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The processing of wild type and mutant forms of rat nuclear pre-tRNA^{Lys} by the homologous RNase P

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ABSTRACT

The 5′ processing of rat pre-tRNA^{Lys} and a series of mutant derivatives by rat cytosolic RNase P was examined. In standard, non-kinetic assays, mutant precursors synthesized *in vitro* with 5′ leader sequences of 10, 17, 24, 25, and 46 nucleotides were processed to approximately equal levels and yielded precisely cleaved 5′ processed intermediates with the normal 7-base pair aminoacyl stems. The construct containing the tRNA^{Lys} with the 46-nucleotide leader was modified by PCR to give a series of pre-tRNA^{Lys} mutants designed to measure the effect on processing by (1) substituting the nucleotide at the +1 position, (2) pairing and unpairing the +1 and +72 bases, (3) elongating the aminoacyl stem, and (4) disrupting the helix of the aminoacyl stem. Comparative kinetic analyses revealed that changing the wild type +1G to A, C, or U was well tolerated by the RNase P provided that compensatory changes at +72 created a base pair or a G·U noncanonical pair. Mutants with elongated aminoacyl stems that were produced either by inserting an additional base pair at +3:a+69:a or by pairing the −1A with a +73U, were processed to yield 7-base pair aminoacyl stems, but with different efficiencies. The efficiency seen with the double insertion mutant was higher than even the wild type precursor, but the −1A−U+73 mutant was a relatively poor substrate. Disrupting the aminoacyl stem helix by introducing a +7G G+66 mispairing or by inserting a single G at the +3:a position dramatically reduced the processing efficiency, although the position of cleavage occurred precisely at the wild type cleavage site. However, the single insertion of a C at the +69:a position resulted in an efficiently cleaved precursor, but permitted a minor, secondary cleavage within the leader between the −6 and −5 nucleotides in addition to the dominant wild type scission.

INTRODUCTION

Ribonuclease (RNase) P is the endonuclease that cleaves tRNA precursors to generate the 5′ termini of mature tRNAs (1,2). RNase P is widespread and has been isolated from prokaryotic cells, eukaryotic cells, mitochondria, and chloroplasts (2–10).

Most examples of RNase P have been shown to be ribonucleoprotein complexes. Among these, only the well-characterized eubacterial enzymes have been found to contain a catalytic RNA (2), able to properly process precursor tRNAs in the absence of the protein subunit. The respective functional roles of the protein and RNA subunits of all other RNases P have not yet been established, and all attempts to demonstrate an RNA-alone reaction among any of these other enzymes have thus far been unsuccessful.

Studies using model RNA substrates have provided evidence that the eubacterial RNase P can process substantially modified precursors. For example, structures composed essentially of only the aminoacyl stem plus the T-arm can be efficiently processed by *Escherichia coli* RNase P (11), suggesting that these two helical elements are sufficient structure for recognition by the eubacterial enzyme. Recent studies by Kirsebom and Svärd (12) indicated that several specific nucleotides clustered around the site of cleavage may act as guide elements for the *E.coli* RNase P reaction as well. For the eukaryotic enzymes, little is known regarding the structural or sequence elements in precursor tRNAs that serve as recognition signals for RNase P interaction.

We have been studying the rat liver cytosolic RNase P using the homologous pre-tRNA^{Lys}. To examine the importance of various structural elements and sequences near the RNase P cleavage site, we have prepared a number of modified tRNA^{Lys} precursors through the use of PCR. The effects of varying the lengths of 5′ leader sequences were evaluated using standard reaction conditions. The relative importance of various structural features and specific nucleotides in the aminoacyl stem was determined in a kinetic analysis comparing a series of mutant tRNA precursors with the wild type pre-tRNA^{Lys}. The specific site of processing of each pre-tRNA was determined as well.

MATERIALS AND METHODS

Enzymes and reagents

Restriction endonucleases and all other DNA modifying enzymes were purchased from Gibco-BRL, New England BioLabs, or Boehringer Mannheim. Each enzyme was used with the

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accompanying buffer, except T4 DNA Ligase which was used with the buffer of Nilsson and Magnusson (13). T7 RNA polymerase, RQI DNase, and pGEM® -4Z were purchased from Promega. Oligo- and polydeoxynucleotides were prepared by the Nucleic Acid Synthesis and Analysis Laboratory (Virginia Commonwealth University). All other standard laboratory chemicals were Molecular Biology-grade or purer.

Preparation of RNase P

The rat liver cytosolic RNase P was isolated as described previously (14). The enzyme fraction obtained after Cs_2SO_4 gradient centrifugation (designated 'Cs_2SO_4 gradient-I') was used throughout this work. This fraction was about 500-fold purified over the initial high speed supernatant step. Although more highly purified preparations were available, they were not used because previous studies showed that additional purification steps dramatically reduced the stability of the RNase P. The Cs_2SO_4 gradient-I fraction used was highly active and sufficiently long lived such that multiple comparative assays could be done with each preparation.

Preparation of precursor tRNA substrates

The wild type precursor used throughout this study was a rat pre-tRNA^{Lys} from the gene locus 1 (15). Both strands of the complete gene sequence were produced by automated DNA synthesis. These included in addition to the tRNA sequence, a *Hin*dIII restriction site and 12 flanking nucleotides on the 5′ side and 10 flanking nucleotides plus an *Eco*RI restriction site on the 3′ side. The complementary strands were annealed, ligated into the *Hin*dIII and *Eco*RI sites of pGEM® -4Z, and introduced into competent JM109 cells. Mini-preps were generated and the cloned plasmids were screened by restriction mapping. A T7 transcript derived from the resultant cloned plasmid contained the wild type tRNA^{Lys} sequence, a 25-nucleotide leader, and a 15-nucleotide trailing sequence; and was designated WT-L25 (Fig. 1). This plasmid (designated pWT-L25) was used as the template for production of pWT-L46 using the PCR primers shown in Table I. The pWT-L46 then served as the parent plasmid for all subsequent leader and aminoacyl stem mutants employed in this study. The leader-length mutant plasmids pWT-L17 and pWT-L24 were generated by restriction endonuclease cleavage and mung bean nuclease digestion, followed by blunt-end ligation. The pWT-L10 mutant was produced by substitution via restriction sites of a 37-nucleotide segment of the pWT-L46 leader with a single nucleotide. The sequence of each mutant plasmid was confirmed by Sanger dideoxy sequencing (16).

The aminoacyl stem mutants were all produced by modification of pWT-L46 by PCR using the primers shown in Table I. The PCR products were ligated into pGEM® -4Z, and introduced into JM109 cells. The resulting plasmid mini-preps were screened by restriction mapping, and the sequence of each mutant plasmid was confirmed by Sanger dideoxy sequencing. Large scale preparation of plasmids were obtained from scaled-up cultures processed by alkaline lysis (17) followed by banding in CsCl−ethidium bromide gradients (18).

The standard transcriptions *in vitro* were carried out as follows. The plasmid templates were cleaved at the *Eco*RI site present at the 3′ end of each precursor gene sequence, purified by phenol/chloroform extraction, ethanol precipitated, and resuspended at 1 μg/μl in 10 mM MOPS, 1 mM EDTA, pH 8.0 (ME buffer). The transcription reactions were carried out in a total of 100 μl and contained: 40 mM MOPS, pH 7.5, 10 mM NaCl, 6 mM $MgCl_2$, 10 mM DTT, 2 mM spermidine, 1 U/μl recombinant RNasin, 0.5 mM each of three nonradioactive NTPs, 0.1 mM [α-^{32}P] NTP (0.75 μCi/nmole), 7 μg of *Eco*RI-digested plasmid, and 60 U of T7 RNA polymerase. The reactions were incubated at 37°C for 2 h, 6 U of RQI DNase were added, and the incubation was continued for 30 min. The reactions were extracted with phenol/chloroform and passed through a 0.7 cm×18 cm column of Sephadex G-50 in 10 mM MOPS, 1 mM EDTA, 0.1% SDS, pH 8.0. The labeled peak was phenol/chloroform extracted, ethanol precipitated, and resuspended in 200 μl ME buffer.

For preparation of the larger amounts of labeled pre-tRNA having lower specific radioactivities required for the steady state kinetic analyses, the transcription reactions were scaled up to 400 μl. In addition to the standard concentrations of buffer and salts, the reactions contained 12 μg of *Eco*RI-cleaved plasmid template, 0.5 mM of each of three unlabeled NTPs, 0.4 mM of the [α-^{32}P] NTP (0.25 μCi/nmole), and 160 U of T7 RNA polymerase. The incubation of the reaction and purification of the labeled transcript were performed as in the standard reaction described above.

Standard assay of RNase P activity

The standard RNase P reactions were carried out in a total of 50 μl which contained 50 mM MOPS, pH 8.0, 5 mM DTT, 5 mM $MgCl_2$, 110 mM KCl, 5−10×10^4 cpm of [^{32}P] pre-tRNA^{Lys}, and 5 μl of the RNase P preparation. The reactions were incubated at 37°C for 3 min (or up to 15 min for poorly processed substrates) and terminated by the addition of an equal volume of 94% (v/v) deionized formamide, 10 mM EDTA, 0.05% (w/v) bromophenol blue, and 0.05% (w/v) xylene cyanol FF. The samples were heated for 4 min at 80°C and electrophoresed on a 1.5 mm thick, 10% polyacrylamide/7 M urea gel. The precursor and processed tRNA products were identified by autoradiography.

Determination of the optimal conditions for RNase P activity

Using the standard assay for RNase P activity, the optimal concentrations of $MgCl_2$ and KCl were measured in a series of experiments using a range of KCl concentrations with several different $MgCl_2$ concentrations being held constant, followed by measurement of a range of $MgCl_2$ concentrations with several KCl concentrations held constant. These were repeated using narrowed ranges until the optimum for each reagent was found. The percent of processing was determined by scintillation counting of the precursor and product bands sliced from the polyacrylamide/urea gels.

Steady state kinetic assays

The RNase P activity was assayed in the standard reaction described above, except that the reaction mix (minus enzyme) was pre-incubated for 10 min at 37°C before enzyme addition, and the various pre-tRNA^{Lys} concentrations were typically varied between 0.3 and 5×K_m. The unprocessed precursor, the 5′ processed tRNA, and the leader sequence were quantitated directly from the standard polyacrylamide/urea gel on a Betagen Betascope 603 Blot Analyzer™. The cpm were converted to concentrations of RNA and the kinetic parameters K_m and V_{max} were calculated from Hanes−Woolf plots to diminish excessive weighting of values from low substrate concentrations (19). For each assay, the amount of enzyme activity in units, defined as the amount of enzyme required to form 1 pmole of product per

min (14), was determined using the WT-L46 pre-tRNA^{Lys} as substrate. The value 'relative V_{max}' for each substrate was calculated as V_{max} per unit of enzyme. This value is similar to k_{cat} calculated for homogeneously purified enzyme preparations. An efficiency value was then calculated as 'relative V_{max}/K_m'.

Mapping the site of RNase P processing

For RNA fingerprint analyses, tRNA^{Lys} precursors, the 5′ processed products, and the leader sequences were separated on 10% polyacrylamide/7 M urea gels and eluted as described (7). The isolated RNAs were digested to completion with RNase T_1 and fingerprinted as previously described (7, 20, 21). The resulting fingerprints were interpreted according to the rules of Sanger and Brownlee (22).

The 5′ terminal nucleotide determination of each 5′ processed product was done according to published procedures (23). The 5′ processed products, labeled at G, C, or A, were purified on 10% polyacrylamide/7 M urea gels and divided into halves. One half was treated with alkaline phosphatase (18). Both halves were then subjected to complete digestion with RNases A and T_2 prior to separation of the Nps and pNps on PEI-cellulose thin layer chromatographic plates.

To clarify any ambiguities derived from the above methods, RNase P-digested products from standard reactions were also analyzed on sequencing gels which effectively separated RNA sequences that differed in length by a single nucleotide (24).

RESULTS

The wild type rat precursor tRNA^{Lys} (pre-tRNA^{Lys}) (Fig. 1) used in this study is a characteristic type I tRNA sequence that carries a 46-nucleotide leader composed primarily of vector sequence, and a 7-nucleotide trailing sequence. The precursor contains neither intervening sequences nor an encoded 3′ CCA, consistent with the known natural genes for this tRNA (15). The probable secondary structure of the mature tRNA^{Lys} exhibits a (G+C)-rich, 7-base pair aminoacyl stem that includes two G·U wobble base pairs. The +1 nucleotide of the mature tRNA^{Lys} sequence is a G residue, consistent with most of the known tRNAs from higher organisms. However, A, C, and U residues have been found at the +1 position of subpopulations of higher eukaryotic tRNA species (25). The wild type gene for the pre-tRNA^{Lys} was produced by automated DNA synthesis, and the mutant derivatives were made by site-directed mutagenesis using PCR. Precursor transcripts were synthesized *in vitro*.

The rat RNase P used in these studies was approximately 500-fold purified over that of an initial high speed supernatant fraction, and was previously designated the 'Cs_2SO_4 gradient-I' fraction (14). The ranges of optimal conditions for this enzyme preparation using the wild type pre-tRNA^{Lys} as substrate were 4.5−5.5 mM Mg^{++} and 70−130 mM K^+ at pH 8.0. Thus all kinetic assays were carried out within these ranges at pH 8.0 in 5 mM $MgCl_2$ and 110 mM KCl. A typical electrophoretic gel assay of the processing reaction products for wild type pre-tRNA^{Lys} is shown in Fig. 2, lane 9.

The general effects of varying the length of the 5′ leader sequence was examined initially by processing several mutants with leaders of 10, 17, 24, 25, and 46 nucleotides in length. These mutants are designated in Fig. 1 as WT-L10, WT-L17, WT-L24, WT-L25, and WT-L46, respectively. In the leader sequence of WT-L25, the twelve nucleotides immediately 5′ to the RNase P cleavage site correspond to the wild type gene. In the remaining leader sequences, only nucleotides −1 to −3 were wild type.

Table I. The PCR primers used to produce WT-L46 and its substitution and insertion mutants

Precursor	Forward Primer[a]	Reverse Primer[a]
	Bam HI +1	Eco RI +72
WT-L46 (+1G-C+72)	5'- CGCGGATCCGCAGCCCGGCTAGCTC -3'[b]	5'- CGGGAATTCTACGCCCAACGTGGGG -3'[b]
Substitution Mutants		
+1C-G+72	AGA.....C.....	TAC.....C.....
+1A-U+72	AGA.....A.....	TAC.....A.....
+1U-A+72	AGA.....T.....	TAC.....T.....
+1G G+72	.....	TAC.....C.....
+1G·U+72	.....	TAC.....A.....
+1G A+72	.....	TAC.....T.....
+1C C+72	AGA.....C.....	.....
+1A C+72	AGA.....A.....	.....
+1U C+72	AGA.....T.....	.....
+7G G+66	AGA.....G.....AGT	.....
-1A-U+73	.....	TAC.....A.....
Insertion Mutants		
+3:a G-C+69:a	AGA.....G.....	TAC.....G.....
C+69:a	.....	TAC.....G.....
+3:a G	AGA.....G.....	.....

[a]The forward and reverse primers for each PCR experiment are listed next to the corresponding mutant.
[b]*Dot* designates an unchanged nucleotide.

As shown in Fig. 2 (lanes 9, 16–19) each of the 5′ leader mutants was well processed under the standard assay conditions, indicating that the leaders, as modified, exerted little effect on the processing reaction. The site of cleavage for WT-L46 and WT-L25 was shown to be at the 5′ end of the mature tRNA sequence by fingerprint analysis (data not shown) and by 5′ terminal nucleotide identifications of the 5′ processed intermediates (Table II). Evidence that the cleavage of WT-L24, WT-L17, and WT-L10 also occurred at the correct site was shown by comparing the migration of the 5′ processed intermediates of each precursor with the corresponding band from WT-L46 on the sequencing gel (Fig. 2). Since all the leader mutants appeared to be processed at approximately equivalent rates, the species with the longest leader (WT-L46) was chosen as the parent precursor for the production of the aminoacyl stem mutants to facilitate the quantitative analyses in subsequent kinetic assays.

Comparison of the kinetic constants of RNase P using wild type pre-tRNA^{Lys} and mutant precursors altered at the +1 site

The goal of this portion of the study was to examine the effect on the RNase P reaction resulting from mutating the +1 nucleotide (G) of the pre-tRNA^{Lys} and to determine the importance of base pairing at +1+72 as well. As shown in Table III, conversion of the wild type +1G–C+72 to A–U, C–G, or U–A base pairs had little effect on the efficiency of cleavage of the mutant species, indicating that the enzyme is nonspecific for the +1 nucleotide. However, comparison of the K_m values of the four paired bases suggests that there may be some binding preference for a +1 purine. Substitution of the +1G–C+72 with the wobble base pair +1G·U+72 gave kinetic values consistent with the wild type precursor as well, suggesting that strict adherence to Watson–Crick base pairing at +1+72 was not required for efficient cleavage.

However, introducing the mispaired bases +1C C+72, +1G G+72, +1G A+72, +1U C+72, or +1A C+72 dramatically reduced the utilization of these mutants, yielding efficiencies that were 1/7 to 1/60 of those of the wild type precursors. The inefficiency of processing of the mispaired +1+72 mutants appeared not to be uniquely dependent upon an altered K_m or a diminished relative V_{max}, but rather appeared to reflect changes in both values. However, although the utilization of the +1+72 mispaired mutants was dramatically diminished, the position of cleavage corresponded to the normal scissile bond of the wild type pre-tRNA^{Lys} (Table II).

Processing of pre-tRNA^{Lys} mutants having elongated aminoacyl stems

The double-insertion, mutant precursor +3:aG–C+69:a resulted in elongation of the aminoacyl stem by a base pair. Based upon the kinetic data (Table III), this precursor was the most efficiently processed of all precursors tested. The enhanced activity was

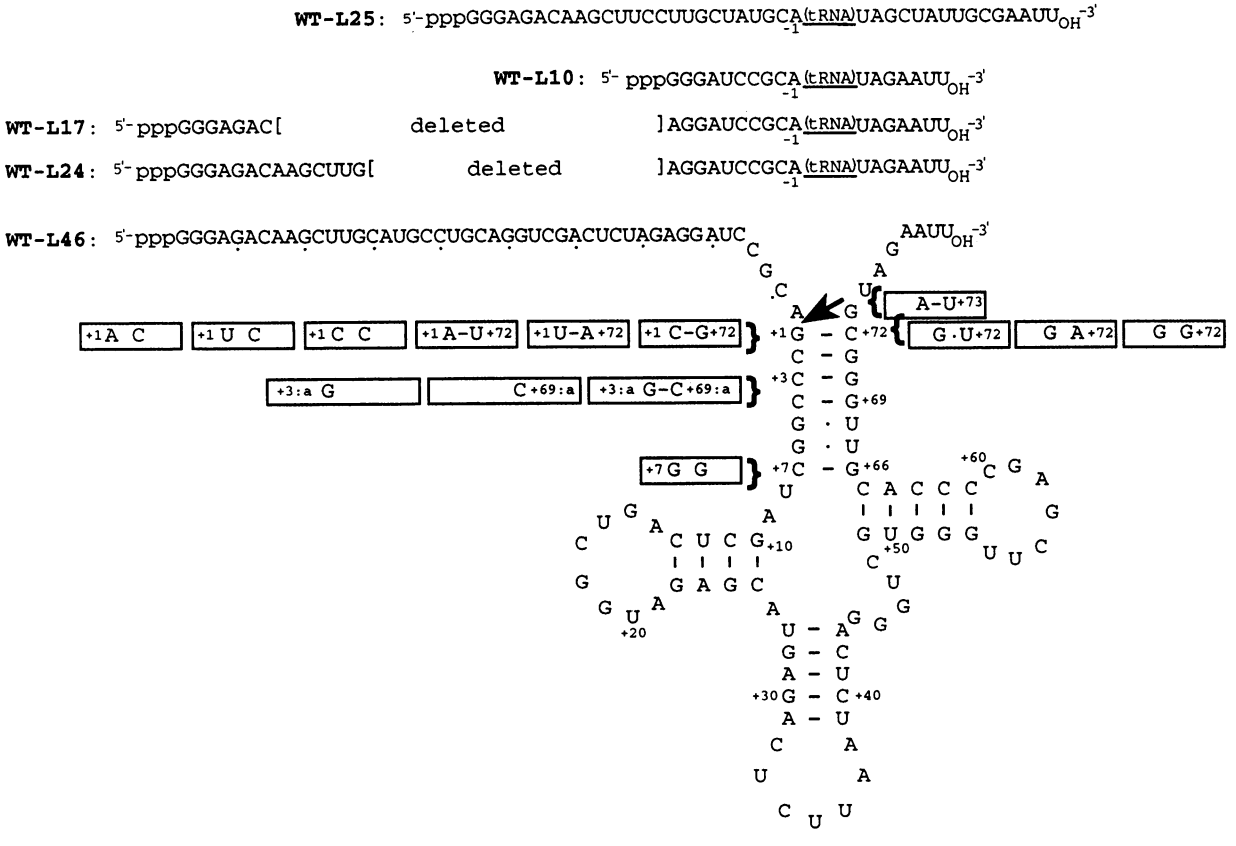

Figure 1. The sequence and secondary structure of the WT-L46 pre-tRNA^{Lys} showing leader and aminoacyl stem mutations. The mutations designated WT-L10, WT-L17, WT-L24, WT-L25, and WT-L46 contain leader lengths of 10, 17, 24, 25, and 46 nucleotides, respectively. The mutations in the aminoacyl stem are indicated in boxes. The numbering system is consistent with that of Sprinzl *et al.* (25). The *arrow* indicates the wild type site of RNase P cleavage.

derived from a low K_m of 0.22 μM, which was less than half that of the WT-L46 precursor. In addition, the site of processing was shifted to the bond between the +1 and +2 position, yielding the characteristic 7-base pair stem (Fig. 2, lane 6; Table II).

In the mutant precursor −1A−U+73 precursor, the length of the aminoacyl stem was elongated by one base pair by mutating the G+73 to a U+73 thus giving rise to a paired A−U in the −1+73 position. This precursor was precisely processed at the wild type cleavage site producing the normal 7-base pair aminoacyl stem (Table II). However, unlike the +3:aG−C+69:a mutant described above, introducing the paired −1A−U+73 caused a decrease in the efficiency of cleavage to about 1/6 that of the wild type precursor (Table III). A similar result has been obtained with *Schizosaccharomyces pombe* RNase P (26).

Thus based upon our two elongated mutants, it appears that elongating the helical aminoacyl stem by different means can produce substantial and opposite effects on the processing reaction, depending on the specific sequence of the altered helix. The poor efficiency with the −1A−U+73 mutant was surprising since natural mammalian pre−tRNAs with −1A−U+73 have been found in, for example, human $tRNA^{Gln}$ (27, 28).

The effects on the pre-$tRNA^{Lys}$ of disrupting the aminoacyl stem helix

In three of the mutants, the integrity of the aminoacyl stem helix was disrupted by (1) a single G insertion at +3:a, (2) a single C insertion at +69:a, and (3) mispairing the bottom of the aminoacyl stem helix by introducing a +7G G+66 mismatch. The mutant having the single G insertion at +3:a was so poorly utilized by the RNase P that processing products were not detectable under kinetic assay conditions. In contrast, the mutant having the single C insertion at +69:a, on the opposite side of the helix, was relatively efficiently utilized (Table III), but the processing occurred at two different sites (Table II; and Fig. 2, lane 5). One site corresponded to the wild type processing site, and the other occurred upstream between the −6U and −5C yielding a 5′ pC-containing product. The ratio of the two products, measured either as the 5′ processed intermediate or as the leader sequence was about 2:1, respectively, indicating that the wild type site predominated.

The mutant containing the mispaired +7G G+66, which lies at the base of the aminoacyl stem helix and serves as an interface between the aminoacyl helix and the T-stem, was processed at the wild type processing site (Table II), but the efficiency of processing was low apparently due to a very high K_m value.

In conclusion, the data indicate that the mutants tested exerted little effect on the accuracy and precision of the cleavage site such that all, except the helix-disrupting mutant C+69:a, yielded only the characteristic 7-base pair aminoacyl stem. Furthermore, the specific base at the +1 position, which is bound to the normal scissile bond, seemed virtually irrelevant to the processing reaction. However, a major effect was seen in the mutants having mispaired bases at the +1+72 site. In each case, a decline in the efficiency of processing was observed. These results will be discussed further below.

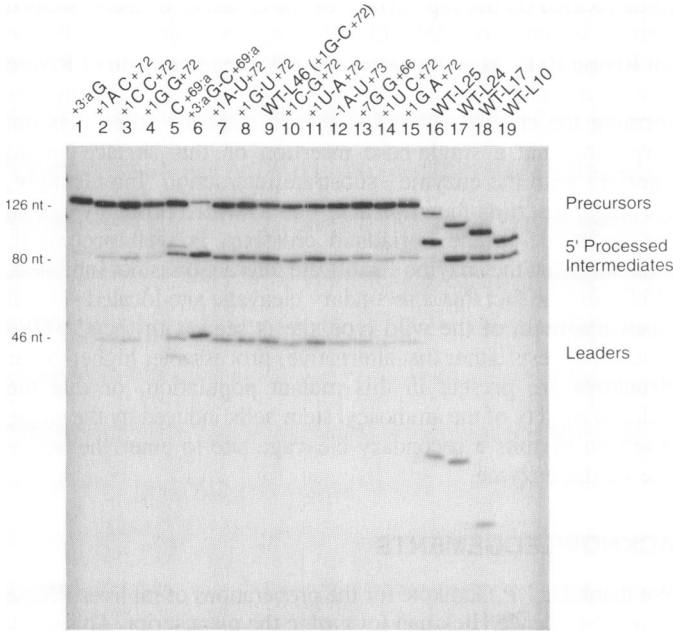

Figure 2. Autoradiograph of a sequencing gel containing the precursor and products generated under standard reaction conditions with wild type and each mutant pre-$tRNA^{Lys}$. Samples of 2.5×10^5 cpm of identically labeled precursors plus products, generated with 0.48 U of RNase P for 7 min at 37°C, were analyzed on a sequencing gel as described under 'Materials and Methods.' The polynucleotide sizes designated on the left side of the figure refers to the WT-L46 precursor (126 nt), the 5′ processed intermediate (80 nt), and the leader (46 nt).

Table II. Determination of the 5′ terminal nucleotide of the processed products

Precursor		5′ Nucleotide	[α-^{32}P] NTP Incorporated
Leader Mutants			
WT-L25		+1G	GTP
WT-L46		+1G	GTP
		+1G	CTP
WT-L24		+1G[a]	GTP
WT-L17		+1G[a]	GTP
WT-L10		+1G[a]	GTP
Substitution Mutants			
+1C-G+72		+1C	CTP
+1A-U+72		+1A	CTP
+1U-A+72		+1U	CTP
+1G G+72		+1G	CTP
+1G·U+72		+1G	CTP
+1G A+72		+1G	CTP
+1C C+72		+1C	CTP
+1A C+72		+1A	CTP
+1U C+72		+1U	CTP
+7G G+66		+1G	CTP
-1A-U+73		+1G	CTP
Insertion Mutants			
+3:aG-C+69:a		+2C	CTP
		-[b]	GTP
+3:aG		+1G[a]	GTP
C+69:a	Primary Product	+1G	GTP
		+1G	CTP
	Secondary Product	-5C	CTP
		-[b]	GTP
		-[b]	ATP

[a]Determined from comparison gel only.
[b]5′ nucleotide is not labeled.

DISCUSSION

RNase P in both prokaryotes and eukaryotes appears to process a broad spectrum of different tRNA precursors *in vivo*. Among the known nuclear tRNAs, each of the four natural base pairs as well as the G·U wobble pair have been identified at the +1+72 position (25). This broad spectrum of potential substrates led us to attempt to identify signals proximal to the cleavage site in tRNA precursors that are recognized by the rat RNase P we have isolated. Our results with mutants of a homologous pre-tRNA^{Lys} indicate that this enzyme can accurately process mutants with G, A, C, or U bases at the +1 position and that +1+72 pairing, or G·U noncanonical pairing, is important for the high efficiency of the process. Thus the RNase P we are studying may be the only RNase P necessary for the 5′ maturation of all nuclear-encoded tRNAs in the cell.

Our studies using mutant precursors with elongated aminoacyl stems produced only products trimmed to the characteristic 7 base pairs. Similar results with *Xenopus laevis* RNase P led Carrara *et al.* (29) to postulate that the eukaryotic enzyme locates its cleavage site, in part, by a mechanism that measures along the aminoacyl stem from its base. Our results are consistent with such a model. In contrast to these eukaryotic enzymes, *E.coli* RNase P processing of two different mutants having an additional base pair at the base of the aminoacyl stem yielded products with 8-base pair stems, and a mutant with an addition of 3 base pairs to the middle of the aminoacyl stem gave a major product with a 10-base pair stem (30). Thus in comparing the eukaryotic RNases P with that of *E.coli*, it appears that the choice of cleavage site by the eukaryotic enzymes is relatively insensitive to changes in the length of the aminoacyl stem, suggesting that the mechanisms that guide the active sites of the two classes of enzymes to the scissile bond are different.

The wild type pre-tRNA^{Lys} from rat, as well as from most other higher eukaryotes, normally contains two G·U wobble pairs at the +5+68 and +6+67 positions. Thus in our +7G G+66 mispaired mutant, approximately the lower half of the aminoacyl stem is substantially devoid of the normal stabilizing bonds. Nonetheless, the overall result with this mutant was precise processing at the wild type cleavage site, albeit with poor efficiency. We suggest that such relatively unstable aminoacyl helices are able to be maintained with sufficient integrity in the overall tRNA structure to permit accurate measuring and RNase P processing. A similar conclusion was drawn from recent studies of the substrate specificities of a plant mitochondrial RNase P (10). Studies by others with the *S.pombe* RNase P examined the effects of eliminating a G·U wobble pair in one case (31) and introducing two G·U pairs in another case (32). In both examples no effect on RNase P cleavage was observed. Furthermore, since the aminoacyl stems of numerous different tRNAs have been found to contain one or two wobble pairs or even an A C mispair naturally (25), we conclude that moderate instability in the aminoacyl helix is probably beneficial to some tRNA function and does not adversely affect the processing reaction. Interestingly, the natural human pre-tRNA^{Lys} has lost the +5G·U+68 wobble pair which has been replaced with G−C, indicating that the presence of two adjacent G·U pairs is not crucial to the function of tRNA^{Lys} in all higher eukaryotes.

Perhaps the most revealing results were obtained with the single-insertion mutants. The +3:aG mutation, which is predicted to be located on the top surface of the crossbar of the Γ-shaped tertiary structure of tRNA (33), is virtually unprocessed by the rat RNase P. If, as is postulated (3, 34), the interaction of RNase P with its substrates is along the upper surface of the helices forming the crossbar of the Γ-shaped structure, then it is not surprising that a single-base insertion on this surface would interfere with the enzyme−substrate interaction. Interestingly, the single insertion mutation at C+69:a, which probably lies on the underside of the horizontal crossbar, is well processed, indicating that the enzyme−substrate interaction is not inhibited. However, the fact that a secondary cleavage site located several bases upstream of the wild type site is seen with the C+69:a mutant suggests either that alternative, processable, higher-order structures are present in this mutant population, or that the reduced rigidity of the aminoacyl stem helix induced by the single insertion permits a secondary cleavage site to enter the active site of the enzyme.

Table III. Kinetic analyses of wild type and mutants of pre-tRNA^{Lys}

Precursor	K_m (μM)	Relative V_{max} (nM/min/unit)	Efficiency (min^{-1}/unit)
WT-L46	0.61 ± 0.14[a]	46.2 ± 11.8[a]	0.080 ± 0.032[a]
+1+72 Mutants			
+1A-U+72	0.52[b]	28.1[b]	0.054[b]
+1C-G+72	2.1	73.8	0.035
+1U-A+72	1.1	36.1	0.032
+1G·U+72	0.96	47.0	0.049
+1G G+72	2.3	4.36	0.0019
+1G A+72	1.2	8.5	0.0071
+1C C+72	1.3	14.0	0.011
+1U C+72	5.1	11.1	0.0022
+1A C+72	8.2	10.5	0.0013
Elongated Aminoacyl Stem Mutants			
+3:aG-C+69:a	0.22	48.4	0.22
-1A-U+73	2.0	24.1	0.012
Helix-Disrupted Aminoacyl Stem Mutants			
+7G G+66	15	77.7	0.0053
C+69:a	0.51	16.1	0.032
+3:aG	Not Determined		

[a]Mean value ± one standard deviation for three different enzyme preparations.
[b]Values for the mutants in each column are from single kinetic determinations from points obtained in duplicate.

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