1980

The Solution Properties of Transfer Ribonucleic Acids and Their Distribution in Tumor Cells.

Kathryn Seyler Aultman

Louisiana State University and Agricultural & Mechanical College

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THE SOLUTION PROPERTIES OF TRANSFER RIBONUCLEIC ACIDS
AND THEIR DISTRIBUTION IN TUMOR CELLS

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THE SOLUTION PROPERTIES OF TRANSFER RIBONUCLEIC ACIDS
AND THEIR DISTRIBUTION IN TUMOR CELLS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Biochemistry

by

Kathryn Seyler Aultman
B.A., University of New Orleans, 1974
August 1980
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<tr>
<td>mA</td>
<td>1-methyladenosine</td>
</tr>
<tr>
<td>Cm</td>
<td>2'-O-methylcytidine</td>
</tr>
<tr>
<td>m5C</td>
<td>5-methylcytidine</td>
</tr>
<tr>
<td>D</td>
<td>Dihydrouridine</td>
</tr>
<tr>
<td>m2G</td>
<td>N2-methylguanosine</td>
</tr>
<tr>
<td>m2G</td>
<td>N2,N2-dimethylguanosine</td>
</tr>
<tr>
<td>m7G</td>
<td>7-methylguanosine</td>
</tr>
<tr>
<td>Gm</td>
<td>2'-O-methylguanosine</td>
</tr>
<tr>
<td>Q</td>
<td>Queine</td>
</tr>
<tr>
<td>T</td>
<td>Ribothymidine</td>
</tr>
<tr>
<td>ψ</td>
<td>Pseudouridine</td>
</tr>
<tr>
<td>Y</td>
<td>Wybutine</td>
</tr>
<tr>
<td>Yr</td>
<td>Wybutoxine</td>
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**Reagents**

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<tr>
<td>NTA</td>
<td>Nitrilotriacetic Acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecylsulfate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>Na2EDTA</td>
<td>Disodium (ethylenedinitrilo) tetraacetic acid</td>
</tr>
<tr>
<td>PPO</td>
<td>2,5-diphenyloxazole</td>
</tr>
<tr>
<td>POPOP</td>
<td>1,4-Bis-2-(5-phenyloxazoly1)benzene</td>
</tr>
<tr>
<td>Adogen 464</td>
<td>Methyltrialkyl (C8-C10) ammonium chloride</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
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<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid</td>
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Chromatography

DEAE

RPC-5

Other

SV40

tRNA\textsuperscript{Leu} \textsubscript{3} (N)

tRNA\textsuperscript{Leu} \textsubscript{3} (D)
eIF-2

nmr

Ci

v

nm

cpm

psi

A\textsubscript{260} unit

Diethylaminoethyl

Reversed Phase Chromatography-5

Simian virus-40

Native conformer of tRNA\textsuperscript{Leu}\textsubscript{3}

Denatured conformer of tRNA\textsuperscript{Leu}\textsubscript{3}

Eukaryotic initiation factor-2

Nuclear magnetic resonance

Curie

volts

nanometer

Counts per minute

Pounds per square inch

that amount of material which has an absorbance of 1.0 at 260 nm at 25°C in a one centimeter light path
ABSTRACT

Partial digestion of native tRNA molecules with P₄ nuclease under conditions which take advantage of the differences in kinetic parameters of this enzyme for various substrates has been performed. Under these conditions the two conformers of yeast tRNA₃^Leu give different digestion patterns, indicating that P₄ nuclease is sensitive to structural features of its substrate. Comparison of the digestion pattern of tRNA₃^Phe from yeast with its known three-dimensional structure shows that stem and loop regions can be distinguished and that twelve of the twenty residues involved in tertiary hydrogen bonding interactions can be detected.

Extrapolation of the tertiary structure of tRNA₃^Phe to yeast tRNA₃^Leu(N) is borne out by the digestion patterns of the D-arm, T-arm and anticodon loop, but results for the variable arm are difficult to interpret. The digestion pattern of yeast tRNA₃^Leu(D) is compatible with models of its secondary structure.

Populations of transfer RNA from human placenta and leiomyoma were compared by reversed-phase chromatography (RPC-5). The isoacceptors of tRNA₃^Phe, tRNA₃^Ser, tRNA₃^Asp and tRNA₃^Tyr included extra species in the tumor tRNA population. Quantitative differences in the isoacceptors of tRNA₃^Lys were observed between the two populations.
These data are interpreted in terms of the theories about regulation of gene expression at the level of translation by transfer RNAs.
Chapter I

PARTIAL P₁ NUCLEASE DIGESTION AS A PROBE OF tRNA STRUCTURE
INTRODUCTION

Transfer RNA was first discovered in 1957 (1) as a fraction of soluble RNA which could accept radioactively labeled amino acids in the presence of a "pH 5 enzyme" preparation and transfer those amino acids to proteins during translation. In a normal cell there are many species of tRNA molecules: one or more for each amino acid with a separate set for mitochondria (2). Transfer RNAs evolved early as the adaptors between nucleic acid and protein sequences, and their sequences have been highly conserved (3). These molecules are now known to have a wide variety of roles which range from ribosome-mediated protein synthesis (4) to attenuation of transcription (5-7). They have been implicated in a variety of regulatory processes.

Structure of tRNA

The first tRNA sequence was elucidated by Holley (9) in 1965. Since that time vast improvements in the technology of RNA sequencing (10-12) have allowed the determination of over 200 sequences (13). Several general features of tRNA structure have emerged (Figure 1; see reference 14 for a review.). First the sequence of tRNA can be folded into a cloverleaf structure which possesses five arms. Second, the acceptor stem and anticodon and T arms are of constant length, having seven base pairs and five base pairs with seven residues in the loops, respectively. Third, in almost all of the sequences which are known at this time there are fifteen invariant residues, four semivariant
Figure 1. Generalized cloverleaf structure of tRNA (except initiator tRNAs). Invariant and semivariant bases are shown: R stands for purines and Y stands for pyrimidines. This Y is distinct from the hypermodified residue Y (wye) of phenylalanine tRNAs. H stands for a hypermodified purine. The dotted regions, α and β of the D loop and in the variable loop contain different numbers of nucleotides in different sequences.
and four correlated semivariant residues. Semivariant residues are restricted to being either a purine or a pyrimidine; correlated semivariants are pairs of one purine and one pyrimidine which are further restricted in that they must be able to form Watson-Crick base pairs with one another. Fourth, transfer RNA molecules have a high proportion of modified nucleosides. There are exceptions to these rules, of course, which include, but are by no means limited to the specialized tRNA\textsuperscript{Gly} which is used for bacterial cell wall synthesis (15) and various initiator and tumor tRNAs (14).

The aspects of tRNA structure which vary are the number of nucleotides in the α and β regions of the D loop, the number of base pairs in the D stem and the number of residues in the variable arm. Of course the identities of the bases at the variable positions of the tRNA are unique for each tRNA.

Two classification schemes have been proposed for grouping the tRNAs according to structure (16, 17). They are based on the number of base pairs in the D stem (D\textsubscript{3} or D\textsubscript{4}) and the number of nucleotides in the variable arm (V\textsubscript{4-5} or V\textsubscript{n}). A set of rules for numbering the residues so that direct comparisons among the tRNA sequences can be made was proposed at the tRNA meeting at Cold Spring Harbor in 1978. Figure 2 shows that system (13).

**Yeast Phenylalanine tRNA**

Yeast tRNA\textsuperscript{Phe} was sequenced by RajBhandary et al. (18). It is a D\textsubscript{4}V\textsubscript{4-5} tRNA with seventy-six nucleotides (Figure 3). Its three dimensional structure has been resolved to 2.5 Å (19-21). Figure 4 shows a flattened, L-shaped molecule whose acceptor and T stems make up the horizontal limb while the anticodon and D stems
Figure 2. The numbering system of Gauss et al. (13). This numbering system follows the rules proposed at the 1978 tRNA meeting in Cold Spring Harbor. It is designed to allow direct comparison with tRNAPhe, whose three-dimensional structure is known.

Heavy circles represent invariant or semivariant residues; light circles represent variable residues. Ovals stand for residues which do not appear in every tRNA sequence. In tRNAs with only four residues in the variable loop, for example, the number 47 is omitted.
Figure 3: Cloverleaf structure of yeast tRNA\textsuperscript{Phe} (18).
Figure 4. Tertiary structure of yeast tRNA^Phe (19-21).

This drawing was adapted from slides supplied by Dr. Kim.
form the vertical member. The greatest contribution to the stability of this conformation comes from the extensive base stacking. The acceptor and T stems make an almost continuous and colinear helix; the bases of the anticodon and D stems are stacked (14). There are ten tertiary interactions which also stabilize this conformation (Table 1 and Figure 5): they involve hydrogen bonds between the bases themselves, between the bases and the sugar residues and between bases and phosphate groups. Only one of these ten, C15-C48, is a normal Watson-Crick base pair. All of these interactions involve invariant or semivariant residues of the tRNA sequence, so it is generally assumed that the structure of tRNA_Phe can be extrapolated to other sequences. The D and variable loops, where variations in chain length are possible among the different tRNAs are in regions of the structure which can "bulge out," so little strain would be introduced by increases in the number of residues. It is important to note that the T loop and variable loop are extensively involved in these tertiary interactions; the anticodon loop is highly exposed and contains a sharp bend which is stabilized by the interaction between Cm32 and A38. In the D loop both G18 and G19 are involved in tertiary hydrogen bonds, but G20 is not.

Various chemical modification studies have been carried out to probe the structure of tRNA_Phe (22-30). In general they have shown the following residues to be highly reactive: D16, D17, G20, G34, A35, U37, C74, C75, and A76. The N1 position of A38 is exposed, but N6 (which is involved in the interaction with Cm32) is inaccessible to the reagents. Cm32 and U33 are only partially reactive, G18, G19, G45, G57 and the rest of the T loop are all unreactive. These findings
### TABLE I

**Bases Involved in Tertiary Hydrogen Bonding**

**Interactions in tRNA\[^{Phe}\] (14)**

<table>
<thead>
<tr>
<th>Segment</th>
<th>Base-base Interaction</th>
<th>Base-backbone Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D Stem</strong></td>
<td>( m^2 G_{10} ) ((G_{45}))</td>
<td>( C_{11} ) ((A_9))</td>
</tr>
<tr>
<td></td>
<td>( G_{22} ) ((m^2 G_{10}))</td>
<td>( G_{18} ) ((m^A_{58}))</td>
</tr>
<tr>
<td></td>
<td>( A_{23} ) ((A_9))</td>
<td>( A_{21} ) ((U_8))</td>
</tr>
<tr>
<td><strong>D Loop</strong></td>
<td>( A_{14} ) ((U_8))</td>
<td>( A_{36} ) ((U_{33}))</td>
</tr>
<tr>
<td></td>
<td>( G_{15} ) ((C_{48}))</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( G_{18} ) ((C_{56}))</td>
<td></td>
</tr>
<tr>
<td><strong>Anticodon Loop</strong></td>
<td>( C_{m32} ) ((A_{38}))</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( U_{33} ) ((A_{36}))</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( A_{38} ) ((C_{m32}))</td>
<td></td>
</tr>
<tr>
<td><strong>Variable Loop</strong></td>
<td>( A_{44} ) ((m^2 G_{26}))</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( G_{45} ) ((m^2 G_{10}))</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( m^7 G_{46} ) ((G_{22}))</td>
<td></td>
</tr>
<tr>
<td><strong>T Loop</strong></td>
<td>( T_{54} ) ((m^A_{58}))</td>
<td>( \psi_{55} ) ((m^A_{58}))</td>
</tr>
<tr>
<td></td>
<td>( \psi_{55} ) ((G_{18}))</td>
<td>( G_{57} ) ((T_{54}, G_{18}, G_{19}))</td>
</tr>
<tr>
<td></td>
<td>( C_{56} ) ((G_{19}))</td>
<td>( C_{60} ) ((C_{61}))</td>
</tr>
<tr>
<td></td>
<td>( m^A_{58} ) ((T_{54}))</td>
<td></td>
</tr>
<tr>
<td>&quot;Bridges&quot;</td>
<td>( U_8 ) ((A_{14}))</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( A_{9} ) ((A_{23}))</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( m^2 G_{26} ) ((A_{44}))</td>
<td></td>
</tr>
<tr>
<td></td>
<td>( C_{48} ) ((G_{15}))</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5. Tertiary interactions in yeast tRNA$^{\text{Phe}}$. Adapted from a drawing in (14).
bear out the three-dimensional structure suggested by the X-ray
diffraction studies.

**Yeast tRNA$^{\text{Leu}}_3$**

Yeast tRNA$^{\text{Leu}}_3$ was sequenced independently in two different lab-
oratories (31,32) (Figure 6). It is one of several tRNAs which
possess a stable denatured conformation (33). Various circular
dichroism (34, 35) and high resolution nmr (36) studies have shown
that the two conformers have different numbers of base pairs:
tRNA$^{\text{Leu}}_3$ (N) has 21 ± 2 while tRNA$^{\text{Leu}}_3$ (D) has only 18 ± 2. On the
basis of computer assisted analysis of the nmr spectra a model for
the secondary structure of the denatured conformer was proposed
(Figure 7) (36). The model predicts that the acceptor, variable and
T stems are all retained but that the D and anticodon stems are all
disrupted. In their place is a new helix between the bases $G_{30}^{5}C_{34}^{5}$
and $G_{66}^{5}C_{70}^{5}$, the bases of the T and anticodon loops. Chemical
modification studies (37, 38) support this model, showing that the
bases of the D stem and anticodon stem are more reactive and those
of the anticodon loop less reactive than the corresponding residues
of the native conformer.

**Partial Nuclease Digestion as a Structural Probe**

The accessibility of the various regions of a tRNA molecule
to nuclease has been used as an indicator of overall tRNA structure.
Partial $T_1$ ribonuclease digestion of tRNA$^{\text{Ala}}$ was performed by Holley
in 1965 (39). Since that time at least twelve tRNAs have been
examined by this technique (see reference 14 for a review), including
yeast tRNA$^{\text{Ala}}$, tRNA$^{\text{Ser}}$, tRNA$^{\text{Phe}}$, tRNA$^{\text{Met}}_f$, tRNA$^{\text{Met}}_m$, tRNA$^{\text{Val}}$ and tRNA$^{\text{Tyr}}$,
E. coli tRNA$^{\text{Glu}}$, tRNA$^{\text{Met}}_m$, tRNA$^{\text{Trp}}_m$ and tRNA$^{\text{Tyr}}$, and mammalian
Figure 6. Sequence and cloverleaf structure of yeast $tRNA_{3}^{Leu}$ (31, 32).
Figure 7: Secondary structure of yeast tRNA$^\text{Leu}_3$ (D) (36).
tRNA$^{\text{Met}}_f$. Various enzymes have been employed. Ribonucleases A, T$^1$ and T$^2$ (cleavage only after certain bases) and S$^1$ nucleases (single-strand specific) (40) have all been applied to the problem.

In general these studies have shown that 1) stem regions of the cloverleaf are generally resistant to nucleolytic attack, 2) the anticodon loop and CCA terminus are highly susceptible to digestion, 3) the T and variable loops are, for the most part resistant, and 4) the variable ($\alpha$ and $\beta$) regions of the D loop are accessible to nucleases while the constant ones are protected.

These procedures have produced somewhat limited information about the overall structure of the tRNAs because they involve cleavage at so few positions and because the relative susceptibilities of residues which are attacked by different nucleases cannot be compared. Digestions must be of a very limited nature because cleavage of more than one position in any single tRNA molecule may alter the conformation of the molecule. This was shown for tRNA$^{\text{Phe}}$ when scission of the D loop at a single position altered its $K_m$ for the cognate aminoacyl-tRNA synthetase (41). In order to ensure that the digestion pattern be reflective of the native conformation of the tRNA Wurst et al. (40) have used conditions in which no more than 3% of the tRNA molecules are cut.

It would also be useful to be able to perform kinetic studies of the course of nuclease digestion (42). This would allow determination of the order of cleavage at the various sites, thus providing information about subtle differences in the accessibility of the residues. This has been difficult to achieve because of the harsh conditions needed to inactivate most of the nucleases used. Boiling,
phenol extraction, and treatment with chelating agents or alkylating agents can present a problem to recovery of tRNA fragments and to obtaining very early time aliquots. Rich's group have recently shown that $S_1$ nuclease can be inhibited with ATP, so for this enzyme at least, kinetic studies may soon be possible (42).

$P_1$ Nuclease

$P_1$ nuclease was isolated from *Penicillium citrinum* by Fujimoto et al. (43). It is a zinc-containing enzyme (44) which possesses both phosphomonomesterase and phosphodiesterase activities (45). It catalyzes the removal of the phosphate group from 3'-mononucleotides and cleaves polynucleotides in such a way that the fragments possess 3'-hydroxyls and 5'-phosphates. It is active against both RNA and heat-denatured DNA strands, but is essentially inactive against duplex molecules (46); it will dephosphorylate 3'-mononucleotides but not 2'- or 5'-nucleotides. It will eventually cleave almost any position of a polynucleotide chain.

In spite of this lack of absolute base specificity $P_1$ nuclease acts on different substrates or at different positions of a polynucleotide chain with different rates (46). The pH optima for various synthetic homopolymers and 3'-nucleotides vary from 4.3 to 8.5; for intact tRNA and DNA chains the optimum pH is 5.3. Values for $V_{\text{max}}$ were obtained for the natural and synthetic substrates which were hydrolyzed at their individual pH optima and ranged from 14 (poly G) to 1440 (3'-GMP) μmoles/min./mg protein at 37°C. The $K_m$ values for all of these substrates were obtained during digestion at pH 5.3 and ranged from $7 \times 10^{-5}$ (3'-AMP) to $6 \times 10^{-4}$ M (3'-dTMP). Besides these differences in kinetic parameters the various substrates
are affected differently by changes in the ionic strength.

P₄ nuclease has been extensively used in the sequence analysis of tRNAs (11, 47). This process involves digestion of 5'-³²P-labeled tRNAs or their fragments in such a way that every position is cleaved. Time aliquots are removed from the reaction mixture and recombined in such a way that the fragments will be present in nearly equimolar amounts. The pooled digestion products are subjected to homochromatography and the sequence is deduced. The nuclease has also been used in 5'-end analysis of tRNAs (48). These digestions involve large amounts of nuclease for extended incubations under optimal conditions for the enzyme. This minimizes the effects of differences in kinetic parameters for different substrates.

Rationale for this Study

The rationale for this study is based on the fact that P₄ nuclease does exhibit differences in the rate of cleavage at various positions in a polynucleotide chain. The largest difference in synthetic substrates is between single and double stranded molecules (46); the magnitude of the differences among specific bases varies with pH and ionic strength and may be minimized.

Cleavage of native tRNAs under conditions which take advantage of these differences in cleavage site preference should allow visualization of all of the positions in the tRNA chain. These conditions are a high enzyme:substrate ratio and a very short incubation time. Under these circumstances the frequency with which cleavage takes place at any particular site will reflect the structure of the tRNA at that position. The restriction that only a very small proportion of the total number of tRNA molecules
be cleaved can also be met, assuring that, on the average, any tRNA molecule will be cleaved only once. This, along with the fact that the digestion will be carried out at a neutral pH, will help to ensure that the digestion pattern will reflect the native conformation of the tRNA.

Hydrolysis products can be resolved on polyacrylamide gels according to chain length. Since the tRNAs are labeled at their 5'-ends prior to nuclease treatment all fragments of the same chain length will have the same primary sequence. They can be identified by electrophoresis in parallel with tRNAs which have been digested with $T_1$ ribonuclease (which cleaves after guanidine residues) or dilute NaOH. The radioactive fragments are visualized by autoradiography and the band intensities on the film will be proportional to the number of fragments of each chain length. The proportion of total fragments which is included in any particular band will reflect the relative preference of $P_1$ nuclease for cleavage at that position.

Since yeast tRNA$^{\text{Leu}}_3$ has two stable conformations at 28°C partial digestion of the two conformers followed by electrophoresis to resolve the fragments should give different digestion patterns if the nuclease is sensitive to differences in structure. The three-dimensional structure of tRNA$^{\text{Phe}}_3$ is known and can be used to compare the cleavage pattern predicted from the kinetic parameters to the one which is actually obtained. Finally, if the predictions about the digestion profile of tRNA$^{\text{Phe}}_3$ are borne out some inference may be made about the structure of the denatured conformer of tRNA$^{\text{Leu}}_3$. 
MATERIALS AND METHODS

Materials

P₁ nuclease was purchased from Sigma Chemical Company and had a specific activity of 400 U/mg. Polynucleotide kinase (E.C. 3.1.4.22) was obtained through P/L Biochemicals; bacterial alkaline phosphatase (E.C. 3.1.3.1.) came from Boehringer-Mannheim and T₁ ribonuclease (E.C. 3.1.4.8.) was from Sankyo.

Yeast tRNA^Phe was purchased from Boehringer-Mannheim. [γ-³²P]-ATP (>3000 Ci/mmole) was obtained from ICN. Ultrapure reagents for gel preparation (acrylamide, methylene bisacrylamide, N,N,N',N'-tetramethylenediamine and ammonium persulfate) were purchased from BioRad. Nitrilotriacetic acid (NTA) was gold label grade and was purchased from Aldrich Chemical Company. Kodak XR-5 x-ray film and Cronex "Lightning Plus" intensifying screens were purchased locally through Evangeline X-Ray, Inc. A previously purified preparation of yeast tRNA^Leu was used (49).

Methods

Labeling of tRNAs

Labeling of intact tRNAs at their 5'-ends involved 1) removal of the 5'-terminal phosphates with bacterial alkaline phosphatase in the presence of 0.1% SDS as described by Shinagawa and Padmanabhan (50), 2) phosphorylation with [γ-³²P]-ATP by polynucleotide kinase in the presence of 40 mM TrisCl pH 8.5, 10 mM MgCl₂ and 8 mM DTT. Polynucleotide kinase was present as 3.5 U for every 6 ug of tRNA (11). Incubation was at 37°C for 30 min., after which the material was frozen in powdered dry ice, lyophilized to dryness and resuspended in a mixture of 8M urea, 0.5% bromophenol blue and 0.5% xylene cyanol
blue \{loading dye (9) \).

Purification of Labeled tRNA

The 5'-end labeled tRNA was separated from the remaining radioactive ATP by electrophoresis on a 20 x 20 x 0.3 cm polyacrylamide slab gel (20% acrylamide with 3.3% crosslinking, 7M urea, 50 mM Tris-borate pH 8.3 and 1 mM Na$_2$EDTA) at 300 volts for fifteen hours at room temperature (51). The band of intact tRNA was located by autoradiography using 2-3 minutes exposure at room temperature. The band was cut out of the gel.

Labeled tRNA was eluted from the gel by the method of Knecht and Busch (52). In this process tube gels were prepared in shortened Pasteur pipettes. The band from the slab gel which contained the labeled tRNA was placed on top of the 5% acrylamide in the tubes, along with 1 A$_{260}$ unit of unlabeled crude yeast tRNA. A tied off dialysis bag filled with running buffer (50 mM Tris-acetate pH 8.0) was placed over the end of the pipette and the tube gel was electrophoresed for several hours at 100 volts and 4°C until all of the radioactivity was in the dialysis bag. Migration of the radioactively labeled tRNA was followed with a survey meter. After electrophoresis the dialysis bag was removed and tied off. The tRNA was exhaustively dialyzed against 1 mM Na$_2$EDTA followed by dialysis against three changes of distilled water. It was then lyophilized to dryness, resuspended in 100 ul of distilled water and stored at -20°C.

Denaturation of Labeled tRNA.

The tRNA was denatured just before being used by heating in a 60°C water bath for five minutes in the presence of 1 mM Na$_2$EDTA.
and then plunging the tube immediately into ice. The native

conformers were also heated to 60°C for five minutes but in the presence

of 10 mM MgCl₂; they were slowly cooled to room temperature. Under

these conditions tRNA<sub>Leu</sub><sup>Λ1</sup> assumes its two stable conformations (53).

**T₁ Ribonuclease Digestion.**

Digestion of tRNAs with T₁ ribonuclease was according to (11).

<sup>32</sup>P-labeled tRNA (2 x 10⁴ Cerenkov cpm) was combined with an amount

of unlabeled crude yeast tRNA sufficient to bring the total to 10 μg,

and lyophilized to dryness. It was resuspended in 10 μl of a
cocktail which contained 20 mM NaCitrate pH 5.0, 1 mM Na₂EDTA, 0.4% each xylene cyanol blue and bromophenol blue dyes, 7 M urea and

6.8 A<sub>260</sub> units/ml of crude unlabeled tRNA. The reaction mixture was

placed in a boiling water bath for 60 seconds and then cooled rapidly

in dry ice. It was then incubated with either 0.075 or 0.150 U of

T₁ ribonuclease for fifteen minutes at 55°C. The digests were loaded

onto the gels immediately.

**Partial NaOH Digestion**

Partial NaOH digestions were performed according to the procedure

of Wrede and Rich (42). Labeled tRNA (2 x 10⁴ Cerenkov cpm) was com­

bined with unlabeled crude yeast tRNA to give a final concentration

of 10 A<sub>260</sub> units/ml. The solution was made 50 mM in NaOH, immersed

in a boiling water bath for 20 or 40 seconds, chilled and neutralized

with an equal volume of ice cold 50 mM HCl, frozen and lyophilized.

It was resuspended in 5 μl of loading dye.

**Partial P₁ Nuclease Digestion.**

Partial P₁ nuclease digestions were carried out in a 10 or 20 μl
reaction mixture which contained the following: 50 mM potassium
caccodylate pH 7.0, 10 mM MgCl₂, 2 x 10⁴ cpm of labeled tRNA and an
amount of crude unlabeled tRNA sufficient to give a final concentration of 10 A_{260} units/ml. Incubations were at 28°C for the times and with the amounts of P_1 nuclease shown in the legends to the figures.

The reaction was stopped by the addition of one volume each of 50 mM NTA and 50 mM Na_2EDTA, followed by heating in a boiling water bath for sixty seconds, immediate freezing in powdered dry ice, lyophilization to dryness and resuspension in 5 ul of loading dye.

**Gel Electrophoresis.**

5'-$^{32}$P-labeled fragments were separated according to chain length by electrophoresis on a 30 x 55 x 0.03 cm polyacrylamide slab gel (54). The gels were poured in two segments, a 2-4 cm "plug" and then the body of the gel. The gels were 20% acrylamide with 3.3% crosslinking in 7 M urea, 50 mM Tris-borate pH 8.3, and 1 mM Na_2EDTA. The running buffer contained no urea. Two gels were run for each digest, one for approximately 21 hours or until the fast moving dye had migrated 45 cm down the gel and the other for 42 hours or until the slow moving dye had traveled the same distance. Electrophoresis was at 1000 volts, 7-9 mA/gel (constant voltage) and room temperature. This allowed resolution of sixty fragments in the digest.

**Autoradiography and Densitometry.**

The fragments were visualized by autoradiography with Kodak XR-5 X-ray film using Cronex Lightning Plus intensifying screens at -70°C in a dry ice chest (11). The intensity of the bands was determined by scanning the films with a Helena Quick Scan densitometer. Data from the two gels of each digest were normalized by comparing the intensities of the bands they had in common. Data were expressed as the percentage of the total readable band intensity found in each fragment.
RESULTS

General Comments

Parallel electrophoresis of $P_1$, $T_1$ and NaOH digests allows the identification of the fragments in the gels. Because fragments produced by $P_1$ nuclease have 3'-terminal hydroxyls while those from $T_1$ or NaOH digestion have 3'-terminal phosphates, $P_1$ fragments migrate more slowly on the gels. Their positions are staggered with respect to those of the $T_1$ and NaOH fragments (42).

Sensitivity of $P_1$ Nuclease to Structural Features

Comparison of the digestion patterns in Figures 8 and 9 shows that $P_1$ nuclease is sensitive to differences in tRNA structure. In Figure 9 the digestion patterns of the two conformers of yeast tRNA$^{\text{Leu}}_{3}$ are clearly different. The fragments produced by cleavage in the D loop are much more intense in the autoradiogram of the denatured conformer than in that of the native one. Conversely the anticodon fragments are more abundant in the native than in the denatured lanes. In contrast, when tRNA$^{\text{Phe}}$ is treated with the same conditions which produced the two conformations of tRNA$^{\text{Leu}}_{3}$, the only difference in the digestion patterns is the overall extent of digestion. More of the tRNA molecules are hydrolyzed in the "tRNA$^{\text{Phe}}$ (D)" than in the "tRNA$^{\text{Phe}}$ (N)" reaction mixtures, but the relative band intensities within any lane of the autoradiogram remain the same. This is in
Figure 8. $P_n$ nuclease digestion pattern of yeast tRNA$^{Phe}$.

Yeast tRNA$^{Phe}$ was denatured and renatured (33) and then incubated with 200 ng $P_n$ nuclease per $A_{260}$ unit of tRNA at 28°C. The digests were electrophoresed at 1000 v for 18 (A) or 42 (B) hours.

Lanes 1-3: "Native tRNA" was incubated for 5, 10, and 16 minutes respectively with the $P_n$ nuclease.

Lanes 4-6: "tRNA$^{Phe}$ (D)" was incubated for 5, 10, and 15 minutes respectively with $P_n$ nuclease.

Lane 7: Partial NaOH digestion.

Lane 8: Partial $T_1$ ribonuclease digestion.

Lane 9: No enzyme control.

Dotted circles refer to the positions of the marker dyes.
Figure 9. Partial P₁ nuclease digestion pattern of tRNA₃^{Leu}.

Yeast tRNA₃^{Leu} was incubated with 50 ng P₁ nuclease per A₂₆₀ unit tRNA after being converted to the two conformers described by Lindahl (31). The digests were electrophoresed for 18 (A) or 42 (B) hours at 1000 v.

Lanes 1: No enzyme control for tRNA₃^{Leu} (N).

Lanes 2-4: The native conformer was incubated with 50 ng nuclease per A₂₆₀ unit of tRNA for 1, 2, and 5 minutes respectively.

Lanes 5-7: The native conformer was incubated with 100 ng nuclease per A₂₆₀ unit of tRNA for 1, 2, and 5 minutes respectively.

Lanes 7 & 8: Partial NaOH digestion of the tRNA for 20 and 40 seconds respectively.

Lanes 9 & 10: Partial T₁ nuclease digestion of the tRNA with 0.075 and 0.150 U of nuclease respectively.

Lanes 11-13: As lanes 2-4 above except that the denatured conformer was used.

Lanes 14-17: As lanes 5-7 above except that the denatured conformer was used.

Lane 18: No enzyme control for the denatured conformer.

Dotted circles are the positions of the dye markers.
agreement with the observation that yeast tRNA$^{\text{Phe}}$ has only one
stable conformation (33) and serves as a control to rule out the
possibility that the differences in the digestion patterns of the
two conformers of tRNA$^{\text{Leu}}_2$ are due to methodological artifacts.

Figure 10 shows a representative densitometer tracing of a
portion of the gel shown in Figure 8. The data from these tracings
(Table II and Table III) were used to construct the histograms shown in
Figures 11 and 12. Figure 11 shows the average band intensities
(as a percent of the total) for different groups of fragments. Bars
a through d represent fragments whose 3'-terminal residues are A, G,
U or C respectively. When this average value is multiplied by the
number of fragments whose 3'-termini are A, G, U or C and the four
multiplication products added together, the total is 100%. Those
labeled e through h show the relationship between stem and loop
residues. Finally, bars i through q show the relative cleavage of
each of the different segments of the tRNA chain, e.g. the acceptor
stem (i) or the anticodon loop (m).

In Figure 12 the band intensities of each fragment are
represented as a percentage of the total band intensity in the
readable portion of the films. The fragments are labeled according
to their 3'-terminal nucleotides, which are numbered according to the
system of Gauss (13) and aligned so that direct comparisons can be
made among the different digestion patterns.
Figure 10. Representative densitometer tracing of a portion of the gel in Figure 8.
# TABLE II

Average Percentages of the Total Band Intensity for Fragment Groups in Yeast tRNA^Phe

<table>
<thead>
<tr>
<th>3'-Termini</th>
<th>Average % Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, mA</td>
<td>2.49</td>
</tr>
<tr>
<td>G, modified G, Y</td>
<td>1.78</td>
</tr>
<tr>
<td>U, D, T,</td>
<td>1.34</td>
</tr>
<tr>
<td>C, m^5°C, ac^4°C</td>
<td>0.95</td>
</tr>
<tr>
<td>Loop Purines</td>
<td>2.80</td>
</tr>
<tr>
<td>Stem Purines</td>
<td>1.80</td>
</tr>
<tr>
<td>Loop Pyrimidines</td>
<td>1.40</td>
</tr>
<tr>
<td>Stem Pyrimidines</td>
<td>0.90</td>
</tr>
<tr>
<td>Acceptor Stem</td>
<td>2.98</td>
</tr>
<tr>
<td>D Stem</td>
<td>0.64</td>
</tr>
<tr>
<td>D Loop</td>
<td>1.11</td>
</tr>
<tr>
<td>Anticodon Stem</td>
<td>0.57</td>
</tr>
<tr>
<td>Anticodon Loop</td>
<td>0.79</td>
</tr>
<tr>
<td>Variable Loop</td>
<td>0.34</td>
</tr>
<tr>
<td>T Stem</td>
<td>0.24</td>
</tr>
<tr>
<td>T Loop</td>
<td>0.89</td>
</tr>
<tr>
<td>&quot;Bridges&quot;</td>
<td>1.27</td>
</tr>
</tbody>
</table>
Figure 11. The average percent band intensity for the following groups of fragments:

a. Fragments whose 3'-termini are A or m^1A.
b. Fragments whose 3'-termini are G, modified G or Y.
c. Fragments whose 3'-termini are U, D, T, or T.
d. Fragments whose 3'-termini are C, m^5C, or ac^4C.
e. Fragments whose 3'-termini are loop purines.
f. Fragments whose 3'-termini are stem purines.
g. Fragments whose 3'-termini are loop pyrimidines.
h. Fragments whose 3'-termini are stem pyrimidines.
i. Fragments from the Acceptor stem.

j. Fragments from the D stem.
k. Fragments from the D loop.
l. Fragments from the anticodon stem.
m. Fragments from the anticodon loop.
n. Fragments from the variable loop.
o. Fragments from the T stem.
p. Fragments from the T loop.
q. Fragments from the "bridges."
TABLE III

Percent of the Total Band Intensity in Each Fragment

<table>
<thead>
<tr>
<th>Residue</th>
<th>% of Total Intensity</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe</td>
<td>Leu(N)</td>
<td>Leu(D)</td>
<td>Phe</td>
<td>Leu(N)</td>
</tr>
<tr>
<td>1</td>
<td>7.2</td>
<td>1.2</td>
<td>6.4</td>
<td>34</td>
</tr>
<tr>
<td>2</td>
<td>4.3</td>
<td>11.1</td>
<td>4.0</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>4.3</td>
<td>-0-</td>
<td>0.7</td>
<td>36</td>
</tr>
<tr>
<td>4</td>
<td>2.4</td>
<td>2.4</td>
<td>0.7</td>
<td>37</td>
</tr>
<tr>
<td>5</td>
<td>8.2</td>
<td>4.3</td>
<td>0.7</td>
<td>38</td>
</tr>
<tr>
<td>6</td>
<td>8.0</td>
<td>1.5</td>
<td>1.9</td>
<td>39</td>
</tr>
<tr>
<td>7</td>
<td>2.5</td>
<td>1.6</td>
<td>1.7</td>
<td>40</td>
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TABLE III (continued)

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<thead>
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<th>% of Total Intensity</th>
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<td>Leu(N)</td>
<td>Leu(D)</td>
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<td>17</td>
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<td>---</td>
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<tr>
<td>18</td>
<td>2.6</td>
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<td>-0-</td>
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<tr>
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<td>3.3</td>
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</tr>
<tr>
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</tr>
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<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
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<td>0.2</td>
<td>-0-</td>
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<tr>
<td>33</td>
<td>0.04</td>
<td>0.4</td>
<td>0.7</td>
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Figure 12. Percent of Total Readable Band Intensity in Each Fragment.

A. Digestion Pattern of $tRNA_{\text{Phe}}$

B. Digestion Pattern of $tRNA_{\text{Leu}}^N$

C. Digestion Pattern of $tRNA_{\text{Leu}}^D$
Partial P₁ Nuclease Digestion of tRNA\textsuperscript{Phe}

Figure 11 and Part A of Figure 12 show the partial P₁ nuclease digestion profile of yeast tRNA\textsuperscript{Phe}. It can be seen that the cleavage on the 3'-side of G and A residues took place preferentially to the corresponding event after pyrimidines. The nucleotides D, Cm and Gm are only weakly hydrolyzed by the enzyme. Within the context of nucleotide preference, however, the fragments produced by cleavage in loop regions are more abundant than those from the stem regions. Nevertheless there are exceptions to this stem/loop rule. There are loop fragments which seem to be relatively scarce, and stem fragments which seem to be anomalously over-represented in the profile. They are those with 3'-terminal residues G₁ through U₇ (the acceptor stem) and A₃₁, which are overabundant, and G₁₅, A₂₁, m₂G₂₆, U₃₃, A₃₈, A₄₄, G₄₅, m₇G₄₆, U₄₇, T₅₄, ₅₅, C₅₆, G₅₇, and C₆₀, which are all too scarce (Figure 12 and Table IV).

Partial P₁ Nuclease Digestion of Yeast tRNA\textsuperscript{Leu}

The digestion pattern of tRNA\textsuperscript{Leu} (N) is shown in Part B of Figure 12. Like its phenylalanine-accepting counterpart it is cleaved at purines in preference to pyrimidines; loop regions are digested by the nuclease more frequently than stem regions. Also similar to the tRNA\textsuperscript{Phe} pattern is the existence of anomalously light or dark bands in the autoradiogram (Table V). Unlike the digestion profile of tRNA\textsuperscript{Phe}, the pattern for tRNA\textsuperscript{Leu} (N) shows relatively good digestion of the D stem and poorer cleavage of the anticodon and T loops.
### TABLE IV

Anomalous Fragments in the Profile of tRNA\textsuperscript{Phe}

<table>
<thead>
<tr>
<th>Under-represented Loop Residues</th>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>D Loop</strong></td>
<td></td>
</tr>
<tr>
<td>[G\textsubscript{15}, D\textsubscript{16}, D\textsubscript{17}, A\textsubscript{21}]</td>
<td></td>
</tr>
<tr>
<td><strong>Ant. Loop</strong></td>
<td></td>
</tr>
<tr>
<td>[Cm\textsubscript{32}, U\textsubscript{33}, Cm\textsubscript{34}, A\textsubscript{38}]</td>
<td></td>
</tr>
<tr>
<td><strong>Var. Loop</strong></td>
<td></td>
</tr>
<tr>
<td>[A\textsubscript{44}, G\textsubscript{45}, \textsuperscript{7}G\textsubscript{46}, U\textsubscript{47}]</td>
<td></td>
</tr>
<tr>
<td><strong>T. Loop</strong></td>
<td></td>
</tr>
<tr>
<td>[T\textsubscript{54}, \textsuperscript{5}U\textsubscript{55}, C\textsubscript{56}, G\textsubscript{57}]</td>
<td></td>
</tr>
<tr>
<td><strong>&quot;Bridges&quot;</strong></td>
<td></td>
</tr>
<tr>
<td>[\textsuperscript{2}m\textsubscript{2}G\textsubscript{26}, C\textsubscript{48}]</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Over-represented Stem Residues</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acc. Stem</strong></td>
<td>All residues</td>
</tr>
<tr>
<td><strong>Ant. Stem</strong></td>
<td>A\textsubscript{31}</td>
</tr>
</tbody>
</table>

*Accounted for by tertiary interactions

+Accounted for by base specificity of P\textsubscript{1} nuclease
TABLE V

Anomalous Fragments in the Profile of \textit{tRNA}^{\text{Leu(N)}}\textsuperscript{3}

<table>
<thead>
<tr>
<th>Under-represented Loop Residues</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>D Loop</td>
<td>D_{20}^{+}, D_{20:2}^{+}, Gm_{18}^{+}</td>
<td></td>
</tr>
<tr>
<td>Ant. Loop</td>
<td>C_{38}^{*}, mG_{37}</td>
<td></td>
</tr>
<tr>
<td>Var. Loop</td>
<td>A_{47:4}^{*}</td>
<td></td>
</tr>
<tr>
<td>T Loop</td>
<td>T_{54}, \psi_{55}, C_{56}, G_{57}, A_{58}, A_{59}</td>
<td></td>
</tr>
</tbody>
</table>

| Over-represented stem residues |  |  |
| Acc. Stem                      | G_{2}, G_{3} |   |
| Ant. Stem                      | A_{31} |   |

*Accounted for by tertiary interactions.

+Accounted for by the base specificity of P_1 nuclease.
The digestion pattern for tRNA\textsubscript{\text{Leu}}\textsuperscript{3}(D) contrasts markedly with that of the native conformer. Although the same general base preferences still apply the D loop is much darker and the anticodon loop much lighter than the corresponding regions on the autoradiograms of the native structure. The patterns are similar, however, in the regions of the variable arm, T stem and T loop.
DISCUSSION

General Features of Partial $P_1$ Nuclease Digestion Profiles

The general features of the partial $P_1$ nuclease digestion profile of yeast tRNA$^{\text{Phe}}$ (Figure 11) show that the enzyme is sensitive to three aspects of tRNA structure. First, it reflects the sequence of nucleotides in the chain by exhibiting a gross preference for purines over pyrimidines. Second, within this context of nucleotide preference, it cleaves phosphodiester bonds in loop regions more rapidly than those from stem regions of the tRNA cloverleaf. Third, hydrolysis is inhibited by the presence of hydrogen bonds which stabilize the tertiary structure of the tRNA molecule. The degree of sensitivity to each of these structural features is greatest for the nucleotide preference and least for the inhibition by tertiary hydrogen bonds, but each effect can be seen within the context of the others.

Bars a through d of Figure 11 show the nucleotide preferences of $P_1$ nuclease. Cleavage takes place at the 3'-ends of adenylates approximately 2.5 times faster than the corresponding event after cytidylate residues. This sequence is consistent with data which were published previously by Fujimoto et al. (46). These authors studied the phosphodiesterase activity of $P_1$ nuclease with various homopolymers. They found that, at pH 7.0, the maximum rate of hydrolysis of poly A
was much greater than that of poly C or poly U, which were nearly equal. Although they could demonstrate no cleavage of poly G with \( P_\perp \) nuclease the enzyme exhibited its greatest phosphomonesterase activity with 3'-GMP. Since poly G is known to have a complex structure in solution (55) the inability of the nuclease to hydrolyze it was interpreted as reflecting sensitivity to structural features. The residues D, Cm and Gm inhibit phosphodiesterase activity substantially (see reference 10) but otherwise cleavage takes place at almost every site.

The preference of \( P_\perp \) nuclease for loop residues (bars e through h of Figure 11) is not as strong as that for purines over pyrimidines, but it is significant nonetheless. This, again, is in agreement with published kinetic parameters. The rate of cleavage of single-stranded poly I or poly C is twenty to thirty times greater than that of duplex poly I poly C (46). In the same publication the authors showed that native DNA was completely resistant to nucleolytic attack while the heat denatured material could be hydrolyzed.

These two aspects of the sensitivity of \( P_\perp \) nuclease to the structure of its substrate are not sufficient to explain the digestion patterns, however. Bars i through q show the average band intensities of each of the segments of the cloverleaf of tRNA\(^{\text{Phe}}\). While it is true that, in general, fragments whose 3'-termini are in the loop regions are more abundant than those from the stems there are individual fragments which are either over-represented or under-represented. The fragments from the four loops of the tRNA (bars k, m, n and p) reflect different extents to which each loop is involved in tertiary hydrogen...
bonding interactions which stabilize the tRNA's three-dimensional structure. Almost all of the nucleotides of the variable loop participate in these hydrogen bonds, while only five of the eight bases of the D loop do so.

In addition the acceptor stem (bar i, Figure 11) is over-represented in the profile. This is probably an artifact of the labelling methods. P₁ nuclease is known to prefer short fragments of RNA to intact tRNA. This low molecular weight material is hydrolyzed at a faster rate and with a higher pH optimum than the intact molecules (46). For this reason a residual level of "double hit" cleavage is difficult to avoid. When only those fragments which contain the 5'-terminal phosphate are counted the effect of this secondary hydrolysis is to increase the number of shorter bands in the autoradiogram. Post-labelling techniques could obviate this bias (see below).

Comparison of the Digestion Pattern of Yeast tRNA_{Phe} with its Known Tertiary Structure

Figure 12 (Part A) shows the relative band intensities for all sixty of the readable fragments in the gel. The general rules that purines are more likely to be cleaved than pyrimidines and that loop fragments are more abundant than those from the stems are borne out. On inspection of the profiles there are, however, several fragments which seem to be over- or under-represented. These are listed in Table IV. The relative abundance or scarcity of these fragments can be understood in terms of the three-dimensional structure of the tRNA molecule.
Thirteen of the eighteen anomalously scarce fragments can be accounted for by tertiary hydrogen bonding. The nucleotides $G_{15}$ and $C_{48}$ are involved in a Watson-Crick base pair in the tertiary structure; their bands are lighter than those of their neighbors. Similarly $A_{38}$ is involved in a base-base interaction with $Cm_{32}$. Its fragment, too, is lighter than those of other adenosines in the anticodon loop. All of the residues of the variable loop except $U_{47}$ are involved in hydrogen bonding with other bases in the tRNA structure. This loop is generally depressed in Figures 11 and 12. The $m^2G_{26}$ residue between the D and anticodon stems interacts with $A_{44}$; its band is almost absent from the autoradiograms. The nucleotides $A_{21}$, $U_{33}$, $T_{54}$, $Ψ_{55}$, $C_{56}$, and $G_{57}$ are also all involved in tertiary interactions (see Table I) and their fragments are all relatively scarce on the gels. Of the five remaining under-represented fragments, the residues $D_{16}$, $D_{17}$, $Cm_{32}$ and $Cm_{34}$ are resistant to $P_1$ nuclease attack in general (see reference 10) and $U_{47}$ is apparently buried in the tRNA structure.

The converse statement is also largely true. Most of the twenty-two loop nucleotides which are involved in tertiary interactions are also the ones whose bands are weaker on the autoradiograms. Two exceptions are $G_{18}$ and $G_{19}$. These residues participate in hydrogen bonding with bases in the variable loop, but their fragments are as abundant as that which terminates with $G_{20}$. Schmidt et al. (56) obtained similar results in their digestion of native yeast tRNA Phe with $T_1$ ribonuclease. The final digestion products were $pG_{1}-G_{18}$, $A_{21}-G_{57}$ and $A_{38}-C_{76}$; $G_{18}$ and $G_{19}$ were missing from the 5'-terminal
quarter molecule. Because $G_{20}$ is more accessible to chemical modification than either $G_{18}$ or $G_{19}$ (30), the data were interpreted (14,56) as representing initial cleavage at $G_{20}$ followed by removal of the two 3'-terminal guanidylates from the resulting fragment. This is likely to be the case for $P_1$ digestion, too. The overabundance of fragments ending in $U_g$ and $A_g$ amy, again, be the result of secondary cleavage, as previously discussed. The only other exceptions are the fragments $pG_1-A_{14}$ and $pG_1-m^1A_{58}$. The explanation for their extraordinary susceptibility to $P_1$ nuclease is less clear. The residue $m^1A_{58}$ is at a sharp bend in the $T$ loop, so the inhibition by its tertiary hydrogen bond may be reduced by its protrusion at the corner of the molecule.

The anomalous stem fragments are those which seem to be overabundant in the profile. The higher intensities of the first seven bands has already been discussed (see the General Comments above). The explanation for the overabundance of fragment $pG_1-A_{31}$ follows the same line of reasoning as that for the fragment $pG_1-m^1A_{58}$ which was discussed in the preceding paragraph. $A_{31}$ is near a sharp bend of the anticodon loop. This may render it more accessible to the nuclease than would be expected from the fact that it is flanked by two hydrogen bonded base pairs, $G_{30}$ and $m^5C_{40}$ of the anticodon stem and $C_{32}$ with $A_{38}$ of the anticodon loop.

In general, then, three rules govern the behavior of $P_1$ nuclease with the yeast tRNA$^{Phe}$ as a substrate. First, cleavage takes place at the 3'-side of purines in preference to the corresponding event after pyrimidines. Second, loop regions are more sensitive than stem regions. Third, the presence of tertiary hydrogen bonds inhibits the
phosphodiesterase activity of $P_1$ nuclease. These three generalizations are adequate to explain all but three of the band intensities observed in the autoradiogram of the $tRNA^{Phe}$ digest. Even the anomalies which these three fragments represent may provide useful information about the structure of tRNA in solution.

Various other digestions of yeast $tRNA^{Phe}$ have been carried out (38,53-61). In general they have shown that (1) stem regions are more resistant to nucleolytic cleavage than loop regions, (2) of the loops the $\alpha$ and $\beta$ regions of the D loop are the most susceptible, followed by the anticodon loop and the CCA terminus, and (3) the variable loop and T loop are relatively inaccessible to nucleases. Like the chemical modification studies (22-31) these experiments bear out the proposed secondary structure, and the data from the $P_1$ nuclease digestions is in good agreement. Unlike them, however, the $P_1$ digestion allows direct comparison of all of the residues in the tRNA chain.

Comparison of the Digestion Pattern of $tRNA^{Leu}_{3}$ with its Structure

A comparison of the partial $P_1$ nuclease digestion profile with the cloverleaf structure of yeast $tRNA^{Leu}_{3}(N)$ shows that loop regions are generally more accessible than stem residues, with the same exception that the acceptor stem fragments are over-represented (Figure 12). An interesting point here is that the nucleotides $G_{13}$ and $A_{22}$ are the fourth pair of bases in the D stem. Rich (14,19,20) has suggested that, even though they are not hydrogen bonded together, they
are stacked on the bases of the D stem and form a continuous part of the D-anticodon helix. If this is true then the susceptibility of a phosphodiester bond to hydrolysis by \( P_1 \) nuclease is inhibited as much by hydrogen bonding as by base stacking. The fragment ending with \( G_{13} \) is only slightly less abundant than neighboring fragments from the D loop; that of \( A_{22} \) is reduced, however.

Because the three-dimensional structure of yeast tRNA\(^{Phe}\) is stabilized by base stacking and by interactions among the twenty-one invariant or semivariant nucleotides its structure is thought to be adopted by other sequences. Extrapolation of the three-dimensional structure of yeast tRNA\(^{Phe}\) to yeast tRNA\(^{Leu}\)\(_3\) (N) allows prediction of the residues which may be involved in tertiary hydrogen bonding interactions (Table VI). Comparison of the under-represented fragments (Table V) with this list shows good agreement for bases outside the variable arm. Fragments ending with \( U_9, G_9, m_2^G_{26}, C_{38}, T_{54}, C_{56}, G_{57} \) and \( A_{58} \) all appear as weak bands while \( pG_{19} \) is relatively abundant. Within the variable loop the data are more difficult to interpret. The bases of the variable loop are certainly less accessible than those of the D loop, as was the case for tRNA\(^{Phe}\), but they are flanked by residues of the variable stem which are even more resistant. The greater availability of the nucleotides in the variable loop as compared to the variable stem agrees with the finding that \( P_1 \) nuclease is more sensitive to the effects of cloverleaf (Watson-Crick) base pairing than to tertiary hydrogen bonds. On the other hand the structural correlation between the variable arms of
<table>
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<th>Location</th>
<th>Bases</th>
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<tr>
<td>D Loop</td>
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</tr>
<tr>
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<td>( A_{22} ) (G_9)</td>
</tr>
<tr>
<td>D Loop</td>
<td>( A_{14} ) (U_8)</td>
</tr>
<tr>
<td></td>
<td>( G_{15} ) (( m^5C_{48} ))</td>
</tr>
<tr>
<td></td>
<td>( G_{m18} ) (( \psi_{55} ))</td>
</tr>
<tr>
<td></td>
<td>( G_{19} ) (C_60)</td>
</tr>
<tr>
<td>Ant. Loop</td>
<td>( U_{32} ) (C_38)</td>
</tr>
<tr>
<td></td>
<td>( C_{38} ) (U_32)</td>
</tr>
<tr>
<td>Var. Loop</td>
<td>?</td>
</tr>
<tr>
<td>T Loop</td>
<td>( T_{54} ) (( m^1A_{58} ))</td>
</tr>
<tr>
<td></td>
<td>( \psi_{55} ) (Gm_{18})</td>
</tr>
<tr>
<td></td>
<td>( C_{56} ) (G_{19})</td>
</tr>
<tr>
<td></td>
<td>( m^1A_{58} ) (T_{54})</td>
</tr>
<tr>
<td>&quot;Bridges&quot;</td>
<td>( U_8 ) (A_{14})</td>
</tr>
<tr>
<td></td>
<td>( G_9 ) (A_{22})</td>
</tr>
<tr>
<td></td>
<td>( m^5C_{48} ) (G_{15})</td>
</tr>
</tbody>
</table>

*Residues in parentheses are the presumed base pairing partners.*
of the two tRNAs is somewhat vague. This ambiguity is reinforced by the fact that the abundance of $pG_{15}$ is not reduced in the digest of the leucine-accepting tRNA as it was in that of tRNA$^{Phe}$. In the latter tRNA $G_{15}$ is involved in the only tertiary Watson-Crick base pairing interaction and hydrogen bonds with $C_{48}$. If a similar interaction takes place in tRNA$^{Leu}_{3}(N)$ then $G_{15}$ should be more resistant to nuclease digestion. Since this is not the case $m^5C_{48}$, whose fragment is reduced, may be hydrogen bonded with some other residue. The same line of reasoning applies to the case of the fragment $pG_{1}-G_{19}$. In tRNA$^{Phe}$ this band should be weak because of the interaction between $G_{19}$ and $\Psi_{55}$. Its lack of inhibition of $P_1$ nuclease was interpreted as reflecting a secondary cleavage which was permitted by the initial cleavage at $G_{20}$. In tRNA$^{Leu}_{3}$ the twentieth residue is a dihydrouridine, which is known to be resistant to $P_1$ nuclease, so this explanation does not apply. An alternative explanation is that its presumed base pairing partner, $C_{56}$ (whose fragment is less abundant) may interact with some other nucleotide.

The digestion data are in general agreement with the chemical modification (37) and oligonucleotide binding data (38). These investigations showed that the residues which were most susceptible to kethoxalation were $G_{18}$ and $G_{19}$. Unlike our results, however, nucleotides $G_{13}$ and $G_{15}$ were inaccessible to the reagent. All stem residues were protected in the native conformer.

In general, then, the loops and stems of the structure of yeast tRNA$^{Leu}_{3}$ fit the conformation of yeast tRNA$^{Phe}$ rather well.
The discrepancies between the profiles of the two tRNAs in the D and variable loops may be the results of differences in structure between the two tRNAs. The D loop of tRNA\textsuperscript{Phe} contains eight nucleotides, with \textit{G}_{18} and \textit{G}_{19} being the fifth and sixth of these respectively. In tRNA\textsubscript{Leu}\textsuperscript{3} these are the fourth and fifth of nine residues when \textit{G}_{13} and \textit{A}_{22} are considered to be stacked onto the D stem. The variable arms contain five and thirteen bases respectively in tRNA\textsuperscript{Phe} and tRNA\textsuperscript{Leu}\textsubscript{3}. These two portions of the cloverleaf are in areas of the overall structure of tRNA\textsuperscript{Phe} which can "bulge out," so they should have little effect on tertiary interactions elsewhere in the molecule. However, the hydrogen bonding pattern within these regions need, by no means, be identical to that of tRNA\textsuperscript{Phe}.

The digestion pattern of tRNA\textsuperscript{Leu}\textsubscript{3} (D) is markedly different than that of the native conformer. In particular the fragments from the D loop are very much more abundant and they are produced at the expense of all of the other fragments. Nevertheless the fragments which terminate in the anticodon and variable loops are reduced to a greater extent than the others. Ketoxalation data support this finding (37). This study showed that G residues in the D stem and anticodon stem are accessible to the reagent in the denatured but not the native conformer. Oligonucleotide binding studies show that the D stem and loop are single stranded in tRNA\textsuperscript{Leu}\textsubscript{3} (D).

These data support the model for the secondary structure which was proposed by Kearns (36). He suggests that there is a long stretch of unpaired bases extending from \textit{U}_{8} to \textit{U}_{29} and that the nucleotides
of the anticodon loop are hydrogen bonded with those of the T loop. The profile of fragments beyond C_{20:1} is essentially flat, showing only the differences expected between the residues of a base paired segment. As was the case for tRNA^{Phe} and tRNA^{Leu(N)} the very short fragments are over-represented. Fragments pG_{1}^{-}U_{3} and pG_{1}^{-}G_{9} are relatively depressed compared to those from the neighboring D loop, suggesting that, although the nucleotides of the D stem do not base pair with each other, the tertiary interactions U_{8}^{-}A_{14} and G_{19}^{-}G_{23} remain intact.

**Effectiveness of P_1 Nuclease Digestion as a Structural Probe**

The general requirements of partial nuclease digestion as a probe of tRNA structure are:

1. that the enzyme be sensitive to the structure of its substrate,
2. that the enzyme be active under conditions where the native structure of the tRNA remain intact, and
3. that it be possible to carry out the digestion in so limited a fashion that, on the average, any tRNA molecule is cleaved only once.

All of these conditions have been met by partial P_1 nuclease digestion. It is desirable that, in addition to these minimum requirements, kinetic studies be performed. In order to carry out these experiments the methods for inactivating or inhibiting the nuclease must be rapid enough and gentle enough to allow collection of very early time aliquots without disruption of the remaining tRNAs. Ideally the digestion would be performed on unlabelled tRNAs so that post-labelling
techniques could be applied. By using this method the possibility of a bias toward shorter fragments could be avoided.

P₁ nuclease could be used in this way. Its activity falls sharply above a pH of 7; at pH 8.5 it should be inactive as a phosphodiesterase. Since post-labelling techniques involve the use of bacterial alkaline phosphatase to remove the 5'-terminal phosphate, inactivation by raising the pH is preferable to the use of chelating agents which would inhibit the BAP.

P₁ nuclease has the added and perhaps more important advantage of being able to hydrolyze almost all of the bonds in a tRNA. Unlike its counterparts, nuclease S₁ and ribonucleases A, T₁, T₂, U₂ etc., it can be used to generate data about each of the residues.

The disadvantages of P₁ nuclease are that it can not (or can only very slowly) hydrolyze the residues D, T, Cm, and Gm. In addition, because of its preference for shorter fragments, a low level of double hit cleavage seems inevitable.

In summary, then, partial P₁ nuclease digestion of tRNAs gives useful information about almost all of the residues in the chains, about cloverleaf base pairing and about tertiary interactions and structure. Further development of the technique to allow kinetic studies and application of post-labelling procedures will increase the amount of information which P₁ nuclease digestion can provide. Although it will never be sufficient by itself, when it is used in conjunction with other enzymes and chemical reagents, partial P₁ nuclease digestion may vastly improve our understanding of the structure of tRNAs in solution.
Chapter II

TRANSFER RNA POPULATIONS IN NORMAL AND TUMOR CELLS
INTRODUCTION

Roles of Transfer RNA

In a living cell transfer RNA molecules perform many functions. The roles in which tRNAs are found range from protein synthesis to regulation of gene expression, and involve either specialized individual tRNA molecules or tRNA populations as a whole. Some of these functions are discussed below.

Transfer RNA has long been known to function as an adaptor in the translation of messenger RNAs (1). In fact this role was postulated to exist even before tRNAs were discovered (64). In the normal translation process tRNAs, as a class, interact with a whole multitude of different proteins and nucleic acids; these interactions depend on structural features common to all tRNAs. Yet these same tRNA molecules are sufficiently distinct to allow a phenomenal degree of fidelity in the translation process (65). This role of tRNA has been extensively reviewed (4).

In spite of the apparently overwhelming constraints on the structure of tRNA molecules, imposed by the dual requirements for common features and uniqueness, bacterial cells make alterations in tRNA sequences for suppression of mutation almost routinely (66). These base substitutions may occur within the anticodon loop (67) or outside of it (68), and result in the suppression of missense (69),
nonsense (70) and frameshift (71,72) mutations. The context which the 
messenger presents to the suppressor tRNAs is known to affect their 
ability to correct the mutation (73,74). The study of this role of 
tRNA is still providing much information about the translation process.

Transfer RNAs can also carry amino acids to molecules other 
than growing polypeptide chains. Aminoacyl-tRNA transferases (75) 
catalyze the addition of amino acids to components of bacterial cell 
walls (76) and plasma membranes (77), and to the amino termini of 
completed proteins (78). The tRNA species which participate in this 
transfer may be specialized molecules for which this is the only 
role (15).

Evidence for the use of tRNAs in viral RNA replication comes 
from many quarters. The Q8 replicase includes prokaryotic initiation 
factors, EF-Tu and EF-Ts (79,80). Bacteriophage and plant virus RNAs 
often have a "tRNA-like" structure either internally or at their 
3'-ends (81,82). Some can be aminoacylated by host aminoacyl-tRNA 
synthetases (81,83,84). In addition some retro-viruses package a host 
tRNA with their genomes; this tRNA acts as a primer in the viral 
replication process (85,86).

Transfer RNAs may also regulate enzymatic activity. In 
Drosophila a tRNA\textsuperscript{Tyr} may act as an inhibitor of the enzyme tryptophan 
pyrrolase (87,88). E. coli endonuclease I activity is converted from 
double-strand scission to single-strand nicking in the presence of 
tRNA (89). Finally, when deacylated tRNAs are bound in the A site of 
ribosomes from enteric bacteria, the relA gene product produces the
"magic spot" compounds ppGpp and pppGpp (90). These phosphorylated nucleotides function in the stringent response of the cells to amino acid starvation (91). There is an absolute requirement for a free 3'-hydroxyl group and for the sequence TTGG in the T loop (92). The critical factor is not the absolute concentration of deacylated tRNA but rather the ratio of acylated to deacylated species (93).

Besides this indirect control of transcription, transfer RNAs can affect the process directly. In bacteria the levels of aminoacyl-tRNA synthetases (94) and of the enzymes for biosynthesis of many amino acids (95) are regulated by the ratio of charged to uncharged tRNAs. One mechanism which has been proposed is attenuation (5,6,7). The mRNAs from attenuable operons possess a leader or "attenuator" sequence which has both a complex secondary structure and a series of identical codons for the amino acid whose biosynthetic enzymes are being made. Transcription and translation are coupled. When the ribosome comes to the series of identical codons the efficiency with which it can translate them depends on the availability of aminoacylated tRNAs. If the level of free amino acids is high there is little need to produce enzymes for their biosynthesis, and a high ratio of charged to uncharged tRNAs results in a high efficiency of translation of the attenuator sequence. The movement of the ribosome beyond this point alters the secondary structure and shuts down transcription.

Other regulatory roles for tRNA have been postulated to exist (8,96,97). They depend on changes in tRNA populations with different circumstances and will be discussed below.
Populations of Transfer RNAs

A normal cell contains approximately fifty-five species of tRNA (98) in its cytoplasm, one or more species for each amino acid. The need for multiple tRNAs for a particular amino acid (isoacceptors) reflects, in part, the degeneracy of the genetic code (99,100). There are instances, however, where one tRNA can read several codons (101) and where there are more isoacceptors than there are codons (102). Thus the relationship between isoacceptors and the genetic code is not a trivial one (103). In addition to the cytoplasmic populations, eukaryotic organelles have their own sets of tRNAs (2) as part of a complete translation apparatus (104).

Populations of tRNAs often reflect the activity of their cells. Smith (105), showed that, in reticulocytes, the relative abundance of different isoaccepting families of tRNAs paralleled the frequency of amino acids in hemoglobin. Vioiti (106) observed the same phenomenon in maize endosperm cells which were actively synthesizing zein. More specific alterations in tRNA populations of bacteria have been observed during sporulation (107) and bacteriophage infection (108,109). Adaptation of tRNA populations in eukaryotes has been observed at the onset of fibroin synthesis in Bombyx mori posterior silk gland (110) and in the response to hormones by chicken liver (111,112) and chick oviduct (113). Changes in the chromatographic profiles of isoaccepting tRNAs have also been demonstrated during differentiation and embryogenesis (114-118) and in response to interferon treatment (119). Although many of these alterations in the elution patterns of tRNA
isoacceptors have been reported only a very few of them have been carefully analyzed (see Discussion). Interpretation of the significance of these adaptations depends on an understanding of both the structure and the biological function of the altered tRNA species.

**Regulation of Translation Based on tRNA Populations**

The theory about the mechanism by which tRNA populations control translation was originally proposed by Ames and Hartman (120) and has been reviewed more recently by Lodish (121) and by Osterman (8). In essence it suggests that the efficiency of translation depends on the tRNAs which are available and that differential rates of translation of mRNAs govern the level of their gene products. In its original form (120,122-124) the theory suggested that differences in codon usage among mRNAs would correspond to differences in the relative abundance of isoaccepting tRNAs. Those messengers which included codons corresponding to scarce tRNAs would be translated more slowly than their counterparts whose codons were read by the more abundant isoacceptors. Three lines of evidence support this theory.

The pattern of codon usage in any particular mRNA depends on the choice of the degenerate ("wobble") base. If the relative abundance of isoaccepting tRNAs is to govern the rate of translation as this theory suggests, then different mRNAs must preferentially employ different codons for the same amino acid. Grantham et al. (125) found this to be the case. They surveyed the known mRNA sequences and found that codon usage within a genome is non-random. Frameshift genes use
codons differently than their wild-type parents; among genomes the pattern of codon usage varies.

A second line of evidence in support of the codon restriction hypothesis is that isoaccepting tRNAs are used differently. It has already been noted that, within a mRNA, different contexts enhance the use of one isoacceptor over another (73,74). In addition, in vitro translation of collagen messenger by chick calvaria preferentially uses one of the four glycine isoacceptors and one of the two alanine tRNAs (126,127).

Finally, deliberate changes in tRNA populations can affect the type and amount of proteins synthesized in vitro. Atkins et al. (128) found that disturbance of the ratios of tRNAs could promote frameshifting of the messenger. A minor species of tRNA$^{\text{Ala}}$ stimulates hemoglobin synthesis in chick embryo cells (129). Other instances are known (130).

In summary, then it has already been shown by many workers that tRNA populations are characteristic of the cells from which they are derived and that the populations can adapt to changing circumstances of the cell. These observations led to the proposal that tRNA populations can act to regulate gene expression at the translational level. This general concept has been reasonably well substantiated by three lines of evidence. First, codon usage is non-random and characteristic of individual genomes. Second, mRNAs use certain isoaccepting tRNAs preferentially, even being able to distinguish among species with the same anticodon. Third, deliberate additions
or substitutions of exogenous tRNA can affect the type and amount of protein synthesized in cell-free translation systems.

Although these findings, when taken together, lend credence to the notion of tRNA-mediated regulation put forward by Ames and Hartman (120), they say nothing about its mechanism \textit{per se}. The original theory that discrimination among tRNAs (and therefore determination of translation rate) depends solely on the choice of degenerate codons has had to be modified in the light of several new observations about protein synthesis.

1. As has already been mentioned the relationship between the tRNA population and the genetic code is not a trivial one. Not only do the numbers of isoaccepting tRNAs and degenerate codons often differ, but the question of mechanism of codon recognition itself has recently been re-opened. The "wobbling" of the tRNA on the messenger was postulated long ago (131). Now the specificity of the codon-reading process has been opened to further debate by Weissenbach's suggestion that a single species of leucine-specific tRNA can read all six leucine codons in extracts of interferon-treated cells (132). Lagerkvist (133) has suggested that the codon-anticodon interaction may involve only "2 out of 3" nucleotides. This seems to be substantiated by work with suppressor tRNAs (68) although the work of Goldman \textit{et al.} (134) seems to contradict the theory.

2. Selection of tRNAs by their aminoacyl tRNA synthetases and by ribosomes can depend on features other than the anticodon sequence itself. Modified bases have been shown to play a role in tRNA structure (14) and in regulation by tRNA of transcription (135) as well as in the aminocacylation (136) and ribosome binding (137) steps of translation. Some of the adaptations of tRNA isoacceptors involve changes only in the extent of modification (138,139; see Discussion also.)

3. Transfer RNAs undergo conformational changes on binding to the messenger RNA in the presence of ribosomes and translation factors (140,141). There is also some reason to believe that conformational changes of tRNAs take place
on binding by aminoacyl-tRNA synthetases (142,143) although there is some debate about this point (144). These changes may be involved in the mechanism of kinetic proof-reading by aminoacyl-tRNA synthetases (145,146) and ribosomes (147,148).

4. Transfer RNA selection mechanisms are presently thought to involve several steps, one of which is irreversible and driven by hydrolysis of nucleoside triphosphates (149). The separation of the selection (editing) process into discrete steps is the phenomenon which allows the very low error rate of translation.

5. There is reason to believe that ribosomes, themselves, may adopt different conformations, particularly in response to extracellular influences (see reference 8). Interferon treatment affects not only the tRNA population (119) but also portions of the translation complex (eIF-2) of the cell (150,151).

In light of these observations Osterman (8) has proposed that the mechanism by which tRNAs can regulate gene expression at the translational level is a multistep process which involves a phenomenon called "pre-selection." He suggests that 1) ribosomal conformations place restrictions on the ability of a tRNA to bind to the A site, 2) tRNA molecules can be classified into families with similar conformations and that 3) an initial step in tRNA-ribosome binding involves selection of isoacceptors on the basis of their conformations. After this pre-selection by ribosomes has taken place the strength of the codon-anticodon interaction plays a second role in determining the efficiency (rate) of translation. This second step, originally proposed by Kurland et al. (140), has been substantiated by Fiers and Grosjean (152).

In summary, populations of tRNA are thought to determine the efficiency of translation of messengers which have different patterns
of codon usage. The mechanism by which this regulation takes place is thought to involve the codon-anticodon interaction as well as the pre-selection by ribosomes of structural classes of tRNA based on transient changes in the structure of the ribosome itself. The existence of two distinct selection steps facilitates both the regulation of translation efficiency and the high fidelity of the process.

Transfer RNA and Cancer

The term, "cancer," refers to a collection of diseases characterized by the uncontrolled growth of cells. This growth may involve solid tumors or disseminated cells, proceeds at rates ranging from slightly faster than normal to wildly proliferative, and may be invasive or not (153). Cancer may be caused in laboratory animals by viruses (154,155), chemical agents (156) or particulate matter (157). Cancer cells have an altered metabolism (158,159) and have been treated with drugs ranging from antimetabolites to metallo-organic compounds (160). One theory likens oncogenesis to de-differentiation (161) because embryonic and neoplastic tissues have common features.

A role for the translational apparatus in neoplastic transformation has been suggested by several observations. Many tumor cells produce specific new proteins (e.g. 162); virus-induced tumors, in particular, have altered antigens on the cell surface (163). Transfer RNAs have been shown to exhibit a greater turnover rate in cancer cells (164) and the activities of tRNA modifying enzymes are often higher (165-170).
It has also been found that crude mixtures of tRNAs from normal and tumor cells have different abilities to support protein synthesis \textit{in vitro} and may lead to the production of proteins with different electrophoretic mobilities (171-174). Many studies have examined the chromatographic behavior of normal and tumor tRNAs in various animal tumors and transformed cells in tissue culture (175-179). They have been reviewed elsewhere (180). In general they involve aminoacylation of a crude mixture of tRNAs with a single radioactively-labelled amino acid and separation of the isoacceptors by column chromatography. Families of isoaccepting tRNAs from two different cell types can be compared directly by labelling one population with the $^3$H- and the other with a $^{14}$C-containing amino acid and co-chromatographing them on a single column.

Rationale for this Study

The tRNA population from a \textit{benign} human tumor (leiomyoma) will be characterized by analyzing the RPC-5 elution profiles of several of its isoaccepting families in comparison with their counterparts from a normal human tissue (placenta). This separation technique is called reversed-phase chromatography because the column packing material is prepared by coating an inert solid support (plaskon beads) with a long chain aliphatic amine (adogen 464). This liquid coating resolves the tRNA species on the basis of both ionic and hydrophobic interactions (181) and can separate a crude mixture of tRNAs into very many species (182). Differences in the relative amounts of the individual
isoacceptors and/or the appearance of novel species of tRNA will be shown in these profiles and will be interpreted in light of the postulated role of tRNA in regulation of translation and in oncogenesis.
MATERIALS AND METHODS

Materials

Uniformly labelled radioactive amino acids were obtained either through ICN Pharmaceuticals or New England Nuclear and had the following specific activities: $[^3\text{H}]-\text{tyrosine}$, 61 Ci/mole; $[^3\text{H}]-\text{phenylalanine}$, 60 Ci/mole; $[^3\text{H}]-\text{lysine}$, 54 Ci/mole; $[^3\text{H}]-\text{aspartic acid}$, 15 Ci/mole; $[^3\text{H}]-\text{serine}$, 3 ci/mole; $[^1\text{C}]-\text{tyrosine}$, 0.43 Ci/mole; $[^1\text{C}]-\text{phenylalanine}$, 0.41 Ci/mole; $[^1\text{C}]-\text{lysine}$, 0.30 Ci/mole; $[^1\text{C}]-\text{aspartic acid}$, 0.20 Ci/mole and $[^1\text{C}]-\text{serine}$, 0.145 Ci/mole.

Aquasol was purchased from New England Nuclear. The fluors, PPO and POPOP, for the toluene-based scintillin were purchased from New England Nuclear. Adogen 464 was purchased from Aldrich Chemicals. The DEAE-cellulose resins, DE-32 and Cellex D, were obtained from Whatman and BioRad respectively.

Trisodium ATP was purchased from Sigma Chemical Company. It was dissolved in distilled water, neutralized with dilute NaOH and stored in small aliquots at -20°C until use. Phenol was re-distilled and saturated with 0.14 M sodium acetate pH 4.5. A crude rabbit liver synthetase preparation, purified as described below, was a generous gift of Dr. Erin Hawkins. Plaskon beads were kindly supplied by the Oak Ridge National Laboratory. Human tissue (placentas and the leiomyoma) were obtained from Dr. Marvin Stuckey at Woman's Hospital in Baton Rouge, La.
A Beckman LS-255 liquid scintillation counter was used for counting column fractions or assays. Two Beckman centrifuges, a J-21 and an L5-75, were used in preparation of tRNAs and aminoacyl-tRNA synthetases. A Gilford spectrophotometer was used to measure ultraviolet absorbance.

Methods

Transfer RNAs were purified according to the method of Bruce Roe (183). The tissue was minced and extracted three times with equal volumes of 0.14 M sodium acetate pH 4.5 and buffer-saturated phenol. After each extraction the aqueous and organic phases were separated by centrifugation at 8,500 x g, 4°C for thirty minutes. The final aqueous phase was applied to a DEAE cellulose column equilibrated with the same buffer and rinsed with that buffer until the absorbance at 260 nm was below 0.05. The second phase of the elution involved 0.14 M sodium acetate with 0.3 M NaCl. When the absorbance at 260 nm had again fallen below 0.05 the third elution buffer was applied: 0.14 M sodium acetate pH 4.5 with 1.0 M NaCl.

Column fractions were assayed for amino acid accepting ability in a 100 µl reaction mixture which contained the following: 100 mM Tris HCl pH 8.5, 5 mM MgCl₂, 50 mM KCl, 0.5 mM EDTA, 2.5 mM ATP, 5 µl of a uniformly labelled mixture of amino acids, 7.5 µl of the rabbit liver enzyme preparation and 60 µl of each fraction. The tubes were incubated for 15 minutes at 37°C. A 75 µl aliquot was placed on 1-cm squares of filter paper and allowed to soak in completely. The papers
were then washed three times with ice cold 5% trichloroacetic acid (TCA) and twice with ice cold 95% ethanol to remove unbound amino acids. The papers were dried, placed in a toluene-based scintillation fluid and counted.

Column fractions which had high amino acid accepting activity were pooled, combined with 2.5 volumes of ice cold 95% ethanol and allowed to stand at -20°C overnight. The precipitated tRNA was collected by centrifugation at 15,000 x g, 4°C for thirty minutes, washed with ethanol, dried under vacuum for a short period and resuspended in distilled water to a final concentration of 100 A\textsubscript{260} units/ml. It was stored at -20°C until use.

Preparation of Crude Aminoacyl-tRNA Synthetases

Preparation of crude synthetases from human placenta was a modification of two procedures (184,185). A fresh placenta (obtained through caesarian section) was obtained from Womans Hospital in Baton Rouge and placed on ice immediately. It was transported to the lab, rinsed with 0.25 M sucrose, 20 mM Tris HCl, 100 mM NH\textsubscript{4}Cl, 1 mM MgCl\textsubscript{2} and 1 mM DTT, pH 7.5, and minced. After being filtered through cheesecloth, it was centrifuged at 15,000 x g for ten minutes at 4°C. The supernatant was centrifuged at 150,000 x g for 80 minutes and 4°C in a Beckman L5-75 ultracentrifuge. This second supernatant was applied directly to a DEAE-cellulose column equilibrated with 10 mM potassium phosphate pH 7.5, 2 mM DTT, 1 mM Na\textsubscript{2} EDTA, 5 mM MgCl\textsubscript{2} and 15% glycerol. The column was rinsed with starting buffer until the
absorbance at 280 nm fell below 0.05. The synthetases were then eluted with 10 mM potassium phosphate pH 6.5, 0.25 M KCl, 2 mM DTT, 1 mM Na₂ EDTA, 5 mM MgCl₂ and 15% glycerol.

Column fractions were assayed for amino acylation activity in a 100 μl reaction mixture which contained the following: 100 mM Tris HCl pH 7.5, 10 mM MgCl₂, 50 mM KCl, 2 mM DTT, 5 mM sodium ATP pH 7.0, 100 μg/ml crude placenta tRNA, 2 μl of a uniformly ¹⁴C-labelled mixture of amino acids, and 20 μl of column fraction material. Incubation was at 37°C for 15 minutes. The amount of aminoaacylated tRNA was determined as in the tRNA assays described above. Active fractions were pooled and concentrated by dialysis against 63% glycerol to give a final glycerol concentration of 50% (v/v). They were stored at -20°C in small aliquots.

**Determination of Optimum Conditions for Aminoacylation of tRNA**

Optimum conditions for aminoacylation of crude tRNA preparations with individual radioactively labelled amino acids were determined in the following manner. 1) The optimal pH and length of incubation were determined by carrying out four 400 μl assays which contained the following: 10 mM MgCl₂, 5 mM sodium ATP pH 7.0, 16 μl of the radioactive amino acid in question, 20 μl of either rabbit liver or placenta synthetase, 40 A₂₆₀ units of crude placenta tRNA and 10 mM Tris HCl of pH 7.0, 7.5, 8.0 or 8.5 respectively. Incubation was at 37°C. Aliquots of 50 μl were removed after 0, 2, 5, 10, 15, 20 or 30 minutes of incubation and counted as usual. The pH and time of incubation
which showed the highest activity were used for subsequent assays.

2) Once the optimal pH and time of incubation were determined the second step involved choice of buffer and of the MgCl₂:ATP ratio. Assays were carried out as above except that Tris HCl, HEPES or cacodylate buffers were tried, and the concentration of MgCl₂ was varied while the ATP concentration was held at 4 mM. 3) The last assay measured the effects of various additions on aminoacylation activity. The following compounds were added to the reaction mixture and the activity compared to a control tube which contained no additions: 0.5 mM Na₂ EDTA, 2 mM 2-mercaptoethanol, and 50 mM KCl.

Preparative Scale Aminoacylation

Once the optimal conditions had been worked out for each isoaccepting family of tRNAs, a large scale aminoacylation was performed (186). These preparative-scale reactions typically involved a 2-3 ml volume.

The tRNA samples were denatured and reannealed by heating them to 65°C for five minutes in 10 mM Tris HCl pH 7.0, 10 mM MgCl₂ and allowing them to cool slowly. They were then aminoacylated under conditions determined above. At the end of the reaction the solution was acidified with sodium acetate pH 4.5, extracted twice with buffer-saturated phenol and three times with buffer-saturated ether. The tRNA was precipitated with two volumes of ice cold absolute ethanol. After standing at -80°C for 90 minutes, the precipitate was spun down, dried under vacuum for a few minutes, resuspended in 50 mM sodium
acetate and dialyzed against one change of 0.5 M NaCl in 50 mM sodium acetate pH 4.5, and then against two changes of 2 mM sodium acetate pH 4.5. A 5 µl aliquot of each dialysate was removed for determination of total radioactivity.

**Preparation of RPC-5 Packing Material**

The packing material for the RPC-5 column was prepared according to modifications of two methods (187,188). Adogen 464 (15 mls) was combined with 250 mls of 0.4 M NaCl, 10 mM sodium acetate pH 4.5, 10 mM MgCl$_2$ and mixed in a blender for five minutes at room temperature. Then 100 grams of plaskon beads were added and the mixture was blended for five two-minute intervals; sufficient time was allowed between mixings to avoid overheating. The resin was then poured into a buchner funnel and washed with, first, four liters of 1.5 M NaCl in 10 mM sodium acetate pH 4.5, 10 mM MgCl$_2$ and second, two liters of 0.4 M NaCl in the acetate-magnesium chloride buffer. The column was packed in this low salt buffer and then compacted by having 2 M NaCl, 10 mM sodium acetate pH 4.5 and 10 mM MgCl$_2$ pumped through it at 300 psi overnight.

**RPC-5 Chromatography**

Approximately 80,000 cpm of $^3$H-labelled tRNA and 40,000 cpm of $^{14}$C-labelled tRNA were combined with 50 A$_{260}$ units of unlabelled yeast tRNA as an OD marker, and injected into a 0.9 by 52 cm column. It was eluted with a concave gradient of 0.45 to 0.85 M NaCl in 525 ml of 10 mM sodium acetate pH 4.5, 10 mM MgCl$_2$. The column was operated at 100 psi
to generate a flow rate of 27 ml/hr. Fractions of 1.8 ml were collected and 1.5 ml of each were counted in 10 ml of Aquasol in a Beckman LS 255 liquid scintillation counter. Each fraction was counted three times, and the average, corrected for background and overlap, was normalized to picomoles of aminoacyl tRNA.
RESULTS

Preparation of Aminoacyl-tRNA Synthetases

Figure 13 shows the DEAE-cellulose elution profile for the purification of a mixture of aminoacyl tRNA synthetase from human placenta. Fractions 40-55 were pooled and, after concentration, gave 80 $A_{280}$ units of protein. The activity of this enzyme preparation was similar to that of rabbit liver synthetase preparations (data not shown). As little as $4 \times 10^{-3} A_{280}$ units of the synthetase preparation gave a linear response of activity vs. tRNA concentration up to 500 µg/ml of tRNA. (Figure 14) This enzyme was also similar to the rabbit liver preparation when used in preparative-scale aminoacylations. The RPC-5 profiles of placenta tRNA$^{Phe}$ aminoacylated with either enzyme preparation are identical (data not shown).

Optimal Conditions for Aminoacylation

The optimum conditions for aminoacylation of placenta tRNA are shown in Table VII. These conditions were also used for the tumor tRNA preparation in order to avoid any differences which would make comparison of normal and tumor tRNA elution profiles more difficult.

RPC-5 Profiles

The RPC-5 profile of tRNA$^{Ser}$ is shown in Figure 15. The tRNA from the normal tissue has one minor and two major peaks. The tumor profile has two extra early-eluting peaks.
Figure 13. Elution profile of crude aminoacyl-tRNA synthetases from a DEAE-cellulose column.
Figure 14. $^{14}$C-labelled amino acids incorporated in 15 minutes, with 10 µl of aminoacyl tRNA synthetase preparation, as a function of tRNA concentration.
TABLE VII
Optimal Aminoacylation Conditions for Placenta tRNAs

<table>
<thead>
<tr>
<th>Isoaccepting Family</th>
<th>Length of Incubation</th>
<th>pH</th>
<th>Buffer</th>
<th>[MgCl$_2$]</th>
<th>Additions</th>
</tr>
</thead>
<tbody>
<tr>
<td>tRNA$^{\text{Phe}}$</td>
<td>15 minutes</td>
<td>7.0</td>
<td>Tris Cl</td>
<td>20 mM</td>
<td>0.5 mM Na$_2$EDTA</td>
</tr>
<tr>
<td>tRNA$^{\text{Asp}}$</td>
<td>30 minutes</td>
<td>7.5</td>
<td>Tris Cl</td>
<td>20 mM</td>
<td>None</td>
</tr>
<tr>
<td>tRNA$^{\text{Tyr}}$</td>
<td>30 minutes</td>
<td>7.0</td>
<td>Tris Cl</td>
<td>12 mM</td>
<td>None</td>
</tr>
<tr>
<td>tRNA$^{\text{Lys}}$</td>
<td>20 minutes</td>
<td>7.0</td>
<td>Tris Cl</td>
<td>4 mM</td>
<td>None</td>
</tr>
<tr>
<td>tRNA$^{\text{Ser}}$</td>
<td>20 minutes</td>
<td>8.5</td>
<td>Tris Cl</td>
<td>8 mM</td>
<td>0.5 mM Na$_2$EDTA</td>
</tr>
</tbody>
</table>
Figure 15. RPC-5 chromatographic profile of human placenta ••
and leiomyoma ○○ tRNA<sub>Ser</sub>.
For tRNA^{Asp} (Figure 16) there is a single major isoacceptor in the placenta tissue along with 3-4 minor species. The profile of the tumor tRNAs differs from its normal counterpart both qualitatively and quantitatively. The major peak is much higher in the tumor profile and an extra slow-moving peak appears.

The profiles of tRNA^{Tyr} are shown in Figure 17. The normal tRNA has four to five isoacceptors. The same peaks are present in the tumor profile, but there are two additional isoacceptors of tumor tRNA^{Tyr}. These extra peaks are eluted at a higher salt concentration than those from the placenta tissue. There also seems to be much more tRNA^{Tyr} in the leiomyoma than the normal cells.

The RPC-5 profiles of tRNA^{Lys} are shown in Figure 18. They differ in the relative amounts of the two isoacceptors and in the absolute levels of total lysine-accepting activity.

The RPC-5 profile of tRNA^{Phe} is shown in Figure 19. The normal tissue has only one isoacceptor. The tRNA of the leiomyoma is in four peaks, one corresponding to that from the placenta and three extra, early-eluting isoacceptors.

Comparison of these five sets of profiles shows quantitative or qualitative differences between the normal and tumor tissue in each instance. In two cases, those of tRNA^{Tyr} and tRNA^{Lys}, there were also differences in the absolute amounts of tRNA between the two types of tissue.
Figure 16. RPC-5 profiles of human placenta •—• and leiomyoma

O- - O tRNA$^{\text{Asp}}$. 
Figure 17. RPC-5 profile of placenta ●● and leiomyoma ○ ○

Tyr.

tRNA
Figure 18. RPC-5 profile of human placenta •—• and leiomyoma ○ —○ tRNA Lys.
PICOMOLE PLACENTA tRNA

PICOMOLE TUMOR tRNA

FRACTION NUMBER
Figure 19. RPC-5 profile of human placenta •—• and leiomyoma ○—○ tRNA Phe.
DISCUSSION

General Comments

Reversed-phase chromatography is a technique by which tRNAs can be very highly resolved (182). Although columns used in this work can separate crude yeast tRNA into more than twenty well-resolved peaks (data not shown), these profiles are not entirely reproducible in my hands. Two problems come into play here. The first is one of recovery. When tRNA is loaded onto the column some of it is "lost," presumably bound irrevocably to the column material. The other problem which complicates interpretation of RPC-5 elution profiles is one of column capacity. Through successive uses of the column some of the adogen 464 coating is rinsed off (189). As the coating is lost the column packing material is less and less able to bind tRNAs. While it is assumed that the "excess" tRNAs are washed through the column non-specifically the possibility remains that some isoacceptors may be sloughed off to a greater extent than others. Neither of these problems is insurmountable; each of the profiles presented here has been reproduced at least twice, and in some cases as many as four times. Nevertheless, in view of these limitations caution must be exercised in interpretation of subtle differences in profiles.
A second consideration which must be made concerns the aminoacylation conditions. These were identical for any isoaccepting family of tRNAs in the two populations. They were chosen so as to give the maximum rate and extent of aminoacylation. They may or may not reflect the conditions which obtain in vivo. Since the two tRNA preparations, that from placenta and that of leiomyoma, were treated identically, differences between their two profiles are assumed to be valid. However, the differences within any one profile, for example among the isoacceptors of tRNA$^{Asp}$ from human placenta, may be dramatically affected by subtle changes in the magnesium concentration or in the ionic strength of the solvent (190). This consideration makes comparisons of the profiles obtained in different laboratories more difficult. Unless the aminoacylation and elution conditions are precisely the same the isoacceptors which appear as major peaks in one profile may be somewhat reduced in another. Within the context of these precautions, however, such comparisons will be attempted. In general, the agreement is good, although this is not always the case.

Studies of the differences between serine-accepting tRNAs from normal and tumor tissues have uniformly shown the existence, in cancer cells, of extra, early-eluting species (191-198). Kuchino and Borek (198) also found that one of the isoacceptors of normal tissue was missing from the tumor tRNA$^{Ser}$ profile. The results shown in Figure 15 are in good agreement with these. The nature of the extra
tRNA peaks is unknown. In another system, however, Randerath et al. (199) analyzed the sequence of one of the extra species of tRNA\textsubscript{Ser}.

In Morris hepatoma the tumor-specific isoacceptor has a parent sequence identical to one of those from rat liver; it differs only in the extent of its modification. The early-eluting tRNA species from leiomyoma may also be undermodified versions of their normal counterparts.

**Tumor-specific tRNA\textsubscript{Asp} and tRNA\textsubscript{Tyr}**

Studies of the chromatographic behavior of tyrosine and aspartic acid-accepting tRNAs from normal and tumor cells have almost uniformly observed increases in the late eluting isoacceptors associated with cancer (200-207 and Figures 16 and 17). In normal cells these tRNAs often contain the hypermodified base Q (208), which is synthesized in the cytoplasm and then inserted into the tRNA at specific positions by a tRNA transglycosidase, a "G insertion enzyme" (209). The absence of this residue has been implicated in the appearance or increase of the late-eluting tRNA species (210) as have differences in the extent of methylation (211,212). These results, when taken together, suggest that the alterations in the profiles of these two families of isoacceptors reflect accumulation of precursors of the mature tRNA species.

**Tumor-specific tRNA\textsubscript{Lys}**

Lysine-accepting tRNAs have been extensively studied, not only in tumor cells, but also in developing and differentiating
ones (213-218, Figure 18). Although the absolute number of peaks in any particular profile has varied widely the results have usually been in agreement that the relative amounts of each isoacceptor changes between cell types. Two tRNA species, in particular, have been observed to increase with proliferation and cell division and to decrease on cell differentiation (216,218). Raba et al. (219) analyzed the sequences of three isoacceptors from normal rat liver and one of the "tumor-specific" peaks from SV40-transformed mouse fibroblasts. They found that the tumor-specific tRNA differed from one of its normal counterparts only in two positions, D_{20} and T_{m-4}, both of which were undermodified. The variation in the numbers of peaks on the elution profiles of tRNA^Lys reflects the problems inherent in RPC-5 chromatography. The change in distribution of the isoacceptors, however, is again consistent with a precursor-product relationship among the tRNA species. Such a change was observed in the case of leiomyoma (Figure 18).

Tumor-specific tRNA^Phe

Phenylalanine-accepting tRNA is the most frequently studied species in tumor cells, probably because it exists as a single isoacceptor in normal tissue. It contains a hypermodified base, Yr, at the 3'-side of its anticodon (237). In every tumor system examined, including the case presented here, extra early-eluting peaks are present in the profiles of tumor cells (220-231, Figure 19). Mushinski and Marini (227) found that one of the extra peaks may be
an artifact of the tRNA purification methods: unbuffered phenol can have a pH lower than 2.5 and at this pH the Y base can be cleaved away from its tRNA. This does not account for all of the extra peaks, however (228), although it has been taken as an indication that tumor specific tRNAs lack the Y base in vivo. Several of the studies have implicated Y base synthesis in the appearance of extra species of tRNA_Phe from neoplastic tissue (229,230) and the sequence of one of these early peaks indicates that it is an undermodified version of its counterpart in normal cells (226). The profiles in Figure 20 are in good agreement with those described above.

Significance of these Findings

In summary, then, the RPC-5 elution profiles described here are in basically good agreement with those published elsewhere. The presence of additional isoacceptors and the shift in elution position or relative abundance of existing tRNA species coincide with the findings of other workers. These data extend the previous findings in two ways: 1) they are the first case in which several isoaccepting families of tRNAs from a human tumor have been examined, and 2) they refer to a benign tumor.

The observation that alterations in the chromatographic profiles of several groups of tRNA isoacceptors appear concomitantly suggests that they reflect a general effect on tRNA, rather than one which involves particular tRNA species. The proposal that there is a precursor-product relationship among the new and pre-existing
isoacceptors is consistent with the hypothesis that cancer cells are characterized by increased synthesis and degradation of tRNA (164) and that the rate of synthesis exceeds the capacity of the maturation enzyme to modify the primary transcripts. This is not to say that these alterations are by-products of the general increase in growth rate of cancer cells or that the possibility of a regulatory role for tumor specific tRNAs in oncogenesis is ruled out.

The fact that tRNAs from this benign tumor show the same alterations in chromatographic behavior as those from malignant cells is also significant. Benign tumors occupy an intermediate position in the spectrum which ranges from normal differentiated cells to undifferentiated malignancy. If the extent of alterations in tRNA populations were directly related to growth rate, invasiveness or differentiation of the tumor then one might expect fewer extra isoacceptors or a smaller change in the relative abundance of pre-existing tRNA species in the tRNA population from this benign tumor. This is not the case. The observation of alterations of tRNA populations in this benign tumor as great as any from a malignant one suggests that cancer is an all-or-nothing phenomenon in its effect on transfer RNAs.

Compatibility of these Findings with the Theory of tRNA-Mediated Regulation of Translation

The biological significance of these alterations in tRNA populations from cancer cells remains largely a mystery. A few studies have examined the abilities of tumor-specific tRNAs to support protein
synthesis in cell-free translation systems and have obtained conflicting results (209,211,224,232). The absence of a difference between two isoacceptors in the ability to translate globin messenger in a reticulocyte cell-free system may not be indicative of their roles in the homologous cells, but, at the same time, differences which are observed \textit{in vitro} are not necessarily indicative of the \textit{in vivo} situation, either. In short, much work remains to be done on the analysis of the biological significance of tumor-specific tRNAs.

Two explanations for these altered tRNA populations exist. The first is that the new isoacceptors are by-products of a generally increased rate of protein and RNA synthesis in tumor cells. According to this hypothesis the "extra" species are incompletely modified versions of the "normal" tRNAs produced because the rate of tRNA synthesis exceeds the capacity of the maturation system. The second alternative is that these changes in tRNA populations in fact allow the characteristic shifts in the numbers and types of proteins synthesized by cancer cells.

This second alternative seems more likely for several reasons. First the levels of tRNA methylase activities in tumor cells are known to be increased dramatically (166-171). This is inconsistent with a hypothesis that under-modification is the result of weak maturation activity. It cannot be ruled out altogether, though, because changes in the levels of S-adenosylmethionine and the various folate derivatives are known to take place during oncogenesis (233-235) and may play a role in the production of methyl-deficient nucleic acids (236).
Second, adaptation of tRNA populations to the activities of their cells was not only one of the lines of evidence in support of the codon restriction hypothesis, but was also one of the first indications that such a regulatory mechanism might exist. Third and perhaps most important is the fact that the changes in tRNAs which accompany oncogenesis are the reverse of those which are observed during differentiation (118). If the differentiation process is enhanced or directed to any extent by increases in the overall level of tRNA modification, then decreases in this modification must work against the control of gene expression.

Understanding of the relevance of tumor specific tRNAs to the cancerous state will depend on three areas of research: careful analysis of all of the isoaccepting tRNAs of a cell's population, determination of the structural basis for the appearance of these new isoacceptors, and examination of the roles of these tRNAs and their modified bases in protein synthesis. The work presented here is just one step in this process.
REFERENCES


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VITA

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