LETTERS TO NATURE

Mutant tRNA^{His} Ineffective in Repression and Lacking Two Pseudouridine Modifications

TRANSFER RNA has been implicated in the regulation of a number of amino-acid biosynthetic operons[1-4]. Histidyl-tRNA^{His} has been shown to be involved in regulation of the histidine operon by analysis of six genes (*hisO*, *hisR*, *hisS*, *hisT*, *hisU*, *hisW*), mutation of which causes derepression of the enzymes of the histidine biosynthetic pathway in *Salmonella typhimurium*[5-7]. A class of derepressed mutants (*hisR*) has only about 55% as much tRNA^{His} as the wild type[4], and in the one example sequenced, contains tRNA^{His} with a structure identical to that of the wild type[8]. Studies of mutants of the gene for histidyl-tRNA synthetase (*his*S) indicated that the derepressed phenotype was associated with defects in the charging of tRNA^{His} *in vitro*[3]. The amounts of charged and uncharged tRNA^{His} present *in vivo* during physiological derepression of the wild type, and in the six classes of regulatory mutants, have been determined[9]. This work has shown that repression of the histidine operon is correlated directly with the concentration of charged histidyl-tRNA^{His} *in vivo*, and not with the ratio of charged to uncharged or the absolute amount of uncharged tRNA^{His}. The derepression observed in mutants, of *hisS* (the gene for histidyl-tRNA synthetase), *hisR* (the presumed structural gene for the single species of tRNA^{His}), and *hisU* and *hisW* (genes presumably involved in tRNA modification) may be explained by the lower cellular concentration of charged tRNA^{His} which these mutants contain.

The *hisO* mutants, on the other hand, are derepressed, yet have a wild-type amount of charged tRNA^{His}. This finding is expected because *hisO* mutants have an altered operator-promoter region[6,10].

Mutants of the sixth class, *his*T, also contain the same amount of charged and total tRNA^{His} as the wild-type organism[9], yet are derepressed. It has been found recently that tRNA^{His} from a *his*T mutant has an altered chromatographic mobility compared with that from wild-type cells[11]. This led us to determine the nucleotide sequence of tRNA^{His} from a *his*T mutant. We show below that this tRNA differs from that of the wild type[12] in having two uridine residues that have not been modified to pseudouridine.

As shown in Fig. 1, tRNA^{His} from wild-type cells[12] contains three pseudouridine nucleotides (ψ-) *, two near the anticodon, G/-U-G-, and one in the G-T-ψ-C- sequence common to the species of tRNA that have been sequenced. The ψ nucleotides were definitively characterized by their chromatographic mobility after isolation by electrophoresis from digests of T_1-RNAase fragments of tRNA^{His} [12]. Two different chromatographic systems which readily distinguish ψ- from U- on Whatman No. 1 paper were used[12]. In the acidic chromatograph system (17.6% concentrated HCl (v/v) and 68% isopropanol (v/v) in H_2O), ψ- had a mobility 0.83 times that of U-. In the basic chromatographic system (1% concentrated NH_3 (v/v) and 70% isopropanol (v/v) in H_2O), ψ- had a mobility 0.65 times that of U-. When isolated from wild-type tRNA^{His} in a variety of ways, the two nucleotides on the 3′-side of A^{m2}- and the nucleotide on the 3′-side of T- behaved as ψ- in both systems[12].

We determined the nucleotide sequence of tRNA^{His} from a strain differing from wild-type *S. typhimurium* LT-2 by the spontaneous mutation, *hisT1504*[6,9,13,14]. This strain was selected by resistance to amino-triazole plus triazolealanine, a selection for *his* derepressed mutants[6], and the mutation was shown to be located between *aroD* and *purF*[14]. ^{32}P-tRNA^{His} was prepared, purified, and sequenced from this organism as reported elsewhere for wild-type tRNA^{His} [12].

The nucleotide on the 3′-side of A^{m2}- was shown to be U- in tRNA^{His} from the *his T* mutant, when it was isolated either from T_1-RNAase fragments or from pancreatic RNAase fragments of the whole molecule. T_1-RNAase fragment, A^{m2}-U-U-C-C-A-G-, was isolated by the standard two dimensional electrophoretic system of Barrell[15]. This fragment was cleaved after each pyrimidine 3′-phosphate with pancreatic RNAase. The resultant dinucleotide, A^{m2}-U-, was isolated by electrophoresis at *p*H 3.5 on DEAE-paper and hydrolysed with alkali. Chromatography in the two systems described above produced two nucleotides in equimolar yield, one with mobilities in the two systems identical to those of A^{m2}- and another with mobilities identical to known U-. The latter nucleotide was also isolated from the pancreatic RNAase fragment, G-A^{m2}-U-, by alkaline hydrolysis and electrophoresis at *p*H 3.5 on 'Whatman 3MM' paper, where it was well separated from G- and A^{m2}-. In the acidic chromatographic system, this nucleotide ran parallel with known U-.

The nucleotide two positions away on the 3 -side of A^{m2}- also was shown to be U-. The T_1-RNAase fragment, A^{m2}-U-U-C-C-A-G-, was digested with pancreatic RNAase and subjected to electrophoresis at *p*H 3.5 on DEAE-paper. The free U- thus produced was distinguishable from ψ- by its mobility in this system. Further proof of its identity was

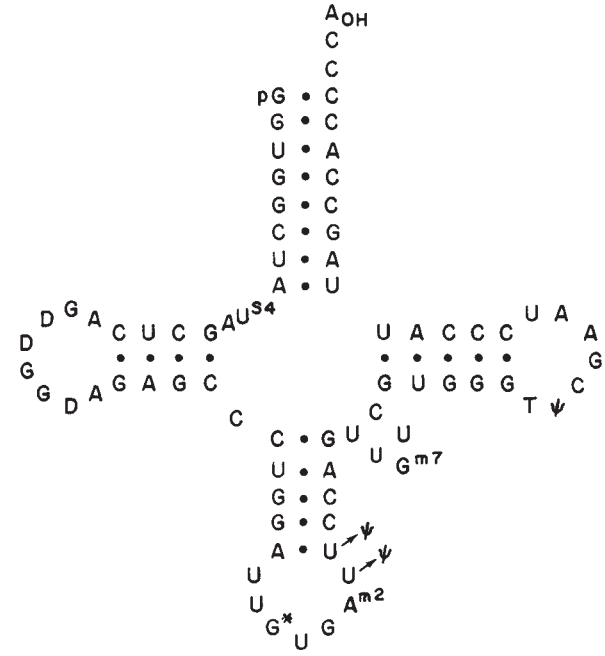

Fig. 1 Nucleotide sequence of tRNA^{His} from *S. typhimurium* with the *hisT1504* mutation. Arrows indicate the positions where pseudouridine nucleotides are found in tRNA^{His} from the wild type organism[12]. U^{s4} is 4-thiouridylate; D is 5,6-dihydrouridylate; G* is a derivative of guanylate; A^{m2} is 2-methyladenylate; G^{m7} is 7-methylguanylate; T is 5-methyluridylate; ψ is pseudouridylate.

* Hyphen is used to indicate the 3′ phosphate.

obtained by chromatography in the acidic and basic systems, where it ran parallel to known U-. The absence of ψ- in this position in the mutant tRNA^{His} also was reflected in a fingerprint of the pancreatic RNAase fragments from the whole molecule. Here, ψ- appeared in visibly reduced yield when compared with the yield of ψ- in a fingerprint of wild-type tRNA^{His}.

These two U- nucleotides following A^{m2}- were also isolated together, separated from all other nucleotides. An alkaline digest of the T_1-RNAase fragment, A^{m2}-U-U-C-C-A-G-, was fractionated by electrophoresis at *p*H 3.5 on 'Whatman 3MM' paper, where ψ- and U- migrate together. Analysis of these U-s by acidic and basic chromatography revealed no ψ-, where we estimate as little as 5% ψ- with respect to U- could have been detected.

The electrophoretic mobilities of A^{m2}-U-U-C-C-A-G-, A^{m2}-U-, and G-A^{m2}-U- were not noticeably different from those of the corresponding products from wild-type tRNA^{His}, which contain ψ-s instead of U-s. This is not unexpected, because the mononucleotides, ψ- and U-, have very similar electrophoretic mobilities[15].

In contrast to the absence of ψ- nucleotides near the anti-codon, the ψ- nucleotide found in wild type in the common sequence, G-T-ψ-C-, is present in tRNA^{His} from the *his T* mutant. This was shown by acidic and basic chromatography of the mixture of T- and ψ- isolated by electrophoresis from an alkaline digest of the T_1-RNAase fragment T-ψ-C-G-. The ψ- had the expected mobilities of 0.83 and 0.65 relative to those of U- in the acidic and basic chromatographic systems, respectively. This ψ- accounts for the ψ- seen in pancreatic RNAase fingerprints of tRNA^{His} from the *his T* mutant.

Similar studies showed that the remainder of the tRNA^{His} molecule from the *his T* mutant is identical to tRNA^{His} isolated from wild type. T_1-RNAase fragments of tRNA^{His} from the *his T* mutant were isolated by two-dimensional electrophoresis and digested with pancreatic RNAase. The resultant products were shown by electrophoresis on DEAE-paper at *p*H 3.5 to be identical to similarly isolated products from wild-type tRNA^{His}. The modified nucleotides A^{m2}-, G*-, and T- isolated from wild-type tRNA^{His} or from *his T*-mutant tRNA^{His} were identical when analysed by acidic and basic chromatography and by electrophoresis at *p*H 3.5 on 'Whatman 3MM' paper. The presence of the D-s and G^{m7}- was demonstrated by electrophoresis at *p*H 3.5 on 'Whatman 3MM' paper after alkaline degradation of the appropriate T_1-RNAase fragments.

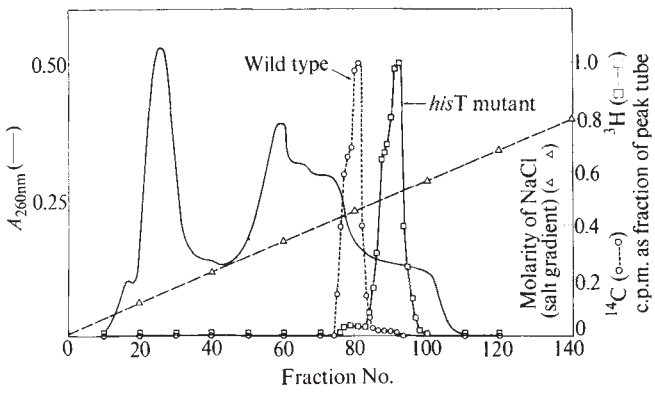

Fig. 2 Comparison of the elution position on reversed-phase column No. 3 (ref. 19) of tyrosyl-tRNA^{Tyr} from wild type (*S. typhimurium* strain LT-2) and from a *hisT* mutant (carrying the *hisT1504* mutation). The applied sample contained 10 mg of tRNA from the wild type charged with ^{14}C-tyrosine, 1.5 mg of tRNA from the *hisT* mutant charged with ^{3}H-tyrosine. The column was developed at 37° C with a linear gradient of NaCl in 10 mM $MgCl_2$ and 10 mM sodium acetate, *p*H 4.5. The flow rate was 50 ml./h, and 10 ml. fractions were collected. The tRNA was precipitated from the fractions and counted as previously described. Peak tubes of ^{14}C and ^{3}H had 25,730 and 15,341 c.p.m., respectively. Recoveries were 65% for ^{14}C and 54% for ^{3}H.

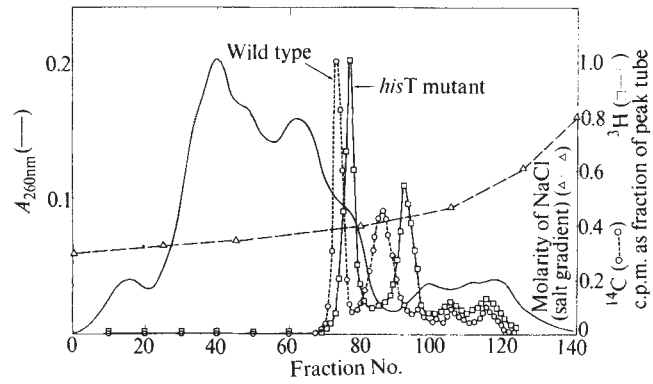

Fig. 3 Comparison of the elution position of leucyl-tRNA^{Leu} from wild type and from the *his T* mutant. Conditions were the same as those in Fig. 2, except that a concave gradient was used. The applied sample contained 4 mg of tRNA from the wild type charged with ^{14}C-leucine and 2 mg of tRNA from the *his T* mutant charged with ^{3}H-leucine. The two peak tubes of ^{14}C contained 10,945 and 4,328 c.p.m. respectively; peak tubes of ^{3}H contained 5,698 and 3,457 c.p.m. Recoveries were 81% for ^{14}C and 77% for ^{3}H.

The presence of U^{s4}- was established by the position of the T_1-RNAase fragment, C-U-A-U^{s4}-A-G-, on the standard two-dimensional electrophoretic system[12].

Thus the only detectable defect in tRNA^{His} from the *his T* mutant is the absence of the two pseudouridine modifications.

Since pseudouridine is present in the anti-codon region also in tRNA$_I^{Tyr}$ and tRNA$_{II}^{Tyr}$ [16] and tRNA$_I^{Leu}$ and tRNA$_{II}^{Leu}$ [17,18], we compared the chromatographic mobility of these tRNA species from the wild type with that of the *his T* mutant. We used the column and the double labelling method (Brenner and Ames[11]) that showed that tRNA^{His} from the *his T* mutant had an altered mobility. Fig. 2 shows that tyrosyl-tRNA^{Tyr} from the *his T* mutant has an altered chromatographic mobility. The minor species of tRNA^{Tyr} does not separate well from the major species in this column. As with tRNA^{His} [11], tRNA^{Tyr} from the *his T* mutants is retarded relative to that from wild type. The same result was obtained when the radioactive labels were reversed.

Fig. 3 shows that leucyl-tRNA^{Leu} I and II from the *his T* mutant are also altered. Too few counts were recovered in the other peaks (species III and IV) to make a valid comparison between the wild type and the mutant. We did not resolve a fifth peak which is found using another type of chromatography[17]; with our column ('Kelmers' No. 3) only 4 peaks were found by Weiss *et al.*[19]. As with the other tRNA species from the *his T* mutant, tRNA^{Leu} species are retarded relative to the wild-type species.

Unpublished experiments of Brenner show that valyl-tRNA^{Val} from the *his T* mutant and from the wild type have identical elution profiles using this same chromatographic system. This is in agreement with the absence of ψ in the anticodon region of tRNA^{Val} [20,21] and supports our conclusion that the altered chromatographic behaviour of tRNA from the *his*T mutant is correlated with the absence of ψ.

Of the *Escherichia coli* tRNA sequences reported, tyrosine[16], leucine[17,18], glutamine[22], phenylalanine[23] and methionine[24] tRNA species contain a pseudouridine in the anticodon region, while the valine[20,21], glycine[25], isoleucine[26], formyl-methionine[27], serine[28] and tryptophan[29] species do not. Presumably the *his T* enzyme modifies U in all of the tRNA species that have ψ in this region. In a separate study (Cortese, R., and Ames, B. N., unpublished results) it has been found that *his T* mutants are resistant to 3-aminotyrosine, trifluoro-leucine, β-hydroxyleucine and thialysine. The relation of the tRNA alteration to the analogue resistance is not yet understood.

Johnson and Soll[30] have presented evidence that pseudouridine nucleotides in mycoplasma tRNA are formed by enzymatic modification of uridine nucleotides at the macromolecular level.

Our results provide strong confirmation of this mechanism. Our data suggest that the wild-type *his T* gene specifies an enzyme which converts U- nucleotides in tRNA to ψ- nucleotides. In a separate study (Cortese, R., Spengler, S., Kammen, H., and Ames, B. N., unpublished results) evidence has been obtained that the *his T*-mutant cells lack an enzyme activity found in wild-type cells which converts U- to ψ- in unfractionated tRNA isolated from the *his* T mutant.

The tRNA sequences reported to date contain a pseudouridine in the G-T-ψ-C loop[16-29,31]. Our analysis shows that this ψ is still present in the $tRNA^{His}$ from a *his T* mutant, and M. Yaniv (personal communication) has found that $tRNA^{Val}$ from this mutant also contains G-T-ψ-C. Thus, the *his T* enzyme does not appear to be responsible for synthesizing the ψ in the G-T-ψ-C loop which then would be done by a different enzyme.

The protein specified by the *his T* gene is not essential for the life of the cell, as indicated by the isolation of amber mutations and frameshift mutations in the *his T* gene[14]. The *his T* mutants grow very well in minimal medium, though with a slightly decreased growth rate (62-min doubling time compared with the wild type 47-min doubling time)[9]. Strains with the amber mutation *his T2890*, or with the *his T1504* mutation, or with various frameshift mutations in *his T* all have about the same extent of derepression of the histidine operon and about the same doubling time[9,14]. This slight, but reproducible, decrease in the growth rate is not due to the derepression of the histidine operon *per se*, since *his O* mutants with the same degree of derepression have the wild type doubling time. This decrease is presumably due to another effect of the pleiotropic *his T* mutation.

The tRNA that lacks pseudouridines thus must function reasonably well in protein synthesis in *his T* mutants. The total amount of $tRNA^{His}$ and the ratio of charged to uncharged $tRNA^{His}$ *in vivo* are the same in the wild type and in *his T* mutants[9]. In addition, the $tRNA^{His}$ isolated from a *his T* mutant is charged normally and has the same affinity for the histidyl-tRNA synthetase as does the modified tRNA from the wild type[11].

The derepression of the histidine operon in a *his T* mutant thus appears to be due to the lack of modification of the $tRNA^{His}$ molecule, and this is consistent with the known role of $tRNA^{His}$ in regulation. The possibility that the *his T* protein itself has a role in regulation, unrelated to the modification of $tRNA^{His}$, cannot, however, be ruled out. This pseudouridine modification would also have to be accounted for in any theory in which the histidyl-tRNA synthetase[5,32] or the first enzyme of the biosynthetic pathway[33,34] or another protein is the coregulator of the histidine operon. Unmodified charged $tRNA^{His}$ could be unable to induce a conformational change in a protein required for regulation.

The pseudouridine modifications, in addition to aminoacylation, appear to be necessary for the $tRNA^{His}$ to be competent in repression. This, and the viability of *his T* mutants, suggest the possibility of a regulatory role for these pseudouridines in tRNA.

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Phage DNA Directed Enzyme Synthesis in *in vitro* System from Yeast Mitochondria

MITOCHONDRIA possess their own apparatus for protein synthesis[1] with rather bacteria-like properties[2-8]. DNA dependent RNA polymerase from *Neurospora crassa* mitochondria was reported to be sensitive to rifampicin as is bacterial RNA polymerase[9] (although work on other mitochondrial polymerases described rifampicin resistance[10,11]). The molecular weight of this mitochondrial RNA polymerase was found to be surprisingly low (64,000)[9] compared with bacterial polymerases (such as *E. coli* polymerase, 450,000). Does the sensitivity to rifampicin indicate a general similarity of mitochondrial polymerase and bacterial polymerases? We show here that RNA polymerase from mitochondria is able to mediate enzyme synthesis *in vitro* directed by DNA from the *E. coli* phages T3 and T7, and thus show that transcriptional signals for *E. coli* RNA polymerase can be recognized by the mitochondrial enzyme.

In a protein synthesizing system from yeast mitochondria, T3 phage DNA directed the synthesis of the phage specific enzymes lysozyme and S-adenosylmethionine cleaving enzyme (Table 1). Enzyme synthesis (Table 1) was inhibited by fusidic acid, a potent inhibitor of peptide chain elongation factor G,