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Rare Transfer Ribonucleic Acid Essential for Phage Growth. Nucleotide Sequence Comparison of Normal and Mutant T4 Isoleucine-Accepting Transfer Ribonucleic Acid†

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ABSTRACT: One of the eight tRNA species coded by bacteriophage T4 is unique in that (1) it is found in a yield lower by three- to fourfold than that of any other tRNA and (2) while dispensable for growth in standard laboratory hosts, it is essential for phage propagation in a natural isolate of *Escherichia coli* (strain CT439). We report here the nucleotide sequence of this tRNA and of several mutationally altered forms. The molecule is 77 nucleotides in length and has the anticodon N-A-U. Depending on the pairing properties of the "wobble" nucleotide N, this sequence could correspond to one or more of the isoleucine-specific codons A-U-$\begin{matrix}U\\A\end{matrix}$ or to the methionine-specific codon A-U-G. Since a T4-specific acceptor activity for isoleucine which is stimulated in ribosome binding by A-U-A but not A-U-U has been reported previously, we infer that we have sequenced a $tRNA^{Ile}$ species which preferentially recognizes A-U-A. Mutant HA1 is unable to grow in CT439; it produces no $tRNA^{Ile}$. The primary mutational alteration is a transition four residues from the 5′ terminus which converts a C·G to a U·G base pair. The consequences of this lesion can be partially reversed by second-site mutations nearby in the acceptor stem. Unexpectedly, the $tRNA^{Ile}$ synthesized in these revertants still retains two unusual structural features found in the wild-type molecule: the opposition of two Up residues in the amino acid acceptor stem and the opposition of an Ap and a Gp residue in the anticodon stem. Implications of these structural anomalies for a possibly unique physiological role of this minor tRNA species are discussed.

Bacteriophage T4 codes for eight unique species of transfer RNA (McClain et al., 1972). In the past several years this phenomenon has been profitably exploited for the investigation of tRNA structure–function relationships and biosynthesis (Guthrie et al., 1974). Yet it remains unanswered why T4, which infects a host already containing a complement of tRNAs, should direct the synthesis of an additional population. Several years ago (Guthrie & McClain, 1973), we isolated a set of T4 point mutants which are viable on standard laboratory hosts but unable to grow on a strain of *Escherichia coli* (CT439) recently isolated from nature. These point mutants define five complementation groups, two of which

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affect the production of T4 tRNAs. One group defines the structural gene for a single tRNA species, previously designated δ. Mutant HA1 produces none of this species, while revertants of HA1 which have regained the ability to grow on CT439 were shown to make this tRNA in amounts correlated with their efficiency of growth on this host.

We have now determined the nucleotide sequence of this tRNA. The molecule is 77 nucleotides in length and can be arranged in a cloverleaf configuration with the anticodon N-A-U. Np is an unidentified modified nucleotide. Depending on the pairing properties of the "wobble" nucleotide N, this anticodon could correspond to one or more codons specific for isoleucine A-U-$\begin{matrix}U\\A\end{matrix}$ or methionine (A-U-G). Several lines of evidence, including hybridization to T4 DNA of aminoacylated tRNAs from T4-infected cells (Scherberg & Weiss, 1970), have suggested that T4 codes for an isoleucine acceptor activity. The nucleotide sequences of the other seven T4 tRNAs have now been determined and include species specific for serine (McClain et al., 1975), proline (Seidman et al., 1975), glutamine (Seidman et al., 1974), leucine (Pinkerton et al., 1973), glycine (Barrell et al., 1973; Stahl et al., 1974), arginine (Mazzara et al., 1977), and threonine (Guthrie et al., 1978). We thus conclude that the molecule we have sequenced corresponds to the acceptor activity identified by Scherberg & Weiss (1970) and infer that it is a tRNA^{Ile}.

Even in wild-type T4 infections of standard hosts, tRNA^{Ile} comprises a minor species relative to the other seven T4 tRNAs (McClain et al., 1972). It is present in only 20–30% the amount of tRNA^{Thr}, with which it is processed from a common dimeric precursor (Guthrie et al., 1973; Guthrie and Scholla, unpublished experiments). The determination reported here of the nucleotide sequence of tRNA^{Ile} and of several mutationally altered forms reveals several structural anomalies which may be related to the low yield of this tRNA and/or an unusual physiological role for this molecule.

Experimental Procedures

Isolation of ^{32}P-Labeled RNA. ^{32}P-Labeled low molecular weight RNA was isolated from *E. coli* strain B/5 infected with wild-type T4D or derivatives of this phage carrying various mutations in tRNA^{Ile}, and the tRNA^{Ile} was purified following two successive steps (10 and 20%) of polyacrylamide gel electrophoresis, all as described previously (Guthrie & McClain, 1973). Isolation and characterization of the T4 strains are detailed elsewhere (Guthrie & McClain, 1973).

Nucleotide Sequence Analysis. In general, the techniques, methods, and materials used were those described in detail by Barrell (1971). Analysis of modified nucleotides following complete digestion with RNase T_2 was as described and reported previously (Guthrie & McClain, 1973). Reactions with the water-soluble carbodiimide CMCT[1] and product analysis were performed as detailed elsewhere (Seidman et al., 1974). Conditions of digestion with silkworm nuclease and fractionation and analysis of these products are detailed in the legend to Table II. Limited digests of tRNA^{Ile} to obtain partial products, as shown in Figure 4, were performed with an enzyme (RNase A or T_1) to substrate ratio of 1:2500 in the presence of 20 mM $MgCl_2$ for 8 min at 0 °C. Products were fractionated by electrophoresis on cellulose acetate at pH 3.5 in the first dimension and by homochromatography on DEAE plates in the second dimension.

Digestion of tRNA^{Ile} with S1 Nuclease. Approximately 3 × 10^6 cpm of tRNA^{Ile} together with 20 μg of carrier tRNA was digested with 10 units of S1 nuclease from *Aspergillus crysae* prepared essentially according to the method of Vogt (1973). Conditions of digestion and fractionation of products were as described elsewhere (Manale et al., 1979) except that incubation was carried out for 2 h at 15 °C.

Results

Sequence Analysis of T4 tRNA^{Ile}. ^{32}P-Labeled tRNA^{Ile} can be purified to apparent homogeneity by two successive steps of polyacrylamide gel electrophoresis of low molecular weight RNA from T4-infected wild-type cells [McClain et al. (1972); Plates I and III of Guthrie & McClain (1973)]. After elution, tRNA^{Ile} is approximately 90% pure and was used as such for sequence analysis.

(*a*) *Ribonuclease T_1 Products.* The pattern of oligonucleotides generated by digestion of tRNA^{Ile} with RNase T_1 is shown in Figure 1A. Each product was quantitated, and its composition was determined by alkaline hydrolysis as reported in Table I. The identity of minor nucleotides was established by fractionation of RNase T_2 digestion products by two-dimensional thin-layer chromatography. All RNase T_1 oligonucleotides were further characterized by analysis of RNase A digestion products. This information, together with the electrophoretic mobility, allowed the determination of the sequences of T1, T3, T4, T5, T6 and T8. Sequence analysis of the larger oligonucleotides is described in Tables I and II and is summarized below.

T2 (U-C-A-C-C-A_{OH}). Digestion with RNase A gave the products A-Cp(1), Cp(2), and Up(1), while RNase U_2 products were (Up,Cp)Ap and C-C-A_{OH}. The latter sequence was established by alkaline hydrolysis, which yielded Cp, and digestion with snake venom phosphodiesterase, which gave pC(1):pA(1). This yielded the partial sequence (Up,Cp)A-C-C-A_{OH}. The order was established by obtaining the product U-Cp upon RNase A digestion of CMCT-modified U-C-Ap.

T7 (C-C-C-U-Gp). The composition Cp(3),Up(1),Gp was determined by alkaline hydrolysis. The CMCT product U-Gp established the sequence, which was supported by analysis of the products of limited digestion with the 5′-exonuclease spleen phosphodiesterase.

T10 (m^7G-U-U-A-C-C-A-Gp). Digestion with RNase A gave the products m^7G-Up, Up, A-Cp, Cp, and A-Gp. RNase U_2 products were (m^7G-Up,Up)Ap, C-C-Ap, and Gp. This yielded the partial sequence (m^7G-Up,Up)A-C-C-A-Gp. The order of m^7G-Up and Up was established by the sequence analysis of the RNase A oligonucleotide containing this modified base (see P16 and P16′ below).

T11 (C-U-C-A-A-U-Gp) and T11′ (C-U-C-A-A-U-Gm$^{2'}$-Gp). Oligonucleotide T11 apparently arises as a result of undermethylation of the Gm$^{2'}$ p residue of T11′, which allows cleavage by RNase T_1. Digestion with RNase A gave the products A-A-Up, Cp(2), Up(1), and Gp(T11) or Gm$^{2'}$-Gp (T11′). After RNase U_2 treatment, the products identified were [Cp(2),Up]Ap, Ap, and U-Gp or U-Gm$^{2'}$-Gp. Limited digestion of [Cp(2),Up]Ap with spleen phosphodiesterase gave the products C-U-C-Ap, U-C-Ap, and C-Ap. These results thus yielded the complete sequence C-U-C-A-A-U-Gm$^{2'}$-Gp.

T12 (T-Ψ-C-A-A-A-U-C-U-Gp). The sequence determination of this oligonucleotide is detailed in Table II. Digestion with RNase A gave the products A-A-A-Up, Cp(2–3), Tp + Ψp + Up(4–5), and Gp. Two products plus free Ap were

[1] Abbreviation used: CMCT, *N*-cyclohexyl-*N*′-(β-morpholin-4-yl-ethyl)carbodiimide–methyl *p*-toluenesulfonate.

Table I: Sequence Determination and Molar Yields of RNase T_1 Oligonucleotides of $tRNA^{Ile}$

oligo-nucleotide[a]	sequence	molar yield: exptl	molar yield: theo-retical	composition[b]: C	A	G	U	other	products of digestion with[c]: RNase A	RNase U_2	CMCT + RNase A	other
T1	Gp	5.97	6			+						
T2	U-C-A-C-C-A_{OH}	0.87	1	3.1	1.1		1		A-Cp(1.2), Cp(2.6), Up(1)	(Up,Cp)Ap, C-C-A_{OH}	U-Cp, A-Cp	
T3	C-A-Gp	1.93	2	1.6	1.1	1			A-Gp(1), Cp(1.3)	C-Ap, Gp		
T4	A-A-A-Gp	0.83	1		3.4	1			A-A-A-Gp			
T5	pGp	0.6	1					pGp				
T6	U-A-Gp	0.93	1		0.8	1	1.0		A-Gp(1), Up(1.4)	U-Ap, Gp		
T7	C-C-C-U-Gp	0.97	1	3.1		1	1.1				U-Gp, Cp	SP'ase:[d] C-Cp(Cp,Up)Gp Cp(Cp,Up)Gp
T8	D-D-A-Gp	0.99	1		0.9	1		Dp(2.8)	A-Gp(1), Dp(2.2)			
T9	U-C-U-Gp	(1.0)	1	1.3		1	2.3		Up(2), Cp(1), Gp(1)		U-Cp, U-Gp	SP'ase:[d] U-C-U-Gp C-U-Gp
T10	m^7G-U-U-A-C-C-A-Gp	0.9	1	2.1	1.8	1	2.2	m^7Gp(0.9)	m^7G-Up(0.9), Up(1.7), A-Cp(1.4), Cp(0.5), A-Gp(1)	(m^7G-Up,Up)Ap, C-C-Ap, Gp		
T11	C-U-C-A-A-U-Gp	0.56	1	2.8	2.8	1	2.6		A-A-Up(1.2), Cp(2.4), Up(1.1), Gp(1)	C-U-C-Ap, U-Gp	U-Cp, A-A-U-Gp, Cp	(Cp,Up)C-A-A-U-Gp C-A-A-U-Gp A-A-U-Gp A-U-Gp
T11′	C-U-C-A-A-U-$Gm^{2'}$-Gp	0.4		3.0	3.3		2.8	$Gm^{2'}$-Gp(1)	A-A-Up(1.4), Cp(1.9), Up(1.3), $Gm^{2'}$-Gp(1)	U-$Gm^{2'}$-Gp, (Cp,Up,Cp)Ap		(Cp,Up,Cp)A-A-U-$Gm^{2'}$-Gp A-A-U-$Gm^{2'}$-Gp A-U-$Gm^{2'}$-Gp
T12[e]	T-Ψ-C-A-A-A-U-C-U-Gp	0.93	1	2.7	3.6	1		Up + Tp + Ψp(4.7)	A-A-A-Up(1.1), Up(4.5), Cp(3.2), Gp(1)	Tp(Ψp,Cp)Ap, Up(Cp,Up)Gp	U-Gp, (Tp,Ψp)Cp, A-A-A-U-Cp	
T13[f]	U-C-C-C-C-U-N-x-A-U-A-A-Gp	0.77	1	4.0	3.2	1	3.2	Np, Ap	A-A-Gp/A-A-Gp, (Np,Ap)Up, Cp, Up	U-N-Ap, U-A-Ap	U-Cp	

[a] Oligonucleotides are the digestion products shown in Figure 1A. The theoretical molar yield is based on the final sequence shown in Figure 5. The experimental molar yield was calculated by normalizing the radioactivity in each oligonucleotide to product T9, which was assumed to occur only once in the molecule. These calculations are the average of three tRNA preparations. [b] The composition of each oligonucleotide was determined by alkaline hydrolysis, followed by electrophoretic fractionation of the products. The molar yield of each nucleotide is given relative to Gp. In addition, each oligonucleotide was subjected to complete digestion with RNase T_2 and analyzed by two-dimensional thin-layer chromatography to allow identification of modified nucleotides. Chromatographic properties of these nucleotides have been reported in Table 7 and Plate IV of Guthrie & McClain (1973). [c] Sequence determination was as follows. *RNase A*. Figures in parentheses are the molar yields relative to the Gp-containing products. Products were identified by their electrophoretic mobility and composition as determined by alkaline hydrolysis. The mobility on DEAE paper at pH 3.5 of A-A-Gp relative to A-A-Gp (T13) is 2.8; the mobility of N-A-Up relative to xylene cyanol (R_B) is 0.95. *RNase U_2*. Products were characterized by their composition. The sequence C-U-C-Ap of T11 was determined by partial spleen phosphodiesterase digestion, yielding products C-U-C-Ap, U-C-Ap, and C-Ap. The partial sequence of Tp(Ψp,Cp)Ap (T12) was established by loss of the Tp residue after digestion of dephosphorylated material with snake venom phosphodiesterase. The partial sequence of Up(Cp,Up)Gp (T12) was determined by treatment with spleen phosphodiesterase. The sequence of U-N-Ap (T13) was determined following complete digestion with RNase A which yielded Up and the dinucleotide N-Ap. The sequence of U-A-Ap was established, following dephosphorylation, by comparison of products of alkaline hydrolysis (Ap,Up) with those of digestion with venom phosphodiesterase (pA,pA). *CMCT + RNase A*. Products were identified by their composition and in some cases (e.g., A-A-A-U-Cp of T12) by complete digestion with RNase A following removal of the CMCT blocking group. The presence of Tp and Ψp in T12 was verified by chromatography after complete digestion with RNase T_2. [d] SP'ase is spleen phosphodiesterase. Products were identified by their composition. [e] Oligonucleotide T12 frequently appears as a double spot in the fingerprint. No differences in the sequence or in the state or degree of modification have ever been detected; we suspect that the product migrating faster in the first dimension is a cyclic form. [f] Further sequence information on T13 is reported in Table II. The data reported here leave unordered (m^7G-Up,Up) for T10; see the text for the explanation.

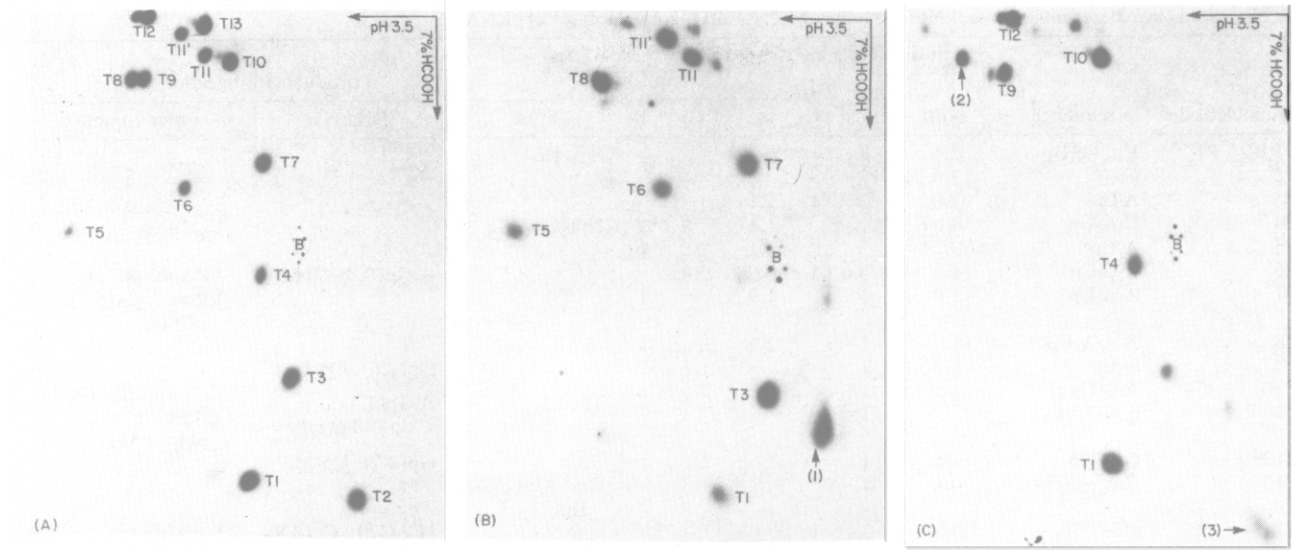


FIGURE 1: Products of RNase T_1 digestion of T4 tRNA^{Ile}. (A) Intact molecule; (B) the 5′ half of the tRNA; (C) the 3′ half of the tRNA. Products were fractionated on cellulose acetate, pH 3.5, in the first dimension and on DEAE paper with 7% HCOOH in the second dimension. Sequences of the T-labeled oligonucleotides are given in Table I. The half-molecules were generated by incubation of the tRNA with S1 nuclease, and the products were fractionated as shown in Figure 3. These oligonucleotides were quantitated and characterized following digestion with RNases A, T_2, and, in some cases, with venom phosphodiesterase. Both half-molecules essentially lack T13, consistent with its location spanning the anticodon (see text and Figure 5). The arrows indicate new oligonucleotides generated by S1 cleavage. Spot 1 is derived by internal cleavage of T13 and is tentatively identified as U-C-C-C-C-U-N-A-U_{OH}. Spot 2 appears to be the complementary half of this cleavage event since it has the sequence $\overset{x}{p}$A-A-Gp. Spot 3 is generated from the 3′-terminal oligonucleotide (T2) and is a mixture containing the sequences U-C-$\overset{x}{A}$-C_{OH} and U-C-A_{OH}.

Table II: Products of Digestion of T13 with Silkworm Nuclease[a]

U-C-C	
U-C-C-C	
U-C-C-C-C	
C-C-C - U	
C - U	
C-[U - N]-A	
U-[N - A]-U	
C-[U - N - A]-U	
	U-A-A - G
	A-A - G
C-[U - N - A -]U-A-A]-G	
U-C-C-C-C - U - N - A - U-A-A - Gp deduced sequence	

[a] RNase T_1 digestion product T13 was digested for 2 h at 37 °C with 0.2 unit of silkworm nuclease and 250 μg/mL bacterial alkaline phosphatase in 0.5 M bicarbonate, pH 10.5, 0.1 M NaCl, and 5×10^{-3} M $Mg(OAc)_2$. The sample was spotted on a strip of cellulose acetate and electrophoresed for 70 min at 5 kV at pH 3.5. The strip was then "blotted" onto DEAE paper and electrophoresed at pH 3.5 for 19 kV h. Products were localized by autoradiography, eluted, and subsequently digested with venom phosphodiesterase and RNase T_2, respectively. The resulting nucleotides were then quantitated by scintillation counting. This procedure usually allows unique determination of the 5′- and 3′-terminal nucleotides. Nucleotides within brackets cannot be ordered by this procedure. In many preparations, T13 resolves into two spots on the two-dimensional RNase T_1 fingerprint; this reflects the presence or absence of the $\overset{x}{A}$ modification 3′ to the anticodon (see Table I and Figure 5). The results reported here were obtained with the unmodified form of the oligonucleotide.

obtained following digestion with RNase U_2. One product gave Tp, Ψp, Cp, and Ap after hydrolysis with alkali; since Tp was not present after dephosphorylation followed by digestion with snake venom phosphodiesterase, the order must be Tp(Ψp,Cp)Ap. The second RNase U_2 product had the composition Up(2),Cp(1),Gp(1); analysis of products following limited digestion with spleen phosphodiesterase determined the order Up(Cp,Up)Gp. These results allow the partial sequence Tp(Ψp,Cp)A-A-A-U(Cp,Up)Gp. After CMCT modification, RNase A digestion products included (Tp,-Ψp)Cp, U-Gp, and (Ap,Ap,Ap,Up)Cp. The sequence of the latter product was determined by RNase A digestion of material which had been deblocked and gave the products A-A-A-Up and Cp. These operations established the sequence as T-Ψ-C-A-A-A-U-C-U-Gp.

T13 (*U-C-C-C-C-U-N-A-U-*$\overset{x}{A}$*-A-Gp*). This product could often be found in two positions in the fingerprint, apparently depending on the state of modification of $\overset{x}{A}$ (see below). The presence of the N residue was a great hindrance to sequence analysis; it is resistant to cleavage by nucleases and exhibits a strong tendency toward degradation, yielding very streaky products upon electrophoretic fractionation. As shown in Table II, alkaline hydrolysis yielded the composition Cp(4),Up-(3),Ap(3),Np(1),Gp(1). Digestion with RNase A gave two products in addition to Up and Cp. One had the partial sequence (Np,Ap)Up. The second varied in electrophoretic mobility; in the absence of modification, the position and composition gave the sequence A-A-Gp. When the Ap residue is modified, this product has a faster mobility on DEAE paper at pH 3.5 and has the sequence $(\overset{x}{A}p,Ap)Gp$. Digestion of T13 with RNase A following CMCT modification gave, in addition to an unresolved smear, the discrete product U-Cp. Analysis of RNase U_2 digestion products was also complicated by the presence of the modified N residue, with the apparent production of internal cleavage products. However, several of these anomalous products proved useful for the sequence determination. One such product had the composition, from alkaline hydrolysis, Up,Np,Ap. After RNase A treatment of the latter product, we obtained N-Ap and Up, yielding the sequence U-N-Ap. A second RNase U_2 product had the composition $\overset{x}{U}p,Ap,Ap$. This product was dephosphorylated with alkaline phosphatase and subsequently analyzed after either alkaline hydrolysis, which gave $\overset{x}{A}p$ and Up, or digestion with snake venom phosphodiesterase, which yielded pA and

Table III: Sequence Determination and Molar Yields of RNase A Oligonucleotides of $tRNA^{Ile}$

oligonucleotide[a]	sequence	molar yield: exptl	molar yield: theoretical	composition[b]: C	A	G	U	other	products of digestion with: RNase T_1	other enzymes
P1, P1′, P1″[c]	Up,Ψp,Dp	7.6	8				+	Ψp, Dp		
P2	Cp	12.0	12	+						
P3	A-Cp	2.0	2	1	1.1					
P4	N-A-Up	0.6	1		1.1		1	Np(0.3)		U_2:N-Ap, Up
P5	A-Up[e]	0.7			1.1		1			
P6	A-G-Cp	3.0	3	1	0.9	0.8			A-Gp(0.8), Cp(1)	VP′ase: pC, pG
P7	A-A-Up	(1.0)	1		1.9		1			VP′ase: pA(0.7), pU(1)
P8	A-A-A-Up	(1.0)	1		2.4		1			
P9	G-Up	1.3	1			1.1	1		Gp(1.6), Up(1)	
P10	A-G-Up	2.5	1		2.1	2.1	1	Tp(0.8)	A-Gp(1.1), Up + Tp(1)	T_2: Ap(1.0), Gp(1) VP′ase: pG(1.0), pU + pT(1)
P10′[c]	A-G-Tp		1							
P12[d]	G-G-Up	1.5	1			2.1	1		Gp(2.2), Up(1)	
P13	$Gm^{2'}$-G-Dp	0.4	1					$Gm^{2'}$-Gp(1.0), Dp(1)		
P14	pG-G-Cp	0.9	1	1		1.4		pGp(1.8)	pGp(1.0), Cp(0.8), Gp(1)	T_2: Gp VP′ase: pG(0.7), pC(1)
P15	G-G-G-Up	(1.0)	1			3.1	1		Gp(2.8), Up(1)	
P16	A-A-G-G-G-A-A-A-G-m^7G-Up[f]	0.7	1		5.2	4.6	1	m^7Gp		
P16′	N-A-U-A-A-G-G-G-A-A-A-G-m^7G-Up[f]	0.3			3.2	3.0		Ap, m^7Gp		

[a] Oligonucleotides are the digestion products shown in Figure 2A. The theoretical molar yield is based on the final sequence shown in Figure 5. The experimental molar yield was calculated relative to the average yields of products P7, P8, and P15, each of which were assumed to occur only once in the molecule. These calculations are the average of two tRNA preparations. [b] Composition was determined as described in the legend to Table I. RNase T_1 products were identified by their nucleotide composition and electrophoretic mobility. The molar yields in parentheses are based on the pyrimidine-containing product. Products of digestion with RNase U_2 were identified by their composition. Prior to digestion with RNase T_2 or venom phosphodiesterase (VP′ase), oligonucleotides were treated with bacterial alkaline phosphatase. [c] P1, P1′, P1″ and P10, P10′, respectively, are not resolved in the two-dimensional fingerprint. [d] Undermodification of P13 results in an increased molar yield of P12. [e] The sequence A-Up (P5) is not present in the final sequence shown in Figure 4; it may be derived from P4 when the N residue is not modified or is cleaved by RNase A. [f] The sequence determination of P16 and P16′ is reported in Table IV.

pA; this defines the sequence U-A-Ap. The combined results allow the partial sequence (U-Cp,Cp,Cp,Cp)U-N-A-U-A-A-Gp.

The final determination of the sequence was established by use of a unique nuclease isolated from the silkworm *Bombyx mori* (Mukai, 1972; Funaguma & Mukai, 1973). This enzyme exhibits a fairly random endonucleolytic activity, generating products on the average of four to six nucleotides in length. The nuclease generates 3′ hydroxyls and 5′ phosphates. When used together with bacterial alkaline phosphatase, useful sequence information of the tetra-, penta-, and hexamers is derived by comparing the products of alkaline hydrolysis with those yielded following digestion with snake venom phosphodiesterase. In this way the 5′- and 3′-terminal residues of the oligonucleotide can usually be uniquely determined. Selected products of such a digestion are shown in Table II. These results confirm the partial sequence suggested above and establish the order as U-C-C-C-C-Up. Thus, the complete sequence of T13 was determined to be U-C-C-C-C-U-N-A-U-A-A-Gp.

(*b*) *Ribonuclease A Products.* The tRNA was also subjected to complete digestion with RNase A, as shown in Figure 2A. The molar yield and composition of each product were determined as described in section a above. As detailed in Table III, the composition, electrophoretic mobility, and spectrum of digestion products obtained with RNase T_1 and other enzymes allowed the sequence determination of all of the oligonucleotides with the exception of one.

P16 (A-A-G-G-G-A-A-A-G-m^7G-Up) and P16′ (N-A-U-A-A-G-G-G-A-A-A-G-m^7G-Up). This oligonucleotide could be found in two positions in the fingerprint, apparently depending on the state of modification of the A residue (see below). Most of the sequence determination was performed on the unmodified oligonucleotide, P16; the analyses of both P16 and P16′ are outlined in Table IV. The composition determined from alkaline hydrolysis was Ap(5),Gp(4-5),m^7Gp(1),Up(1). The products of RNase T_1 digestion were A-A-A-Gp, A-A-Gp, m^7G-Up, and Gp. Limited digestion with RNase T_1 gave a product that on complete RNase T_1 digestion yielded A-A-A-Gp and m^7G-Up, suggesting that the majority of the Gp residues are located in the 5′ portion of the oligonucleotide. From these results, the partial sequence (A-A-G,Gp,Gp)A-A-A-G-m^7G-Up can be inferred. (It should be noted that this information also defines the sequence of RNase T_1 product T10 as m^7G-U-U-A-C-C-A-Gp.)

The remainder of the sequence was established by an analysis of products resulting from digestion with silkworm nuclease plus bacterial alkaline phosphatase, as described above and in Table IV. The crucial products for the determination were G-A-A-A, which confirms the presence of the RNase T_1 product A-A-A-Gp at the 3′ rather than the 5′ end of the oligonucleotide, and G-G-G-A, which confirms that the free Gp residues generated by RNase T_1 are located in the internal portion of the oligonucleotide.

Oligonucleotide P16′ differs from P16 in that digestion with RNase T_1 produces no A-A-Gp but instead yields a major

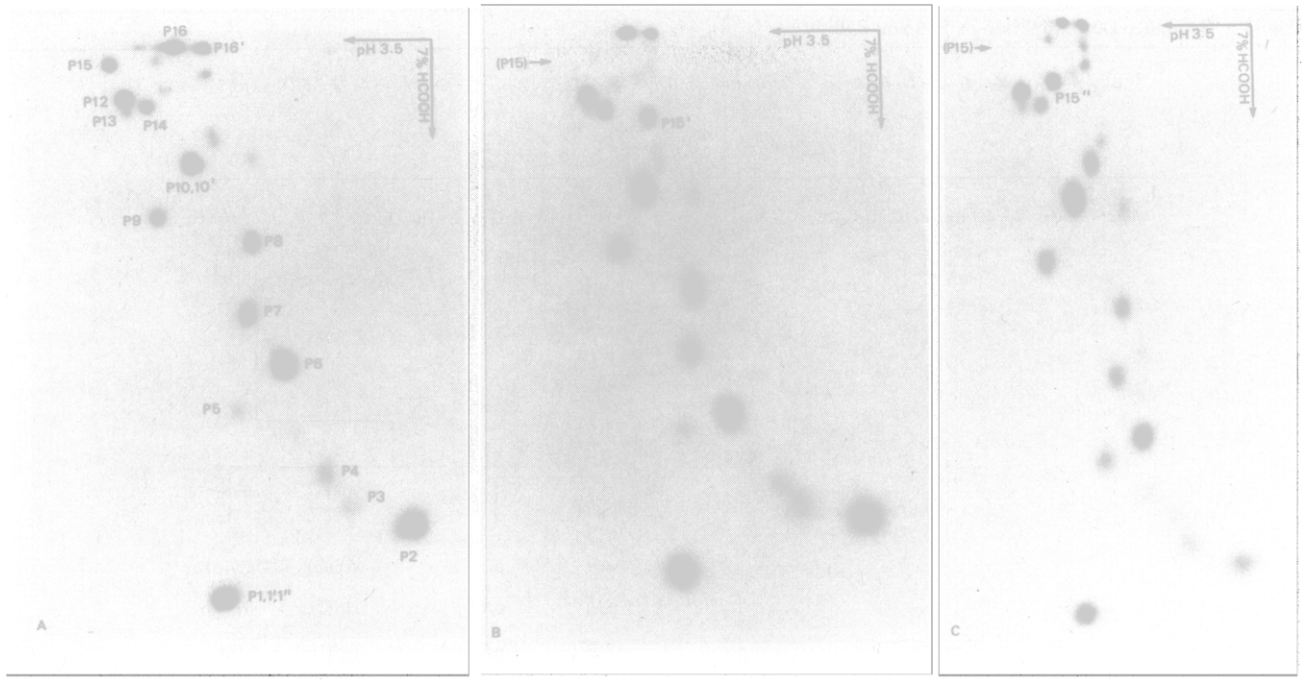


FIGURE 2: Products of RNase A digestion of tRNA^{Ile} from T4 (A) and HA1 revertants HA101 (B) and HA103 (C). Products were fractionated as described in Figure 1. Sequences of the P-labeled oligonucleotides in (A) are given in Table III. Oligonucleotides relevant to the determination of mutant changes are indicated in (B) and (C); the sequence analyses are reported in Table V and discussed in the text.

FIGURE 3: Autoradiograph of 20% polyacrylamide gel electrophoresis of tRNA^{Ile} digested with the single-strand specific S1 nuclease as described under Experimental Procedures. Lane a contains undigested material. The identification of the fragments was based on the analyses shown in Figure 1B,C.

product with the composition Ap(3),Up(1),Np(1),Gp(1). The loss of the Gp residue after treatment with alkaline phosphatase, followed by alkaline hydrolysis, confirms the location of Gp at the 3′ terminus of this product. Digestion with RNase U_2 gave the products N-Ap, (Up,Ap)Ap, and Gp. From these results, together with information from the analysis of P16 and RNase T_1 product T13, the sequence of this oligonucleotide can be inferred as N-A-U-A-A-Gp. Thus, the complete sequence of P16′ would be N-A-U-A-A-G-G-G-A-A-A-G-m^7G-Up. This was corroborated by an analysis of silkworm nuclease digestion products (data not shown). Oligonucleotide P16′ presumably arises because the Ap residue inhibits RNase A cleavage of the neighboring Up residue.

(*c*) *Modified Nucleotides.* These were characterized previously (Guthrie & McClain, 1973). All residues could be correlated with standard modified nucleotides, except residues Np and Ap. We have not been able to determine the identity of the parent residue of Np.

(*d*) *Determination of Sequence Overlaps.* In order to establish the relative order of RNase A and T_1 oligonucleotides in the molecule, we subjected tRNA^{Ile} to limited digestion with either RNase A or RNase T_1, and the products were fractionated by electrophoresis on cellulose acetate and by homochromatography. The products were then analyzed after complete digestion with RNases T_1 and A, respectively. In addition, tRNA^{Ile} was also digested with S1 nuclease. This single-strand specific enzyme has been shown by Harada & Dahlberg (1975) to attack tRNAs at the 3′ terminus and endonucleolytically in the anticodon loop, cleaving the molecule into two fragments. The two half-molecules were separated on a gel of 20% polyacrylamide as shown in Figure 3 and

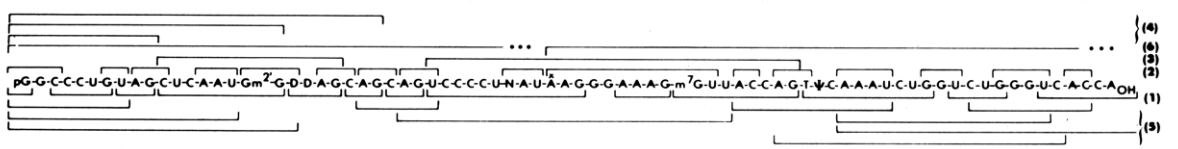
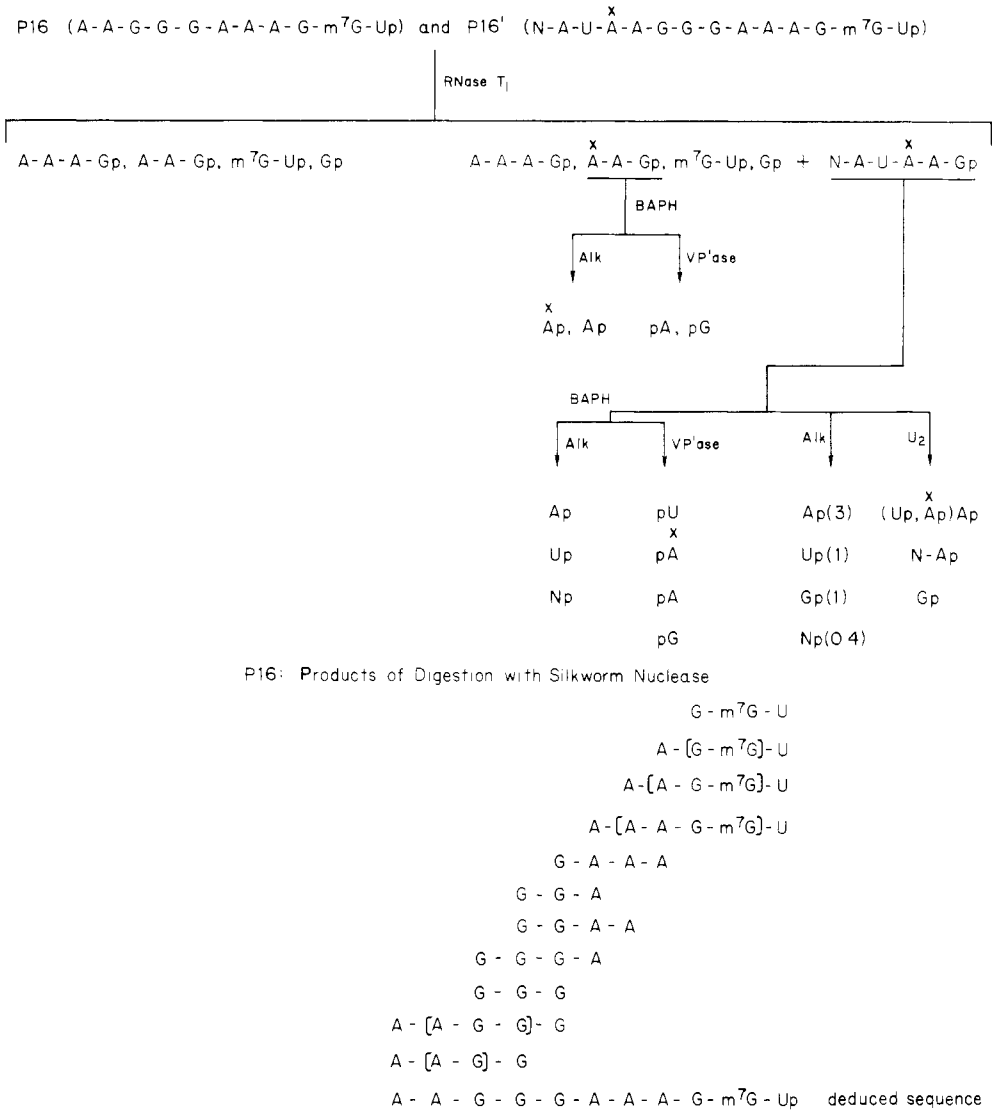


FIGURE 4: Nucleotide sequence of tRNA^{Ile} showing digestion products used to determine the linear sequence. Indicated products were obtained by (1) complete digestion with RNase T_1, (2) complete digestion with RNase A, (4) limited digestion with RNase T_1, (5) limited digestion with RNase A, and (6) digestion with S1 nuclease. The dotted lines extending from the solid lines in (6) indicate heterogeneity. Products indicated in (3) are overlaps generated from consideration of products obtained in (1) and (2).

Table IV: Sequence Determination of P16 and P16′[a]



[a] RNase A oligonucleotides P16 and P16′ were digested with RNase T_1, and the resultant digestion products were characterized by their composition and electrophoretic mobility. A-A-Gp of P16 is replaced in P16′ by X-A-A-Gp, which was identified (after dephosphorylation with bacterial alkaline phosphatase) by alkaline hydrolysis (alk) and treatment with venom phosphodiesterase, respectively. In addition, RNase T_1 digestion of P16′ yields N-A-U-X-A-A-Gp (R_B = 0.03), which was characterized by digestion with RNase U_2 and by alkaline hydrolysis before and after dephosphorylation. P16 was subjected to digestion with a mixture of silkworm nuclease and alkaline phosphatase as described in the legend to Table II. Selected products are shown.

subsequently digested with RNase T_1. Products of this digestion are shown in Figures 1B and 1C and were analyzed further as described in the legend to Figure 1.

Figure 4 shows the sequences of the partial products used to deduce the final structure. The sequence of $tRNA^{Ile}$ is presented in the cloverleaf configuration in Figure 5.

Characterization of $tRNA^{Ile}$ Mutants. As mentioned above, we have previously (Guthrie & McClain, 1973) isolated mutants of T4 which are viable on standard laboratory hosts (e.g., *E. coli* strain B) but are unable to grow on a strain of *E. coli* isolated from nature, CT439. These T4 mutants define five complementation groups, which have been analyzed with respect to their tRNA production in permissive infections. Mutant HA1, which arose after hydroxylamine mutagenesis, is a member of group I and synthesizes no detectable amounts of $tRNA^{Ile}$, while production of the other seven T4-coded tRNA species appears normal. Revertants of HA1 which have regained at least partial ability to grow on the restrictive host have been isolated. They are of two types: one class arose spontaneously; the second arose after mutagenesis. Two revertants of the former type have now been analyzed in detail in order to determine the mutational alteration which prevents the production of $tRNA^{Ile}$ in HA1. The spontaneous revertants HA101 and HA102 have regained the ability to grow on CT439 with efficiencies of plating comparable to T4 [Table 5 of Guthrie & McClain (1973)]. HA102 synthesizes $\simeq$100% wild-type levels of $tRNA^{Ile}$; the production in HA101 is 30–50% [Plate IV of Guthrie & McClain (1973)]. The $tRNA^{Ile}$ from these mutants was subjected to digestion with RNases A and T_1, respectively, and the oligonucleotide patterns shown in Figures 2B and 6A and Plate V of Guthrie & McClain (1973) were compared with those of T4 $tRNA^{Ile}$ by quantitation and redigestion with RNases T_1 or A, respectively.

By these criteria, the sequence of $tRNA^{Ile}$ from HA102 appears indistinguishable from the wild type. In contrast, there

Table V: Sequence Determination of Oligonucleotides from Mutant tRNA^{Ile} [a]

mutant	oligonucleotide	sequence
HA101	T2′	C-C-A-C-C-A$_{OH}$ —RNase A→ Cp(3), A-Cp(1); —RNase U$_2$→ C-C-Ap, C-C-A$_{OH}$
	T7′	C-U-C-U-Gp —SP'ase→ C-U-C-U-Gp; U-C-U-Gp; C-U-Gp; U-Gp
	P15′	G-G-G-Cp —RNase T$_1$→ Gp(3), Cp(1)
Ha103	T2	U-C-A-C-C-A$_{OH}$ —RNase A→ Up(1), Cp(2), A-Cp(1); —RNase U$_2$→ U-C-Ap, C-C-A$_{OH}$
	T7′	C-U-C-U-Gp —SP'ase→ C-U-C-U-Gp; U-C-U-Gp; C-U-Gp; U-Gp
	T9	U-C-U-Gp —SP'ase→ U-C-U-Gp; C-U-Gp; U-Gp
	P15″	G-A-G-Up —RNase T$_1$→ Up(1), A-Gp(1), Gp(1); —SP'ase→ G-A-G-Up; A-G-Up; G-Up

[a] Oligonucleotides are those shown in Figure 2B,C and Figure 6A,B. Products were characterized by their mobility on DEAE paper and by composition as determined by RNase T_2 digestion, followed by two-dimensional thin-layer chromatography. The RNase U_2 product C-C-A$_{OH}$ was also characterized by digestion with venom phosphodiesterase. SP'ase is spleen phosphodiesterase.

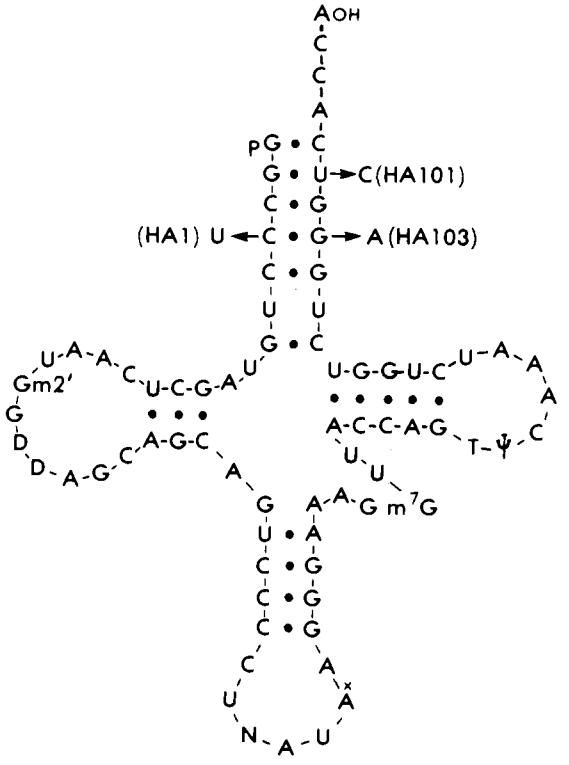


FIGURE 5: Nucleotide sequence of tRNA^{Ile} arranged in the cloverleaf structure. The sequence changes identified in mutant HA1 and revertants HA101 and HA103 are indicated. The following are the differences from the wild-type sequence: HA1, C to U at position 4; HA101, C to U at position 4 plus U to C at position 72; HA103, C to U at position 4 plus G to A at position 70.

are several alterations seen in tRNA^{Ile} from HA101. As can be seen in Figure 6A, oligonucleotides T7 (C-C-C-U-Gp) and T2 (U-C-A-C-C-A$_{OH}$) are absent from an RNase T_1 digest of tRNA^{Ile} from HA101. This is accompanied by the appearance of two novel oligonucleotides. As described in Table V, these sequences are C-U-C-U-Gp (T7′) and C-C-A-C-C-A$_{OH}$ (T2′). Comparable analyses of RNase A digests of tRNA^{Ile} from HA101 shown in Figure 2A reveal the absence of P15 (G-G-G-Up) and the concomitant appearance of a new sequence G-G-G-Cp (P15′). Taken together, these results suggest that the sequence of HA101 tRNA^{Ile} differs from that of T4 at two positions:

T4: pG-G-C-C-C-U-G · · · G-G-G-U-C-A-C-C-A$_{OH}$

HA101: pG-G-C-U-C-U-G · · · G-G-G-C-C-A-C-C-A$_{OH}$

The next question is which of these two changes represents the primary HA1 lesion and which represents the secondary, compensating alteration.

Since HA102 appears to produce tRNA^{Ile} which is quantitatively and qualitatively indistinguishable from the wild type, we infer that HA102 has arisen by a back-mutation at the original site.

We then analyzed a third revertant, HA103, which was induced by hydroxylamine mutagenesis of HA1. In contrast to HA101 and HA102, revertant HA103 shows a marked reduction in efficiency of growth on the restrictive host [Table 5 of Guthrie & McClain (1973)] and synthesizes amounts of tRNA^{Ile} ≤10% that made by T4. Digestion of this tRNA with RNase T_1, as shown in Figure 6B, reveals an oligonucleotide pattern similar to that of HA101 in that T7 (C-C-C-U-Gp) from wild type is missing and replaced by an oligonucleotide

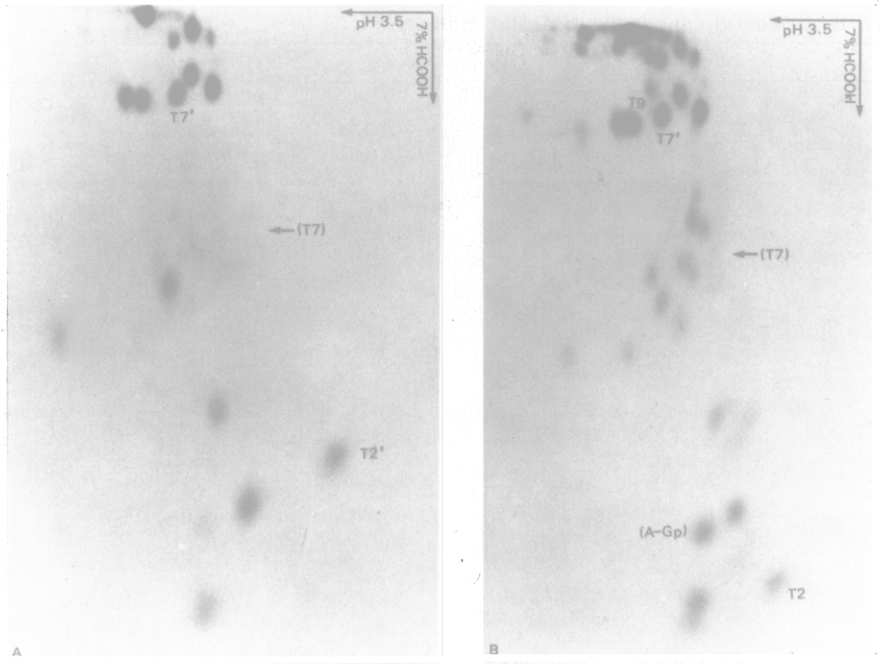


FIGURE 6: Products of RNase T_1 digestion of $tRNA^{Ile}$ from HA1 revertants HA101 (A) and HA103 (B). Products were fractionated as described in Figure 1. Oligonucleotides relevant to the determination of mutant changes are indicated (cf. Figure 1A); the sequence analyses are reported in Table V. The complexity of the digestion pattern of HA103 $tRNA^{Ile}$ results from its low yield and altered polyacrylamide electrophoretic mobility [see text and Plate IV of Guthrie & McClain (1973)]; oligonucleotides from T4 $tRNA^{Gly}$ and $tRNA^{Arg}$ account for the contamination. Taking this into account, the high molar yield of A-Gp in $tRNA^{Ile}$ from HA103 is consistent with the generation of 1 mol of AG (which is not found in T4 $tRNA^{Ile}$) expected from the G → A transition in HA103 (see text and Figure 5).

with the same sequence as T7′ (C-U-C-U-Gp). In contrast, as described in Table V, the 3′-terminal oligonucleotide from HA103 has the same sequence as that of the wild type (U-C-A-C-C-A_{OH}). Analysis of the products of RNase A digestion of $tRNA^{Ile}$ from HA103 shown in Figure 2C reveals a pattern different from that of T4 or HA101; neither P15 (G-G-G-Up) of wild type nor P15′ (G-G-G-Cp) of HA101 is present. Instead a unique oligonucleotide (P15″) is found. As shown in Table V, this new RNase A product has the sequence G-A-G-Up and is thus derived from P15 by a G → A transition at the second position in this oligonucleotide. This conclusion is consistent with the sequence of oligonucleotides surrounding G-A-G-Up. Thus, the sequence of HA103 $tRNA^{Ile}$ differs from that of T4 at two positions and from that of HA101 at only a single position.

T4: pG-G-C-C-C-U-G · · · G-G-G-U-C-A-C-C-A_{OH}
HA103: pG-G-C-U-C-U-G · · · G-A-G-U-C-A-C-C-A_{OH}

Taken together, these results indicate that the primary mutational alteration is a C → U transition at the fourth residue from the 5′ terminus of $tRNA^{Ile}$. We also note that the identification of both the T4 → HA1 and the HA1 → HA103 sequence changes is consistent with the specificity of hydroxylamine, the mutagen used to induce nucleotide alterations in these strains.

Discussion

Using hybridization to T4 DNA of aminoacylated tRNAs from T4-infected cells, Scherberg & Weiss (1970) previously identified an acceptor activity for isoleucine. They subsequently reported (Scherberg & Weiss, 1972) ribosome binding studies which indicated that T4 $tRNA^{Ile}$ was stimulated by the trinucleotide A-U-A but not by A-U-U. Binding to methionine codons was not tested. Finally, a minor tRNA species from *E. coli* has been identified (Harada & Nishimura, 1974), which recognizes the A-U-A codon but does not respond to A-U-U, A-U-C, or A-U-G. This tRNA has been partially sequenced and shown to have the anticodon N-A-U. The modified nucleotide in the wobble position has electrophoretic and two-dimensional chromatographic properties which are not distinguishable from those of Np found in the anticodon of T4 $tRNA^{Ile}$. On the basis of these results, we conclude that the T4 molecule we have sequenced is a $tRNA^{Ile}$ species which preferentially recognizes the isoleucine codon A-U-A. Proof of this hypothesis will require direct testing.

A major interest in determining the primary sequence of this tRNA derived from the unique biological properties described above. An examination of the secondary structure of T4 $tRNA^{Ile}$ shown in Figure 5 reveals several unexpected features. The opposition of two Up residues in the amino acid acceptor stem and the opposition of an Ap and a Gp residue in the anticodon stem are unusual in that these positions are normally occupied by complementary base pairs (Barrell & Clark, 1974). The presence of these novel oppositions would be expected to destabilize the conformation of T4 $tRNA^{Ile}$.

To assess the possible significance of these structural anomalies, we took advantage of the conditional lethality of the T4 mutant strain HA1, which does not synthesize $tRNA^{Ile}$, to select secondary mutants which have regained at least partial ability to grow on the restrictive host, CT439. The primary HA1 lesion converts a C·G to a U·G base pair in the acceptor stem and prevents production of the molecule. The consequence of the HA1 defect on $tRNA^{Ile}$ synthesis is partially overcome in HA101 (~30–50% synthesis relative to T4) by conversion of G·U to G·C at a nearby base pair in the acceptor stem. In revertant HA103, the secondary mutation converts the U·G base pair in HA1 to an U·A base pair. The low level of synthesis of $tRNA^{Ile}$ in this strain (≤10% relative to T4) indicates only a minor increase in stabilization resulting from the U·G to U·A substitution. Revertant HA102 produces amounts of $tRNA^{Ile}$ equivalent to T4 and appears to have arisen by back-mutation at the site of the original HA1

lesion. A priori, one might have predicted that compensation for the HA1 lesion could be readily effected by creation of an A·U or G·U base pair at the position of U,U opposition in the acceptor stem. Though too few revertants have been analyzed to critically evaluate this hypothesis, it is tempting to speculate that the U,U opposition is correlated with a function of tRNA^{Ile} that requires destabilization of the acceptor stem.

The unusual structural features of T4 tRNA^{Ile} revealed by sequence and mutant analysis could thus be interpreted as suggesting a unique physiological role for this molecule. A previously proposed rationale (Subak-Sharpe, 1966) for the existence of viral-coded tRNAs is that they provide increased translational efficiency of mRNAs whose composition is biased toward codons synonymous with rather than identical with those of the host. The amount of tRNA$^{Ile}_{AUA}$ in *E. coli* has been estimated to be less than 5% that of the major [A-U-(U,C)] decoding species (Harada & Nishimura, 1974). In light of these findings, we might then have predicted that the inability of tRNA^{Ile}-defective strains of T4 to grow on strain CT439 would be consequent to a deficiency of the cognate tRNA^{Ile} in that host. However, just the opposite is true. Analysis of tRNA in strain CT439 by Nishimura (personal communication) revealed that the levels of tRNA$^{Ile}_{AUA}$ are comparable to or greater than those in wild-type laboratory strains of *E. coli* B.

Thus, we must entertain alternative hypotheses for the physiological role of T4 tRNA^{Ile}. Possibly it is primarily involved not in protein synthesis per se but in some unique regulatory function, precedence for which is provided by the finding that some species of eucaryotic host tRNA function as primers in the replication of viral DNA (Bishop, 1978). Alternatively, it may be involved in cell wall synthesis or in some phage function which is essential only in some hosts and/or conditions of growth. Finally, the possibility might be considered that T4 tRNA^{Ile} is necessary for the efficient decoding of AUA codons in a particular local mRNA environment, i.e., a context-dependent requirement. Several T4 suppressor tRNAs have previously been shown to exhibit a strong variation in the degree of suppression vs. the site of the nonsense codon being read [see, e.g., Colby et al. (1976)]. While there is precedence for the influence of neighboring codons on translation (Salser et al., 1969), it has yet to be shown that preferential codon usage in T4 generates mRNA contexts which are different from those of the host.

It also remains to be seen whether the apparent underproduction of this tRNA species is related to its in vivo function. To address this question, it will first be necessary to determine how this stoichiometry is generated. In addition to the destabilization of tRNA^{Ile} conformation predicted by the observed structural anomalies, the transcription and/or processing of this tRNA should also be considered as potentially providing mechanisms for differential regulation of tRNA^{Ile} gene expression. Experiments to evaluate these possibilities are currently in progress.

Acknowledgments

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