression of the SUP4-o tRNA alleles

The SUP4-o tRNA gene encodes a nonsense suppressor derived from one of the eight yeast tRNA^{Tyr} genes. It contains a small intron which must be removed from the transcript prior to formation of a functional gene product. The SUP4-o tRNA gene cloned into M13 mp19 phage DNA was mutagenised by oligonucleotide directed mutagenesis to generate the three possible base substitutions at position 52 (C 52→ A 52, C 52→T 52, C52→G52, figure1). We have also removed the intron by mutagenesis in order to be able to distinguish more easily splicing defects from end processing defects (−IVS mutants). The mutant SUP4-o tRNA genes were inserted in the multicopy vector pFL1 (20) and introduced into *S.cerevisiae* by transformation. The pFL1 vector which contains the 2,2kb replication origin ORI of the yeast plasmid 2μm circle and the yeast URA3 marker is present at a high copy number per cell and transforms uracil auxotrophic yeast cells to prototrophy. The recipient *S.cerevisiae* strain AB1380 carries an ade2-1 ochre allele suppressible by the SUP4-o tRNA gene. Cells expressing a functional suppressor tRNA form white colonies. An unsuppressed ade2-1 allele leads to a block in the adenine pathway and confers a red pigmentation to the colonies if the cells are grown on limiting adenine plates (7.5 μg/ml). Mutations that reduce the suppressor activity result in pink colonies. The ability of mutant SUP4-o tRNA gene to express functional products can therefore be analysed by monitoring the degree of red color formation which develops in the transformed yeast. The results obtained for the SUP4-o tRNA genes mutagenized at position 52 are summarized in table 1. Only cells transformed with the wild type SUP4-o tRNA gene are able to form white colonies. Cells transformed with mutant alleles

show subtle differences in the level of suppression since the degree of red color formation is not identical for the various mutants. The C52→G52 (−IVS) mutant is clearly red whereas the other mutants are pink. We have next measured the steady state level of SUP4-o transcripts for the various mutants by using an abortive primer extension assay which allows identification of particular tRNA gene products in an homologous background (19). In this assay the primer extension is done in the absence of dCTP so that DNA synthesis arrests at the position of the first G downstream of the primer. The position of the primer is chosen so that there is a clear distinction between the extension products of the SUP4-o tRNA and the $tRNA^{Tyr}$ genes transcripts. With the primer used in this work the extension products are 25 nucleotides in length for spliced SUP4-o transcripts or transcripts from intronless SUP4-o tRNA alleles, 21 nucleotides for spliced Tyrosine transcripts and 20 nucleotides for unspliced Tyrosine or SUP4-o transcripts. The 21 nucleotides band serves as a internal control for the efficacy of RNA extraction and primer extension reaction. The results of an abortive primer extension experiment are shown in figure 2. The steady state level of transcripts is different for each mutant. As mentioned in the Introduction unspliced mutant SUP4-o tRNA precursors accumulate in yeast cells transformed with the C52→A52 (+IVS) allele: in figure 2 (slot f) an intense 20 nucleotides band indicates the presence of large amounts of unspliced tRNA. If the intron is removed from the C52→A52(+IVS) allele by mutagenesis, an accumulation of mutant transcripts in transformed cells is also observed. This is shown in figure 2 (slot c) where the 25 nucleotides band is a measure of the steady state of the C52→A52(−IVS) transcripts. The steady state level of transcripts

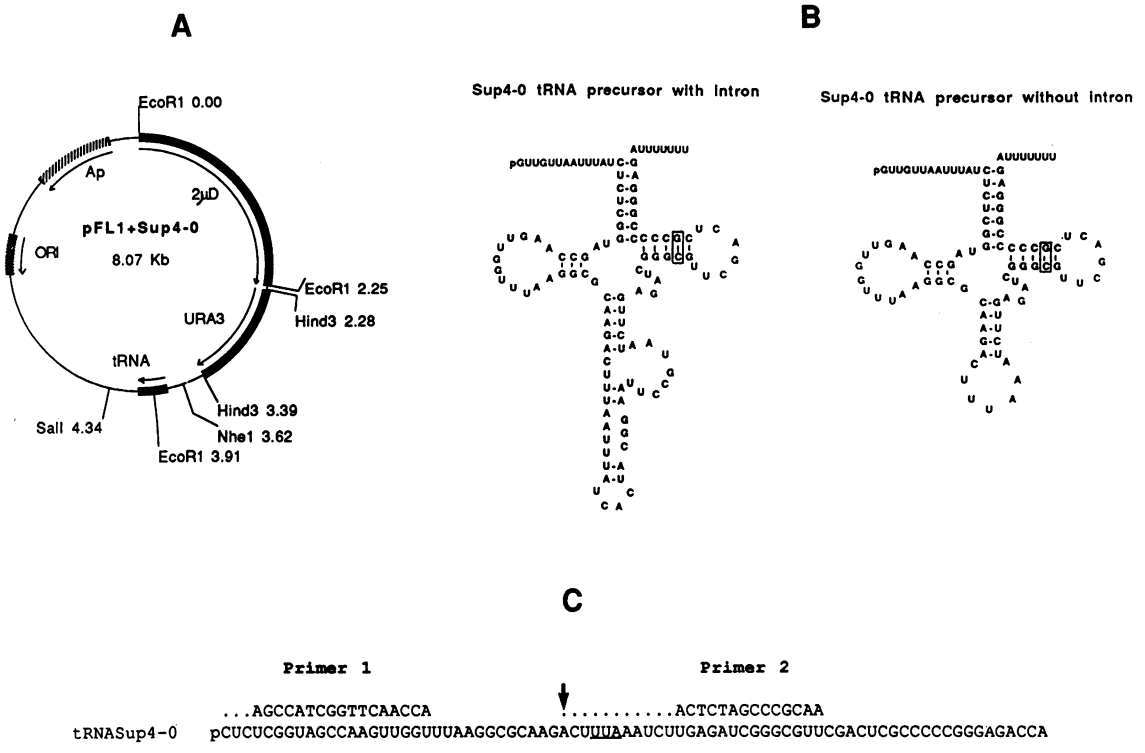

Figure 1. A) Restriction map of the pFL1+SUP4-o vector. The SUP4-o tRNA gene can be retrieved from the plasmid by digestion with Nhe1 and Sal1. Ap, ampicillin resistance gene.Ori, origin of replication in *E.coli*. The thick lines indicate DNA of the 2μm yeast plasmid and of the ura3 marker. The arrows indicate the direction of transcription of the genes. **B)** Secondary structure for precursor SUP4-o tRNA with and without intron. Nucleotides C52 and G52 which are base paired are enclosed in a box. Position 52 was changed to the three possible alternative nucleotides by site-directed mutagenesis. **C)** The nucleotide sequence of the yeast mature SUP4-o tRNA. The position of the oligonucleotide primers used in the primer extension experiments is shown above the tRNA sequence. Primer 1 was used to determine the 5′ end of the transcripts. Primer 2 was used in the abortive primer extension assay, the arrow indicates the 3′ end of the extension product of spliced SUP4-o transcripts.

Table 1. Summary of suppression assay and steady state level of SUP4-o tRNA gene products

SUP4-o allele	IVS in the gene	Suppression of ade2-1	Steady state level of tRNA without intron	Steady state level of precursor	Possible cause for inefficient suppression
WT	+	+++(white)	++	−*	
C52→A52	+	+/− (pink)	−	++	poor 3′ and 5′ processing, poor splicing.
C52→A52	−	+/− (pink)	+++	+++	poor 3′ end processing.
C52→T52	−	+/− (pink)	+	+/−	low level of transcript, poor 3′ end processing.
C52→G52	−	− (red)	+	+/−	low level of transcript, poor 3′ end processing

* Precursor bands can be detected only on overexposed films.

obtained from cells transformed with the two other mutants – C52→T52(−IVS) and C52→G52(−IVS) – was also measured by the primer extension assay. As shown in figure 2 (slots d and e) the accumulation of transcripts is decreased by a factor of 10 to 20 for these two mutants. The level of C52→G52(−IVS) transcripts is lower than the level of C52→T52(−IVS) transcripts (compare the intensity of the 25 and 21 nucleotides bands in slots d and e) and could explain that the yeast cells transformed with the C52→G52(−IVS) allele are red. In figure 2 slots a and b controls obtained with untransformed cells (slot b) and cells transformed with the wild type gene (slot a) are also shown. It is worth noting that the level of wild type transcripts is lower than the level of C52→A52(−IVS) transcripts. Since base C52 is located in box B of the internal promotor region, our results show that mutation of this base can result either in an increase of the promotor strength in the case of the C→A mutation or to a promotor down effect in the case of the C→T or C→G mutation. However the level of expression of the gene is always sufficiently high to allow detection of the transcripts by our primer extension assay. Therefore the reduction or lack of suppression by the different mutants cannot be explained by a lack of transcription of the gene. The results described below show that the reduction of suppression caused by mutations at position C52 is due to a defect in RNA processing.

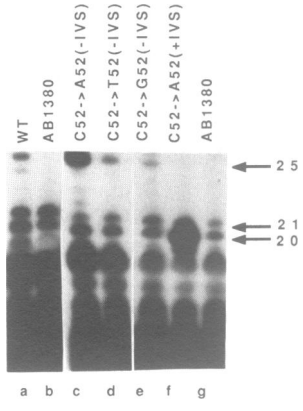

Figure 2. Steady state level of mutant SUP4-o tRNA in transformed yeast cells: An abortive primer extension assay was performed on RNA prepared from yeast cells transformed with the wild type (WT) SUP4-o tRNA gene, the C52→A52(−IVS), C52→T52(−IVS), C52→G52(−IVS) and C52→A52(+IVS) alleles. A control was made with tRNA extracted from untransformed yeast cells (AB1380). The transcripts were assayed by extending a 14 nucleotides primer (primer 2 in figure 1) with the Klenow fragment of DNA polymerase I in the presence of dTTP, dATP and dGTP as described in reference 19. The primer extension products were analysed on a 8% polyacrylamide/7M urea gel. The 25 nucleotides band is a measure of the steady state level of spliced SUP4-o transcripts or transcripts from intronless SUP4-o tRNA alleles, the 21 nucleotides band measures the level of spliced Tyrosine transcripts and the 20 nucleotides band the level of unspliced Tyrosine or SUP4-o transcripts.

Processing defects caused by mutations at position C52

The nature of the defect caused by mutations at position C52 was further characterized by probing Northern blots with a ^{32}P-labelled oligonucleotide complementary to the SUP4-o tRNA (figure 3). Large amounts of precursor tRNA are detected in cells transformed with the C52→A52(+IVS) and C52→A52(−IVS) alleles (figure 3, slots a and c or g). For cells transformed with the C52→T52(−IVS) or C52→G52(−IVS) alleles it is possible to detect precursor bands on the Northern blots after a longer exposure of the films (figure 3, slots e and f). This is consistent with the primer extension results showing that the accumulation of transcripts is decreased by a factor 10 to 20 for these two mutants. In cells transformed with wild type unmutated SUP4-o tRNA gene, precursors can only be detected on overexposed films indicating that the unmutated SUP4-o pre-tRNA is perfectly processed and spliced (figure 3 slot b).

The tRNA precursor band which accumulates in cells transformed with the C52→A52(+IVS) allele has the size of a primary transcript containing the 5′ leader sequence, 3′ trailing sequence and the intron (figure 3, slot a). No intermediate precursor band is observed between the primary transcript and the mature tRNA^{Tyr} transcript. End processing as well as splicing is thus defective for the C52→A52(+IVS) mutant. Surprisingly the C52→A52(−IVS) mutant accumulates two precursors (figure 3, slots c or g): a slow moving band which corresponds to the primary transcript with the 5′ and 3′ extensions and a broad intermediate band moving slightly above the mature tRNA^{Tyr}. Northern blots of tRNA extracted from yeast cells transformed with the C52→T52(−IVS) and C52→G52(−IVS)

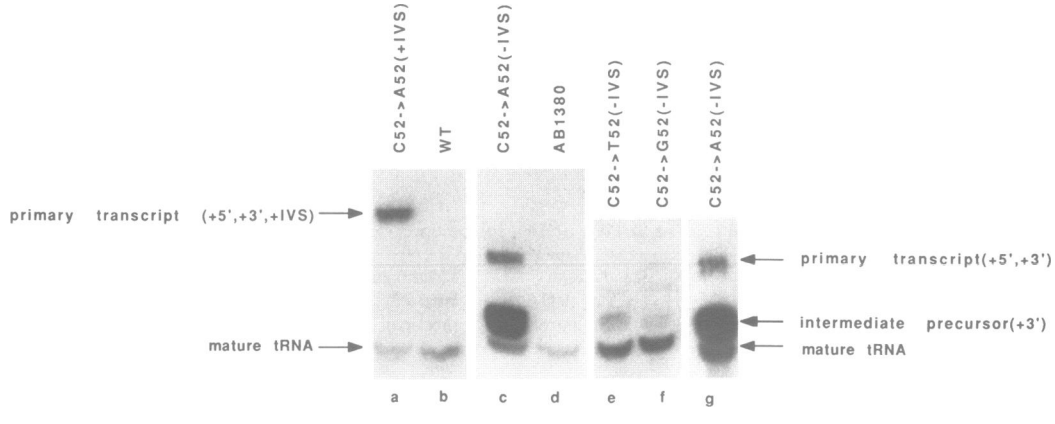

Figure 3. Accumulation of mutant tRNA precursors: Northern analysis was performed as described in Materials and Methods. 20μg of total tRNA was loaded in each lane. Lanes marked AB1380 correspond to tRNA extracted from untransformed cells. The other lanes correspond to tRNA extracted from yeast cells transformed with the wild type (WT) SUP4-o tRNA gene, the C52→A52(+IVS), C52→A52(−IVS), C52→T52(−IVS) and C52→G52(−IVS) alleles.

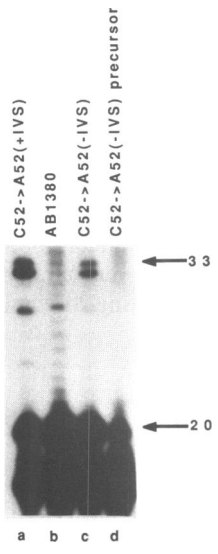

Figure 4. Primer extension analysis of the 5′ termini of tRNA isolated from transformed yeast cells. A ^{32}P-labelled oligonucleotide (primer 1 in figure 1) complementary to nucleotides 4–20 of SUP4-o tRNA or tRNA^{Tyr} was hybridized to 10μg of RNA. The primer extension was performed as described in Materials and Methods and the products of the reaction analysed on a 8% polyacrylamide/7M urea gel. The 20 and 33 nucleotides bands correspond to the addition of 3 and 16 nucleotides to the primer. The primer extension was performed with bulk tRNA extracted from cells transformed with the C52→A52(+IVS) allele (lane a), C52→A52(−IVS) allele (lane c), untransformed cells (lane b), or with the C52→A52(−IVS) precursor fractionated on a polyacrylamide gel (lane d).

alleles reveal that the same intermediate precursor is detected in the tRNA extracted from these cells. The size of this intermediate band suggests that the 5′ leader sequence has been cleaved by RNase P.We have confirmed that this is indeed the case by 5′ end analysis. Total tRNA extracted from yeast cells transformed with the C52→A52(−IVS) allele was fractionated on a denaturing polyacrylamide gel. The fraction containing the intermediate tRNA precursor was eluted from the gel, annealed to an oligonucleotide complementary to nucleotides 4 to 20 of the tRNA (primer 1 in figure 1) and extended with reverse transcriptase. The result of this experiment is shown in figure 4. When the gel purified precursor tRNA is used as a template (slot d), reverse transcriptase adds only three nucleotides to the primer as expected from a 5′ processed tRNA. Total tRNAs extracted from cells transformed with the C52→A52(+IVS) or C52→A52(−IVS) alleles were used as controls (slots a and c). For these two samples, the primer is extended 16 nucleotides to the expected 5′ end of the precursor with a 13 nucleotides leader. It is therefore clear that the C52→A52(−IVS) mutants accumulate a processing intermediate in which the 5′ leader has been removed but has not been trimmed at its 3′ end. The presence of an intermediate precursor band in the Northern blots of the C52→T52(−IVS) and C52→G52(−IVS) mutants shows that the C→T and C→G substitutions also result in an incomplete maturation of the 3′ end of the tRNA. The intermediate 5′ processed precursor band detected on the Northern blots is very broad and probably reflects a size heterogeneity of the transcripts. This was confirmed by a two dimensional gel electrophoresis experiment (figure 5): in the Northern blot of a two dimensional gel of the tRNA extracted from cells transformed with the

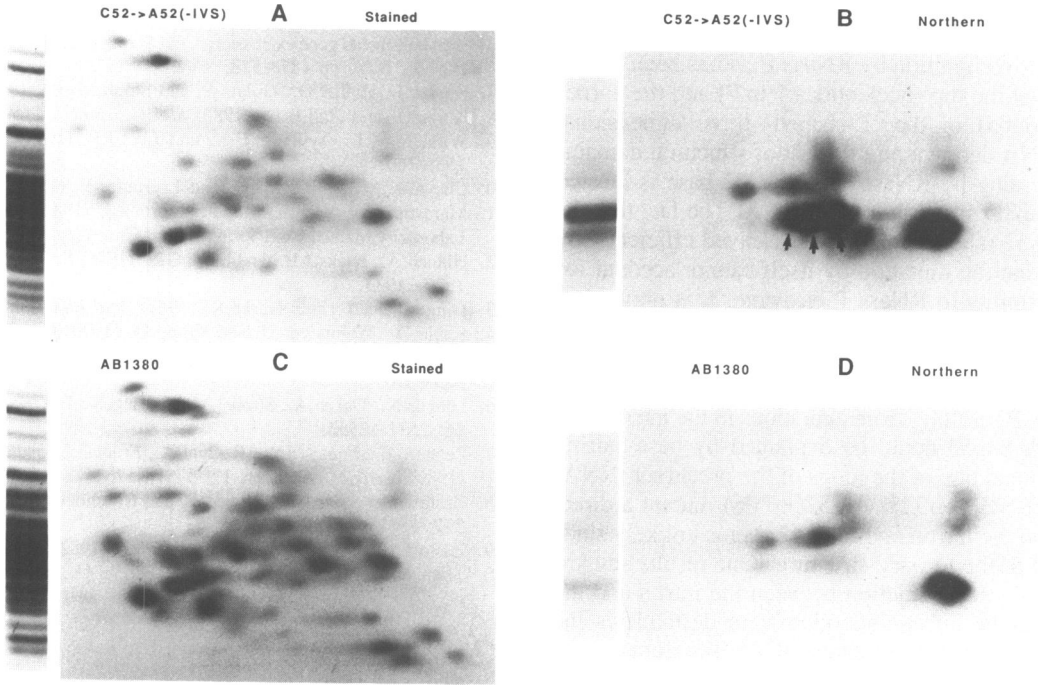

Figure 5. Two dimensional separation of tRNA and their precursors. Electrophoresis in the first dimension was from top to bottom and that of the second dimension from left to right. The two left panels (A and C) show the stained gels. The RNA was subjected to Northern analysis. Autoradiography of the blots are shown in the right panels (B and D). In panel B, the arrows point to the three SUP4-o tRNA precursor species present in cells transformed with the C52→A52(−IVS) allele. Panels A and B: analysis of the tRNA from cells transformed with the C52→A52(−IVS) allele. Panels C and D: analysis of the tRNA from untransformed AB1380 cells.

C52→A52(−IVS) allele, at least three species of precursors can be resolved (figure 5, panel B). This size heterogeneity is consistent with variable amounts of remaining 3′ trailer and reflects partial 3′ exonucleolytic processing slowed down by the C52→A52 mutation.

CONCLUSION

Nuclear tRNA are transcribed by RNA polymerase III and are first synthesized as precursors containing 5′ extended leaders, 3′ trailer sequences and intervening sequences in the case of split genes. In vitro experiments with purified enzymatic activities (1−6) and results of oocytes microinjection studies (7−11) imply that the processing reactions of yeast pre-tRNA occur in a sequencial order. The first pre-tRNA processing reaction is the endonucleolytic removal of the 5′ leader by RNase P. The 3′ extension is subsequently removed by an endonuclease or exonuclease activity. The intervening sequence is then excised and the two half molecules religated to produce mature tRNA. Additional reactions include nucleotide modifications and the addition of CCA to the 3′ terminus. In vivo the order of processing reactions appears to be more flexible since splicing may occur either before or after end trimming. In the case of yeast $tRNA^{Tyr}$ the end processing first pathway seems to be preferred in vivo (14). The results described in this report show that this order is not obligatory since the spliced C52→A52(−IVS) mutant SUP4-o tRNA precursor is a substrate for RNase P whereas the unspliced precursor cannot be trimmed at its 5′ end. This is rather surprising since it has been suggested that intron containing tRNA precursors form a structure consisting of a tRNA-like domain with the conserved secondary and tertiary interactions characteristic of their mature tRNA counterpart and an IVS-containing domain extending from the anticodon stem (intron/anticodon stem). The tRNA-like structure of the precursor provides feature for recognition by RNase P. It has been shown recently (24,25) that the top (nucleotides 1 to 7) and the corner (nucleotides 49 to 65) of the 'L-shaped' three dimensional structure of the tRNA define a minimal set of structural domains required for processing by RNase P. The C52 base is located in the corner of the 3D structure of the tRNA. The fact that the intronless C52→A52 mutant precursor is cleaved efficiently by RNase P implies that the mutation by itself cannot account for the lack of susceptibility to RNase P cleavage. It is only when the intron is present that the mutant precursor tRNA is not processed by RNase P. This result can be compared to the in vitro studies of Leontis et al.(26) who have shown that inefficient cleavage by RNase P resulting from alterations in the intervening sequence of tRNA Sup53 could be explained by base-pairing between the intron and one of the loops of the precursor tRNA-like domain. In the SUP4-o C52→A52(+IVS) mutant a direct interaction between the intron and base 52 seems unlikely since this base is buried in the tertiary structure. Our results suggest that although there is no interaction between the intron and the tRNA-like domain, the intron/anticodon stem destabilizes the overall tertiary structure of the mutant tRNA-like domain and prevents recognition by RNase P.

A second consequence of the C52→A52 mutation is to prevent complete maturation of the precursor at its 3′ end. Only three other mutant tRNA genes with similar processing defects have been reported: a C→T transition at position 61 of the mitochondrial $tRNA^{Asp}$ gene of *S.cerevisiae* (27), a G→T transversion at position 56 of human $tRNA_i^{Met}$ (28,29) and four mutations (T51, A54, A57 and A63) in human $tRNA^{Val}$ (30). It is striking that for all mutants the mutations are located in the T loop or stem. A correct structure of the T loop and stem might therefore define a structural feature required for the recognition of the 3′ processing activity.

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