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Effects of tRNA-intron structure on cleavage of precursor tRNAs by RNase P from *Saccharomyces cerevisiae*

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ABSTRACT

RNase P derived from *S.cerevisiae* nuclei was tested for its ability to cleave a variety of naturally occurring and selectively altered precursor-tRNA molecules to yield matured 5' termini. Precursors were synthesized *in vitro* in order to test which aspects of substrate structure are crucial to recognition and cleavage by RNase P. Base modifications in the precursor substrates are not required for cleavage by the enzyme, but deletion and substitution mutations affecting any portion of the precursor tertiary structure reduce cleavage. In particular, a number of alterations in the intervening sequence (IVS) reduce the susceptibility of the substrate to cleavage by RNase P. The significance of these results is discussed in reference to the contribution of the IVS to the structure of the precursor-tRNA.

INTRODUCTION

Maturation of tRNA primary transcripts in eukaryotes involves a series of processing steps including 1) trimming of extraneous 5'- and 3'-terminal sequences, 2) removal of intron sequences, 3) modification of specific bases, and 4) addition of 3'-terminal CCA. The 5'-leader sequences of precursors are removed by the action of an endonuclease, RNase P, which cleaves its substrates at a single site to generate the phosphorylated 5'-terminus of the mature tRNA. This reaction appears to be independent of the RNA base sequence at the site of cleavage but has been found to be sensitive to the secondary and tertiary structure of its substrates (1-3). The exact sequence of events along the tRNA maturation pathway in eukaryotes is not known, but the removal of 5'-leader sequences appears to occur at an early stage. Yeast mutants deficient in tRNA splicing accumulate end-matured intermediates with some modified bases, indicating that RNase P cleavage in yeast may precede removal of the intervening sequence (4, 5a).

Prokaryotic RNase P is a ribonucleoprotein; the isolated RNA component of the enzyme can itself correctly cleave the 5'-ends of pre-tRNAs in the total absence of protein (6-11). RNase P activity has also been identified in, or partially purified from a number of eukaryotic sources including human KB cells (12), veal heart (13), HeLa mitochondria (14), chick embryos (15), Xenopus oocytes (16), and yeast (11). RNase P isolated from nuclei of the fission yeast *S.pombe* contains two RNA species essential to function (17). The nuclear and mitochondrial RNase P activities of *S.cerevesiae* also appear to contain RNA molecules which are integral to function (18-20).

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A number of studies have shown that mutations designed to disrupt the tertiary structure of the mature tRNA domains reduce the susceptibility of the pre-tRNA to 5'-end maturation by RNase P (21-23). Moreover, it has been reported recently that a mutation which reduces the base-pairing potential between the anticodon and the intervening sequence in a pre-$tRNA^{Ser}$ from *S.pombe* results in decreased cleavage by the RNase P from *S.cerevisiae* (24). Here we report on mutations within the intron of pre-$tRNA_3^{Leu}$ (SUP53 allele) from *S.cerevisiae* that are found to interfere with 5'-end processing by the homologous RNase P, and which also alter the overall tertiary structure of the precursor.

MATERIALS AND METHODS

Enzymes. RNase T1 was obtained from Sankyo, S1 nuclease and cobra venom RNase from Pharmacia, and proteinase K from Beckman. SP6 RNA polymerase was prepared by the method of Butler & Chamberlin (25). The plasmid pSP65 was obtained from Promega Biotec.

Preparation of tRNA precursors. *DNA templates for synthesis of pre-tRNA substrates.* The cloned yeast $tRNA^{SUP53}$ gene and the mutations used in this study have been described (23, 26, 27). Transfer-RNA^{SUP53} is an amber allele of $tRNA_3^{Leu}$. The genes were transferred into the polylinker site of plasmid pUC9 for the preparation of DNA templates to be used for *in vitro* transcription (28).

In order to prepare unmodified pre-tRNA substrates, tRNA genes were cloned into the polylinker of plasmid pSP65 downstream of the promoter for SP6 RNA polymerase. The Rsa I-Alu I DNA fragment containing the $tRNA^{SUP53}$ gene was inserted into the Sma I site of pSP65. The Mbo I fragment containing the yeast mitochondrial $tRNA^{Glu}$ gene (29) (the gift of G. Homison and A. Tzagoloff) was inserted into the Bam H1 site of the same vector.

Synthesis of pre-tRNAs. Pre-tRNAs were synthesized *in vitro* by transcription of cloned genes using a yeast nuclear extract prepared as described previously (30). The transcripts were internally labelled by incorporation of α-^{32}P-UTP (New England Nuclear). The reaction mixtures included 30-50μCi of α-^{32}P-UTP, 0.020mM UTP, 0.25mM ribo-ATP, CTP, and GTP, 150mM KCl, 8mM $MgCl_2$, and 1mM phosphoenolpyruvate.

Unmodified, labelled pre-tRNAs were synthesized by transcribing super-coiled pSP65 plasmids (Promega Biotec) containing tRNA genes with SP6 RNA polymerase essentially as described (31). Transcripts were purified by electrophoresis on 6% polyacrylamide gels containing 8M urea. Precursor bands were identified by autoradiography, cut out of the gel, eluted in 0.1M sodium acetate buffer (pH 6.0) containing 10mM EDTA and 1% phenol, and precipitated with ethanol.

Characterization of pre-tRNA transcripts. The 5' and 3' termini and the RNase P cleavage sites of pre- $tRNA^{SUP53}$ synthesized using yeast nuclear extract have been determined (30) and are summarized in **Figures 1** and **4a.** Transcription *in vitro* by RNA polymerase III initiates 10 base-pairs upstream from the 5'-end of the mature tRNA and terminates within the T-

rich sequence following the gene. RNase P produces the correct 5' terminus *in vitro* by endonucleolytic cleavage (30). S1 nuclease was employed to determine the termination and RNase P cleavage sites of RNA synthesized by SP6 RNA polymerase from the SUP53 and mitochondrial $tRNA^{Glu}$ genes cloned into pSP65 (data not shown). These results are summarized in **Figure 4b.** The SUP53 (SP6 transcript) studied here contains a 91-base 5'-leader sequence. Approximately 10% of the SP6-synthesized SUP53 terminated in the poly-T tract following the gene, and included two extra bases at the 3'-end not found in the SUP53 transcripts prepared from yeast extracts. Approximately 85% of the $tRNA^{Glu}$ transcripts terminated in the poly T site 120 residues downstream of that gene. Transcription products were purified by electrophoresis through polyacrylamide denaturing gels, prior to assay for RNase P cleavage.

Partial Purification of RNase P. Subcellular extracts were prepared from the haploid yeast strains EJ101 (obtained from Elizabeth Jones) and A161 rho^0 petite (the gift of Philip Perlman) as previously described (30). Results presented here were obtained with the enzyme from the EJ101 strain. RNase P from the rho^0 strain (lacking mitochondrial DNA) was subsequently isolated by the same procedure and gave qualitatively identical results using both the nuclear and mitochondrial pre-tRNAs as substrates, although the enzyme was less stable to purification. All fractionation steps were performed at 0-4°C.

RNase P activity was bound to BioRex 70 ion exchange resin (BioRad) in HGMED buffer [20mM HEPES (pH 7.9), 10% glycerol, 8mM $MgCl_2$, 0.1mM EDTA, 2mM DTT] containing 300mM KCl and was eluted in a single peak by raising the KCl concentration to 1000mM. The activity was precipitated from solution by dissolving crystalline ammonium sulfate (Boehringer Mannheim, Ultra-Pure) to the point of saturation. The precipitate was collected at 4°C by centrifugation at 10,000 rpm for 30 minutes in a Sorvall HB-4 rotor, resuspended in HGED buffer (HGMED lacking $MgCl_2$) to a final ammonium sulfate concentration of 50mM and applied to a column of DEAE cellulose (Whatman DE52). The column was washed with HGED buffer containing 50mM and then 100mM ammonium sulfate and the RNase P activity was eluted with HGMED containing KCl to a final ionic strength 5% lower than the previous wash. The overall increase in specific activity was difficult to determine due to the presence of RNase P inhibitors in the crude nuclear extract, but was estimated to be 2000- to 5000-fold, on the basis of protein removal. The enzyme was inactivated by digestion with either proteinase K or micrococcal nuclease (J.Lee, E.Prokhov, & D.Engelke, in preparation) in agreement with the observations made on the RNase P activity from *S. pombe* (11).

RNase P assays. RNase P reactions were performed at 30°C in a total volume of 50 µl containing 2mM HEPES (pH 7.9), 8mM $MgCl_2$, 110mM KCl, 1% glycerol, 0.2mM DTT, 50-75fmol tRNA precursor, and 2.5µl of an RNase P fraction. The time course of RNase P digestion of a given pre-tRNA was studied by taking 10µl aliquots at various times after the addition of RNase P and quenching the reaction by the addition of 2µl stop mix [2% SDS, 100mM EDTA (pH 8.0), 1 mg/ml carrier tRNA, and 1 mg/ml proteinase K]. Digestion with proteinase K was carried out for

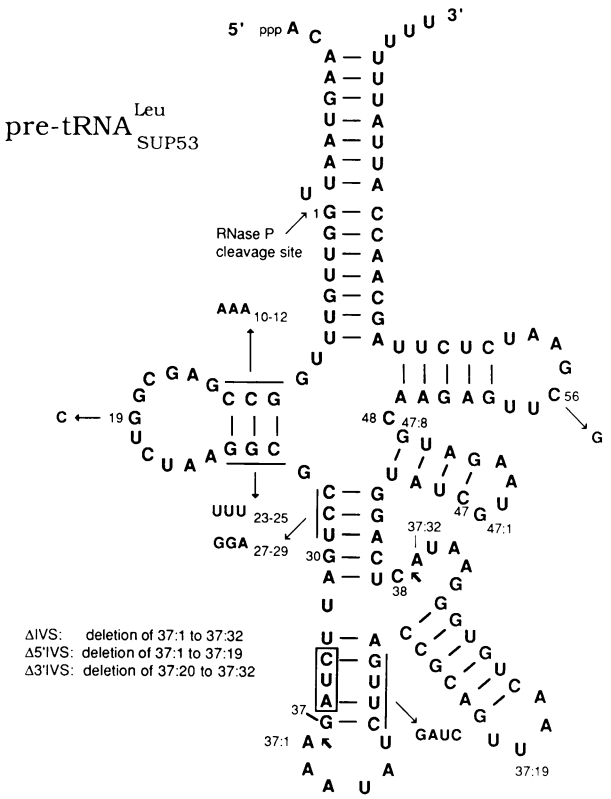


Figure 1: Precursor- tRNA^{SUP53} mutations, according to Lee and Knapp (35). The position of the intron sequence is indicated by two heavy arrows. Nucleotides are numbered according to the conventional numbering of conserved bases. Intron nucleotides are numbered 37:1 to 37:32. Mutations used in this study are indicated in the figure.

90 minutes at 50°C and the RNA was recovered by precipitation with 2.5 volumes of ethanol. The RNA was denatured by heating to 95°C in deionized formamide prior to electrophoresis on denaturing polyacrylamide gels containing 8M urea and 6% or 10% polyacrylamide (32). Radiolabelled RNA was detected by exposure of the gels for 30-40 hours on Kodak XAR5 film with a DuPont Lightning Plus intensifier screen. All results shown in **Figures 2, 3,** and **4b** were obtained with a single enzyme fraction.

Preparation of end-labelled pre-tRNA transcripts. End-labelled pre-tRNAs were prepared for structure analysis by transcribing plasmid DNA using yeast extract in the presence of 300-500μCi of γ-^{32}P-ATP (30). Plasmid pUC9 DNA (1μg) containing the gene of interest was transcribed with 12μl of yeast extract in a volume of 50μl. The reaction mixtures contained 250 μM each of UTP, CTP, and GTP, 50 μM of unlabelled ATP, 8mM $MgCl_2$, and 50mM KCl. The reactions were carried out at 30°C for 8 minutes and quenched by digestion with 10μl stop mix (see

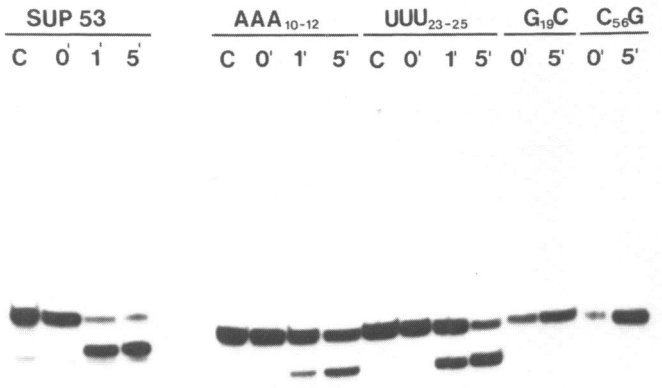


Figure 2: Cleavage of tRNA^{SUP53} precursors with altered D and TΨ arm sequences. Yeast pre-tRNA primary transcripts synthesized by yeast extract were subjected to cleavage with RNase P as described in Materials and Methods. All RNAs were digested with identical aliquots from the same RNase P fraction. Digestions were carried out for the indicated length of time (in minutes) and analyzed by electrophoresis through 10% polyacrylamide denaturing gels. No RNase P was included in control reactions run in the lanes marked "C". Faint bands migrating slightly faster than the product bands correspond to partially degraded precursor tRNAs. The level of RNase P activity used in the reactions shown in Figures 3, 4, and 5 was chosen to highlight the differences in rates of cleavage among the altered precursors.

above). Longer reaction times resulted in the accumulation of end-processed molecules. The RNA products were ethanol-precipitated and purified by gel electrophoresis on 6% polyacrylamide gels containing 8M urea using internally labelled pre-tRNAs as size-markers. The appropriate bands were cut out of the gel and the RNA eluted from the gel slice as described above. The RNA was concentrated by ethanol precipitation, taken up in TE buffer [10mM tris, 1mM EDTA (pH 7.5)] and reprecipitated with ethanol to ensure removal of phenol. The RNA was redissolved in distilled water, renatured as discussed below, and used immediately in the structure-probing reactions. Typically, 2000-6000 cpm (Cherenkov) were recovered per reaction. It was estimated that less than 10% of the full-length precursors retained a 5'-triphosphate terminus, based on the radioactivity of transcripts labelled internally with α-^{32}P-uridine in otherwise identical transcription reactions.

Nuclease sensitivity studies. The structure-specific nucleases S1 and V1 (cobra venom RNase) were employed to probe selected 5' end-labelled pre-tRNAs. Nuclease digestions were carried out under conditions similar to those used in RNase P assays. V1 RNase digestions were carried out in 8 mM $MgCl_2$, 110 mM KCl, and 5 mM HEPES (pH 7.5). The same buffer was used for S1 digestions, except for the addition of 1 mM $ZnCl_2$. The labelled RNA was renatured by incubation in digestion buffer for 20 minutes at 37°C prior to the addition of the appropriate

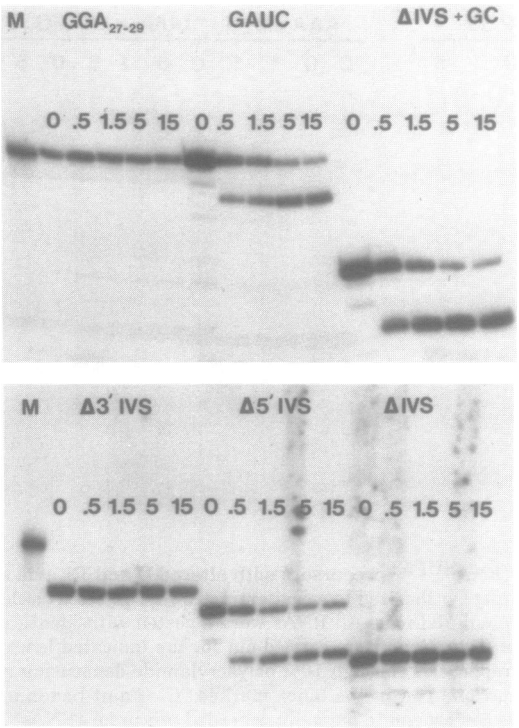


Figure 3: Cleavage of tRNA^{SUP53} precursors with altered anticodon and intron sequences. Yeast extract-transcribed pre-tRNAs were subjected to RNase P cleavage as in **Figure 2.** Identical amounts from the same fraction of RNase P were used.

nuclease. The RNA was digested at 30° or 37°C for 20 minutes and then quickly frozen and lyophilized to dryness. The enzyme concentrations were adjusted so that no more than 30% of the molecules were cut. The RNA fragments were dissolved in 10M urea containing xylene cyanol and bromphenol blue tracking dyes in TBE [89mM tris, 89mM boric acid, 2mM EDTA]. No further digestion takes place in 10M urea (1). Sequence ladders were produced by digesting aliquots of each RNA with T1 RNase as described by Donis-Keller et al. (33). Cleavage products were analyzed on 10% polyacrylamide sequencing gels (40cm x 20cm x 0.3mm) containing 8M urea in TBE. Cleavage patterns were visualized by autoradiography for 7-15 days as described above.

RESULTS

Altered Forms of tRNA^{SUP53}. The altered tRNA^{SUP53} precursors studied here are summarized in **Figure 1**, where they are superimposed upon an illustration of the secondary structure of the pre-tRNA deduced by nuclease sensitivity studies (34). The susceptibilities of the altered molecules to cleavage by RNase P under standard conditions are shown in **Figures 2** and

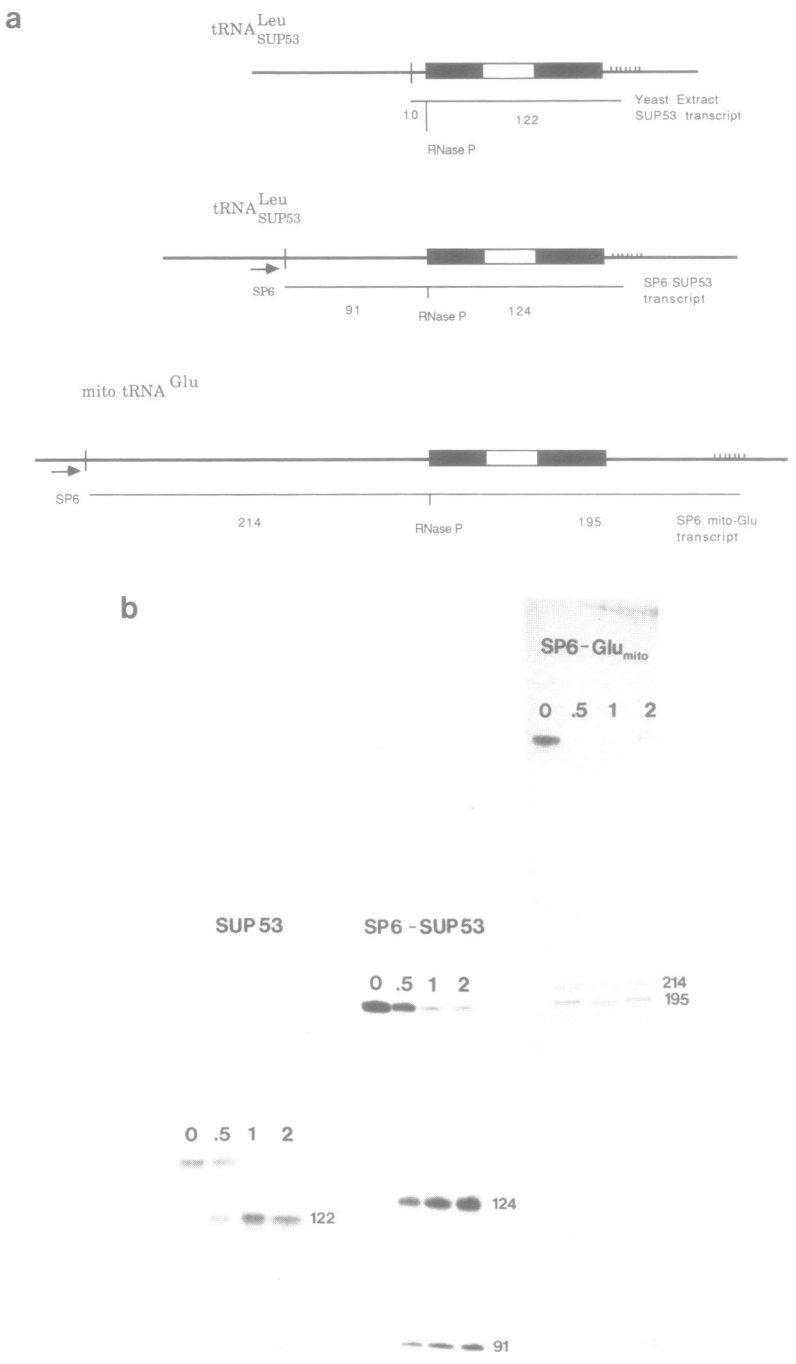
Table 2: Summary of structure probing experiments. Crosses indicate primary cleavage sites observed. Columns correspond to indicated pre-tRNA probed with S1 and V1 nucleases.

	Precursor tRNA's					
	SUP53	ΔIVS	ΔIVS+GC	Δ5'IVS	Δ3'IVS	GAUC
S1 Cleavage sites						
A31					x	
Base 37:1†	x		x	x	x	x
A37:32 (or last base of intron)	x			x	x	x
G57	x		x	x	x	x
V1 cleavage sites						
17-19					x	
28-30	x	x	x	x	shifted to positions 26-30	x
U33				x		
G37:14	x					x

† In the case of ΔIVS this refers to base C38.

3, and are summarized in **Table 1**. All of the experiments presented here were performed with the same RNase P fraction in assays carried out in parallel on the altered molecules with the pre-tRNA^{SUP53} as a standard. The assays were repeated several times using different preparations of altered precursors synthesized in parallel with pre-tRNA^{SUP53}, confirming the qualitative conclusions summarized in **Table 1**. More precise quantitation of cleavage rates was precluded by variations in the absolute rates of cleavage between experiments and by rapid loss of RNase P activity under assay conditions.

Effects of Mutations within the Mature Domain on RNase P Cleavage. Mutations which disrupt the secondary and tertiary structure of the mature domain of pre-tRNA^{SUP53} reduce or eliminate cleavage by our partially purified preparation of RNase P from *S.cerevisiae*, consistent with the results of previous studies (21-24). Mutation of base G19 to a C or of C56 to a G eradicates RNase P cleavage of the 5'-leader sequence (**Figure 2**). These universally conserved bases in the loops of the opposing arms of the molecule hydrogen-bond to each other thus stabilizing the tRNA tertiary structure. Mutations which disrupt the D stem ($AAA_{10\text{-}12}$ or $UUU_{23\text{-}25}$) reduce, but do not block cleavage. The rate of RNase P cleavage of these two mutations was shown to be approximately the same in repeated assays. Only the $AAA_{10\text{-}12}$ mutation falls within the conserved region of the D arm sequence. Therefore it is likely that structural perturbations rather than conserved nucleotide alteration is responsible for the lowered susceptibility to cleavage in


a
tRNA Leu SUP53
10
122
Yeast Extract SUP53 transcript
RNase P
tRNA Leu SUP53
SP6
91
RNase P
124
SP6 SUP53 transcript
mito tRNA Glu
SP6
214
RNase P
195
SP6 mito-Glu transcript
b
SP6-Glu mito
0 .5 1 2
SUP 53
SP6 - SUP 53
0 .5 1 2
214
195
0 .5 1 2
122
124
91

these cases. Combinations of mutations which would have restored the interloop contact (G19 to C and C56 to G) or the D stem structure (AAA_{10-12} and UUU_{23-25}) could not be synthesized in the yeast extract because the transcription promoters are not functional (23). The mutation GGA_{27-29} eliminates base-pairing in the anticodon stem; the affected bases do not constitute a conserved sequence. This mutation also blocks RNase P cleavage (**Figure 3**), providing further evidence that the presence of correct overall structure is crucial to recognition of the substrate by the *S.cerevisiae* nuclear enzyme.

Effect of Mutations in the IVS on RNase P Cleavage. The $tRNA_3^{LEU}$(SUP53) used in the present study is one of 11 yeast tRNAs known to contain an intron. Such introns show no sequence conservation internally or at the splice junctions and vary in length from 8 to 60 nucleotides. All yeast tRNA introns occur between positions 37 and 38 in the standard tRNA numbering system, that is, between the first and second bases following the anticodon (4, 5). The intron sequences extend the anticodon stems of these precursor molecules without appearing to disrupt their core cloverleaf structures (35,36). The only other conserved feature of intron-containing tRNAs is the potential for base-pairing between the anticodon and sequences within the intron (5,35). Intron mutations have been constructed in SUP53 that were designed to interfere with various portions of the extended anticodon stem structure shown in **Figure 1** (26,27). We tested several of these precursors with altered introns for their effect on RNase P cleavage, to determine the contribution of the intron to substrate utilization.

The results shown in **Figure 3** and summarized in **Table 1** indicate that the intron sequences are essential for RNase P cleavage, but that the most deleterious mutations are not necessarily those predicted by the structure shown in **Figure 1** (35). The Δ5'-IVS precursor, for example, is cleaved normally by RNase P even though the anticodon- intron base-pairing shown in **Figure 1** is eliminated by deletion of the first nineteen nucleotides of the intron. Similarly, the three base substitution designed to disrupt anticodon-intron pairing (GAUC, 37:7-37:10) did not substantially alter the rate of cleavage by RNase P. Removal of the last thirteen bases of the intron

Figure 4 (a): Precursor tRNA transcripts. The pre-tRNA substrates derived from yeast extract transcription of the $tRNA^{SUP53}$ gene (upper), SP6 RNA polymerase transcription of the $tRNA^{SUP53}$ gene (middle), and SP6 transcription of the mitochondrial $tRNA^{Glu}$ gene (lower) are shown schematically. The boxes correspond to the tRNA genes, with the open boxes indicating the intron sequences. The transcripts are indicated by thin lines below the DNA. Hatch marks on the DNA at the end of the transcripts represent the poly T sequences that served as terminators for the RNA polymerases. The lengths of the RNase P cleavage-products are given in number of nucleotides. **(b): RNase P cleavage of unmodified nuclear and mitochondrial precursors.** Pre-tRNAs, synthesized by a yeast extract or by SP6 RNA polymerase, were subjected to RNase P cleavage as described in Materials and Methods. Cleavage products were analyzed by electrophoresis through 6% polyacrylamide denaturing gels (8M urea). The lanes are labelled according to the digestion time in minutes. The 10-nucleotide-long cleavage product of the yeast extract SUP53 transcript has migrated off the gels shown here, but has been found to remain intact and to contain a 3'-hydroxyl group as expected (30). The lengths of the other cleavage products are indicated in nucleotides to the right of each panel.

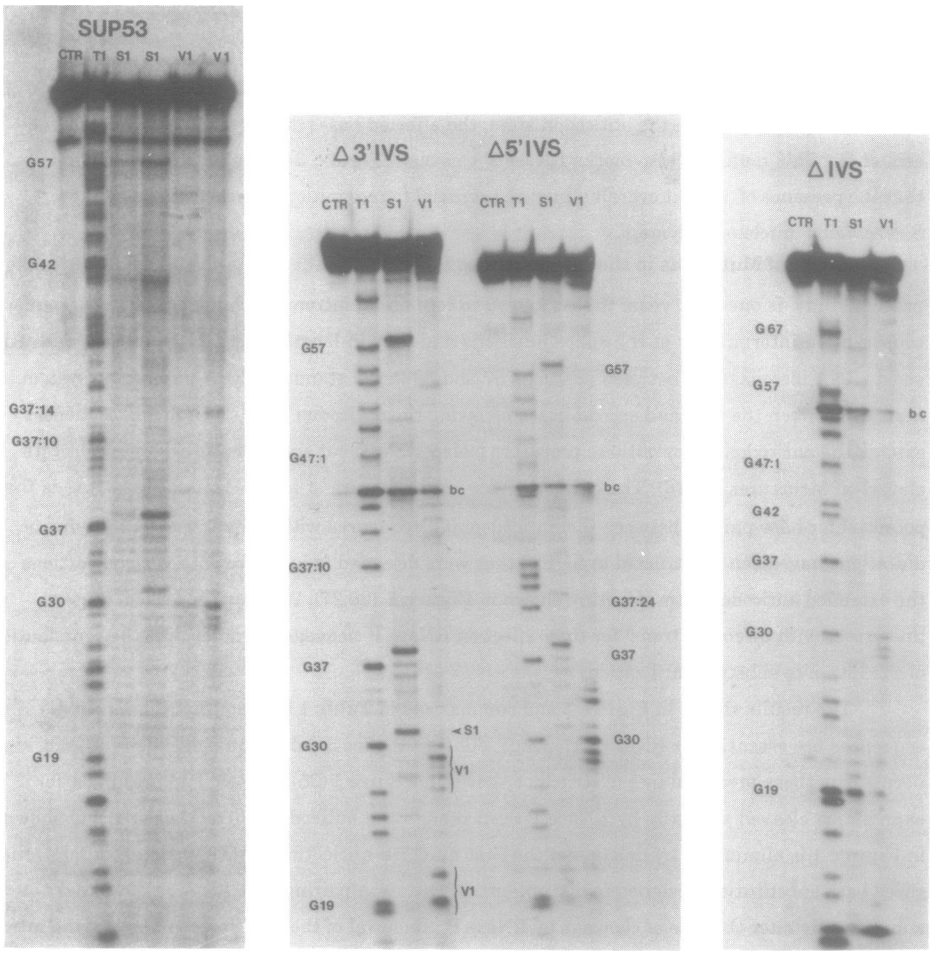


Figure 5: Representative structure probing data. CTR indicates no enzyme. Lanes labelled "T1" indicate treatment of the precursor under denaturing conditions with T1 nuclease to generate a sequencing ladder of G-residues. Lanes labelled S1 and V1 indicate treatment with S1 nuclease and cobra venom RNase under native conditions described in *Methods*. "bc" indicates bands subject to compression. (**SUP53**) Pre-tRNA was treated with S1 nuclease (1un/μg RNA) for 5 min (3rd lane from left) or 20 min (4th lane); with V1 nuclease (0.007un/μg RNA) for 5 min (5th lane) or 20 min (6th lane). (**Δ3' and Δ5' IVS**) Pre-tRNAs were treated with S1 and V1 nucleases as for SUP53 for 20 min. (**ΔIVS**) Pre-tRNAs were treated with S1 and V1 nucleases as for SUP53 for 20 min.

(Δ3' IVS, 37:20-37:32), however, severely reduced RNase P cleavage even though this precursor can be drawn in a form that closely resembles the consensus tertiary structure of Lee & Knapp (35) with only the "extra stem" of the intron removed.

Also unexpected was the finding that deletion of the 32-nucleotide intron from SUP53 (mutation ΔIVS) results in inefficient cleavage by the partially purified RNase P (**Figure 3**). This

Table 1. Susceptibility of precursor tRNAs to cleavage by RNase P. Susceptibility to cleavage is related to that of SUP53 precursor.

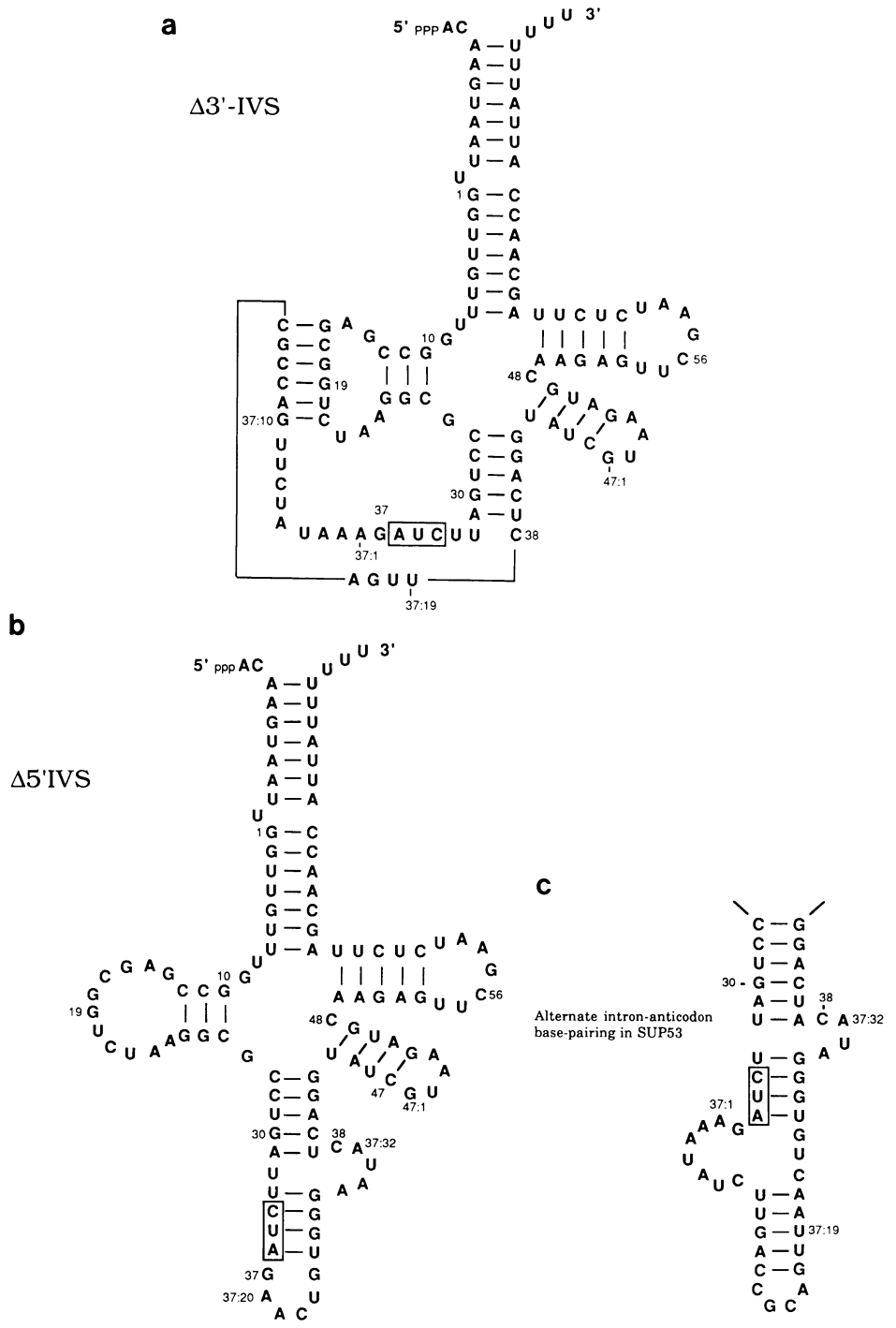
Designation of Mutation	Description of Mutation	Susceptibility to Cleavage by RNase P relative to SUP53
G19·C	3° structure disruption	not cleaved
C56·G	3° structure disruption	not cleaved
AAA(10-12)	D-stem disruption	reduced
UUU(23-25)	D-stem disruption	reduced
GGA(27-29)	anticodon stem disruption	not cleaved
ΔIVS	intron deletion	reduced‡
Δ5' IVS	37:1 to 37:19 deletion	normal
Δ3' IVS	37:20 to 37:32 deletion	not cleaved
ΔIVS + GC	intron replaced by GC	normal
GAUC	anticodon: intron base-pairing disruption	normal

‡Cleavage of ΔIVS depends on the degree of RNase P purification. ΔIVS precursor is cleaved very poorly by more highly purified fractions of the enzyme (as in **Figure 3**). However, ΔIVS is cleaved more efficiently than AAA(10-12) or UUU(23-25) precursors (although less efficiently than SUP53 precursor) by crude RNase P-containing extracts.

effect cannot be attributed to a requirement for any specific intron sequences since replacement of the intron by the dinucleotide GC (mutation ΔIVS + GC) restores normal RNase P processing.

RNase P processing does not require base modifications. It has been shown previously that the presence of modified bases in *E.coli* pre-tRNA does not strongly effect cleavage by RNase P (40). Similarly, it has been shown that *in vitro* transcription of the altered $tRNA^{SUP53}$ genes used in the present study by yeast extract results in precursor molecules containing a subset of the base modifications found in the mature wild-type tRNA (26). The nature of the modifications depends on the particular substrate. To ensure that RNase P cleavage was not strongly influenced by the presence of modifications, pre-tRNA substrates were synthesized *in vitro* using SP6 RNA polymerase. The 5'-end processing of SUP53 precursors transcribed using SP6 RNA polymerase and those made with the yeast extract were compared in parallel assays. A typical experiment is shown in **Figure 4b**. No significant difference in the rate of RNase P cleavage was observed between the two types of transcripts.

To demonstrate that our nuclear RNase P preparation did not display any requirement for introns or other sequences peculiar to nuclear tRNA precursors, yeast mitochondrial $tRNA^{Glu}$ was also synthesized using SP6 RNA polymerase and assayed for RNase P cleavage (**Figure 4b**). It was found consistently that among the RNase P substrates studied here, the mitochondrial pre-tRNA was the most efficiently cleaved. The reason for the exceptional susceptibility of the mitochondrial pre-tRNA to cleavage by our preparation is not known, but is not due to contamination by mitochondrial RNase P activity because comparable results were obtained using enzyme prepared from a rho° petite yeast strain (data not shown). Such strains lack mitochondrial DNA and therefore should lack functional mitochondrial RNase P (18,19).


a
Δ3'-IVS
b
Δ5'IVS
c
Alternate intron-anticodon base-pairing in SUP53

Effect of IVS mutations on pre-tRNA structure. In light of the fact that mitochondrial pre-tRNA^{Glu}, and other precursors lacking introns are cleaved efficiently by RNase P, it seemed unlikely that sequence differences could in themselves account for the differences in susceptibility to RNase P cleavage observed among the intron mutations. The structure of the precursors containing altered introns was therefore compared in nuclease sensitivity experiments to determine whether the reduced cleavage rate was coincident with disruption of the precursor secondary or tertiary structure. Single-strand specific S1 nuclease and double-strand specific V1 RNase were used to probe the native structures of the pre-tRNA^{SUP53} and the precusors bearing altered introns. End-labelled precursors were probed under buffer conditions identical to those used for RNase P cleavage assays. Representative structure-probing data is shown in **Figure 5**. Several sets of experiments were performed in which the five SUP53 precursors with altered introns were probed in parallel. The data is summarized in **Table 2**. The structure-analysis carried out on the SUP53 precursor using these two structure-specific enzymes generally agrees with that carried out by Lee and Knapp on tRNA^{Leu} (CAA). We found that nuclease S1 cuts at A37:1, in agreement with their finding that the primary cleavage sites for single-strand specific nucleases occur at bases A37:2, A37:3, and U37:4. Although, they identified secondary cutting sites for single-strand specific enzymes at bases U37:19, A37:20 (in the intron loop), A37:32 (at the 3'-intron cleavage site), and A47:3 (in the variable loop) using RNases T1, U2, A, and nuclease from mung bean, we consistently observed secondary S1 cutting only in the vicinity of A37:32. They observed T1 cutting at G57 in the TΨ -loop but only in the absence of magnesium, whereas we consistently observed S1 cutting in the vicinity of G57 in the presence of magnesium. In agreement with Lee and Knapp, we observed strong V1 cutting at G37:14 in the stem formed by intron-sequences and also at G30 in the anticodon stem. In addition, we saw secondary cleavages by V1 at C28 and U29.

Those SUP53-derived precursor substrates that were most efficiently processed by RNase P were also most similar in structure to SUP53, as judged by the nuclease-sensitivity data. The GAUC precursor was subject to a more extensive set of Sl cleavages in the region of the molecule surrounding position 37:1, as would be expected from the loss of the anticodon-intron pairing. Nonetheless, the V1 cleavage at 37:14 was observed, consistent with retention of this region of the stem structure. The major S1 and V1 cleavages in the Δ5'-IVS and ΔIVS+GC precursors were the same as those observed in pre-SUP53 except for the deleted positions and the appearance of a new V1 cleavage at position 33 or 34 in the Δ5'-IVS precursor.

In contrast, the structures of the ΔIVS and Δ3'IVS precursors differed markedly from pre-SUP53. ΔIVS, which showed reduced susceptibility to cleavage by RNase P, was found less sensitive to Sl nuclease at position 38 (which corresponds to position 37:1 of the intron-containing

Figure 6 (a): Potential secondary structure of Δ3' IVS precursor. **(b):** Potential secondary structure of Δ5' IVS precursor. **(c):** Possible alternate intron base-pairing in pre-tRNA ^{SUP53} (compare with **Figure 1**).

pre-tRNAs) and position 57, although the pattern of V1 cutting in the anti-codon was the same. Δ3' IVS, which was very poorly cleaved by RNase P, had differences in both S1 and V1 susceptibilities. A new S1 cut appeared at A31 and strong V1 cuts appeared in the D-loop sequence, indicating that this region is base-paired to another part of the sequence (see Discussion). In the extended anticodon stem, the V1 cut at 37:14 was absent and the cluster of V1 cuts in the anticodon stem shifted 2 bases toward the 5'-end.

DISCUSSION

Precursor tRNAs that do not normally contain intervening sequences, including the mitochondrial $tRNA^{Glu}$ used here, are fully active substrates for yeast RNase P (11, 30). Moreover, normal RNase P cleavage has been demonstrated in a precursor transcribed from a gene coding for yeast $tRNA^{Tyr}$ (SUP6) from which the normally present intron sequence was deleted (37,38). In this work, however, we have found that deletion of the intron from SUP53 significantly reduces RNase P cleavage. Furthermore, we have found that insertion of the dinucleotide GC in the place of the intron (ΔIVS+GC) results in a precursor that is processed normally by RNase P. It is clear from our structure-probing studies that the ΔIVS precursor exhibits a tight, nuclease-resistant structure. Insertion of the GC appears to "loosen" up the structure, resulting in a precursor displaying S1 and V1 cleavage patterns similar to those of SUP53. Although it is not clear precisely what conformation the ΔIVS precursor adopts to become resistant to S1 nuclease and RNase P, the insertion of the two-base GC sequence is apparently enough to restore a more normal structure.

RNase P cleavage of the Δ3'IVS precursor is strongly inhibited. This mutation also results in pre-tRNA that is resistant to splicing (26). The fact that the intron sequences complementary to the anticodon remain in the Δ3'IVS precursor (**Figure 1**) suggests that it should fold in a manner similar to SUP53. However, the aberrant nuclease sensitivity data reported here, and the finding that this molecule is not processed by RNase P, support the view that its structure has been drastically altered relative to the parent molecule. Computer calculations using Zuker's program (39) indicate that the Δ3'-IVS precursor should favor a structure in which the remaining intron sequence base-pairs with the D-loop. A hypothetical structure for Δ3'-IVS based on this base-pairing feature which also incorporates our nuclease-sensitivity data is shown in **Figure 6a.**

The Δ5'-IVS precursor has the potential to form an extended anticodon stem involving base-pairing between the 3'-half of the intron and the anticodon, as illustrated in **Figure 6b.** It is possible that this is in fact the favored structure in solution, given that strong S1 cutting is observed at 37:1, just as in SUP53. The new V1 cut at position 33 also supports this interpretation. It is well-known that V1 does not cut all double-helical domains. A change in sequence in the complementary strand could create a new V1 site, as appears to have occurred in the anti-codon of Δ5'IVS. By adopting this structure, Δ5'-IVS would conform to the consensus structure for intron-containing yeast pre-tRNAs derived by Lee and Knapp (35) from structure-probing studies. The

SUP53 structure differs from the consensus model, in that a large helical domain exists in place of the small, bulged loop separating the 3'-splice site from the anticodon base-pairing region. It is conceivable that SUP53 could adopt an alternate structure more similar to the consensus model, which appears to be favored by Δ5'-IVS for lack of the 5'-intron sequence. This alternate structure for the SUP53 extended anticodon stem is illustrated in **Figure 6c**.

In this regard, it is interesting to ask whether the structure of the GAUC (37:7-37:10) mutation (designed to abolish base-pairing between the anticodon and intron sequences) is like that shown in **Figure 1** or like the alternate form in **Figure 6c**. The nuclease sensitivity data we have obtained (data not shown) suggest that this molecule also prefers the pre-SUP53 structure of **Figure 1**. Like pre-tRNA$_3^{Leu}$, the GAUC precursor is cut by V1 at G37:14. GAUC shows increased S1 sensitivity at the bases surrounding A37:1, as would be expected from enlargement of the single-stranded loop due to the disruption of base-pairing between the anticodon and intron sequences. In spite of the minor disruption of its secondary structure, the GAUC precursor is cleaved normally by RNase P. This demonstrates that the anticodon need not be base-paired (in the structure proposed by Lee and Knapp) for recognition by RNase P.

The work presented here suggests that conservation of appropriate structure in the anticodon/intron stem may be important in RNase P substrates primarily because of the contribution of this region to attaining or stabilizing an appropriate overall tertiary structure, thus allowing greater flexibility in the sequence requirements of the mature tRNA domain. It is quite possible that a set intron structure is necessary for other steps in tRNA maturation, consistent with earlier observations that the presence of the intact intron in the SUP53 precursor is essential for correct splicing and modification of certain bases (26, 27). It is clear from the results with the ΔIVS+GC and Δ5'-IVS precursors, however, that no particular intron structure is required for RNase P cleavage, as long as the overall tertiary structure of the molecule is preserved.

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ABBREVIATIONS:

Cpm, counts per minute (Cherenkov); V1, cobra venom nuclease; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, N-2-hydroxyethylpiperazine- N'-2-ethanesulfonic acid; IVS, intervening sequence (intron); PEP, phosphoenolpyruvate; pre-tRNA, precursor tRNA.

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