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Alteration of a mitochondrial tRNA precursor 5' leader abolishes its cleavage by yeast mitochondrial RNase P

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ABSTRACT

A mitochondrial specific RNase P is required to process 5' leaders from mitochondrial tRNA precursors in Saccharomyces cerevisiae. Experiments with a pair of mitochondrial pretRNAs^{Asp} having leaders of different base composition suggest that this enzyme is unexpectedly sensitive to leader sequence or structure. Asp-AU (75% AU leader) is cleaved by the mitochondrial RNase P while Asp-GC (39% AU) is not. Both are substrates for E. coli RNase P. Partial nuclease digestions show that the tRNA portions of the two precursors differ in tertiary structure, while their 5' leaders differ in secondary structure. It is unusual for an RNaseP to have substrate specificity requirements which preclude processing of a pretRNA known to be a suitable substrate for an RNaseP from another species.

INTRODUCTION

RNase P catalyzes the endonucleolytic cleavage of 5' leader sequences from tRNA precursors. RNase P obtained from a variety of organisms depends upon both protein and RNA subunits for activity (1-6). The RNAs from E. coli and B. subtilis RNase Ps have been shown to be catalytically active in the absence of protein in vitro (7). These results suggest that the RNA subunit of RNase P is the catalytically active subunit, although there is a recent report of a 5' tRNA processing enzyme that has no detectable nucleic acid component (8). Progress is being made both on determining structural requirements of the RNase P RNA necessary for catalytic activity (9-11) and on some aspects of the catalysis (9,12). However, the actual mechanism of catalysis has not been fully elucidated.

The subunits of yeast mitochondrial RNase P are produced in separate compartments of the cell. The protein is encoded within the nucleus, translated in the cytoplasm, and imported into the mitochondrion. The RNA subunit is encoded and transcribed within the mitochondrion itself. A combination of genetic analyses and in vitro enzymatic assays have allowed us to identify the gene that codes for the yeast mitochondrial

RNase P RNA (6, 13) The most abundant transcript from this gene is approximately 450 bases long and is presumed to be the catalytic subunit (14). It consists of 88% A+U bases and has no primary sequence homology to either the M1 RNA from the E. coli RNase P nor to the P RNA from the B. subtilis enzyme.

The initial characterization of yeast mitochondrial RNase P has been reported (6), but studies on substrate specificity have not. There are many examples of mutations within the tRNA portion of precursor tRNAs that greatly reduce processing by RNase P both in vivo and in vitro (15-22). We initiated our studies of substrate specificity by comparing a pair of mitochondrial tRNA precursors that were entirely the same except for their distinctly different 5' leaders. The 5' leaders of these two precursors differ in their base composition. One, Asp-AU, is a substrate for the yeast mitochondrial RNase P while the other, Asp-GC, is not. Asp-AU has a 5' leader that is 75% A+U. The leader of Asp-GC, in constrast, is only 39% A+U. The yeast mitochondrial genome is very A+T rich and mitochondrial tRNA precursors have leaders that are at least 75% A+U rich. The radical differences in the suitability of these two precursor tRNAs as substrates demonstrates that a 5' leader of a precursor can determine the absolute substrate specificity of an RNase P holoenzyme in vitro. The results do not allow us to determine if the specificity results from the primary sequence difference or from accompanying alterations in secondary and tertiary structure.

EXPERIMENTAL PROCEDURES

Construction of templates

Two recombinant DNAs were used to produce the tRNA precursors for this study. The vector in both cases was pT3T7.19 (BRL). pAsp-GC was constructed by ligating a Sau3A/HpaII fragment originally from S. cerevisiae strain MH41 mitochondrial DNA to BamHI/AccI cut pT3T7.19. The Sau3A/BamHI ligation restored the first base (G) of yeast mitochondrial $tRNA^{Asp}$. The 3' end of the tRNA had been previously engineered by olionucleotide mutagenesis to contain a BstNI site, CCAGG. Treatment of pAsp-GC with BstNI followed by transcription with T7 RNA polymerase yields a precursor $tRNA^{Asp}$ with a CCA on the 3' end. The 5' leader of the pAsp-GC product is 36 nucleotides long and is derived from the multicloning site of the vector. pAsp-AT was constructed by ligating the same Sau3A/HpaII fragment as above with two complementary

oligonucleotides (5'-AGCTTTAATAATAATAATAAAATAAA-3' and 5'-GATCCTTTATTTTATTATTAT-TATTAA-3') to HindIII/AccI cut pT3T7.19.

Transcription of substrates

Precursor tRNAs were transcribed from BstNI (New England Biolabs) cut pAsp-AT or pAsp-GC using T7 RNA polymerase. Conditions for transcription reactions used to produce uniformly radiolabeled precursors were essentially as described by the polymerase supplier (BRL, NE Biolabs, Promega). Different conditions were used to transcribe precursor in larger quantities. The reaction contained plasmid at 20 nM, 4 mM each of ATP, GTP, CTP, and UTP, 24 mM $MgCl_2$, 8% (w/v) PEG-8000, 1mM spermidine, 5 mM DTT, 40 mM Tris-Cl pH 8.1, and 50 units of T7 RNA polymerase per 50 microliter reaction. The reaction mixtures were subjected to electrophoresis through 10% acrylamide 6M urea gels. The pertinent RNA was identified by autoradiography or uv irradiation over fluorescent plates and excised. The RNA was eluted from the crushed gel slice by shaking in 10 mM Tris-Cl pH 8 0.1 mM EDTA at 4°C overnight.

Preparation of yeast mitochondrial RNase P

Yeast mitochondria were prepared from Saccharomyces cerevisiae as previously described (13). Frozen mitochondrial pellets were thawed in the presence of 100 mM NH_4Cl, 50 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 10% glycerol, 0.2 mM DTT, 0.2 mM PMSF. Protein concentration was determined by the Bradford method (25). The solution was diluted to 5 mg protein per ml and deoxycholate was added to a final concentration of 0.25%. After a 48,000 x g centrifugation, the supernatant was applied to a DEAE sepharose (Pharmacia) column at 4.5 mg protein per ml of resin. The column was washed with one column volume of 100 mM NH_4Cl, 10 mM $MgCl_2$, 50 mM Tris-Cl pH 7.5, 10% glycerol. After extensive (75 column volumes) washing with 150 mM NH_4Cl, 10 mM $MgCl_2$, 50 mM Tris-Cl pH 7.5, 10% glycerol, the activity was eluted with 350 mM NH_4Cl, 10 mM $MgCl_2$, 50 mM Tris-Cl pH 7.5, 10% glycerol. This active fraction could be stored at 4°C with full activity for approximately six months.

Preparation of E. coli RNase P

A 1.5 liter Luria broth culture of E. coli strain HB101 was grown overnight at 37°C with shaking. The cells were harvested by centrifugation for 10 minutes at 6000 x g. Five grams of the cells were ground into a paste with 10 grams of alumina with a chilled mortar and pestle. Five milliliters of 50 mM Tris-Cl pH 7.5, 60 mM NH_4Cl, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol (buffer A) were mixed into the paste to

form a slurry. Fifty micrograms of pancreatic DNase (Cooper Biomedical) were added and the mixture was incubated on ice for 30 minutes. The slurry was subjected to centrifugation at 8,000 x g for 10 minutes. The resulting supernatant was centrifuged for 40 minutes at 29,000 x g. The supernatant from that centrifugation was applied to a DEAE sephadex (A20-120, Sigma) column equilibrated in buffer A. The column was washed with 5 column volumes of buffer A. Further washing was carried out with 15 column volumes of 50 mM Tris-Cl pH 7.5, 200 mM NH_4Cl, 10 mM $MgCl_2$. The RNase P activity was eluted from the column with 500 mM NH_4Cl, 50 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$. The eluted activity was stable at 4^oC for at least one month.

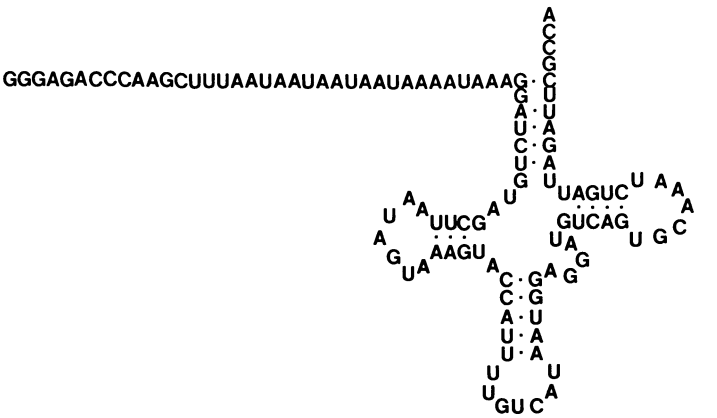
Quantitation of RNase P Activity

RNase P reactions were performed in the presence of 10 mM Tris-Cl pH 8.0, 30 mM $MgCl_2$, and 75 mM NH_4Cl and 0.1 microgram of the mitochondrial extract for 15 minutes at 37^oC. The entire reaction mixture was subjected to electrophoresis in 10% acrylamide 6 M urea gels. The autoradiogram obtained after autoradiography was used as a template for the excision of substrate and product RNAs from the gel. Radioactivity was quantitated by scintillation counting.

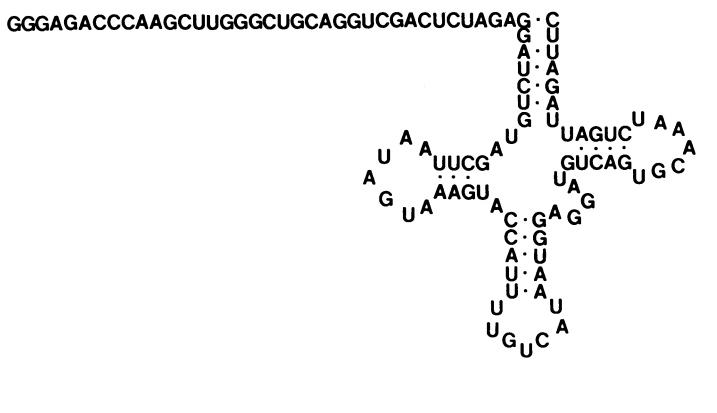
tRNA structure analysis

Purified RNAs were radiolabeled either at the 5' or 3' ends as previously described (6). All of the reactions were carried out at 37^oC for 20 minutes in the presence of one microgram of cold carrier tRNA. Reactions were stopped by the addition of a mixture of formamide and dyes (90% formamide 0.05% (w/v) bromphenol blue 0.05% (w/v) xylene cyanol 0.5 M Tris 0.5 M boric acid 10 mM EDTA). Enzymes were diluted in 10 mM Tris-Cl pH 7.5, 0.1 mM EDTA to appropriate concentrations. RNase T1 (Calbiochem) dilution was 5×10^{-4} units per microliter. RNase V1 (PL Biochemicals) was diluted to 7×10^{-3} units per microliter. Nuclease S1 (BRL) reactions were carried out in the presence of one microgram of cold tRNA for five minutes at room temperature. The reaction was stopped by the addition of an equal volume of 50 mM EDTA and twice the reaction volume of the formamide/dyes. S1 was diluted to 50 units/microliter in 10 mM Tris-Cl pH 7.5, 0.1 mM EDTA, 10 mM $ZnSO_4$. An RNA size ladder was generated by using the BRL RNA sequencing kit according to the manufacturer's instructions. Each set of reactions included one where the radiolabeled RNA was incubated without enzyme as a control for nonspecific nicks in the RNA. Aliquots of each reaction were subjected to

electrophoresis in 8% acrylamide 12 M urea gels that were 0.4 mm thick. After electrophoresis, the gel was transferred to a piece of x-ray film for support and autoradiography was performed.



Asp-AU



Asp-GC

FIGURE 1: Precursors to $tRNA^{Asp}$. Asp-AU is on the right, Asp-GC on the left. The tRNA portions of the precursors have been folded into standard secondary structure (Holley et al 1965).

Sequence and structure analysis

Editing of DNA and RNA sequence was accomplished with the aid of a set of the Seqaid sequence analysis programs by D. D. Rhoads and D. J. Roufa (Kansas State University). Secondary structure and homology analyses were performed with Microgenie by C. Queen and L. Korn (Beckman).

RESULTS

Transcription of substrates

The templates used to make the two precursor $tRNAs^{Asp}$ were designed such that the only difference between them would be the primary structure of the 5' leader. Asp-AU has a 36 base 5' leader that is 75% A+U. The 21 nucleotides immediately adjacent to the tRNA in Asp-AU are the same as found *in vivo*. Asp-GC also has a 36 base 5' leader, but it is only 39% A+U. The cleavage site between the leader and the tRNA, A-G, is the same for both precursors. By computer analysis, it appeared that there was no substantial complementarity between the leaders and the tRNA moiety. Both leaders could have some secondary structure within themselves, but the possible stem/loop regions are in the 5' half of the leader, well away from the RNase P cleavage site. The two precursors are shown in Figure 1, arranged in the standard tRNA secondary structure (23).

Asp-AU and Asp-GC as substrates for RNase P reactions

Radiolabeled Asp-AU and Asp-GC were transcribed from their template DNAs with T7 RNA polymerase as described in Methods. Asp-AU or Asp-GC at concentrations of 3 pM and 4 pM, respectively, were incubated with either *E. coli* or yeast mitochondrial RNase P in the presence of 20 mM Tris-Cl pH 8.0, 30 mM $MgCl_2$, 75 mM NH_4Cl for 15 minutes at 37°C. The reaction products were separated by electrophoresis on 10% polyacrylamide 6 M urea gels. The autoradiogram of the resulting gel pattern is shown in Figure 2. *E. coli* RNase P can process both substrates. The apparent difference in efficiency in processing of the Asp-AU vs the Asp-GC may be due to the fact that although an equal amount of radioactivity was added to each reaction, the Asp-GC had a specific activity 50% higher than that of Asp-AU, resulting in more Asp-GC being present in the reaction. Even so, it appears that the rate of cleavage of Asp-GC is slower than that of Asp-AU by *E. coli* RNase P. The yeast mitochondrial RNase P, however, clearly only processes the Asp-AU under these conditions. While there does appear to be a small amount of RNA generated that comigrates with

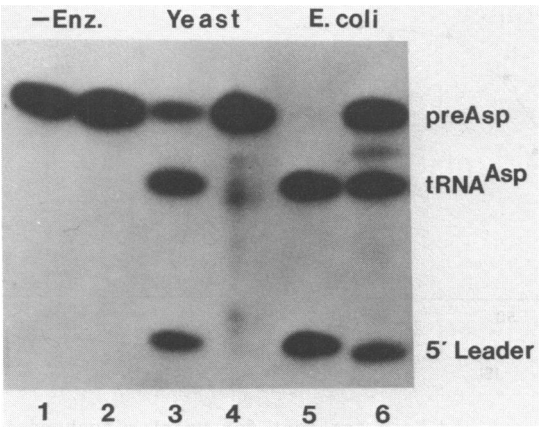


FIGURE 2: Asp-AU and Asp-GC as substrates for yeast mitochondrial and E. coli RNase P. Precursors were mixed with RNase P extracts under conditions given in Methods. Lanes 1, 3, and 5, Asp-AU as substrate. Lanes 2, 4, and 6, Asp-GC as substrate. Lanes 1 and 2, incubation without enzyme. Lanes 3 and 4, yeast mitochondrial RNase P. Lanes 5 and 6, E. coli RNase P. The positions of precursors and products are indicated at the right.

$tRNA^{Asp}$, in the Asp-GC/mitochondrial RNase P lane (lane 4), there is no 5' product. It is necessary to detect both the 5' and the 3' products to conclude that the endonucleolytic action of RNase P has been assayed (6). An autoradiogram was made from this gel that was exposed ten times longer than the original. Even at that exposure, there was no 5' product visible in lane 4 (data not shown). This implies that if the yeast mitochondrial RNase P reacts with Asp-GC at all under these conditions, the velocity of the reaction is very low.

It was possible that the Asp-GC could still be a substrate for the yeast mitochondrial RNase P, but with a much higher Km than the Asp-AU. To test that hypothesis, a series of reactions were performed with yeast mitochondrial RNase P and increasing concentrations of Asp-GC. At no concentration, including 900 nM, which is five orders of magnitude higher than the concentration used in Figure 2, did the yeast mitochondrial RNase P process the Asp-GC (data not shown). Experiments were also performed with Asp-AU at similar concentrations to determine whether these higher concentrations of RNA could be inhibitory to the yeast mitochondrial RNase P under these reaction conditions. There was no inhibition of the enzyme when exposed to Asp-AU concentrations up to 1.2 micromolar (data not shown).

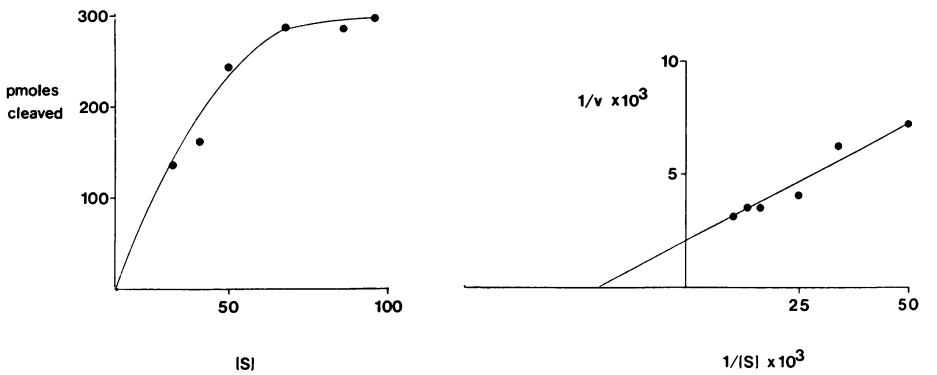


FIGURE 3: Km apparent and Vmax apparent for yeast mitochondrial RNase P and Asp-AU. (Left) Substrate concentration vs. velocity curve. Substrate units are nmolar. Picomoles cleaved are in a 15 minute reaction. (Right) Lineweaver-Burke plot of the graph in A. Units on the X axis are $(nmolar)^{-1}$. Units on the Y axis are minutes/femtomole.

Km apparent and Vmax apparent for Asp-AU

A series of experiments were performed to determine the apparent Km and Vmax of yeast mitochondrial RNase P for the Asp-AU substrate. Mitochondrial extract containing RNase P was incubated with increasing concentrations of Asp-AU under standard reaction conditions. After electrophoresis of the reaction mixture, substrate and product RNAs were excised from the gel and assayed for radioactive content by scintillation counting. A representative graph of the values obtained for velocity vs substrate concentration is shown in Figure 3A. The Lineweaver-Burke plot of that data is shown in Figure 3B. The Km apparent of yeast mitochondrial RNase P for Asp-AU is 60 nM. The Vmax apparent of yeast mitochondrial RNase P for Asp-AU is 300 pm/min/mg protein.

Studies on the Inhibition of RNase P by Asp-GC, $tRNA^{Asp}$, and Asp-AU 5' Leader

Inhibition experiments were performed to determine whether Asp-GC, which is not a substrate for the yeast mitochondrial RNase P, inhibits the enzyme. Asp-AU at an initial concentration of 20 nM was processed by the enzyme in the presence of increasing concentrations of Asp-GC. A graph of the data from a typical series of inhibition reactions is shown in Figure 4. A similar set of experiments were also performed with the products of the reaction, $tRNA^{Asp}$ and the 5' leader from Asp-AU, as inhibitor. The data from a typical set of the $tRNA^{Asp}$ reactions is

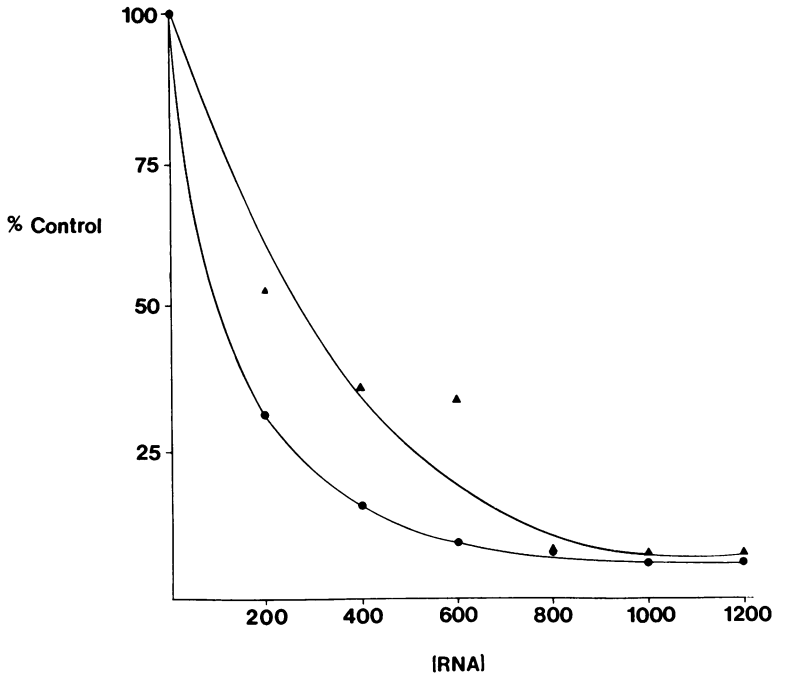


FIGURE 4: Inhibition of yeast mitochondrial RNase P by Asp-GC and $tRNA^{Asp}$. Graph of inhibitor concentration in nanomolar (X-axis) vs. percent inhibition of the reaction (Y-axis). All reactions were in the presence of 20 nM Asp-AU. (●) Asp-GC as inhibitor. (▲) $tRNA^{Asp}$ as inhibitor.

included in the graph in Figure 4. The other product of the RNase P reaction, the 5' leader of Asp-AU, was not an inhibitor of the RNase P reaction under these conditions. The inhibition of yeast mitochondrial RNase P by Asp-GC is kinetically indistinguishable from the product inhibition seen with $tRNA^{Asp}$. Since there is no inhibition of the yeast mitochondrial RNase P by the substrate Asp-AU up to a concentration of 1.2 micromolar, the inhibition caused by the Asp-GC and $tRNA^{Asp}$ are due to these particular RNAs and not a nonspecific inhibition due to a high concentration of RNA.

Structure analysis of Asp-AT and Asp-GC

To determine whether the difference in substrate recognition of these two very similar substrates by yeast mitochondrial RNase P is purely a primary structure phenomenon, structure analysis was performed. The RNAs were treated under mild conditions with single- and

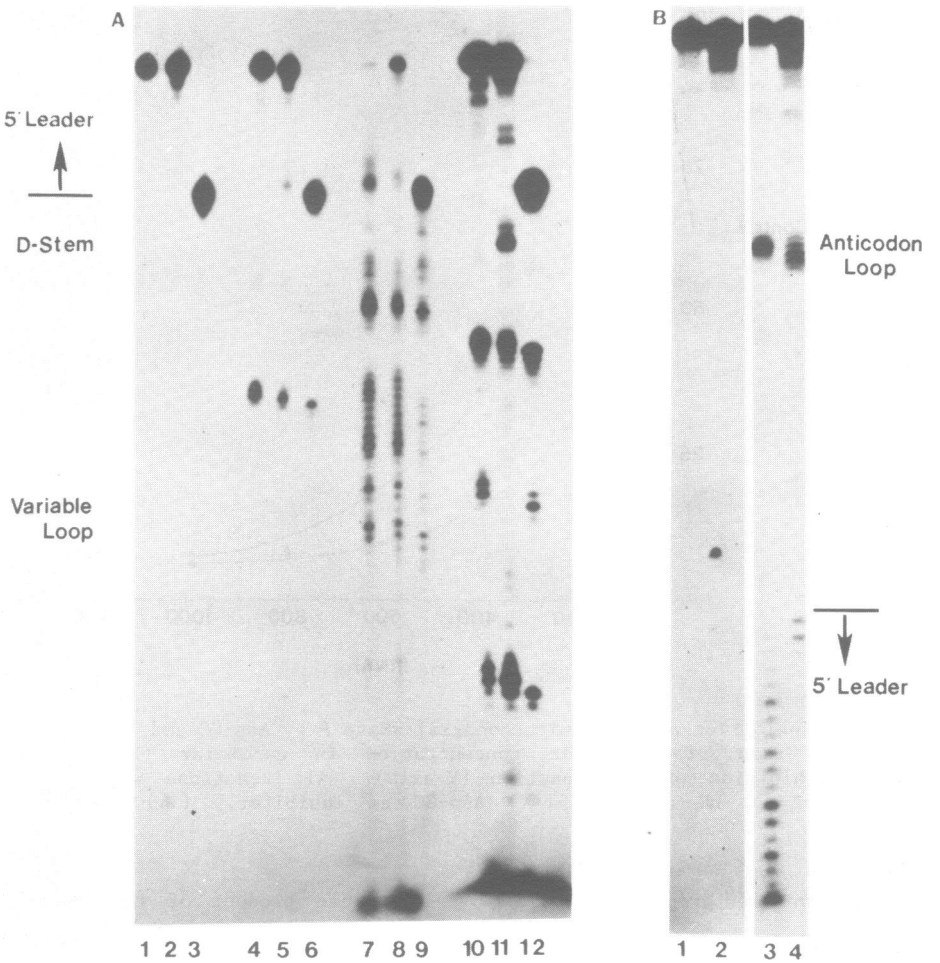


FIGURE 5: Structure analysis of Asp-AU, Asp-GC, and $tRNA^{Asp}$.
A: RNAs were radiolabeled at their 3' ends with (P^{32})-pCp and digested under mild conditions with the enzymes noted. In each set of three lanes, the RNAs are in the following order: Asp-AU, Asp-GC, $tRNA^{Asp}$. Lanes 1-3: RNAs incubated under reaction conditions without enzyme. Lanes 4-6: RNAs digested with 0.0005 units RNase T1. Lanes 7-9: RNAs digested with 50 units of S1 nuclease. Lanes 10-12: RNAs digested with 0.007 units RNase V1. Sizes of products determined by comparison with alkaline digestion ladders (not shown). Location of 5' leader, D-stem, and variable loop indicated to the left. B: S1 analysis of 5'-^{32}P-radiolabeled Asp-AU and Asp-GC. Lanes 1 and 2 are Asp-AU and Asp-GC, respectively, incubated without enzyme. Lanes 3 and 4 are Asp-AU and Asp-GC, respectively, incubated with 50 units S1 nuclease. The positions of the 5' leader and the anticodon loop are indicated at the right.

double-strand specific nucleases. By performing an RNase digestion with very dilute RNase concentrations, it is possible to selectively cleave an RNA at only those sites that are most exposed to the solution. In addition to comparing the relative conformations of the tRNA portions of the precursors, we wanted to determine if there might be secondary structure formed between the leaders and the tRNAs. Digestions with the single strand specific nuclease S1 were performed specifically to probe for such secondary structure.

The RNAs tested were first radiolabeled at either the 5' or 3' termini. Radiolabeled RNA was digested with either RNase T1, which cleaves specifically after G, RNase V1, which is specific for double stranded regions of RNA, or with the single strand specific S1 nuclease. Under the mild conditions used here, T1 is also single strand specific. The results of the T1 and V1 digestions were the same regardless of the position of the ^{32}P, 5' or 3'. Surprisingly, the position of the radiolabel initially appeared to make a difference in the S1 patterns. Further experiments revealed that the only major difference was one of resolution. The S1 patterns in the 5' leader are compressed when the precursor is 3' end labeled. A ^{32}P at the 5' end of the precursor allowed the S1 patterns in the 5' leader to be clearly resolved (see below).

Results from these experiments are in Figure 5. The RNA cleavage products in the S1 lanes migrate as if they are approximately one base longer than the RNase products, due to the presence of a 5' phosphate. The major cleavage products in the $tRNA^{Asp}$ lanes run approximately one base faster than those of the two precursors. The $tRNA^{Asp}$ was isolated from mitochondria while the two precursors were transcribed *in vitro*. Apparently the majority of the *in vitro*-made transcripts are one base longer than the isolated $tRNA^{Asp}$. This could be due to either *in vivo* degradation of the $tRNA^{Asp}$ or to the possibility that the RNA polymerase used to make the precursors *in vitro* doubled back and transcribed one base past the CCA end.

RNase T1 cleaves at essentially the same positions in the tRNA portions of both precursors, as does S1 nuclease. The base most exposed to RNase T1 is the G in the anticodon loop. The D loop, variable loop, and anticodon stem and loop are most accessible to S1 in all three RNAs. In Figure 5A, Asp-AU has been more completely digested by S1 than Asp-GC has. This problem has been corrected in 5B. When both precursors are

digested by S1 to a similar degree, it becomes obvious that the anticodon loop of Asp-GC is more susceptible than the anticodon loop of Asp-AU to S1 nuclease.

Although the cleavage patterns of the tRNA portions of the two precursors by single strand specific enzymes are essentially the same, the RNase V1 cleavage pattern is different. The most exposed area of the precursors and $tRNA^{Asp}$ as detected by RNase V1 is the same, within the T-pseudoU-C loop. All three RNAs have two cleavage sites that are less available than is the primary site, but still easily accessible by the enzyme. They have in common a site in the anticodon stem that is cleaved by V1. Asp-AU and $tRNA^{Asp}$, however, have another secondary V1 site in their variable loops that is not shared by Asp-GC. Asp-GC has, in contrast, a secondary V1 site in the D-stem that is not shared by Asp-AU and $tRNA^{Asp}$. Digestion of 5' end labeled precursors with V1 also showed that the amino acid acceptor stem in Asp-AU is more uniformly exposed than the same area in Asp-GC. Asp-AU is cleaved in that region three times by V1, while Asp-GC has only one major cleavage site in that stem.

T1, S1, and V1 cleavage patterns differ in the 5' leaders of the two precursors. This is to be expected for T1, since it is G-specific and the sequence of the two leaders is not the same. The S1 data, however, indicates a difference in the degree of single stranded nature between the two leaders. S1 digestion of the 3' end labeled precursors does not yield a clear pattern in the leader regions, but it was clear that S1 does digest the Asp-AU leader more extensively than it does Asp-GC. Upon better resolution of this area by S1 treatment of 5' labeled precursors, the pattern from the Asp-AU leader region shows a ladder extending from the RNase P cleavage site to at least 18 bases 5' of that site. In contrast, only the first eight bases immediately adjacent to the 5'end of the RNase P cleavage site in Asp-GC are readily accessible to S1 (Figure 5B). As is predicted from the S1 data, V1 accesible sites within the two 5' leaders also differ. Asp-GC has two sites recognized by V1 within its 5' leader that are not shared by Asp-AU. The V1 cleavage sites in the Asp-GC leader are approximately eleven and fourteen bases away from the RNase P cleavage site, which agrees well with the S1 data.

It is apparent that these two precursors do not have exactly the same structure. We conclude from these observations that the difference between Asp-AU and Asp-GC is not solely limited to the different primary structures of their leaders. There are differences both in the tertiary

structures of the tRNA portions of the precursors and in the secondary structure of their leaders.

DISCUSSION

Yeast mitochondrial RNase P is an enzyme that requires an RNA subunit for activity in vitro (6). The results reported here show that a change in the leader of a mitochondrial tRNA precursor is sufficient to abolish its cleavage by yeast mitochondrial RNase P. The change altered the base composition of the leader so that it became G+C rich. This mitochondrial RNase P RNA is remarkably A+U rich (14). If the RNA plays some role in substrate recognition or positioning, it would not be entirely surprising that the mitochondrial RNase P would prefer substrates with AU rich 5' leaders.

The A+T richness of the mitochondrial genome notwithstanding, the observation that Asp-GC is not a substrate for yeast mitochondrial RNase P under these conditions was unexpected. Previous studies both in vitro and in vivo in other systems have shown that it is necessary to disrupt the base pairing of the tRNA region of the precursor to have an effect on RNase P activity (15-20, 24). The sequence of the tRNA portion of Asp-GC, however, was not changed. In addition, Asp-AU and Asp-GC were designed to be as similar as possible. Their 5' leaders have the same number of bases, 36. The junction between the leader and the tRNA in the two precursors is the same, U-G. The only difference between the two is that the 21 bases immediately 5' to the RNase P cleavage site in Asp-AU are the same as those found in vivo. Despite the similarities between Asp-AU and Asp-GC, only Asp-AU is correctly processed by yeast mitochondrial RNase P.

There are at least three possible explanations for why Asp-AU was processed by yeast mitochondrial RNase P but Asp-GC was not. It was possible that Asp-GC is not a substrate for any RNase P. The fact that Asp-GC is a substrate for E. coli RNase P (Figure 2) eliminates this hypothesis. A second alternative was that the Asp-GC is a very poor substrate and requires more unusual conditions for processing than Asp-AU. The initial experiments reported here demonstrated that at equivalent concentrations, Asp-GC is not cleaved by yeast mitochondrial RNase P but Asp-AU is. If the enzyme has a much higher Km for Asp-GC than Asp-AU, then cleavage would not have been detected at these lower concentrations. Further experiments described in the Results section

demonstrated that this is not the case. Increasing the concentration of Asp-GC by 1500 fold did not result in its cleavage by yeast mitochondrial RNase P. Asp-GC was also incubated with 40 fold higher concentration of RNase P than is needed to cleave Asp-AU. No cleavage was observed (data not shown). We conclude that the third explanation is the correct one, that Asp-GC is not a substrate for yeast mitochondrial RNase P under any of the conditions that we tested.

The kinetics of the reaction with Asp-AU are typical for RNase P, with an apparent Km of 60 nM and Vmax apparent of 300 fm/min/microgram of protein. The Km of the yeast mitochondrial RNase P for a different precursor, $tRNA^{Met}$, is 90 nM. The Vmax (apparent) for this precursor is the same as that for Asp-AU (Hollingsworth, Morales, and Martin, manuscript in preparation). The apparent Km of the mitochondrial enzyme is similar to others that have been determined. Purified E. coli RNase P and M1 RNA, its catalytically active RNA subunit, have a Km for the in vivo made $tRNA^{Tyr}$ precursor of 500 nM (7).

Yeast mitochondrial RNase P is inhibited by Asp-GC to the same extent as it is by one of the products of the reaction, $tRNA^{Asp}$. We could not find conditions where the other RNase P product, the 5' leader, would inhibit the reaction. Since our studies have shown that the 5' leader must somehow play a role in the recognition by the yeast mitochondrial RNase P, the failure of the 5' leader alone to inhibit the reaction implies that this enzymatic recognition probably does not depend solely upon the leader itself. Instead, the specificity of this enzyme for particular types of 5' leaders must either be due to the influence of the leader on precursor tertiary structure or, if a primary structure phenomenon, occur some time after the initial recognition/binding step.

The only primary structure difference between Asp-AU and Asp-GC is within their 5' leaders. By simple inspection, one would not expect these different primary structures to cause major differences in the tertiary structure of the precursors. Both leaders could form some stem/loop structures within themselves, but they would not overlap the RNase P processing site. There did not appear to be a significant amount of complementarity between the 5' leaders and the tRNA sequence itself that might seriously disrupt the conserved structure of the tRNA. Structure analyses were performed to determine whether or not there were any significant differences in the tertiary structures of these two precursors.

We were able to conclude from the tertiary structure analyses that the difference between Asp-AU and Asp-GC is not purely a primary structure phenomenon. There are some differences between these two precursors at the secondary and tertiary structure level as well. It is interesting that the product of the RNase P reaction, $tRNA^{Asp}$, has the same T1-, S1-, and V1-vulnerable sites as does the substrate precursor, Asp-AU. We don't know what combination of the primary, secondary, and tertiary structure differences between Asp-AU and Asp-GC are necessary to cause the specificity that the yeast mitochondrial RNase P has for Asp-AU.

This paper presents a preliminary study of the yeast mitochondrial RNase P mechanism, approached from the standpoint of the enzyme's reactivity with substrate. There are many examples of mutations within the tRNA portion of precursor tRNAs that reduce processing by the RNase P holoenzyme both *in vivo* and *in vitro* (15- 20, 24). In general, tRNA processing endonucleases are thought to recognize the tRNA region of the substrate. RNase P is especially sensitive to changes in the substrate that interfere with the amino acid accepting stem and in the highly conserved D-stem and loop and T-pseudoU-C stem and loop areas. One series of studies using precursors from closely related genes suggests that different rates of processing might occur in response to the nature of the 5' leader sequence. However, there were also differences in the variable loops of these precursors, which made it difficult to draw a firm conclusion (21). It has also been shown that the 5' leader affects the cleavage of a precursor with M1, the RNA subunit of *E. coli* RNase P (22). In the studies reported here, we have shown conclusively that the 5' leader of a tRNA precursor can have a profound effect on the suitability of the precursor as a substrate for the holoenzyme. How much of this effect can be attributed to the primary and secondary sequence of the leader and how much to the alteration in tertiary structure of the precursor caused by the leader remains to be determined.

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