added. After several hours at −20°C, the precipitate was recovered by centrifugation, dissolved in 10 ml of 0.1*M* NaCl, 0.1*M* tris-HCl (*p*H 7.5), and poured onto a 3-ml column of packed diethylaminoethyl (DEAE)-cellulose equilibrated with the same buffer. The column was washed with 60 ml of 0.3*M* NaCl, 0.1*M* tris (*p*H 7.5); tRNA and 5*S* RNA were then eluted with 1.0*M* NaCl, 0.1*M* tris (*p*H 7.5). Fractions (5 ml) were collected and surveyed with a radioisotope monitor; the three peak tubes, which contain the bulk of the tRNA, were pooled. RNA was precipitated with two volumes of 95 percent ethanol at −20°C over several hours and was recovered by centrifugation. The yield was 60 A_{260} units containing 2.6×10^9 count/min. Table 2 gives the recovery of radioactivity at each step of purification.

By means of appropriate procedures individual tRNA's and 5*S* RNA can be isolated from the "crude" tRNA fraction. The $tRNA^{Leu}_3$ was obtained by the following procedure. The crude tRNA was dissolved in 5 ml of 0.01*M* potassium cacodylate (*p*H 7.0), 0.0005*M* EDTA, passed through a short Chelex column (K^+ form) to remove divalent cations, heated at 60°C for 5 minutes, and cooled to 0°C. By sequentially adding 3*M* KCl to give 0.15*M* K^+ and 1*M* $MgCl_2$ to give 0.01*M* Mg^{2+}, then incubating the solution at 25°C for 30 minutes and cooling again to 0°C, $tRNA^{Leu}_3$ was selectively trapped in the denatured state (*4*). In this form, it was fractionated on a column (2 by 100 cm) of Sephadex G-100 at 4°C (*4*), from which it emerges with 5*S* RNA ahead of the bulk of tRNA. The $tRNA^{Leu}_3$-enriched fractions were concentrated by absorption on a 1-ml DEAE-cellulose column and eluted in a small volume with 1*M* NaCl, 7*M* urea, 0.1*M* tris (*p*H 7.0), from which tRNA was precipitated with two volumes of ethanol at −20°C and washed twice with 95 percent ethanol. The precipitated RNA was dried under vacuum and dissolved in 0.15 ml of gel electrophoresis buffer (see Fig. 1). The density of the solution was then increased by adding 0.05 ml of 40 percent sucrose containing 0.02 percent bromophenol blue as running marker. After carefully layering the sample under buffer atop the polyacrylamide gel, electrophoresis was carried out (Fig. 1). A band of pure $tRNA^{Leu}_3$, well separated from both 5*S* RNA and 4*S* RNA, yielded 40×10^6 count/min after extraction from the gel (*5*). This material was used in the sequence determination of $tRNA^{Leu}_3$ (*6*).

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Nucleotide Sequence of the "Denaturable" Leucine Transfer RNA from Yeast

Abstract. *The nucleotide sequence of "denaturable" leucine acceptor transfer RNA ($tRNA^{Leu}_3$) from baker's yeast was determined on ^{32}P-labeled material. The molecule is 85 nucleotides long and can be folded into the "cloverleaf" model for secondary structure. The basis on which the sequence was deduced from the products of complete enzymatic digestion, prior to its unambiguous determination, is presented.*

We report here the nucleotide sequence of $tRNA^{Leu}_3$ (*1*) from baker's yeast (*Saccharomyces cerevisiae*). This organism contains at least three leucine tRNA's, of which $tRNA^{Leu}_3$ is the major species, comprising some 40 percent of the acceptor activity for this amino acid (*2*). This tRNA can be trapped in a metastable denatured state whose biological and physical properties differ significantly from those of the native form (*2–5*). This denaturability has, in fact, been exploited in its purification (*6, 7*). Because of its unusual conformational properties, the sequence of this tRNA is of particular interest.

We determined the sequence with ^{32}P-labeled RNA (*7*), using techniques developed by Sanger *et al.* (*8*). Complete T_1 and pancreatic ribonuclease digests were fractionated (Figs. 1 and 2), and the separated oligonucleotides were sequenced. Larger oligonucleotides produced by partial T_1 ribonuclease digestion were isolated by homochromatography (*9*) and identified on the basis of the products of their complete hy-

Table 1. General features of tRNA structure.

Primary structure (*10*)	Secondary structure (*11*)
1. $-CCA_{OH}3'$ terminus	1. Invariant arrangement of helical regions relative to each other
2. 5′-Phosphate terminus	2. Constant length of stem helical region
3. GTψC sequence starting 24 residues from 3′ terminus	3. Constant size of anticodon loop and supporting helical region
4. Recurrence of either purines or of pyrimidines at homologous sites	4. Constant size of TψC loop and supporting helical region
5. Recurrence of certain nucleotides or modified analogs at homologous sites	5. Dihydro-U containing loop of variable size supported by helical region of 3 to 4 base pairs
	6. Accommodation of additional residues in segment of variable size and helical content between anticodon and TψC arms

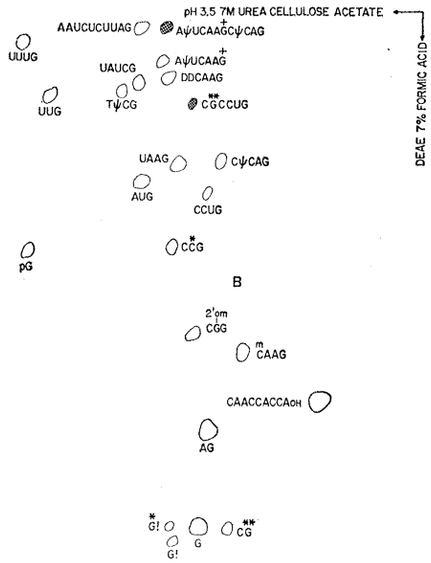

Fig. 1. Two-dimensional fractionation of a T_1 ribonuclease digest of $tRNA^{Leu}_3$. Diagram shows positions and sequences of the fragments. *B* is the blue marker. The fragment CC*G is located where T_1 oligonucleotides containing one U residue occur. Alkaline hydrolysis of this spot and subsequent electrophoresis at *p*H 3.5 showed one G, two C's, and no U. Digestion with T_2 or pancreatic ribonuclease gave one G, one C, and a spot slightly slower than U. Hence, the alkali-labile C* is probably an acylated C derivative, possibly 4-acetyl-C, which occurs at the homologous position in $tRNA^{Ser}$ from yeast (*21*) and $tRNA^{Ser}$ from rat liver (*22*). The fragments shown by cross-hatching are products of incomplete hydrolysis under the conditions used. The ratio of enzyme to substrate was 1 : 2; hydrolysis was for 30 minutes at 37°C.

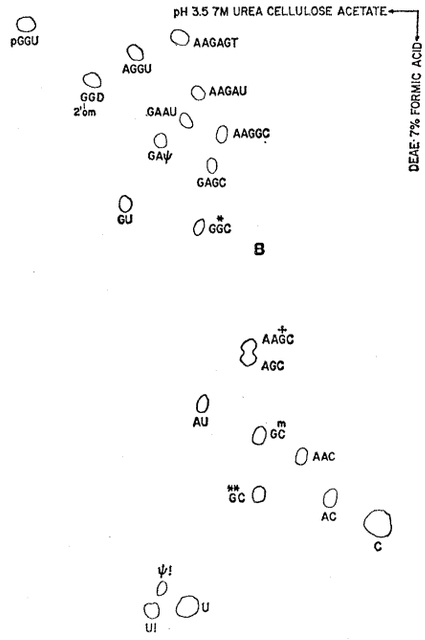

Fig. 2. Two-dimensional fractionation of a pancreatic ribonuclease digest of $tRNA^{Leu}_3$. Diagram shows positions and sequences of the fragments.

drolysis with T_1 and pancreatic ribonucleases.

By taking into account the constant primary structural features of tRNA that have emerged from established sequences (*10*) (Table 1), the characteristics of the "cloverleaf" model for tRNA secondary structure that have also become evident (*11*) (Table 1), and the previously determined coding properties of $tRNA^{Leu}_3$ (*4*), it was possible to assemble the products of complete digestion by T_1 and pancreatic ribonucleases into the sequence shown in Fig. 3. This deduced primary structure was fully confirmed by overlaps provided by partial digestion products (Fig. 4).

Several features of the sequence are worthy of note. The chain length of 85 nucleotides, with which the hydrodynamically determined molecular weight (*5*) is in excellent agreement, puts $tRNA^{Leu}_3$ at the upper end of the molecular weight distribution for tRNA's (*12*). A recently sequenced *Escherichia coli* $tRNA^{Leu}$ (*13*), which has a different anticodon and no significant sequence similarities, contains

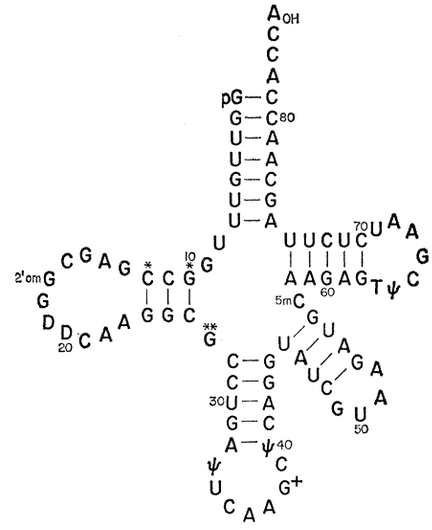

Fig. 3. Nucleotide sequence of $tRNA^{Leu}_3$ folded into the "cloverleaf" pattern. On the basis of electrophoretic and chromatographic mobilities and by homology with other tRNA's, G* is tentatively identified as 2-methyl-G, and G** as 2-dimethyl-G. G^+ may be 1-methyl-G, a residue found at the homologous position in $tRNA^{Asp}$ from brewer's yeast (*23*) and possibly also in a leucine tRNA from *E. coli.* (*13*). C* is probably an acylated C derivative (see Fig. 1).

87 residues. The low mobilities of both these tRNA's on polyacrylamide gel electrophoresis (*7, 13*) as compared to those of other tRNA's is undoubtedly a reflection of their larger size.

In comparison with other tRNA sequences folded in the "cloverleaf" pattern for secondary structure, $tRNA^{Leu}_3$ has an unusually high proportion of A·U base pairs. The TψC loop is identical in its *entirety* to that of $tRNA^{Tyr}$ (*14*) and $tRNA^{Met}_M$ (*15*), both from *E. coli.* This identity emphasizes that any function associated with this loop is common to all tRNA's. In view of the proposal that the first three base pairs in the stem constitute the recognition site for aminoacyl-tRNA synthetases (*16*), it is interesting that the residues at these positions in $tRNA^{Leu}_3$ are identical to those of $tRNA^{Tyr}$ (*E. coli*) (*14*) and $tRNA^{Ile}$ (*Torula utilis*) (*17*).

The CAA anticodon established for $tRNA^{Leu}_3$ is consistent with its previously determined coding properties (*4*).

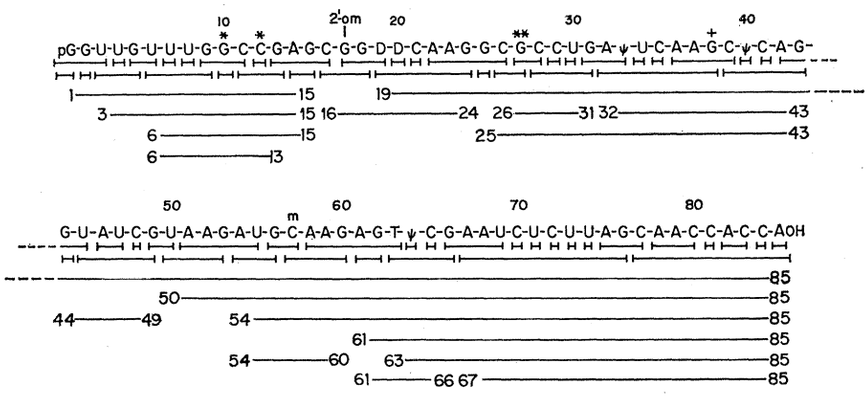

Fig. 4. Overlaps used in establishing the sequence of $tRNA^{Leu}_3$. The first two lines show fragments from complete pancreatic and T_1 ribonuclease digestion. Fragments from partial T_1 ribonuclease digestion (10 minutes at 0°C with enzyme-substrate ratios of 1 : 400 and 1 : 1500) are shown below.

Residue 38, being susceptible to T_1 ribonuclease, is presumably a G (or I) derivative. Its occurrence adjacent to the anticodon is an exception to the proposal that tRNA's whose code words begin with U have a modified A at this position (*18*).

Finally, that it was possible to assemble the fragments from T_1 and pancreatic ribonuclease digests into the correct sequence by means of regularities that have emerged from known tRNA sequences emphasizes not only the essential correctness of these generalities, but also the extent to which they reflect functional requirements (*19*). The similarities among tRNA sequences suggest not only a common evolutionary origin, but also a severely restricted tolerance to mutation. This limited tolerance implies restraints which must derive from the integration of multiple functions into one molecule, with a resultant well-defined tertiary structure: hence, the close relation between tRNA functions and native tertiary structure (*3*), and the marked topographical similarity among tRNA's (*20*).

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References and Notes

1. Abbreviations used: tRNA, transfer ribonucleic acid; $tRNA^{Leu}_3$, "denaturable" leucine acceptor tRNA; A, adenosine; C, cytidine; G, guanosine; U, uridine; D, dihydrouridine; I, inosine; ψ, pseudouridine; T, ribothymidine; mC, 5-methylcytidine; 2′omG, 2′,*O*-methylguanosine; p, on the left (of G) indicates a 5′-phosphate on that residue; OH on the right as in A_{OH} indicates the absence of a 3′-phosphate, that is, a free 3′-hydroxyl group; $tRNA^{Met}$, $tRNA^{Tyr}$, $tRNA^{Ile}$, methionine, tyrosine, and isoleucine accepting tRNA, respectively.
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Striated Muscle Fibers: Facilitation of Contraction at Short Lengths by Caffeine

Abstract. *One of the factors evidently responsible for decreasing the force of muscle contraction with shortening is inactivation of the myofibrils in the core of a muscle fiber. Caffeine antagonizes this inactivation and, correspondingly, changes the length-force relationship at short muscle lengths.*

The relationship between the length of a frog striated muscle fiber and the force it can actively produce shows a plateau of maximum force at striation spacings of 2.0 to 2.2 μm (*1*). As the length is increased above 2.2 μm, force drops linearly toward zero at 3.65 μm (Fig. 1). As explained by the sliding filament theory (*2*), this results from a decrease in overlap between thick and thin filaments. On the other side of the plateau, force decreases to zero at 1.3 μm. One of the reasons for this decrease is that the degree of activation evidently becomes less as a muscle shortens, for during shortening initiated by membrane depolarization the myofibrils in the core of a fiber become wavy, showing that their activation has been inhibited (*3*). To determine the degree to which this factor influences the length-force relationship at short striation spacings, we observed the effect of caffeine, which is known to facilitate activation (*4*), on contractions of frog skeletal muscle.

Single muscle fibers, isolated from the semitendinosus muscle of *Rana temporaria*, were mounted horizontally in a trough filled with Ringer solution sitting on the stage of an ordinary light microscope. Their tendons were fastened to steel hooks, one of which was stationary, the other attached to an RCA 5734 force transducer. The latter was mounted on a rack and pinion that was used to vary the fiber length. For contractions below slack length (about 2.0 μm) the fiber was allowed to hang in a parabola between the hooks. On stimulation it took up the slack until it had reached the predetermined striation spacing before producing force isometrically. Cine micrographs (Fig. 2) were taken while simultaneously force was being measured. Striation spacings were, in general, measured on enlargements made from the cine film. Occasionally, however, striations could not be unequivocally resolved at lengths below about 1.3 μm, and then we calculated the final length

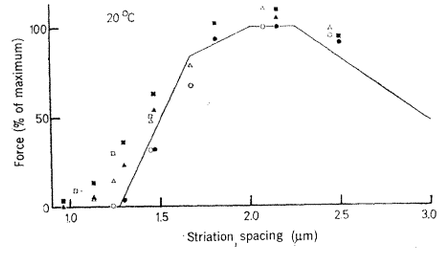

Fig. 1. The influence of caffeine upon the length-force relationship of isolated muscle fibers. Force, on the ordinate, is the maximum amount recorded during contractions elicited by tetanic stimulation (77 hertz) at a particular striation spacing. The open and filled symbols refer to two single fiber preparations. Circles, triangles, and squares are the responses in 0, 2, and 3 m*M* caffeine Ringer solution, respectively. The solid line is a summary of the data taken from Gordon *et al.* (*1*), for comparison with our control responses.