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Structural alterations in mutant precursors of the yeast $tRNA_3^{Leu}$ gene which behave as defective substrates for a highly purified splicing endoribonuclease

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We have produced a highly purified preparation of the *Xenopus laevis* splicing endonuclease (*Xla*I RNase). The purified enzyme correctly cleaves tRNA precursors, creating substrates for subsequent ligation. The 5'-half molecules have a 2',3' cyclic phosphate at their 3' termini. Assuming that splicing enzymes recognize primarily structural elements in the 'mature domain', we have been studying the conformation of three splicing-defective precursors made from mutants of the yeast $tRNA_3^{Leu}$ gene. The mutations alter base-pairing in the D-stem region and two of the mutants are absolute defectives. Enzymatic probing of the structures of the altered tRNA precursors shows that the structural perturbations in these mutants are localized on the 'inside' of the 'L'-shaped three-dimensional structure. The implications of this finding for the recognition process are discussed.

Key words: yeast / tRNA genes / splicing endoribonuclease / mutations

Introduction

Many eucaryotic genes are interrupted by intervening sequences (IVS), or introns, which are removed from the primary transcripts by the RNA splicing process. The molecular mechanisms of this process are not well understood; it is clear, however, that such mechanisms are different for each of the major classes of RNA: tRNA, rRNA and mRNA (reviewed by Abelson, 1979).

In the case of tRNA coded in the nucleus, the introns can vary enormously with respect to size (from 14 to 60 nucleotides) and sequence; however, they are always localized one base 3' to the anticodon. Calculations of free energy minima (Tinoco *et al.*, 1973) and the use of chemical and enzymatic structure-specific probes (Wrede *et al.*, 1979) suggest that there exists a common tertiary structure for all the pre-tRNA examined (Swerdlow and Guthrie, 1984; Lee and Knapp, 1985). In this structure, the tRNA portion of the precursor maintains the 'L'-shaped conformation, stabilized by the interaction between the D and TΨC loops, and an extended anticodon stem is formed by the pairing of nucleotides which include the anticodon triplet with a complementary sequence within the intron. Results obtained with partially purified preparations of splicing endonuclease (Otsuka *et al.*, 1981; Peebles *et al.*, 1983) lend support to the possibility that a single enzyme operates on all the precursors. It seems possible that the specificity of the reaction is based primarily, if not entirely, on the recognition of the conformational features of the precursor which are determined by the portion of the molecule conserved in the mature domain.

To analyze those elements of pre-tRNA structure which are essential for enzyme-recognition, we produced a number of mutated precursors. We have recently reported that in *Xenopus* germinal vesicle (GV) extracts the excision of the IVS from the yeast $tRNA_3^{Leu}$ is dependent on the formation of a D-stem (Baldi *et al.*, 1983). The precursors of two substitution mutants, characterized respectively by the sequence change of GCC to AAA at positions 10, 11 and 12 in the non-coding strand and by the sequence change GGC to TTT at positions 24, 25 and 26, again in the non-coding strand, both of which are unable to form a D-stem, are not spliced. On the contrary, a double mutant characterized by both of the substitutions described above and by the ability to form a D-stem, consisting now of three AT base pairs and not of three GC base pairs as in the wild type, is spliced by the GV extract (Baldi *et al.*, 1983). The fact that the excision of the IVS is dependent on the formation of a D-stem does not, however, necessarily imply that the D-stem plays a direct role in the recognition process. Non-homology in the D-stem could result in changes in the conformation of other parts of the tRNA.

To gain a better understanding of the recognition features of the splicing substrates we subjected the mutant precursors to structural analysis. In order to focus on the interaction enzyme substrate, possible interferences by other proteins present in the GV extract must be distinguished from the enzyme substrate interaction proper. For this reason, we produced a highly purified preparation of the splicing endonuclease.

Here we demonstrate that the highly purified enzyme produces halves and intervening sequences from the wild-type precursor, generating a cyclic phosphate at the 3' terminus. Unlike crude extracts, the purified enzyme excises the IVS from the double mutant very inefficiently. Reconstitution experiments demonstrate the existence of factor(s) that increase the efficiency of cleavage. The factor(s) might have a role in stabilizing the weak D-stem.

Using enzymatic structure-specific probes, we analyzed the conformation of the wild-type and of the three mutant precursors. Interpreted on the basis of the proposed model for the tertiary structure of IVS-containing tRNA precursors, our results indicate that the perturbations due to the mutations are localized in a limited region on the 'inside' of the 'L'-shaped three-dimensional tRNA structure (Ogden *et al.*, 1980). These findings are discussed in relation to the recognition process.

Results

Transcription and processing reactions occur in GV extracts

Xenopus laevis GV extracts can transcribe cloned tRNA genes and accurately process the primary transcript to form mature tRNA (Mattoccia *et al.*, 1979).

An analysis of all the products of the $tRNA_3^{Leu}$ gene transcription *'in vitro'* revealed several RNA processing intermediates: a simultaneous transcription and processing reaction occurs in the test-tube. Processing intermediates include molecules with or without 5' leader and/or 3' trailer, intermediates of IVS excision, unligated and ligated half pre-tRNA molecules, and also the IVS (Otsuka *et al.*, 1981).

We have identified in the GV extract four enzymatic activities

Table I. Purification of RNase *Xla*I

Fraction	U/ml	Vol (ml)	Total units	Yield (%)	Protein (mg)	Sp. act. (U/mg)
I. Nuclear extract	7834	47	368 198	100	265	1384
II. Phosphocellulose	2100	158	331 380	90	32.2	10 290
III. Heparin-Sepharose	8900	15	133 500	36	2.2	60 680
IV. DEAE-Sephadex	5455	15	81 825	22	0.9	90 916
V. CM-Sephadex	11 200	2.4	26 880	7.3	0.120	224 000
VI. Sucrose gradient	1827	3.6	6577	2.0	0.004	1 644 250

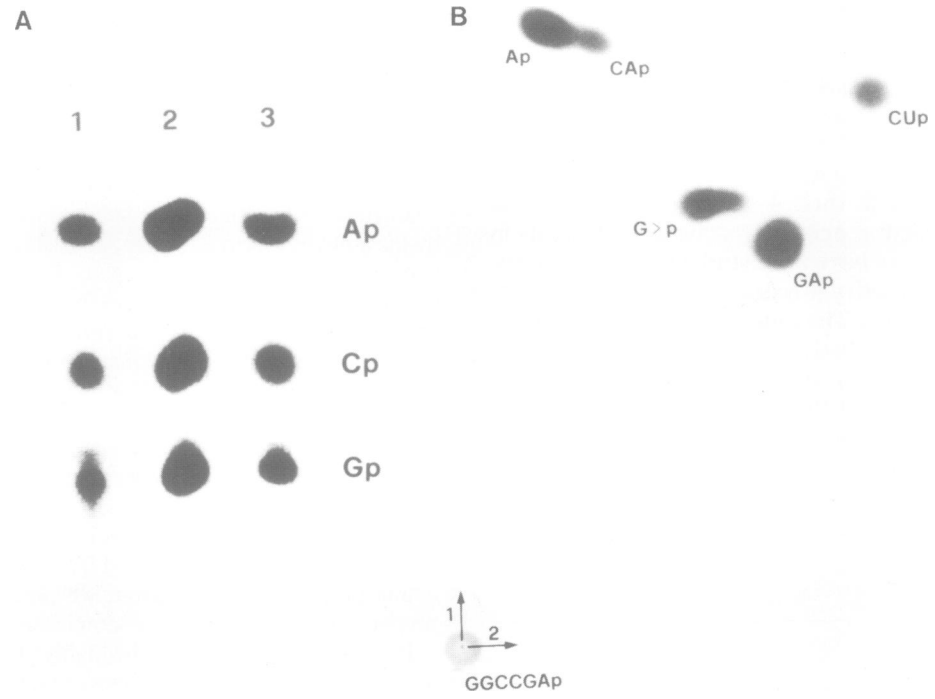

Fig. 1. Analysis of the terminus of the 5′ half molecule. (**A**) [α-^{32}P]ATP labeled 5′ half molecules produced by the GV extract and by the splicing endonuclease (Fraction V) after treatment with alkaline phosphatase, were RNase TI digested and fingerprinted. Oligonucleotide t20 (Otsuka *et al.*, 1981), derived from 5′ half molecules produced by the endonuclease in the absence of ATP (**lane 1**), in the presence of ATP (**lane 2**), and by the GV extract (**lane 3**), was treated with RNase T2 and analyzed on a PEI thin-layer plate as described in Materials and methods. Identification of the nucleotides was based on markers run in parallel. (**B**) [α-^{32}P]ATP labeled 5′ half molecules produced in the absence of ATP by the splicing endonuclease (Fraction V) were cleaved with RNase Physarium M and analyzed by two-dimensional chromatography on a cellulose thin-layer plate together with the unlabeled guanosine 2′,3′-cyclic monophosphate marker. The labeled terminal guanosine 2′,3′-cyclic monophosphate was identified by co-migration with the marker. Identification of the other oligonucleotides was based on sequential digestion with RNase Physarium M and RNase A.

which have a role in the processing of the precursor: the splicing endonuclease (*Xla*I RNase) which precisely excises the IVS; an RNase-P-like activity (*Xla*II RNase) which is responsible for the removal of the 5′ leader; an endonuclease activity (*Xla*III RNase) responsible for the removal of the 3′ trailer; and the ATP-dependent ligase which selectively joins the complementary halves of the tRNA molecules.

To investigate the salient features of each of the processing reactions it was necessary to purify each enzyme. We previously reported a preliminary purification of the splicing endonuclease (Otsuka *et al.*, 1981) and here we present a protocol which yields a highly purified enzyme (see Table I).

A nuclear extract was prepared by low-speed centrifugation from germinal vesicles isolated from stage 6 oocytes (Fraction I). Chromatography of the nuclear extract on a phosphocellulose column resulted in ~7-fold purification (Fraction II). At this stage of purification the endonuclease activity is free of contaminating 5′-processing and 3′-processing activities, and of ligase. The products of the reaction are 5′ half molecules with 5′ leader sequences, 3′ half-molecules with trailer sequences, and the intervening sequence. Further purification involved heparin-Sepharose (Fraction III), DEAE-Sephadex (Fraction IV) and CM-Sephadex (Fraction V) chromatography. Fraction V shows a 206-fold increase in specific activity over the nuclear extract and is stable for months at −70°C. Aliquots of this fraction were further purified by sucrose gradient sedimentation (Fraction VI). The peak of the endonuclease activity co-sedimented with the bovine immunoglobulin G marker (mol. wt. 160 000). Fraction VI shows >1200-fold purification over Fraction I. Since the enucleated stage 6 oocyte has a 10-fold higher protein concentration than the germinal vesicle, Fraction VI shows >12 000-fold purification over the total oocyte.

The purified enzyme requires Mg^{2+} ions in order to cleave the wild-type precursor of $tRNA_3^{Leu}$. The sequences of the products were verified by T1 fingerprinting (data not shown). The halves produced by the purified enzyme are efficiently ligated

Fig. 2. Digestion of wild-type and mutant pM1 and pM3 precursors by the purified splicing endonuclease. Incubations were carried out under standard conditions without (**lanes 1–3**) and with (**lanes 4–6**) addition of purified splicing endonuclease (Fraction V), wild-type (**lanes 1,4**), pM1 (**lanes 2,5**) and pM3 (**lanes 3,6**). Products were analyzed by gel electrophoresis.

Fig. 3. pM2 precursor requires an additional factor for the splicing endonuclease. Wild-type pre-tRNA incubated with splicing endonuclease (Fraction II) (**lane 1**). pM2 pre-tRNA incubated with nuclear extract (Fraction I) (**lane 2**), with splicing endonuclease (Fraction II) (**lane 3**), with 0.15 M eluate from phosphocellulose column (**lane 4**), with both splicing endonuclease (Fraction II) and 0.15 M eluate (**lane 5**). Incubations were carried out under standard conditions; products were analyzed by gel electrophoresis.

by the RNA ligase derived from the GV extract (data not shown).

Chemical structure of the 3′ terminus

To excise the IVS in the GV extract, the pre-tRNA$_3^{Leu}$ is cleaved at two sites (arrows):

$$5'A\underbrace{\text{Um CAAm'Gp}}_{t\ 20}\downarrow AAAUp...IVS...AAUAp\downarrow C\Psi CAGp3'$$

We determined the structure of the 3′ terminus of the 5′ half obtained from a simultaneous transcription processing reaction in which the label was [α-^{32}P]ATP. The purified 5′ half was treated with alkaline phosphatase before RNase T1 fingerprinting. We observed that digestion with RNase T2 of the T1 oligonucleotide t20 (Otsuka *et al.*, 1981), which is the 3′ end of the 5′ half, produced Gp (Figure 1A, lane 3). This finding indicates that the 3′-phosphate is resistant to alkaline phosphatase and that it is probably guanosine 2′,3′-cyclic phosphate (Peebles *et al.*, 1983; Filipowicz and Shatkin, 1983). The phosphate resistant to alkaline phosphatase could be produced either directly by the splicing endonuclease or by the action of an ATP-dependent cyclase (Filipowicz *et al.*, 1983) on the RNA terminal phosphate. An experiment identical to the one reported above, performed with 5′ half molecules produced by the highly purified splicing endonuclease (Fraction V) in the presence (Figure 1A, lane 2) and in the absence of ATP (Figure 1A, lane 1), indicated that Gp is produced in equal amounts in both cases. In another experiment, 5′ half molecules labeled with [α-^{32}P]ATP, produced by the splicing endonuclease (Fraction V) in the absence of ATP, were digested with RNase Physarium M (Up↓N and Ap↓N specific). Analysis of the products using two-dimensional t.l.c. on cellulose plates yielded guanosine 2′,3′-cyclic monophosate (Figure 1B). Nuclease PI digestion of the same material released labeled pA and guanosine 5′ phosphate 2′,3′-cyclic phosphate (data not shown).

It appears, therefore, that the cyclic phosphate is produced directly by the purified endonuclease which operates in the absence of ATP. The role of the cyclase (Filipowicz *et al.*, 1983) could be that of actively regenerating the cyclic terminus which might be opened by the phosphodiesterases of the extract.

What is the fate of the cyclic phosphate? In *Xenopus* extracts, as in HeLa extracts, the 3′-terminal cyclic phosphate of the 5′ half molecule is incorporated into the splice junction 3′,5′-phosphodiester linkage (Filipowicz and Shatkin, 1983). This conclusion is based on an analysis of the t20 oligonucleotide of mature tRNA, which had been synthesized in the GV extract in the presence of [α-^{32}P]ATP. In this case, digestion of oligonucleotide t20 with RNase T2 produced Gp (data not shown), indicating that the phosphate at the 3′ terminus of the 5′ half was conserved.

pM1, pM2 and pM3 precursors are defective substrates for the purified endonuclease

The pM1 and pM3 precursors, which are unable to form a D-stem, are not spliced in the GV extract; they behave as absolute defectives (Baldi *et al.*, 1983). As might have been expected on the basis of these results, the IVS is not excised from both mutant precursors by the purified endonuclease (Figure 2). The double mutant pM2, characterized by the ability to form a D-stem which now consists of three AT base pairs and not of three GC base pairs like in the wild-type, is spliced in the crude extract but, surprisingly, is a very poor substrate for the highly purified enzyme. To find an explanation for this fact, we carried out a purification of the enzyme according to the protocol reported here, assaying at each step with wild-type and pM2 precursors. Fraction II, resulting from phosphocellulose chromatography, is very active on the wild-type precursor (Figure 3, lane 1) but cleaves the pM2 precursor only very inefficiently (Figure 3, lane 3). This finding indicates that a factor present in the crude extract was removed during phosphocellulose chromatography. To substantiate this conclusion we attempted a reconstitution experiment. The fractions eluting from phosphocellulose at ~0.15 M KCl do not show any endonuclease activity when assayed with the wild-type (data not shown) or the pM2 precursor (Figure 3,

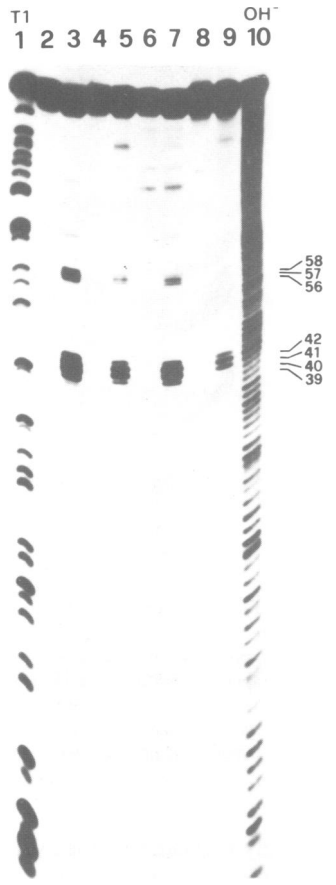

Fig. 4. Partial nuclease S1 digests of 5′-^{32}P-end labeled wild-type and mutant precursors. Wild-type (**lanes 2, 3**), pM1 (**lanes 4,5**), pM3 (**lanes 6,7**), pM2 (**lanes 8,9**) precursors, were digested with (**lanes 3,5,7,9**) or without (**lanes 2,4,6,8**) nuclease S1 (10 U/μg RNA) under native conditions, as described in Materials and methods, for 3 min at 22°C. **Lane 1** (T1) is a partial digestion of wild-type pre-tRNA with RNAse T1 under denaturing conditions. **Lane 10** (OH^-) is a partial alkaline digest. Nucleotides have been numbered taking into account the fact that S1 bands run between 1 and 1½ bands slower than the corresponding bands which have an additional phosphate at the 3′ end. The reaction mixtures were electrophoresed on a slab gel of 8% polyacrylamide-7 M urea.

lane 4). However, when the same fractions are added to Fraction II (eluting between 0.25 and 0.40 M KCl) the pM2 precursor is cleaved (Figure 3, lane 5). The factor(s) does not complement the purified endonuclease in the cleavage of the two single mutants pM1 and pM3 and stimulates only slightly (less than a factor of two) the cleavage of the wild-type precursor. Spermidine cannot substitute for the factor and the latter is non-dialysable and heat-sensitive. It is possible, since the naked pM2 precursor does not assume the wild-type conformation (see following section), that the factor(s) acts by stabilizing the weak D-stem constituted by three AT base pairs. Further purification will be necessary in order to clarify the role of the factor(s).

Accessibility of the intron in the mutant precursors

The analysis of solution conformations of intron-containing tRNA precursors using enzymatic probes has led to the conclusion that the structure of such precursors is dominated by the presence of structural domains which are conserved in the mature tRNA product. Swerdlow and Guthrie (1984) and Lee and Knapp (1985), have reached this same conclusion while investigating several mature pre-tRNA/pairs.

Assuming that splicing is achieved *via* the recognition of structural elements which are primarily in the 'mature domain', we compared the conformation of the wild-type precursor with that of the pM1, pM2 and pM3 precursors which are defective in splicing.

Wild-type and mutant pre-tRNAs were synthesized *in vitro* in the presence of cold ribotriphosphates using the GV system (Mattoccia *et al.*, 1979). The primary transcripts, after alkaline phosphatase treatment, were labeled at the 5′ end with the T4 polynucleotide kinase. The terminally labeled molecules are identical, except for the 5′ termini, to the ones labeled with [α-^{32}P]-GTP. The only labeled product resulting from the cleavage of the terminally labeled precursor, is, as expected, the 5′ half molecule (data not shown).

The native structures of wild-type and mutant precursors were probed using limited RNase digestion. The probing conditions were identical to those employed when the precursors were used as substrates for the endonuclease, since we were interested in obtaining information on the structures recognized by the enzyme.

The structures of the precursors were probed using the following enzymes: S1 nuclease, under conditions in which the enzyme cleaves single-stranded regions of RNA without sequence specificity; RNase T2, which shows no sequence specificity but a preference for adenosine bonds; RNase T1, a guanosine-specific nuclease; RNase A, a pyrimidine-specific enzyme.

In this kind of experiment it is important to try to generate only single-cleavage products. Primary cleavage sites permit the identification of the most accessible portion of the molecule and provide information about the tertiary structure. Secondary sites may be the result of structural perturbations caused by a primary hit at another site. They do provide information, however, about the secondary structure.

To generate only single-cleavage products, low RNase/RNA ratios are used. S1 nuclease will then cleave wild-type and mutant precursors in the anticodon-intron loop and, though less efficiently, in the extra-arm. Figure 4 shows that all four precursors were cleaved in a region localized between nucleotides 39 and 42 and in correspondence to nucleotides 56 – 58. The patterns of cleavage sites obtained using RNase T2 and RNase A at low enzyme/RNA ratios (data not shown) correspond to those obtained with S1. The primary sites described above are not cleaved by RNase T1 since there is no G either in the intron-anticodon loop or in the extra-arm loop. The fact that the primary cleavage sites are located in the intron in all four precursors indicates that this region represents the most accessible portion of the molecule. The intron is presumably on the surface, available for interaction with the splicing nuclease. However, since the mutant precursor is not cleaved by the splicing endonuclease, accessibility in itself does not assure excision of the IVS by the endonuclease; the recognition elements must reside somewhere else in the molecule.

Primary cleavage in the D and TΨC regions

The absence of G residues in the intron-anticodon loop and in the extra-arm loop allows for the use of RNase T1 to generate primary cleavages in other parts of the molecule. This fact enabled us to focus on the D-region of the molecule, where the mutations that we are studying are localized. Figure 5 shows a time-course analysis of the conformations of wild-type and mutant precursors at low RNase/RNA ratios. In all four cases cleavage occurs at G98, a nucleotide of the TΨC loop which is not involved in tertiary interactions. G15 is cleaved in the wild-

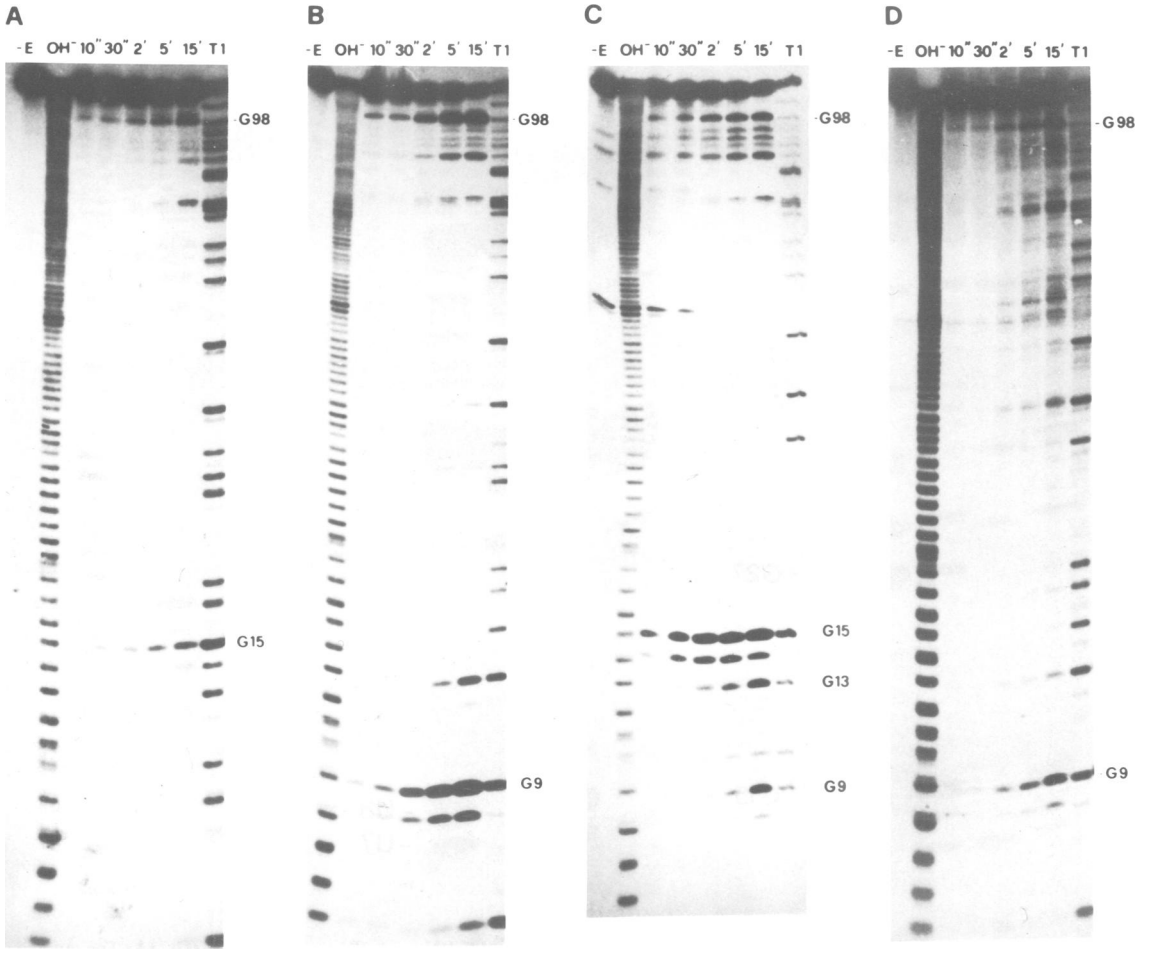

Fig. 5. Time course of RNase T1 digestion on 5′-^{32}P-end labeled wild-type (**A**), mutant pM1 (**B**), mutant pM3 (**C**) and mutant pM2 (**D**) precursors. From left to right: (−E) pre-tRNAs incubated under RNase T1 digestion conditions, with enzyme omitted. (OH^-) partial alkaline digest. Other lanes contain RNase T1 digests, obtained under native conditions as described in Materials and methods. The enzyme RNA ratio was 5×10^{-3} U/μg RNA, for times indicated in the Figure. T1, partial digestion with RNase T1 under denaturing conditions. Sites which were preferentially cleaved are indicated. The reaction mixtures were electrophoresed on slab gels of 16% polyacrylamide-7 M urea.

type; G9 in pM1 and pM2; G15, G13 and G9 in pM3. Of the G-residues present in the D loop (G13, G15, G17 and G18), only G15 is cleaved in the wild-type precursor. G17 and G18 are involved in the tertiary interaction with the TΨC loop, and G13 is apparently not within easy reach of RNase T1. The structural perturbations caused by the mutations might explain why the splicing endonuclease is on the whole inactive on the pM1 and pM3 precursors and only very inefficiently active on the pM2 precursor. In the case of pM1 and pM3 we are dealing with mutations which alter base-pairing, critical for the maintenance of the conformation of the mature domain; in the case of pM2, the D-stem, constituted by three AT base pairs which substitute the three GC base pairs of the wild-type, is evidently not stable enough to allow for the formation of the wild-type structure.

Secondary cleavages

To obtain information about the secondary structure, we performed experiments at high RNase/RNA ratios. Figure 6A shows the cleavages produced by RNase T1 at high RNase/RNA ratios. It is clear that the differences between the cleavage patterns of the wild-type and the mutant precursors are all localized in the 5′ portion of the molecule, up to position G27, which is preferentially cleaved only in pM3 and pM2. When RNase A is used to probe the secondary structure, the pM1 and pM2 precursors show striking cleavages at U7 and U8 (Figure 4B). Experiments with RNase T2 show that A14 is susceptible only in the wild-type and pM3 precursors (Figure 4C). The results of the conformational analysis are represented in the linear map in Figure 7. In summary: (i) the three mutants differ from the wild-type only in the 5′ portion of the molecule up to G27. The interaction between the D and TΨC loops appears to be maintained in all the cases since the bases involved in the mutual tertiary interaction are not, as in the wild-type, susceptible to the enzymatic probes. (ii) The double mutant does not assume the wild-type conformation. (iii) Three distinct regions of altered sensitivity (U7, U8, G9; G13, A14; G27) are visible in the linear representations of the molecules (see Figure 7). When the molecules are folded according to the model for the tertiary structure of the tRNA precursors, the three regions are located near one another on the 'inside' of the angle in the 'L'-shaped conformation (Figure 8).

Discussion

In eucaryotes, intervening sequences interrupt the coding regions of many genes specifying mRNA, tRNA and rRNA (reviewed by Abelson, 1979). In all cases examined the intervening sequences are transcribed as part of a precursor RNA and are subse-

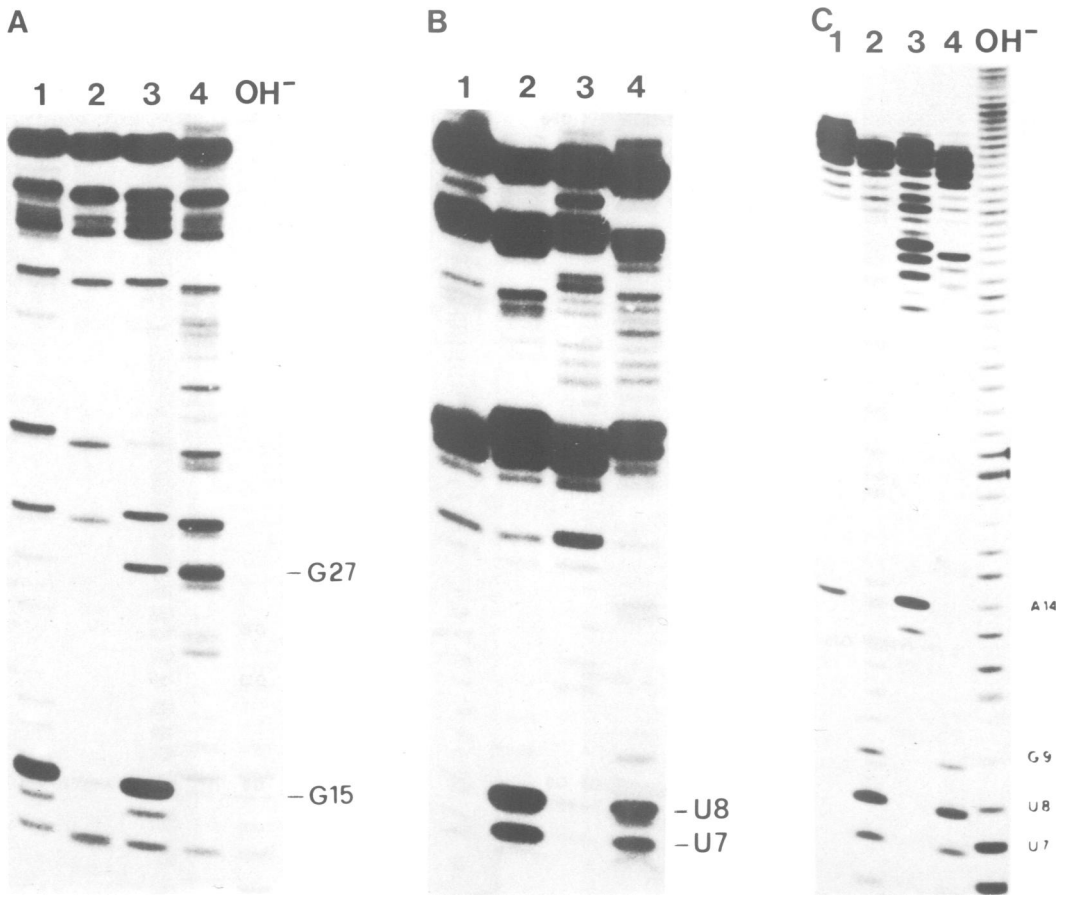

Fig. 6. Structure-probing reactions of wild-type and mutant precursors with RNase T1 (**A**), RNase A (**B**) and RNase T2 (**C**). (**A**) The autoradiograph shows a portion of a 16% polyacrylamide-7 M urea slab gel. 5′-^{32}P-end labeled wild-type (**lane 1**), pM1 (**lane 2**), pM3 (**lane 3**), pM2 (**lane 4**) precursors were digested under native conditions with RNase T1 at an enzyme/RNA ratio of 5×10^{-2} U/μg RNA for 3 min at 22°C. (**B**) The autoradiograph shows a portion of a 16% polyacrylamide-7 M urea slab gel. 5′-^{32}P-end labeled wild-type (**lane 1**), pM1 (**lane 2**), pM3 (**lane 3**), pM2 (**lane 4**) precursors were digested under native conditions with RNase A at an enzyme/RNA ratio of 10^{-6} U/μg RNA for 10 s at 22°C. (**C**) The autoradiograph shows a portion of a 16% polyacrylamide-7 M urea slab gel. 5′-^{32}P-end labeled wild-type (**lane 1**), pM1 (**lane 2**), pM3 (**lane 3**), pM2 (**lane 4**) precursors were digested under native conditions with RNase T2 at an enzyme/RNA ratio of 0.5 U/μg RNA for 3 min at 22°C. Residue assignments were made by alignment with sequencing reactions on portions of the autoradiograph (not shown).

quently removed by a process termed RNA splicing. There is no single, general mechanism involved in the splicing of the different types of RNA. It is expected, however, that both secondary and tertiary structures of the nucleic acid play a crucial role in all cases. Both in *Xenopus* and in yeast a single nuclease can cleave several tRNA precursors to create substrates for subsequent ligation (Otsuka *et al.*, 1981; Peebles *et al.*, 1983). The enzyme, therefore, must be recognizing features that are shared by all the precursors. Two features seem to be common to all tRNA precursors: first, the intervening sequences, when present, are always located one nucleotide to the 3′ side of the anticodon; second, all of the precursors probably present a tRNA-like tertiary structure in the mature domain of the molecule.

The purified splicing endonuclease generates cyclic phosphodiester termini

To investigate the interaction between enzyme and pre-tRNA, we produced a highly purified preparation of splicing endonuclease.

The enzyme, purified >12 000-fold from oocytes, requires Mg^{2+} ions to cleave the wild-type precursor of $tRNA_3^{Leu}$. The purified enzyme sediments in glycerol gradients containing 150 mM salt with a sedimentation coefficient of 7S. The activity is insensitive to micrococcal nuclease (data not shown). This negative evidence suggests that the enzyme does not contain an RNA component.

GV extracts produce, in the simultaneous transcription processing reaction, 5′ half molecules with 2′,3′-cyclic phosphate termini. The cyclic phosphate might be directly produced by the endonuclease, as has been demonstrated in yeast (Peebles *et al.*, 1983), or, alternatively, could be the result of the action of the RNA 3′-terminal phosphate cyclase present in GV extracts (Filipowicz *et al.*, 1983). The experiments with the purified endonuclease reported here indicate that the cleavage of tRNA precursors by the enzyme generates 5′ halves with cyclic phosphodiester termini. The cyclase may have a role in maintaining the cyclic structure at the terminus of the ligation substrate. The presence in the GV extracts of enzymes capable of converting the 2′,3′-cyclic phosphate into a 3′-phosphate group, would require the action of the cyclase since the RNA ligase involved in tRNA processing utilizes substrates with 2′,3′-cyclic phosphate ends (Fourneaux *et al.*, 1983).

The purified enzyme requires Mg^{2+} ions to cleave the wild-type precursor of $tRNA_3^{Leu}$. In contrast to the situation observed with the crude extract, cleaving of the pM2 precursor occurs very inefficiently with the purified endonuclease. In a reconstitu-

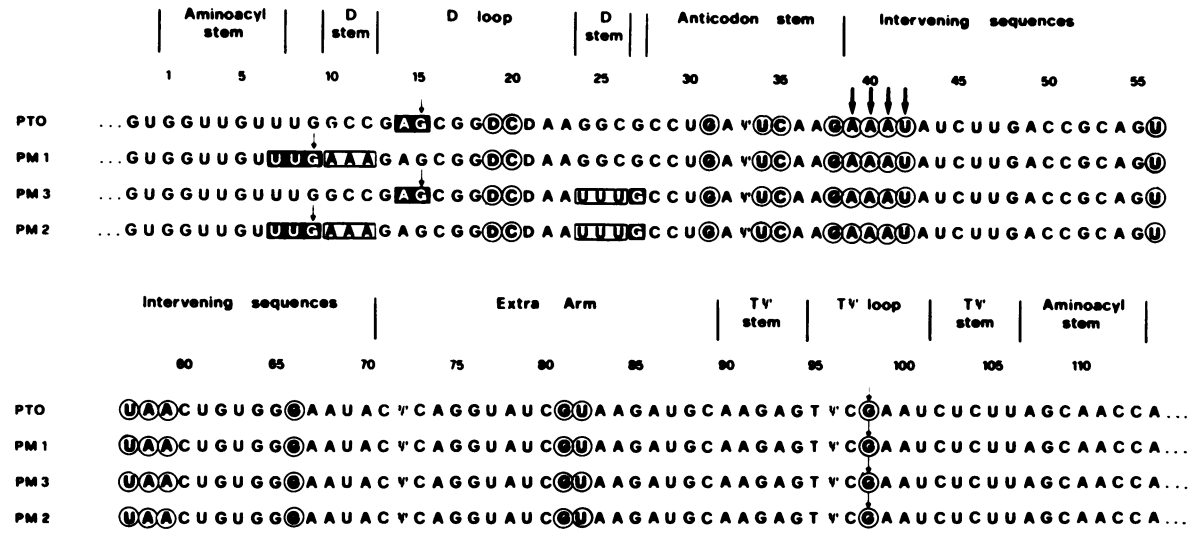

Fig. 7. Sequences of wild-type and mutant pM1, pM3 and pM2 precursors. Sequence changes in the mutants are boxed. Upper line indicates the regions of the secondary structure model of the pre-tRNA corresponding to the sequences shown below. The limits of the 5′ leader and 3′ trailer sequences of the precursors are not indicated. The RNA sequence shows the base modifications present in the mature tRNA and not necesarily those present in the precursors. Primary cleavage sites are indicated by arrows. Circled nucleotides correspond to preferential cleavage sites. Black-boxed nucleotides indicate sites of cleavage which differ among the precursors.

tion experiment we have shown that a factor(s) present in the GV extract complement the enzyme when the pM2 precursor is the substrate. The factor(s) might act by stabilizing the weak D-stem of the double mutant. In this case the binding of the factor(s) presumably contributes to the formation of a correct structure of the precursor.

What are the structural features recognized by the splicing endonuclease?

An analysis of the conformations of several IVS-containing tRNA precursors indicates that they have a common structure, dominated by domains which resemble corresponding elements in the mature tRNA with respect to both secondary and tertiary structure (Swerdlow and Guthrie, 1984; Lee and Knapp, 1985). A single enzyme, presumably, recognizes common structural features in the mature domain. The structural analysis of defective splicing substrates may provide information about the elements involved in the recognition process. Here we report a study of the conformation of a class of precursors, having found these to be the most defective in our collection of mutants. In these mutants the excision of the IVS is dependent on the formation of a D-stem (Baldi *et al.*, 1983); this does not necessarily imply, however, a direct role of the D-stem in the recognition process. We have found that non-homology in the D-stem results in alterations in the conformation of other parts of the tRNA. The interaction between D and TΨC loops seems to be conserved in the mutants described here. The enzymatic probes we have used are able to detect new sensitive sites in the D and TΨC loops when the tertiary interaction is affected, as in the case of the mutants in the same gene where G18 is substituted with C and C56 with G (E.Mattoccia, M.I.Baldi, S.Ciafré and G.P. Tocchini-Valentini, unpublished data). Our results in general indicate that the overall 'L'-shaped conformation is maintained in the mutants; the perturbations due to the mutations are localized on the 'inside' of the 'L'-shaped three-dimensional tRNA structure (Figure 8). The endonuclease could bind at the inside angle of the 'L'-shaped precursors and then proceed to cut at the 3′ site and subsequently at the 5′ site. With the *Xenopus* enzyme the production of molecules cleaved exclusively at the 5′ site has never been observed, on the contrary, molecules lacking the 3′ half are frequently found (Otsuka *et al.*, 1981; Baldi *et al.*, 1983). M.C.Lee and G.Knapp (unpublished data) have observed in the yeast system that 3′ cleavage can occur independently of 5′ cleavage and that the modification of sequences at the 3′ site significantly alters the rate of precursor cleavage at both sites.

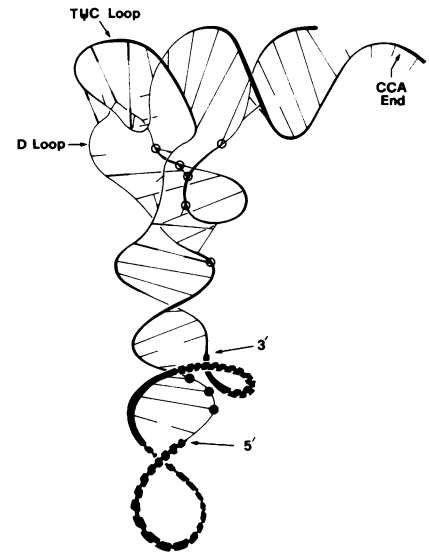

Fig. 8. Model for the tertiary structure of the yeast tRNA precursors (Lee and Knapp, 1985). The tRNA portion (thin line) of the precursor retains the conventional 'L'-shape conformation with tertiary interaction between different regions of the molecule. The intron (thick line) is inserted into the anticodon loop (filled circles indicate the anticodon triplet). Intron segments that form part of the consensus secondary structure are shown by thick black lines. Postions of variable structure are shown by the hatched thick lines. Circled positions correspond to the sites of cleavage descibed in the text.

Several mutants, in addition to the absolute defectives described in this paper, are inefficiently spliced. The mutations have been mapped in the D-loop, the intron and the extra-arm (Willis *et al.*, 1984) and in the vicinity of the intron (Nishikura *et al.*, 1982);

the induced perturbations could affect phases of the process that occur after the binding of the enzyme.

Since the binding of aminoacyl-tRNA synthetases occurs along and around the 'inside' of the 'L', it is tempting to hypothesize that a similarity might exist between the mode of binding of synthetases (Starzyk *et al.*, 1982) and that of the splicing endonuclease. Over 150 cytoplasmic tRNAs sequenced to date contain uridine (or 4-thiouridine) at position 8, which lies on the inside vertex of the 'L' formed by the folded nucleic acid. A transient Michael adduct between the uracyl ring and the active site of *E. coli* Ala-tRNA synthetase has been observed and, since aminoacyl tRNA synthetases catalyze H-5 exchange at the common uridine 8, this residue is presumably the site of interaction (Schoemarker and Schimmel, 1977; Koontz and Schimmel, 1979). In agreement with this conclusion, Starzyk *et al.* (1985) reported that the selective reduction of the 5,6 double bond of 4-thiouridine at position 8 in *E. coli* tyrosine-tRNA prevents aminoacylation.

Substitution of U8 in $tRNA_3^{Leu}$ with A, G or C produces precursors that are defective in splicing (M.I.Baldi, E.Mattoccia, S.Ciafré and G.P.Tocchini-Valentini, unpublished data); however these results could derive either from a conformation change of the molecule as a whole, or from the modification of the site *per se*.

Two other endonucleases which act on the pre-tRNAs have been partially purified from GV extracts (G.Carrara, P.Fruscoloni, D.Civitareale and G.P.Tocchini-Valentini, unpublished data). An RNase-P-like enzyme (*Xla*II RNase) cleaves the 5′ leader and presumably contains an RNA moiety, since the activity is sensitive to micrococcal nuclease and bands as a ribonucleoprotein in Cs_2SO_4 gradients (Akaboshi *et al.*, 1980).

The second endonuclease (*Xla*III RNase) removes the 3′ trailer and is insensitive to micrococcal nuclease treatment. It should be interesting to see how these two nucleases act on mutant precursors and to determine which part of the nucleic acid molecule plays a role in the recognition process.

Materials and methods

Plasmids

Plasmid pTO was constructed by subcloning the 2.5 kb *Eco*RI DNA fragment containing the $tRNA_3^{Leu}$ gene from pJB 2k (Beckman *et al.*, 1977) into pBR322 at the *Eco*RI site as described by Johnson *et al.* (1980). Mutant plasmids pM1, pM2 and pM3 were derived from plasmid pTO as described by Mattoccia *et al.* (1983) and by Baldi *et al.* (1983).

Enzymes and chemicals

[α-^{32}P]GTP (410 Ci/mmol), [α-^{32}P]ATP (410 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from Amersham. RNase T1 (Sankyo) was purchased from Calbiochem. RNase A, RNase Physarium M, nuclease S1 and guanosine 5′-phosphate 2′,3′-cyclic phosphate were obtained from P.L.Biochemicals. RNase T2 was purchased from Bethesda Research Laboratories. Nuclease P1 and guanosine 2′,3′-cyclic monophosphate were obtained from Sigma.

Buffers

J buffer contained 70 mM NH_4Cl, 7 mM $MgCl_2$, 0.1 mM EDTA, 2.5 mM dithiothreitol, 10% (v/v) glycerol, and 10 mM Hepes (pH 7.5). JB75 and JB150 are J buffers with respectively 75 and 150 mM NH_4Cl. TEMG contained 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1.4 mM 2-mercaptoethanol and 16% (v/v) glycerol.

Transcription of tRNA genes and purification of RNAs

Germinal vesicle extract from stage 6 oocytes was prepared according to Mattoccia *et al.* (1979). The standard transcription reaction mixture contained, in 200 μl, 10 μg of DNA, 0.2 mM of the three non-radioactive ribonucleotide triphosphates, 0.04 mM [α-^{32}P]GTP at a sp. act. of 40 Ci/mmol, 7 mM $MgCl_2$, 0.1 mM EDTA, 2.5 mM dithiothreitol, 10% glycerol (v/v), 10 mM Hepes (pH 7.5) and 70 mM NH_4Cl. After incubation for 2 h at 22°C the reaction was stopped and the RNA products were purified as previously described (Baldi *et al.*, 1983).

Endonuclease assay

The standard reaction mixture contained, in 50 μl of J buffer, 10 000 c.p.m. of precursor and 10 μl of enzyme. In order to identify the chromatographic fractions with endonuclease activity, the assay contained 10 μl of each column fraction in a volume of 60 μl of J buffer such that the salt contribution of the fraction did not inhibit the enzyme activity. Reactions were incubated for 2 h at 22°C and stopped by addition of SDS to 0.5% and Proteinase K to 0.1 mg/ml. After incubation for 30 min at room temperature and addition of 1 μg of yeast tRNA and NaCl to 0.2 M, the samples were precipitated with 3 vol ethanol at −70°C for 30 min.

Quantitation of endonuclease activity was performed in 50 μl of J buffer containing 2×10^{-2} pmol of ^{32}P-labeled precursor and 10 μl of purification fractions I−V. Fraction VI was assayed in 100 μl of JB75 containing 4×10^{-2} pmol of labeled precursor and 50 μl of sucrose fraction. All fractions were assayed in the same experiment; the products were analyzed by gel electrophoresis, as described, and the reactants and products were Cerenkov counted. One unit of endonuclease is defined as the amount of enzyme that cleaves 1×10^{-3} pmol of precursor in 2 h at 22°C.

Purification procedure

All procedures were carried out at 4°C. The nuclear extract (47 ml, 265 mg) was prepared from 350 ml of collagenase-treated oocytes, divided into two equal batches, and frozen at −70°C until use. Each batch of extract was dialyzed against TEMG buffer pH 7.5 (Fraction I) and applied to a 40 ml phosphocellulose column equilibrated with TEMG buffer pH 7.5. The column was washed with the equilibration buffer and eluted with a 600 ml non-linear gradient from 0 to 1.5 M KCl in the same buffer (flow-rate 50 ml/h). The endonuclease activity eluted between 0.25−0.40 M KCl. The pool of the active fractions derived from the two phosphocellulose columns was dialyzed against TEMG buffer pH 7.5 (Fraction II) and divided into two batches. Each batch was applied separately onto a heparin-Sepharose column (4 ml), equilibrated with TEMG buffer pH 7.5. The column was washed and eluted with a 40 ml linear gradient from 0 to 0.8 M NaCl in the same buffer (flow-rate 6 ml/h). The endonuclease activity eluted between 0.4 and 0.5 M NaCl. The pool of the active fractions derived from the two heparin-Sepharose columns was dialyzed against TEMG buffer pH 8.0 (Fraction III) and applied to a DEAE-Sephadex column (4 ml), equilibrated with the same buffer. The column was washed and eluted with a 50 ml linear gradient from 0 to 0.5 M KCl in the same buffer (flow-rate 6 ml/h). The endonuclease activity eluted between 0.1 to 0.2 M KCl. The active fractions were dialyzed against TEMG buffer pH 7.5 (Fraction IV) and applied to a CM-Sephadex column (5 ml), equilibrated with the same buffer. The column was washed and eluted with a 60 ml linear gradient from 0 to 1 M KCl in the equilibration buffer (flow-rate 6 ml/h). The active fractions at ~0.25 M KCl were dialyzed against J buffer (Fraction V) and stored in aliquots at −70°C. 0.3 ml aliquots of Fraction V were applied onto 5 ml sucrose gradients (5−20% sucrose in JB150) and centrifuged for 22 h at 48 r.p.m. in a SW 55.1 rotor at 3°C. A total of 32 fractions were collected from the bottom of the tube. The standard proteins used for sedimentation were bovine immunoglobulin G (160 000), bovine serum albumin (66 000), ovalbumin (45 000) and myoglobin (17 000).

5′-^{32}P-end-labeling and purification of precursor tRNAs

To prepare unlabeled precursors, the transcription reaction was scaled-up four times in the presence of 0.2 mM of the four non-radioactive ribonucleotide triphosphates. Purified wild-type and mutant precursor tRNAs were dephosphorylated and labeled with ^{32}P using T4 polynucleotide kinase and [γ-^{32}P]ATP sp. act. 1500 Ci/mmol. After labeling, the precursors were ethanol precipitated and repurified onto a 10% polyacrylamide gel-4 M urea.

RNA structure analysis

Structure probing reactions of 5′-^{32}P-end-labeled precursors under native conditions were performed at 22°C in J buffer (4 μl) using RNase T1 (guanosine specific), RNase A (pyrimidine specific) and RNase T2 (non-specific with a preference for adenosine bonds). Reactions with nuclease S1 (no known sequence specificity) in J buffer contained 1 mM Zn^{2+}. Enzyme concentrations and times of incubation are specified in the figure legends. Reactions were stopped by addition of 2 μl of a solution containing 9 m urea, 10% glycerol (v/v) 0.05% (w/v) xylene cyanol and 0.05% (w/v) bromophenol blue and immediately loaded on and electrophorized through polyacrylamide slab gels. Slab gels of different polyacrylamide concentrations (8% polyacrylamide-7 M urea or 10% polyacrylamide-7 M urea or 16% polyacrylamide-7 M urea) were used to resolve different regions of the precursor RNA molecules. The RNA sequences were determined by partial digestion with alkali and, under fully denaturing conditions, with RNase T1 and RNase A according to Donis-Keller *et al.* (1977). The specific cleavage sites in the structure-probing reactions were assigned by comparing their mobilities with those produced by base-specific sequencing reactions and alkali hydrolysis.

RNA sequence analysis

RNase T1 digestion of 5′ half molecules of pre-tRNA were analyzed by finger-

printing according to Brownlee (1972). The sequences of T1 oligonucleotides have been previously described (Otsuka *et al.*, 1981). Oligonucleotides were eluted with 30% triethylamine carbonate (pH 10) and digested with RNase T2 (0.04 U/μg RNA) in 50 mM Na acetate pH 4.5, 4 mM EDTA for 15 min at 37°C. RNase T2 digests were analyzed by one-dimensional chromatography on thin-layer PEI-plates in isobutyric acid-NH_4OH-H_2O (57.7:3.8:38.5). Ribonuclease Physarium M digests of 5′ half molecules were analyzed by two-dimensional chromatography on cellulose thin-layer plates (20 × 20 cm; Merck) in isobutyric acid-NH_4OH-H_2O (57.7:3.8:38.5) in first dimension ,and saturated $(NH_4)_2SO_4$-1 M NaOAc-isopropanol (80:18:2) in second dimension (Konarska *et al.*, 1985).

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