S1 Nuclease as a Probe for the Conformation of a Dimeric tRNA Precursor†

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ABSTRACT: We have employed S1 nuclease to probe the structure of an intermediate in tRNA biosynthesis available only in radiochemical purity. The dimeric precursor to $tRNA^{Gln}$ and $tRNA^{Leu}$ from bacteriophage T4 was digested with the single-strand specific nuclease, and the products of the reaction were compared with the S1 digestion products of the mature cognate tRNAs. Quantitation and sequence analysis of the products revealed that the location and accessibility of S1 cleavage sites in the precursor were substantially identical with those in the mature forms. Based on these conclusions, it is argued that the dimer is comprised of two domains in which the specific features of both secondary and tertiary conformation closely resemble those found in the mature molecules; at the same time we noted small but apparently significant differences in certain regions of the molecule which may reflect signals for various maturation events. Finally, we have determined that the sites of precursor cleavage by RNase P, the endonuclease which generates the mature 5′ termini of these tRNAs, were completely inaccessible to S1 digestion.

It is now apparent that the production of most if not all cellular tRNA species proceeds via biosynthetic intermediates which are longer in nucleotide sequence than the mature, functional molecules. In both *Escherichia coli* (Altman & Smith, 1971; Schedl et al., 1974; Carbon et al., 1974) and the bacteriophage T4 (Guthrie et al., 1974; Abelson et al., 1974), each of these tRNA precursors is cleaved endonucleolytically by RNase P (Robertson et al., 1972) to generate the mature 5′ termini. However, comparison of nucleotide sequences at and around RNase P cleavage sites on a number of these molecules reveals no similarity among them (Guthrie, 1975). This clear absence of sequence-specific recognition thus points to the fundamental importance of conformation in the interaction between processing enzyme and RNA substrate.

Considerable data consistent with this notion have now been accumulated. Genetic and biochemical studies of mutationally inactivated suppressor tRNAs from both *E. coli* (Smith, 1974; Altman et al., 1974) and T4 (Seidman et al., 1974; McClain et al., 1975) suggest that mutations which disrupt the secondary or tertiary structure of the molecule can result in accumulation of the precursor at the expense of the mature tRNA. Data based on chemical modification (Chang & Smith, 1973) and thermal denaturation (cited in Altman et al., 1974) tend to support this interpretation. More recently (McClain & Seidman, 1975), modification analysis of mutationally altered tRNAs which arise from dimeric precursors demonstrated that the two tRNA sequences must act essentially independently at the time nucleotide modifications are introduced into the precursor RNA. While a "paired cloverleaf" model (McClain & Seidman, 1975) is clearly the simplest and most appealing secondary structure *consistent* with these data, the problem remains the same: how can one obtain more direct information about the specific secondary and tertiary conformation of precursors which are currently available in only radiochemical purity?

In seeking a new approach to this challenging problem, we have taken advantage of the recent demonstration by Harada & Dahlberg (1975) that the single-strand specific nuclease S1 attacks *E. coli* tRNAs at only two regions, the 3′ terminus and the anticodon loop, to produce oligonucleotides with 5′-phosphate termini. Their results are entirely consistent with the current three-dimensional model of tRNA structure (Kim et al., 1974; Robertus et al., 1974), which predicts that all other single-stranded regions in the cloverleaf are involved in secondary and/or tertiary structure. We thus reasoned that an analysis of the S1 digestion products of a precursor should reveal whether the regions of single strandedness in the dimer are the same as those in the mature tRNAs and furthermore whether the precursor-specific regions containing the RNase P cleavage sites are "exposed" or are involved in base interactions.

We chose to analyze the T4 dimeric precursor to glutamine and leucine tRNAs[1] (Guthrie, 1975). This precursor, which is normally efficiently processed and therefore undetectable in a wild-type host (Guthrie et al., 1973), can be isolated in high purity from T4 infections of an *E. coli* mutant (Schedl & Primakoff, 1973) carrying a temperature-sensitive lesion in a gene coding for RNase P. We considered this an additional advantage over the previous studies of precursor structure cited above, all of which employed mutationally altered substrates which are matured at greatly reduced rates.

Materials and Methods

(*1*) *Preparation of Substrates.* The Gln-Leu tRNA precursor[1] was obtained from infections of *E. coli* strain A49 (Schedl & Primakoff, 1973), a temperature-sensitive mutant of RNase P which accumulates tRNA precursors at the nonpermissive temperature, with Δ27, a strain of T4 deleted for all T4 tRNAs except those specific for glutamine and leucine (Comer et al., 1974). Conditions of ^{32}P labeling, RNA extraction, polyacrylamide gel electrophoresis, and gel elution were exactly as described by Guthrie (1975). Mature $tRNA^{Gln}$ and $tRNA^{Leu}$ were prepared from infections of *E. coli* strain B/5 with Δ27 as above, except that the cells were grown at

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[1] Abbreviations used: the dimeric precursor to the mature molecules $tRNA^{Gln}$ and $tRNA^{Leu}$ is referred to as the Gln-Leu tRNA precursor. The precursor-specific nucleotides at the 5′ terminus of this molecule are termed the 5′ leader; the junction between the two tRNA sequences is called the interstitial region. Modifications are indicated as follows: m^2A, 2-methyladenosine; Ψ, pseudouridine; T, ribothymidine; Â, 2-(methylthio)-6-isopentenyladenosine; GmG, 2′-*O*-methylguanosine.

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37 °C and the temperature inactivation step was omitted.

(*2*) *Nuclease S1 Purification and Digestion Conditions.* Nuclease S1 was purified according to the procedure of Vogt (1973) through the DEAE-cellulose chromatography stage; the sulfo-Sephadex step was omitted. The specificity of the enzyme used in these experiments was determined by comparing the rates at which it converted native or heat-denatured calf thymus DNA to acid-soluble products. Native DNA was degraded at less than 5% the rate for denatured DNA.

The digestion buffer, that of Harada & Dahlberg (1975), contained 0.3 M NaCl, 0.03 M NaOAc (pH 4.5), 0.001 M $ZnCl_2$, and 5% glycerol. Each reaction contained, in a total volume of 0.2 mL, 20 μg of carrier tRNA and 10 units of nuclease S1. Gln-Leu tRNA precursor (8×10^6 cpm), $tRNA^{Leu}$ (5×10^6 cpm), and $tRNA^{Gln}$ (6×10^6 cpm) were digested. One-tenth this amount of radioactivity was present in the undigested controls. Incubation was for 30 min at 15 °C. The reaction was terminated by diluting the samples to 1 mL in 10 mM EDTA and immediately extracting with phenol. The aqueous phases were ethanol precipitated. Recovery of input RNA following S1 treatment was in all cases greater than 90%.

Fractionation and Sequence Analysis of S1 Digestion Products. The cleavage products were fractionated by electrophoresis in a 20% polyacrylamide slab gel (Ikemura & Dahlberg, 1973) (30 mA, 300 V for 8 h) in a water-cooled apparatus. After autoradiography, the desired bands were eluted according to Guthrie & McClain (1973).

The techniques, materials, and methods used for sequence analysis were those described in detail by Barrell (1971). Primary analyses were performed with RNase T_1; secondary analyses, with RNases A, T_2, and P_1.

Results

(*1*) *Determination of Reaction Conditions.* In order to faithfully ascertain the conformation of the dimer as revealed by nuclease S1 sensitive sites, several parameters of the reaction must be controlled. It is necessary to chose conditions which maximize the yield of primary digestion products, that is, of products of the initial cleavage reaction rather than secondary or subsequent reactions. This was accomplished by varying the ratio of enzyme to tRNA carrier (the ^{32}P-labeled substrate is essentially carrier free) and comparing the pattern of reaction products. A ratio of enzyme to carrier was chosen which left residual undigested substrate (see legend to Figure 1). A twofold increase in time of incubation produced no significant qualitative changes in the cleavage pattern.

Another important consideration is the choice of digestion conditions which favor the preservation of the native forms of the substrates. Previous studies (e.g., Reeves et al., 1970) have implicated both heat and low $[Mg^{2+}]$ as factors which can promote tRNA denaturation. Thus, trial digestions were carried out in the presence (5 mM) and absence of $MgCl_2$ and with and without prior heat treatment (20 min at 45 °C). Again, no qualitative differences in the banding pattern of reaction products on a 20% polyacrylamide gel were observed. Furthermore, there were no overall differences in the extent of digestion of the substrate.

(*2*) *Analysis of S1 Digestion Products.* Digestion products were fractionated and analyzed as described under Materials and Methods. Components present in less than 2% yield (where 100% is the sum of the residual unreacted substrate plus all cleavage products, as determined by densitometer tracings of the autoradiograph) are not reported. The data are given in Table I. The digestion products are shown in Figure 1. G refers to $tRNA^{Gln}$, L to $tRNA^{Leu}$, and K to the Gln-Leu tRNA precursor; the numbers designate bands of increasing electrophoretic mobility.

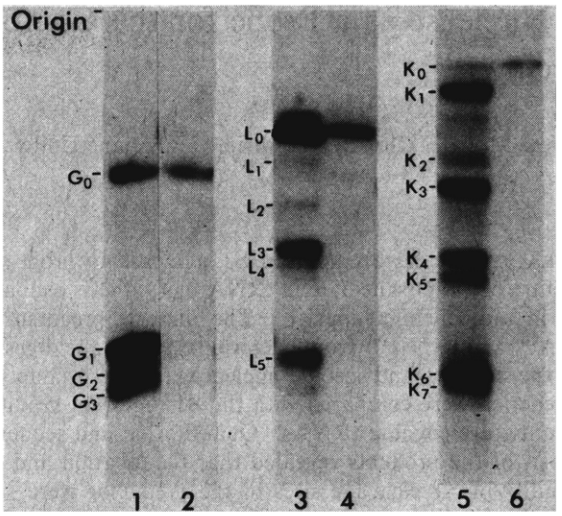

FIGURE 1: Autoradiograph of polyacrylamide gel fractionation of S1 digestion products. ^{32}P-labeled RNA substrates were prepared and digested with nuclease S1 as described under Materials and Methods and separated on a slab gel of 20% acrylamide. Lanes 2, 4, and 6 contain undigested controls; treated samples are in lanes 1 ($tRNA^{Gln}$), 3 ($tRNA^{Leu}$), and 5 (the Gln-Leu tRNA precursor). The indicated products were eluted from the gel and analyzed as described in Table I. In two experiments, the amount of substrate which was unaffected by S1 digestion (residual substrate) varied as follows. Experiment I: $tRNA^{Gln}$, 10%; $tRNA^{Leu}$, 30%; Gln-Leu tRNA precursor, 34%. Experiment II: $tRNA^{Gln}$, 9%; $tRNA^{Leu}$, 36%; Gln-Leu tRNA precursor, 2%. The gel pattern did not vary.

(*a*) *G1–G3.* As can be seen in Figure 1 (lane 1), S1 digestion of $tRNA^{Gln}$ yields three cleavage products. Band G1 is a fragment containing the 3′ half of the molecule, extending from the anticodon to the 3′ terminus. Cleavage occurred primarily between Up and Gp in the anticodon and to a lesser extent between Gp and m^2Ap adjacent to the anticodon. Neither CCA_{OH} nor CC_{OH} was detected. Further digestion by RNase T_2 of the terminal RNase T_1 product UCCUU-UAUUCCCAGp produced 1 mol of Gp, indicating that the 3′ terminus of the tRNA was cleaved between the two C residues. Bands G2 and G3 are both 5′ fragments that extend from the mature 5′ terminus pUp to slightly different positions in the anticodon.

(*b*) *L1–L5.* After cleavage of $tRNA^{Leu}$ with S1, five products could be distinguished (Figure 1, lane 3). Bands L1 and L2 both contain fragments extending from the dihydrouridine loop to the 3′ terminus. In all, three different S1 cleavage sites within the dihydrouridine loop were identified. In L1, cuts occurred between the second and third Ap residues, which is the predominant site, and at one or both of the two Gp residues; in L2 the 5′ terminus pAGp was generated.

L3 is a fragment extending from the anticodon to the 3′ terminus. Only one cleavage site is apparent, as evidenced by the presence of only one new RNase T_1 oligonucleotide pAAAΨGp.

L4 and L5 comprise four fragments, three of which result from cleavages within the variable loop. L4, a very minor product, includes two complementary fragments generated by a cut at an undetermined site in the variable loop. L5 also comprises two comigrating species. The 5′ fragment extends from the 5′ terminus of the tRNA to the anticodon. The 3′ fragment contains sequences from the variable loop to the 3′ terminus of the tRNA; this product is generated by a unique cut within the variable loop, but we have not been able to

determine whether the 5′ terminus is pGp or pAp (see footnote *c*, Table I). In summary, then, although there are several S1-sensitive sites within the variable loop, one (at either pGp or pAp) is predominant.

The intact 3′ terminus CCA_{OH} was found present in molar yields ranging from 0.2 to 0.5 among these fragments. Molecules lacking the terminal A residue were detected in 0.05–0.25 molar yield.

(*c*) *K1–K7.* K1 is a 3′ fragment extending from the $tRNA^{Gln}$ anticodon to the 3′ terminus of the dimer. Two cleavage sites within the anticodon loop can be identified; they are exactly the same as those found in mature $tRNA^{Gln}$ (G1 above).

The 5′ terminus of K2 is also within the $tRNA^{Gln}$ anticodon, but this fragment terminates somewhere beyond the $tRNA^{Leu}$ anticodon. While it seems likely that the 3′ terminus of K2 is generated by an S1 cut analogous to that which produced L4, the low yield of this fragment precluded further analysis.

K3 extends from the $tRNA^{Gln}$ anticodon to the $tRNA^{Leu}$ anticodon. As above, pGp and pm^2Ap are the predominant 5′ termini; however, two new termini, pCp and pΨp, are detected in low yield.

K4 extends from the $tRNA^{Leu}$ anticodon to the 3′ terminus of the dimer and is analogous to L3. However, we now find, in addition to the 5′ terminus pAAAΨGp of L3, a new terminus, pAAΨGp, present in equivalent molar yield.

K5 extends from the $tRNA^{Gln}$ anticodon to the dihydrouridine loop of $tRNA^{Leu}$. There are multiple 5′ termini, which are identical with those identified in K1 and K3. The sequence at the 3′ terminus is consistent with the predominant S1 cleavage site observed in the dihydrouridine loop in L1.

K6 and K7 both contain fragments extending from the 5′ leader sequence of the dimer to the $tRNA^{Gln}$ anticodon. None of these molecules contain the intact 5′ leader; within this region there are multiple S1 cleavage sites distributed around a predominant site as indicated in Figure 2. Of particular importance is the finding that no S1 cuts can be detected at the RNase P cleavage site.

Comigrating with K7 is a fragment containing sequences extending from the variable loop of $tRNA^{Leu}$ to the 3′ terminus of the dimer. We were not able to determine whether the S1 cut in the variable loop which generated this fragment is identical with that which produced L5.

The 3′ termini of those Gln-Leu tRNA precursor fragments which extend to the 3′ terminus of the dimer comprise several sequences. As found for the cognate $tRNA^{Leu}$ products, the intact CCA_{OH} sequence predominates, while lesser yields of CC_{OH} are also found.

(*3*) *Interpretation of Cleavage Patterns.* The data in Table I indicate that, in most cases, both fragments generated by S1 cleavage are recovered. In the case of $tRNA^{Gln}$, which is cut only in the anticodon loop, 5′ and 3′ halves of the molecule are equally represented. Similarly, the molar yields of complementary sequences generated by S1 digestion of the Gln-Leu tRNA precursor are nearly identical (5′:3′ = 1.05). There is, on the other hand, a considerable excess (3:1) of 3′ over 5′ fragments among the $tRNA^{Leu}$ species. The overrepresentation of 3′ fragments primarily reflects the absence of short-chain oligonucleotides generated by S1 cleavage in the dihydrouridine loop. These species are apparently lost during gel electrophoresis; the predicted fragments are 14 nucleotides in length, whereas the smallest species we have recovered contains 32 residues.

Table II presents a summary of S1 cleavage sites as determined primarily from an analysis of 3′ fragments. This

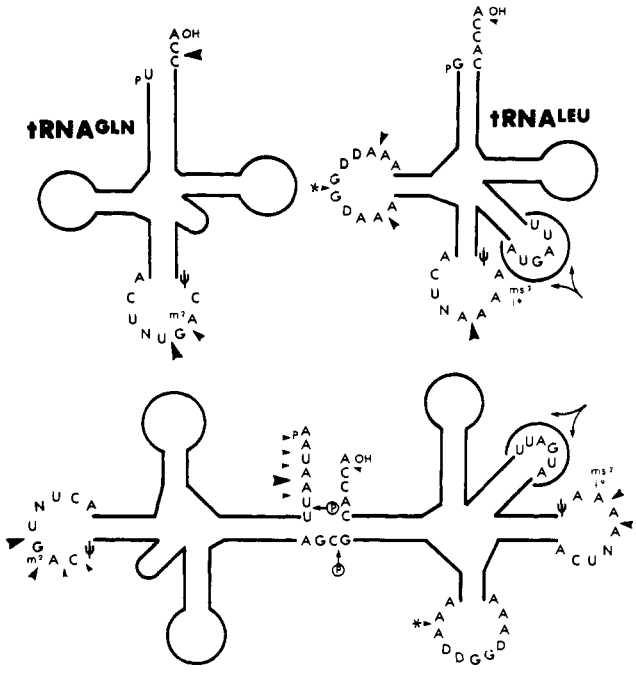

FIGURE 2: Schematic summary of S1 cleavage sites. $tRNA^{Gln}$, $tRNA^{Leu}$, and the Gln-Leu tRNA precursor have been drawn in cloverleaf form. The polynucleotide chain in the portions of these molecules resistant to S1 has been denoted by a solid line. In the regions susceptible to S1 attack, and in the interstitial region, the nucleotide sequences are shown and cleavage is indicated by an arrow. The size of the arrows is proportional to the frequency with which cleavage by S1 occurs at the indicated loci and is based on the data of Table I. An asterisk preceding an arrow indicates a cut which has not been rigorously proven, but which is suggested by the available evidence (see footnote *d*, Table II). A P attached to an arrow denotes an RNase P cleavage site (Guthrie, 1975); no cleavage by S1 was found to have occurred at these sites. The curve accompanied by two diverging arrows which surrounds the variable loop of $tRNA^{Leu}$ and the respective sequence in the dimer indicates that cleavage occurs at several locations within the loop.

rationale stems from the observation by us and by others (Vogt, 1973; Harada & Dahlberg, 1975) of an apparent 3′ exonucleolytic activity associated with S1. Since 5′ fragments (i.e., terminating with 3′ hydroxyls) may thus have undergone subsequent exonucleolytic degradation, we have utilized these data primarily to corroborate rather than actually to determine sites of cleavage.

S1 splits $tRNA^{Gln}$ endonucleolytically at two adjacent sites in the anticodon loop; the predominant cleavage (66%) is between the second and third nucleotides in the anticodon triplet. The same results are seen in the dimer, though here two new S1 sites can be detected in a combined total of less than 10% molar yield.

We have identified three specific sites of cleavage within the dihydrouridine loop of $tRNA^{Leu}$. One of these sites is also observed in the dimer. Since 3′ fragments generated by cleavage in this region are not recovered, susceptibility at the other two sites may have gone undetected. While the overall difference in S1 sensitivity between the mature tRNA and the precursor varies most in the dihydrouridine loop, that difference is less than twofold (see Table II). Only a single cleavage site is accessible in the anticodon of $tRNA^{Leu}$. This site is also available in the dimer; however, a new cut, displaced by a single nucleotide, is observed in equal frequency in the precursor. There is a single predominant S1 cleavage site in the variable loop of $tRNA^{Leu}$. This loop is also cut by S1 in the precursor, although to a somewhat lesser extent, but we

Table I: Sequence Analysis of S1 Digestion Products.[f] Molar Yields of Oligonucleotides Generated upon Further Digestion with RNase T_1

	Band[a]	G1	G2	G3	L1	L2	L3	L4	L5	K1	K2	K3	K4	K5	K6	K7
	% of total cleavage product	50	14	36	7	7	45	6	35	12	5	19	19	11	23	11
	T_1 Oligonucleotides															
	leader (precursor) pAAUAAUUGp														pAUAAUUGp <.05 pUAAUUGp <.05	pAAUUGp 0.24
tRNA^{Gln}	pUGp (tRNA^{Gln})		pUGp 1.14	pUGp 0.80											pAUUGp 0.98	pUUGp 0.39
	Gp		[1.13]	[1.41]											[0.63]	[1.44]
	AAUUAGp		0.91	0.87											1.00	1.00
	CCAAGp		1.10	0.96											0.93	0.85
	DDGp		1.01	0.98											1.51	1.00
	Gp		[1.13]	[1.41]											[0.63]	[1.44]
	DAAGp		0.84	0.81											1.27	0.61
	Gp		[1.13]	[1.41]											[0.63]	[1.44]
	CAUAGp		0.98	1.01											0.47	0.97
	CACUNUGp	pGp 0.66	CACUNU$_{OH}$ 1.02 CACUN$_{OH}$ 0.23	CACUN$_{OH}$ 0.64 CACU$_{OH}$ 0.37						pGp 0.39	pGp 0.80	pGp 0.76		pGp 0.89	CACUN$_{OH}$ 0.07 CAC$_{OH}$ 1.41	CAC$_{OH}$ 0.84
		I 0.76								I 0.50		I 0.70		I 0.81		
	m^{2}ACΨGp	pm^{2}ACΨGp 0.16								pm^{2}ACΨGp ND	0.71	pCΨGp 0.09 pΨGp 0.06		pm^{2}ACΨGp 0.36 pCΨGp 0.16		
	CUAGp	0.66								0.88	1.07	1.07		0.86		
	AUGp	1.07								1.29	1.14	1.01		1.00		
	CAAAGp	0.93								1.17	ND	0.80		ND		
	Gp	[1.84]								[1.33]	[0.92]	[1.25]		[1.40]		
	TΨCGp	1.07								[1.16]	[0.98]	1.53		1.97		
	AGp	1.30								[1.80]	[1.15]	[1.00]		[1.13]		
	UCCUUUAUUCCCAGp	1.10								1.16	0.97	1.38		1.41		
	CCA$_{OH}$ (tRNA^{Gln})							pGp	pGp	CGp [1.07]	CGp [0.77]	CGp [1.24]		CGp [1.02]		
	CGp (precursor)							1.80	1.15							
tRNA^{Leu}	pGp (tRNA^{Leu})															
	CGp							[1.20]	0.67	[1.07]	[0.77]	[1.24]		[1.02]		
	AGp							[1.15]	[0.72]	[1.30]	[1.13]	[1.00]		[1.13]		
	AAUGp							1.00	0.65	[0.99]	[1.04]	[1.42]		1.41		
	Gp							[1.14]	[1.11]	[1.33]	[0.92]	[1.25]		[1.40]		
	UCAAADDGGp[e]				pADDGGp 0.70 pGp 0.30			1.22	1.08	1.17	0.85	1.12		UCAA$_{OH}$ 0.37		
	DAAAGp				1.04	pAGp 0.70		1.30	0.83	0.98	0.89	1.02				
	Gp				[0.94]	[1.58]		[1.14]	[1.11]	[1.33]	[0.92]	[1.25]				
	CACAGp				0.95	1.00		0.96	1.00	0.77	0.71	1.20				
									CACUNA$_{OH}$ 0.31		I 0.40	CACUNA$_{OH}$ 0.23	pAA*AΨGp 0.27			
	CACUNAAA*AΨGp				0.95	1.11	pAA*AΨGp 0.84	1.06	CACU$_{OH}$ 0.68	0.60	CACU$_{OH}$ 0.30	CACUN$_{OH}$ 0.34 CACU$_{OH}$ 0.33	pA*AΨGp 0.33			
	CUGp				0.80	0.76	1.05	1.20		0.55	0.84		0.78			
	CGp				1.38	1.17	0.66	[1.20]		[1.07]	[0.77]		0.88			
	Gp				[0.94]	[1.58]	[1.35]	[1.14]		[1.33]			[1.20]			
	AAUGp				1.19	1.41	0.79	( )[b]		0.99			0.81			
	AUUCCUUGp				1.14	1.41	1.06	0.50	0.84[c]	0.97			1.22			1.16[d]
	UGp				1.19	1.18	0.67	ND	0.43	1.37	0.62		0.93			0.99
	Gp				[0.94]	[1.58]	[1.35]	[1.14]	[1.11]	[1.33]			[1.20]			[1.44]
	TΨCGp				1.14	1.00	0.92	0.80	0.47	[1.16]			1.04			0.63
	AGp				1.24	1.47	1.00	[1.15]	[0.72]	[1.80]			0.66			0.84
	UCCCACUUCUCGp				1.07	1.00	1.22	0.50	0.73	1.32			0.74			0.77
					I 0.48				I 0.32				I 0.33			0.46
	CACCA$_{OH}$				CACC$_{OH}$ 0.05	0.44	0.19		CACC$_{OH}$ 0.25	0.63			CACC$_{OH}$ 0.14			

Table I (*Continued*)

[a] The percentages of total cleavage products were calculated in the following manner. The values for each cleavage band obtained after densitometric reading of the autoradiogram seen in Figure 1 were adjusted for the chain length. The ratios thus obtained were then expressed as the percentages of the total product material found in each reaction. [b] Because $tRNA^{Leu}$ contains a number of reiterated T_1 oligonucleotides, the exact site of S1 cleavage in band L4 cannot be determined. The low molar yield of the T_1 product AUUUCCUUGp and the presence of only 1 mol of AAUUGp, which occurs twice in the intact tRNA, suggest cleavage(s) in the variable loop in one or both of these sequences. [c] Due to the low yield of the S1 fragment L5 we have been unable to determine whether this T_1 oligonucleotide contains a 5′-phoshate, indicating S1 cleavage at that A, or a 5′-OH, indicating cleavage by S1 at the adjacent G. [d] Due to the low yield of the S1 fragment K7 we have been unable to distinguish among three possible 5′ termini for this T_1 oligonucleotide, pAp, Ap, or pUp. [e] 20% of the mature $tRNA^{Leu}$ molecules contain a GmG modification in the D loop. The precursor is not modified at this site. The data for the adjacent nucleotides UCAAADAGp and Gp have been reported together. [f] T_1 oligonucleotides are written 5′ to 3′ with nucleotides represented by terminal OH groups are shown. N, shown in the anticodon sequence of both tRNAs, is in each case an as yet unidentified hypermodified uracil residue. Molar yields were determined by standardizing to representative T_1 oligonucleotides for each region of the precursor. T_1 oligonucleotides occurring in less than 0.3 molar yield are not reported unless they occur as termini of S1 digestion products. T_1 oligonucleotides which occur more than once in a sequence have been assigned average values, indicated by brackets, obtained by dividing the total counts found for that species of oligonucleotide by the number of times it appears. ND indicates that the molar yield was not determined. I indicates that an intact T_1 oligonucleotide was found in addition to partial products. The nucleotide sequences of $tRNA^{Gln}$, $tRNA^{Leu}$, and the Gln-Leu tRNA precursor have been determined by Seidman et al. (1974), Pinkerton et al. (1973), and Guthrie (1975), respectively.

Table II: Summary of S1 Cleavage Sites[d]

		substrate					
		$tRNA^{Gln}$		Gln-Leu tRNA precursor		$tRNA^{Leu}$	
region of cleavage		rel amt of substrate cleaved[a] (%)	termini[b]	rel amt of substrate cleaved (%)	termini	rel amt of substrate cleaved (%)	termini
leader				100	pAUAAUUGp- (<0.01) pUAAUUGp- (<0.01) pAAUUGp- (0.05) pAUUGp- (0.88) pUUGp- (0.08)		
$tRNA^{Gln}$ anticodon		100	$pGm^2AC\psi Gp$- (0.80) $pm^2AC\psi Gp$- (0.20)	100	$pGm^2AC\psi Gp$- (0.72) $pm^2AC\psi Gp$- (0.18) $pC\psi Gp$- (0.07) $p\psi Gp$- (0.02)		
interstitial region				0			
	D loop			23	$-UCAA_{OH}$ (1.0)	39	pADDGp- (0.41) pGp- (0.18) pAGp- (0.41)
	anticodon loop			69	$pA\overset{*}{A}A\psi Gp$- (0.45) $p\overset{*}{A}A\psi Gp$- (0.55)	78	$pA\overset{*}{A}A\psi Gp$- (1.0)
$tRNA^{Leu}$	variable loop			24	nd[c]	28	pGAUp- or pAUp- (1.0)
	3′ terminus			15	$-CCA_{OH}$ (0.85) $-CC_{OH}$ (0.15)	18	$-CCA_{OH}$ (0.82) $-CC_{OH}$ (0.18)

[a] The relative amount of substrate cleaved is the ratio of the percent product which terminates in a given region of the tRNA or precursor to the sum of the percent products which terminate in or continue through that region. [b] For each region of S1 cleavage, the representation of each terminus has been determined by multiplying the percentage of the product which is found in the band(s) containing the terminus by the molar yield of the terminus. These values were normalized to 1.0 for each region of cleavage. [c] nd = exact cite not determined. [d] This summary has been prepared by abstracting from Table I those oligonucleotides which occur at the termini of fragments generated by S1 cleavage. For simplicity and for theoretical reasons (see text) we have listed only the termini of the 3′ fragment of any cleavage with the exception of the cleavage of the precursor $tRNA^{Leu}$ D loop, where only the 5′ fragment (band K5) was recovered.

have been unable to determine the precise location of cleavage. The susceptibility of the 3′ CCA_{OH} terminus in both the dimer and the mature tRNA was found to be limited but equivalent; greater than 30% of all molecules retained the intact sequence.

The leader sequence of the dimer was found to be sensitive to S1 at several sites. The predominant cleavage occurred two nucleotides to the 5′ side of the site of RNase P action, which in no case was cleaved by S1. Nor was there any cleavage seen within the interstitial region of the precursor. Such a cleavage should have been easily detected, since fragments equivalent to $tRNA^{Gln}$ and $tRNA^{Leu}$ would have been generated.

Discussion

Our control experiments on the S1 sensitivity of mature tRNAs are in good agreement with the data of Harada & Dahlberg (1975), who first suggested this single-strand specific nuclease as a probe of tRNA structure in solution. Consistent with the three-dimensional crystal structure proposed for $tRNA^{Phe}$ (Kim et al., 1974; Robertus et al., 1974), we found that S1 nuclease preferentially attacks the anticodon loop. Furthermore, the enzyme shows a high preference for the phosphodiester bond between the second and third nucleotides of the anticodon, although significant cleavage is also seen one nucleotide to the 3′ side of this site. The 3′ terminus of the tRNA is also accessible to nuclease action, although we found the terminal adenosine of $tRNA^{Leu}$ to be relatively refractory.

The major difference between our results and those of Harada and Dahlberg is our finding that both the dihydrouridine and variable loops of $tRNA^{Leu}$ and of the dimeric precursor are available (20–40%) to S1 attack. Although the proposed three-dimensional model predicts that these loops

will be involved in secondary and tertiary structural interactions, it is important to note that the $tRNA^{Phe}$ on which the model is based has only a short variable loop, while the variable region of the $tRNA^{Leu}$ we have examined contains a long, base-paired stem. On the other hand, Harada and Dahlberg analyzed an analogous $tRNA^{Leu}$ from *E. coli*, also having a variable loop 15 nucleotides in length, and did not find that loop sensitive to S1 cleavage. We note an interesting correlation which may explain the apparent discrepancy in our results. The dihydrouridine loops of both the T4 and the *E. coli* $tRNA^{Leu}$ contain a 2′-*O*-methylguanosine modification. The T4 tRNA is commonly undermodified; only 20% of that material used in our study contained this modification, while the cellular tRNA in our hands is fully modified (Colby et al., 1976). The 2′-*O*-methylguanosine modification is completely absent in the dimeric precursor (Guthrie, 1975). We suggest that the absence of this modification might destabilize the secondary/tertiary interactions in which this region is involved, making it available to S1 attack. Furthermore, since the crystal structure has been interpreted as showing tertiary interactions between the dihydrouridine and variable loops, it is conceivable that the lack of modification in the dihydrouridine loop could also influence the accessibility to S1 of the variable loop.

Figure 2 depicts the major sites of S1 cleavage in the dimer. These are in most cases identical with those observed in the mature molecules. Furthermore, as shown in Table II, the extent of reactivity of each of the S1 sites in the dimer and mature tRNAs is, with one exception, comparable. These results argue that the precursor assumes secondary and tertiary conformations which are nearly equivalent to those of the cognate tRNAs and thus that the dimer can appropriately be viewed as comprising two domains within which the details of specific residue interactions must closely resemble those found in the cognate mature molecules.

We would like, however, to draw attention to the minor qualitative and quantitative differences in S1 sensitivity between precursor and mature tRNA species. In $tRNA^{Leu}$ a single site in the anticodon is susceptible to cleavage; an additional site, displaced by a single nucleotide, is available in the dimer. Two new sites also become available in the anticodon loop of the $tRNA^{Gln}$ moiety of the precursor, though only to a slight extent. These results indicate that the region 3′ to the anticodon is less involved in protective interaction in the precursor than in the mature tRNAs. We find, in contrast, that the dihydrouridine loop of the $tRNA^{Leu}$ moiety of the precursor is apparently more structured than in the mature molecule, since the extent of S1 cleavage of this loop in $tRNA^{Leu}$ is twice that seen in the dimer.

These indications of conformational differences are consistent with observations of Chang & Smith (1973) on a mutationally altered precursor of *E. coli* su^{+}_{III} $tRNA^{Tyr}$. Comparing the levels of methoxyamine modification of cytidine residues in precursor to those in mature $tRNA^{Tyr}$, they found an increase in reactivity of the anticodon loop and a decrease in that of the dihydrouridine loop. While interpretation of their results was complicated by the destabilizing effect of the mutational alteration in the dihydrouridine loop (Cashmore, 1971), it would now appear that their conclusion can be extrapolated to normal precursor structure.

What can be said about the structural features of the dimer with respect to precursor-specific regions? No detectable cleavage occurred at the site of RNase P action between the leader and the $tRNA^{Gln}$ sequence, although we did observe cleavage at several sites in the six-nucleotide leader, including at low frequency of hydrolysis a single nucleotide 5′ to the RNase P site. As pointed out by Bothwell et al. (1976), there is the opportunity for formation of a Watson–Crick base pair between the 3′ terminal U residue of the 5′ leader and the G residue at the juncture between the two tRNA moieties (see Figure 2). This is consistent with chemical modification studies (Chang & Smith, 1973) on the *E. coli* $tRNA^{Tyr}$ precursor, which have indicated the existence of several base pairs between the aminoacyl terminus and the 5′ leader of the precursor. Furthermore, we found no hydrolysis at the second RNase P cleavage site in the interstitial region joining the two tRNA sequences. Since we have detected cleavage products at the level of 2%, if such cleavage occurs it must be a rare event. These results thus suggest that the nucleotides comprising *both* RNase P cleavage sites participate in secondary and/or tertiary interactions. While available data (Shenk et al., 1975) suggest that S1 nuclease can cleave DNA heteroduplexes containing a single base mismatch, albeit at a reduced rate, comparable information on RNA is lacking. Thus we cannot rule out the possibility that under our conditions the sensitivity of S1 is insufficient to distinguish a single unpaired nucleotide between two extensively hydrogen-bonded regions. Nevertheless, the absence of S1 cleavage at either of the RNase P sites in the dimeric precursor argues that simple accessibility of these regions is not sufficient to direct processing by RNase P.

The predominant structural feature of the precursor is thus its close resemblance to mature tRNA, while closer examination reveals several subtle differences within the regions occupied by the tRNA moieties. Our ultimate desire, of course, is to inquire into the biological significance of both types of structural characteristics. The existence of conformational similarities between precursor and product would have several useful consequences. By taking advantage of the apparent universality of tRNA structure, this feature would presumably minimize the number of different components required for tRNA processing. At the same time, a mechanism for biosynthetic control of product fidelity would be generated by requiring that efficient recognition of the precursor by maturation enzymes depends upon some of the same structural properties which are required for ultimate tRNA function.

Conformational dissimilarities between precursor and mature tRNA, such as those in the dihydrouridine and anticodon regions detected in our study, may reflect subtle rearrangements of the RNA structure which influence the specificity and/or order of maturation reactions. For example, Schaefer et al. (1973) have shown that the $tRNA^{Tyr}$ moiety derived by RNase P processing of the undermodified precursor is a more efficient substrate for the modification of several residues, including a Ψ in the anticodon stem, than is the uncleaved precursor. While the T4 precursor is generally more highly modified by comparison, the 2′-*O*-methylguanosine modification is completely lacking. The implication is thus that efficient enzymatic recognition in these cases requires some conformational rearrangement in the dihydrouridine and anticodon regions of the tRNA moieties consequent to RNase P cleavage of the precursor.

The biological relevance of such conformational dissimilarities may well extend to other processing events, such as maturation of the 3′ CCA_{OH} terminus. Individual precursors differ with respect to both the number and types of reaction required and the order in which they are performed (cf. Figure 3 of Guthrie, 1975). While the Gln-Leu tRNA precursor is mature at the 3′ terminus and therefore cannot provide us with

information about this aspect of processing, a number of other precursors, immature at the 3′ terminus, are available for study. Clearly more information is needed to determine whether structural variations among tRNA precursors or between a given precursor and its cognate tRNA reflect important differences in the biosynthetic regulation of individual tRNA expression.

We are encouraged to extend the approach described in this study to an analysis of precursors to other tRNA species as well as to mutationally altered variants of the Gln-Leu tRNA dimer, which are inefficient substrates for RNase P in vivo, with the hope of obtaining more specific details as to those conformational features which are prerequisite for efficient maturation.

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