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Initiator tRNAs have a unique anticodon loop conformation

(S1 endonuclease/stem–loop interaction/conformational probe/chain elongation tRNA)

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ABSTRACT **Transfer RNA (tRNA) molecules have been labeled with ^{32}P at the 5′ end and subjected to S1 nuclease digestion. The products were analyzed by high-resolution gel electrophoresis. Three initiator tRNAs and six chain-elongating tRNAs were examined. S1 nuclease cleaved *Escherichia coli* $tRNA_f^{Met}$, yeast $tRNA_f^{Met}$, and mammalian $tRNA_f^{Met}$ at the same two positions in the anticodon loop. In contrast, S1 nuclease cleaved the anticodon loop of *E. coli* $tRNA_m^{Met}$, yeast $tRNA_m^{Met}$, yeast $tRNA^{Phe}$, *Schizosaccharomyces pombe* $tRNA^{Phe}$, *E. coli* $tRNA_2^{Glu}$, and *E. coli* $tRNA^{Trp}$ (su^+) at four positions generally, except where a modified nucleotide in the wobble position inhibited the enzyme. The marked contrast between these cleavage patterns suggests a different conformation for the anticodon loops of these two classes of tRNA molecules. It is suggested that the specialized conformation in the anticodon loop of initiator tRNAs may be due to a special sequence of GC base pairs in the adjoining anticodon stem.**

One of the interesting features of the genetic code is the fact that one codon, AUG, has two distinct functions. It codes for the methionine residues that initiate protein synthesis in both prokaryotic and eukaryotic systems and it also codes for methionine residues that are internal in the polypeptide chain sequence. These distinct functions are carried out by two different tRNA isoacceptors. One of these is the initiator tRNA ($tRNA_f^{Met}$), which acts solely to initiate polypeptide chain synthesis, while the other ($tRNA_m^{Met}$) is used for internal methionine positions. Both of these tRNAs for methionine have the anticodon CAU, which is complementary to the methionine codon AUG. However, what distinguishes an initiator $tRNA_f^{Met}$ from a chain elongating $tRNA_m^{Met}$ is unclear.

Considerable information has accumulated regarding the nucleotide sequence of these two classes of tRNA coding for methionine (1). The results are far from instructive. The major prokaryotic difference is the lack of one complementary base pair on the acceptor stem of the initiator in comparison to chain-elongating tRNAs (see Fig. 1 *A* and *D*). Several investigators have pointed out that there are unusual sequences associated with eukaryotic initiator tRNAs (2–4). In a lower eukaryote, such as yeast, the familiar sequence in the T-loop, T-ψ-C-G, is replaced by the sequence A-U-C-G (Fig. 1*B*). However, the tRNA acceptor stem has a normal complement of seven base pairs. In higher eukaryotes, such as mammals, the initiator tRNA contains the sequence A-U-C-G in the T-loop but has an additional puzzling substitution of a cytosine for uracil in position 33, which is at the 5′ side of the anticodon in all tRNAs (Fig. 1*C*). A uracil residue is found there in all other tRNAs. There appears to be no simple correlation between nucleotide sequence and initiator functions. Nonetheless, initiator tRNA molecules form a functionally distinct class; in many instances one initiator can be substituted for another in an *in vitro* protein synthetic system (5). The principle difference between an initiator tRNA and chain-elongating tRNAs is the ability of the former to bind at the ribosomal P site and thus initiate polypeptide chain synthesis while the latter bind initially only at the ribosomal A site (6).

In an attempt to understand some aspects of this system, we have carried out experiments in which both initiator and chain-elongating tRNA molecules have been subjected to nucleolytic cleavage by the endonuclease S1 from *Aspergillus oryzae*. This nuclease is a strict single-strand-specific endonuclease which has been used in a number of studies as a probe of secondary structure, specifically, to detect loop regions in DNA as well as RNA (7–11).

Here we report that despite considerable differences in the nucleotide sequences of prokaryotic and eukaryotic initiator tRNAs, there is a common S1 nuclease digestion pattern for the anticodon loop for initiator tRNAs. Furthermore, this pattern is quite different from that seen in chain-elongating tRNAs, including the tRNAs that code for internal methionine residues in protein biosynthesis. These findings suggest that the anticodon loop conformation of initiator tRNAs, as a class, differs from that of chain-elongating tRNAs in a manner that is detected by the structurally sensitive S1 nuclease probe.

MATERIALS AND METHODS

Escherichia coli and human placenta initiator tRNA were a gift from B. A. Roe. *E. coli* $tRNA_m^{Met}$ was donated by L. H. Schulman. The yeast initiator tRNA was a gift from U. L. RajBhandary. Yeast $tRNA_m^{Met}$ was a gift from H. Feldmann (Munich, West Germany). *Schizosaccharomyces pombe* $tRNA^{Phe}$ was a gift of D. Söll and *E. coli* $tRNA^{Trp}$ (CA 16) was a gift of R. Buckingham (Paris). *A. oryzae* S1 nuclease was purchased from Boehringer Mannheim, and a purified preparation was provided by J. Vournakis. The commercial S1 nuclease was found to be contaminated by trace amounts of T1 nuclease activity. However, a comparison of the results obtained with this enzyme preparation with those given by the enzyme furnished by J. Vournakis did not show significant differences in the anticodon loop cleavages.

All the tRNAs examined were labeled at the 5′ end with ^{32}P by standard techniques (8, 9). After digestion, the results were analyzed by a modification of the rapid gel sequencing method (12) described by Wurst *et al.* (8) and Wrede *et al.* (9). Nucleotide cleavage sites were identified by comparison with a "ladder" produced by alkaline hydrolysis, in conjunction with various sequence-specific enzymatic cleavages. The alkaline hydrolysis was performed in 50 mM NaOH/1 mM EDTA at 100°C for 20 sec. The sequence-specific nucleases used to assign the alkaline hydrolysis fragments were T1 nuclease from *A. oryzae*, pancreatic RNase A (both purchased from Boehringer Mannheim), and *Physarum* I nuclease (a gift from H. Donis-Keller).

All S1 nuclease digestions were performed in a standard buffer containing 25 mM NaOAc (pH 4.5), 5 mM $MgCl_2$, 50 mM KCl, and 1 mM $Zn(OAc)_2$, at 37°C, except where specifi-

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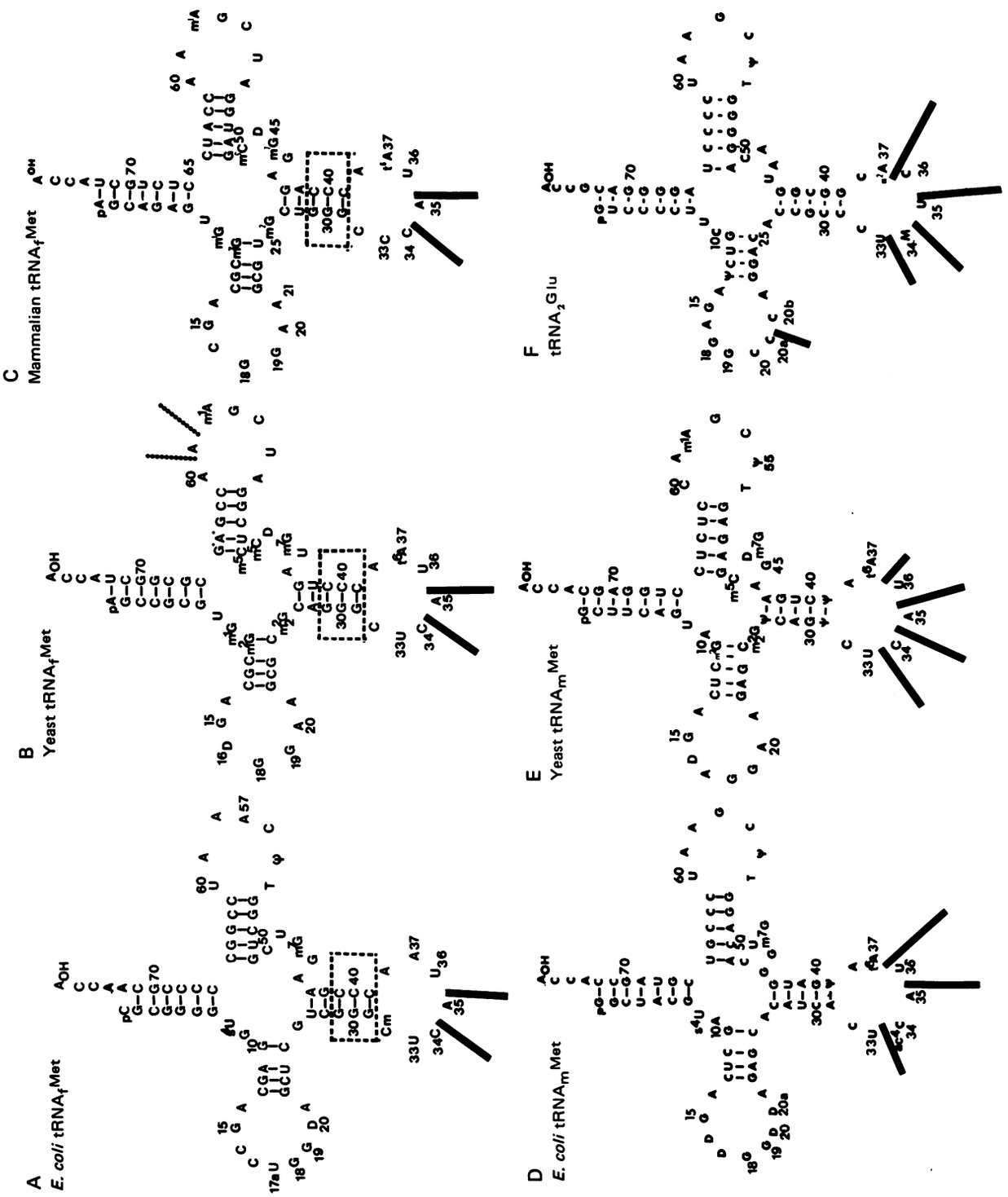

FIG. 1. Nucleotide sequences of three initiator and three chain-elongation tRNAs are shown together with S1 nuclease cleavage sites (black bars). The length of the bar indicates the extent of cleavage. The dotted lines in *B* indicate uncertainty regarding the site of cleavage. The numbering system is that of Gauss *et al.* (1).

cally noted. In order to determine precisely the initial cleavage sites, the digestions of each tRNA were carried out for varying intervals as noted in Fig. 2. The reactions were stopped by adjusting the reaction mixture to 4 mM ATP (pH 7.0). All tRNAs were preincubated in the reaction mixture for 10 min at 37°C prior to addition of the enzymes.

The tRNA numbering system used here is that adopted by Gauss *et al.* (1) in their collation of tRNA sequences. In this system, the constant uridine in the anticodon loop is labeled as U33 irrespective of the total number of nucleotides in a particular tRNA sequence (see Fig. 1). It is important to note that S1 nuclease digestion fragments terminate in a 3′-hydroxyl group while alkaline hydrolysis fragments have a 3′-phosphate. Therefore, S1 nuclease fragments migrate slightly more slowly than the corresponding alkaline hydrolysis fragments on sequencing gels. In particular, the bands of S1 nuclease fragments often have a position between two adjacent bands of alkaline hydrolysis fragments. In order to assign uniquely the 3′ base of S1 fragments, we have used sequence-specific nucleases—namely, pancreatic RNase A, *Physarum* I nuclease, and T1 nuclease. A complete description of the procedure we have devised to unambiguously analyze S1 nuclease cleavage sites is in preparation.

RESULTS

The method used to establish cleavage sites by S1 nuclease involved an initial labeling of the tRNA 5′ end with ^{32}P and subsequent chemical and enzymatic cleavage of the molecule, which was analyzed by high-resolution acrylamide gel electrophoresis. The results of a study on *E. coli* $tRNA_f^{Met}$ is shown in Fig. 2*a*. Ten lanes were run in parallel, which provided the information needed to identify the S1 cleavage sites in the anticodon loop. Alkaline hydrolysis produced a complete ladder of fragments, the identity of which can be determined most directly through the use of *A. oryzae* endonuclease T1, which cleaves after guanosine residues, and *Physarum* I nuclease, which cleaves predominantly after adenine and uracil residues. These two enzymatic digestions were performed in the presence of 7 M urea, which denatures the tRNA but does not inhibit either enzyme. The combination of these results (lanes 2 and 3, Fig. 2*a*) allows one to assign a numbering system to the alkaline hydrolysis ladder (lanes 5 and 10). Partial S1 nuclease digestions under nondenaturing conditions are shown in lanes 4, 6, and 8 in Fig. 2*a*. Various incubation times were used in order to determine the initial cleavage site.

S1 nuclease produces two principle cleavage sites. The precise identification of these cleavage sites is complicated by the fact that S1 nuclease fragments have a 3′-hydroxyl group while alkaline hydrolysis fragments terminate in a 3′-phosphate group. Consequently, S1 nuclease fragments migrate more slowly than the corresponding alkaline hydrolysis fragments. Pancreatic RNase A was used under nondenaturing conditions to facilitate the identification of the 3′ terminus of S1 nuclease fragments (lane 7, Fig. 2*a*). Pancreatic RNase A cleaves after pyrimidines, especially those 5′ to an adenosine, yielding fragments with a 3′-phosphate. The digestion shows two cleavage sites, C34 and U36, in the anticodon loop. A comparison of the two S1 nuclease bands with the pancreatic RNase bands allows one to make a definite assignment of C34 and A35 to the S1 nuclease bands.

The S1 nuclease digestion in Fig. 2*a*, lanes 4, 6, and 8, shows the cleavage after 2 min, 5 min, and 30 min, respectively. The relative intensity of the two bands in the anticodon region remains about the same over this extended digestion period. Further, one does not see the introduction of new cleavage sites in the anticodon loop over this time period. The S1 digestion

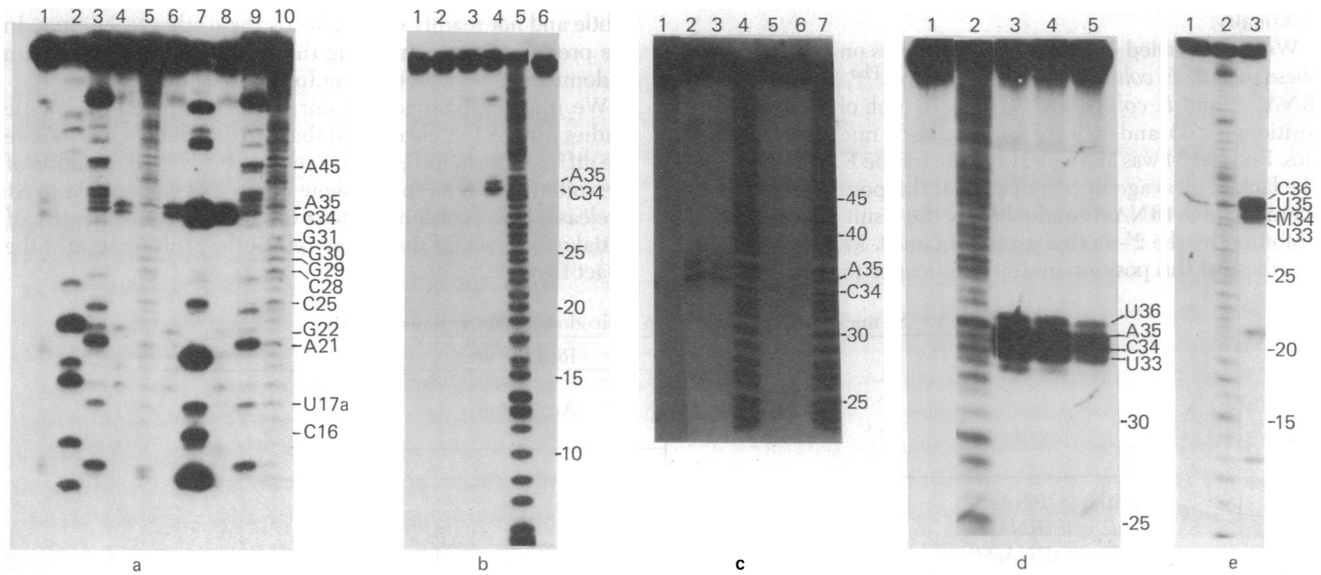

FIG. 2. Autoradiograms of partial S1 nuclease digestions of 5′-labeled [^{32}P]tRNAs. The sequencing lanes are shown only in *a* for *E. coli* $tRNA_f^{Met}$. All reaction mixtures contained the standard buffer except when noted. S1 nuclease digestions were carried out at 0.5 unit/μg of tRNA at 37°C for varying time periods. All alkaline hydrolysis (NaOH) are as described in *Materials and Methods*. (*a*) *E. coli* $tRNA_f^{Met}$: (Lane 1) no enzymes added, 30 min, 37°C; (lane 2) T1 nuclease in 7 M urea/1 mM EDTA, 0.5 unit of enzyme per μg of tRNA, 62°C, 30 min; (lane 3) same as lane 2 except *Physarum* I nuclease was used; (lane 4) S1 nuclease, 2 min; (lane 5) NaOH; (lane 6) S1 nuclease, 5 min; (lane 7) pancreatic RNase A, 4×10^{-4} unit/μg of tRNA, 10 min, 37°C; (lane 8) S1 nuclease, 30 min; (lane 9) same as lane 3; (lane 10) NaOH. (*b*) Yeast $tRNA_f^{Met}$: (Lane 1) no enzyme added; (lanes 2–4) S1 nuclease at 10 sec, 30 sec, and 1 min, respectively; (lane 5) NaOH; (lane 6) no S1 nuclease added, 30 min. (*c*) Human placenta $tRNA_f^{Met}$: (Lane 1) no enzyme added, 30 min; (lanes 2 and 3) S1 nuclease, at 10 min and 5 min, respectively; (lanes 4 and 7) NaOH; (lanes 5 and 6) S1 nuclease at 1 min and 30 sec, respectively. (*d*) Yeast $tRNA_m^{Met}$: (Lane 1) no enzyme; (lane 2) NaOH; (lanes 3, 4, and 5) S1 nuclease at 1 hr, 20 min, and 10 min, respectively. (*e*) *E. coli* $tRNA_2^{Glu}$: (Lane 1) no enzyme added, 30 min; (lane 2) NaOH; (lane 3) S1 nuclease, 5 min. Ten micrograms of tRNA were used in each slot except in the experiments with yeast $tRNA_m^{Met}$, where 20 μg/slot were used. All digestions were run on 20% polyacryalmide gel/7 M urea as described (9).

lanes also show that weak cleavage sites exist at G23, G20, and G19. These are due to a small amount of contaminating T1 nuclease activity in the commercial S1 nuclease; purified S1 nuclease preparations did not show these additional T1 cleavage sites. The diffuse bands near the top of the gel in the S1 lanes are found in lane 1, where no enzyme was added. Thus, the only cleavages produced by S1 nuclease digestion in *E. coli* $tRNA_f^{Met}$ are the two found in the anticodon loop.

A study of the type shown for *E. coli* $tRNA_f^{Met}$ was carried out on several other tRNA molecules. Some of the results are shown for yeast $tRNA_f^{Met}$ (Fig. 2*b*), mammalian $tRNA_f^{Met}$ (Fig. 2*c*), yeast $tRNA_m^{Met}$ (Fig. 2*d*), and *E. coli* $tRNA_2^{Glu}$ (Fig. 2*e*). In Fig. 2*b*, the partial S1 nuclease digestion of yeast $tRNA_f^{Met}$ shows two prominent cleavage sites in the anticodon loop and two slightly weaker sites in the T-loop. In contrast to the results with *E. coli* $tRNA_f^{Met}$, the lower cleavage site, identified as C34, is somewhat more intense than the cleavage at A35. However, the two bands appear simultaneously in the kinetic experiment.

The results with the mammalian $tRNA_f^{Met}$ (Fig. 2*c*) show two S1 nuclease cleavage sites in the anticodon loop. Again, these have been identified as C34 and A35. Both sites appear to be equally susceptible since they have approximately identical intensities for all of the digestion intervals taken.

The results of partial S1 nuclease digestion on yeast $tRNA_m^{Met}$ are shown in Fig. 2*d*. In contrast to the initiator tRNA digestion pattern, there are four cleavage sites in the anticodon loop. The identification of the four cleavage sites can be made by comparing the S1 nuclease bands with the alkaline hydrolysis ladder shown in lane 2. A similar pattern is seen in Fig. 2*e*, which shows the results of S1 nuclease digestion of *E. coli* $tRNA_2^{Glu}$. The anticodon loop contains four cleavage sites. Results similar to these have been presented in refs. 8 and 9. In both tRNAs, the cleavage sites are residues 33, 34, 35, and 36, with the initial cleavage sites being 34, 35, and 36 as determined kinetically.

We have carried out similar experiments on other tRNAs. These include *E. coli* $tRNA_m^{Met}$, yeast $tRNA^{Phe}$, *Schizo. pombe* $tRNA^{Phe}$, and *E. coli* $tRNA^{Trp}$ (su^+). In each of these tRNAs, positions 33, 35, and 36 were found to be S1 nuclease cleavage sites. Residue 34 was also a cleavage site for the *E. coli* $tRNA^{Trp}$. The lack of cleavage by S1 nuclease at this position in the two phenylalanine tRNAs is undoubtedly the result of S1 nuclease inhibition by the 2′-methyl group of Gm34. Modifications in the ribose at this position inhibit S1 nuclease (8, 9). Likewise, the lack of cleavage at ac^4C34 in *E. coli* $tRNA_m^{Met}$ probably reflects inhibition of the enzyme due to the modification of the base. In all these tRNAs, the cleavage at U33 appears to be a secondary cleavage site. The results of S1 nuclease digestion on all the tRNAs tested are summarized in Table 1. Similar results were also obtained with digestion at pH 6.0.

DISCUSSION

The results of this investigation, summarized in Fig. 1 and Table 1, are quite striking. The three initiator tRNAs, which differ considerably in their nucleotide sequence, nonetheless have a common pattern of digestion by the S1 endonuclease, showing two susceptible sites in the anticodon loop. In contrast, the chain-elongating tRNAs, including two of the internal methionine tRNAs, show a markedly different pattern of S1 nuclease susceptibility in the anticodon loop. Except for nucleotides that are resistant to S1 digestion because of modification, the digestion patterns of all the chain-elongating tRNAs are similar to each other with four susceptible sites, one of which, U33, may be a secondary site. These results suggest that the conformation of the anticodon loop is similar in all initiator tRNAs and that this conformation differs from that seen in elongation tRNAs. As judged by this criterion, there appears to be substantial similarity in the conformation of the anticodon loop of all chain-elongating tRNAs.

It is interesting that the results given by this enzymatic structural probe differ considerably from earlier attempts to find initiator–elongation tRNA differences. For example, there have been a number of chemical modification studies of both initiator and chain-elongating tRNAs (for review, see refs. 13 and 14) whose results are quite complex, but they cannot be interpreted to indicate conformational differences. Likewise, oligonucleotide binding studies (15) of these tRNA molecules do not show significant differences. Thus, the nature of the conformational change that we have detected is likely to be subtle and not readily observable by these other techniques. In the present case we are using the structural specificity of an endonuclease to detect the conformational difference.

We must emphasize that our results are based on kinetic studies using S1 nuclease, and that the differences we observe are differences in initial cleavage sites. Prolonged digestion of the initiator tRNAs might show other sites susceptible to S1 nuclease. However, our interest lies only with the identity of initial cleavage sites since these reflect the conformation of the intact tRNA.

Table 1. S1 nuclease cleavage sites in anticodon loop of various tRNAS

	Residue no.						
	32	33	34	35	36	37	38
			Anticodon				
Initiator tRNAs							
E. coli $tRNA_f^{Met}$	Cm	U	C↓	A↓	U	A	A
Yeast $tRNA_f^{Met}$	C	U	C↓	A↓	U	t^6A	A
Mammalian $tRNA_f^{Met}$	C	C	C↓	A↓	U	t^6A	A
Elongation tRNAs							
E. coli $tRNA_m^{Met}$	C	U↓	ac^4C	A↓	U↓	t^6A	A
Yeast $tRNA_m^{Met}$	C	U↓	C↓	A↓	U↓	t^6A	A
Yeast $tRNA^{Phe}$	Cm	U↓	Gm	A↓	A↓	Y	A
Schizo. pombe $tRNA^{Phe}$	Cm	U↓	Gm	A↓	A↓	Y	A
E. coli $tRNA_2^{Glu}$	C	U↓	M↓	U↓	C↓	m^2A	C
E. coli $tRNA^{Trp}$ (CA 16)	Cm	U↓	C↓	C↓	A↓	ms^2i^6A	A

S1 nuclease cleavage sites are indicated by ↓ after the base. M is 2-thio-5-methylaminomethyl uridine. Y is wybutosine. Other abbreviations are standard (1).

What then provides the physical basis for these differences in nucleotide sensitivity? It is clear that the sequence of nucleotides in the anticodon loops cannot be responsible for this difference since the anticodon loop sequence of yeast $tRNA_f^{Met}$ and yeast $tRNA_m^{Met}$ are identical (Fig. 1). However, the anticodon stems do provide a suggestion regarding the source of the differences. As shown in Fig. 1 *A–C*, the three base pairs in the anticodon stem proximal to the loop in initiator tRNAs are all the same: there are three guanosine residues on the 5′ side and three cytosine residues on the 3′ side. No chain-elongating tRNAs have this pattern of base pairing in the anticodon stem (1). Furthermore, this is the only feature common to initiator tRNA molecules. As discussed above, there are significant differences in the TψC loop, in the hydrogen bonding of the acceptor stem, and in the nature of the pyrimidine in position 33 of initiator tRNAs. The conformation of the loop may influenced by the type of base pairs that are found in the adjoining stem. Preliminary experiments that we have carried out with fragmented initiator tRNA molecules from yeast appear to support this suggestion. The tRNA fragments are made by cleaving the molecule in the T-loop and removing the 3′ fragment. The nuclease susceptibility of the remaining three-quarter molecule is identical to that of the intact molecule. This is consistent with the idea that the anticodon stem fixes the conformation of the anticodon loop.

The influence of stem sequences on the loop conformation is further supported by nuclease digestion experiments on *Schizo. pombe* $tRNA^{Phe}$ (to be reported elsewhere). This RNA has the same D-loop as yeast $tRNA^{Phe}$, but the D stem sequence is different. The susceptibility of *Schizo. pombe* $tRNA^{Phe}$ in the D-loop to T1 nuclease differs markedly from that of yeast $tRNA^{Phe}$.

Our results with the three-quarter fragment yeast $tRNA_f^{Met}$ are consistent with those reported by Dube *et al.* (16). They found that fragments of *E. coli* initiator tRNA could bind to the ribosomal P site in the presence of the codon AUG. Among the fragments that could bind were those containing just the anticodon loop and part of the anticodon stem. Specifically, fragments containing at least two of the GC pairs in the anticodon stem suffice to fix an anticodon loop conformation that is recognized as belonging to an initiator tRNA.

An interesting case that should be examined further is the initiator tRNA from the mitochondria of *Neurospora crassa*. Unlike the other initiators discussed here, this tRNA shows only two GC base pairs in the anticondon stem. It will be interesting to see if this initiator has the anticodon loop conformation reported here, particularly in light of the fact that it is known to function as an initiator in an *in vitro E. coli* protein synthetic system (17).

It is possible that in tRNAs with only two anticodon stem GC base pairs adjacent to the loop, the anticodon loop may assume two conformations: that characteristic of elongation tRNAs and that characteristic of initiators. In this regard, it will be useful to examine the nuclease digestion pattern of *E. coli* or yeast $tRNA^{Arg}$ and yeast $tRNA^{Lys}$, all of which have two GC pairs proximal to the loop.

These studies indicate that there is a conformation of the anticodon loop that is unique to initiator tRNA molecules. By implication, this is likely to be an important consideration in targeting the initiator for the ribosomal P site. Although we have no evidence of the nature of the conformational difference from the present study, the fact that chemical modification and oligonucleotide binding studies do not show significant differences between initiators and elongation tRNAs suggests that the conformational distinction may be small in magnitude. Accordingly, one may ask whether it is possible that tRNA molecules, in going from the A site to the P site of a ribosome, undergo this conformational shift. Is it possible that in the ribosomal P site all tRNA molecules have an anticodon loop conformation similar to that seen here for the initiator tRNAs? We suggest that the energy barrier between these different conformations is not great and that the shift could be associated with translocation inside the ribosome. Experiments can be carried out to test this hypothesis directly. In particular, it may be possible to change the environment of chain-elongating tRNAs in such a way as to modify the conformation of the anticodon loop so that the change can be detected by nuclease structural probes. It would be of great interest to examine tRNAs such as *E. coli* and yeast $tRNA^{Arg}$ or yeast $tRNA^{Lys}$, which may be able to shift conformations more readily as a result of the two GC pairs in their anticodon stems.

Initiator and chain-elongation tRNA molecules appear to be subtle structures that are grossly similar but nonetheless have distinct anticodon loop conformations with important functional consequences. It will be of great interest to define the physical nature of the conformation differences between these two classes of tRNA molecules.

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