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Pleiotropy of *hisT* Mutants Blocked in Pseudouridine Synthesis in tRNA: *Leucine* and *Isoleucine-Valine* Operons

(amino-acid regulation/*Salmonella typhimurium*/tRNA chromatography)

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ABSTRACT **The *hisT* gene codes for an enzyme responsible for the conversion of uridine to pseudouridine (Ψ) in the anticodon region of many tRNA species in *Salmonella typhimurium*. We have previously shown that a *hisT* mutant has $tRNA^{His}$ which lacks pseudouridine in this region and as a consequence has an altered chromatographic behavior. We show here a similar alteration in chromatographic behavior of all $tRNA^{Leu}$ and one $tRNA^{Ile}$ species from a *hisT* mutant. By contrast, $tRNA^{Val}$, which contains no pseudouridine except for the one in the TΨCG sequence, is chromatographically unaltered in a *hisT* mutant.**

The absence of pseudouridine in the anticodon region of tRNA in *hisT* mutants has been previously shown to cause derepression of the histidine operon. We show here that in *hisT* mutants the regulation of the leucine and the isoleucine and valine operons is also affected: the enzymes of these operons are refractory to repression by the branched chain amino acids. However, there is no difference between *hisT* and wild type in the pattern of derepression caused by isoleucine or valine limitation and only a slight difference in the enzyme levels in cells grown on minimal medium.

The alteration in the regulation of branched chain amino acid operons may also explain why *hisT* mutants are resistant to inhibition of growth by the amino acid analogues 5,5,5-trifluoroleucine, β-hydroxyleucine, and norleucine and by the oligopeptides glycylglycylnorleucine and norleucylnorleucine.

hisT mutants, originally isolated as being derepressed for the histidine operon in *Salmonella typhimurium* (1, 2), have an altered $tRNA^{His}$ which has been sequenced and which differs only in having two uridines in place of two pseudouridines (Ψ) in the anticodon region (3). In contrast, the pseudouridine in the "TΨCPu" loop was present in both the wild-type and mutant $tRNA^{His}$ (3). Direct evidence has been obtained that the *hisT* gene product is a pseudouridylation enzyme. An enzyme that can modify specific uridine residues of bulk *hisT* tRNA (4) has been identified and purified using wild-type strains of *S. typhimurium* and *Escherichia coli*. This activity is not detectable in extracts derived from *hisT* strains and is heat-labile in temperature-sensitive *hisT* strains (4).

Other tRNAs which have Ψ in the anticodon loop also appear to have Ψ replaced by U in the *hisT* mutant: $tRNA^{Tyr}$, $tRNA^{Leu}$, and $tRNA^{His}$ species from *hisT* are altered in chromatographic behavior (3). *hisT* mutants are also resistant to some amino-acid analogs of histidine, leucine, and tyrosine (3). As the growth rate of *hisT* amber mutants was almost normal (2, 5), we concluded (3) that these Ψs in tRNA (about half the known tRNA species) may have a function in regulation rather than in protein synthesis.

Histidyl-$tRNA^{His}$ is necessary for the repression of the histidine operon in *S. typhimurium* (5). Studies on the regulation of the leucine, isoleucine, and valine operons have also implicated the amino acyl tRNAs in regulation, although this evidence, involving the respective tRNA synthetases (6–10), was only circumstantial. Recently, however, an involvement of leucine tRNA as an element in repression of the leucine biosynthetic enzymes was strongly implied by the finding that the *hisT* mutant phenotype included resistance to several leucine analogues (3) and an alteration of $tRNA^{Leu}$ (3, 11). The present work extends these findings.

MATERIALS AND METHODS

Chemicals. The chemicals and their sources were as follow: sodium salts of dihydroxyisovalerate, dimethylcitraconate, and 3-isopropylmalate (Reef Laboratory, Lafayette, Ind.); 5,5,5-trifluoro-DL-leucine (PCR, Inc., Gainesville, Fla.); DL-norleucine and L-amino acids employed (Calbiochem); L-norleucyl-L-norleucine, L-lysyl-L-lysyl-L-lysine, and glycylglycyl-L-norleucine (Cyclo Chemicals); β-hydroxy-leucine (D,L *threo* and *erythro*) (a gift from Dr. T. Otani); Adogen 464 (Ashland Chemical Co.); Plaskon CTFE 2300 powder (Allied Chemical Corp.); [^{14}C]isoleucine, [^{3}H]leucine, and [^{3}H]valine (New England Nuclear Corp.); [^{3}H]isoleucine (Schwarz/Mann), [^{14}C]leucine (Amersham Searle), and [^{14}C]valine (ICN).

Bacterial Strains. The bacterial strains were derivatives of *S. typhimurium* LT2. Strain SB560 (*aroD5 purF145*) was the parent of strain TA265 (*hisT*$^+$), strain TA253 (*hisT1504*), and strain TA1309 (*hisT2890*). *hisT2890* is an amber mutation. Strain TR1 (aroD5 *purF145 ilv-542*) was the parent of strain TA3002 (*hisT1504 ilv-542*) and TA3003 (*hisT*$^+$ *ilv-542*). The indicated *hisT* allele was transduced into the parent strain with phage P22, selecting for *aroD*$^+$ *purF*$^+$ recombinants.

Media. The minimal growth medium (12) used in the enzyme assay experiments was made 0.5% in glucose and modified by omitting citrate. The enriched medium was L-broth (13). In the analogue-inhibition experiments the medium employed contained, per liter: 1.0 g of K_2SO_4, 13.5 g of K_2HPO_4, 4.7 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, and 15 g of agar. The cells were pregrown in nutrient broth (0.8% Difco Bacto with 0.5% NaCl).

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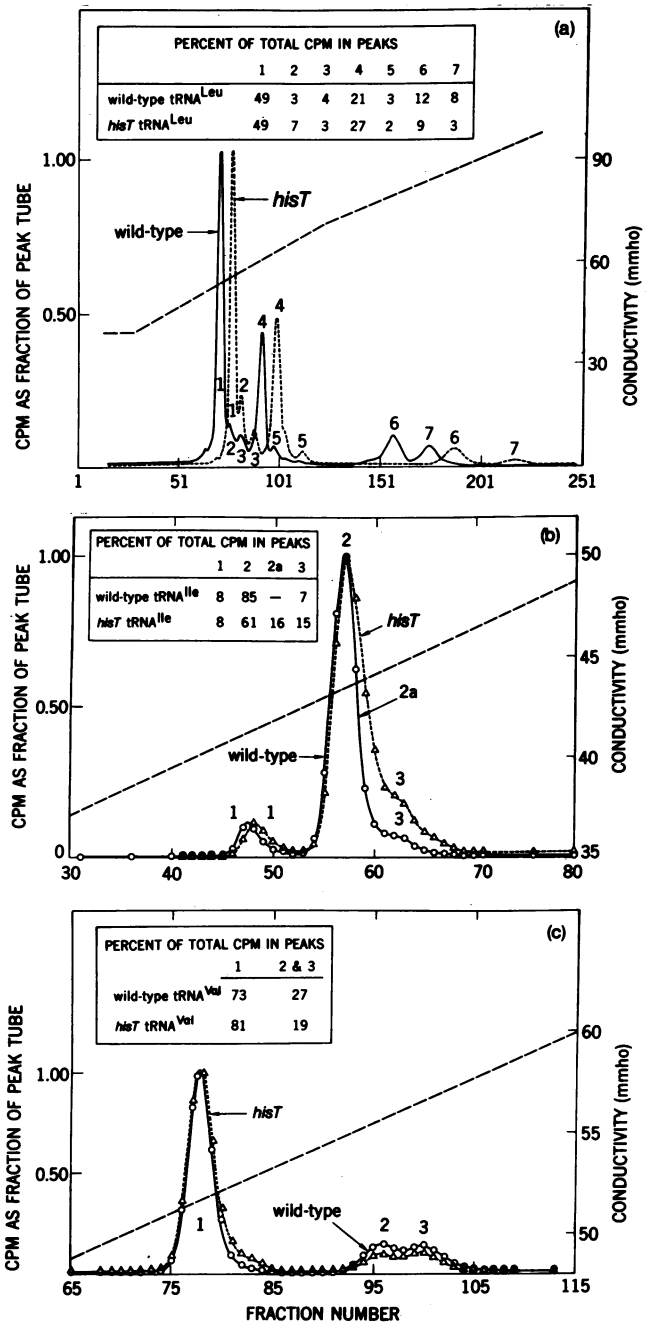

FIG. 1. Co-chromatography on RPC-5 of aminoacyl-tRNA from wild type (TA265) and *hisT1504* (TA253). The columns were developed and analyzed as described in *Materials and Methods*, with a linear gradient of NaCl in 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 10 mM sodium acetate, pH 4.5. (*a*) Leucyl-$tRNA^{Leu}$. The sample contained 90 μg of wild-type tRNA charged with [^{14}C]leucine and 8.5 μg of *hisT* tRNA charged with [^{3}H]leucine. Peak tubes of ^{14}C and ^{3}H had 976 and 1029 cpm, respectively. Recoveries were about 90% for ^{14}C and 75% for ^{3}H. An experiment with the labels reversed gave a similar pattern. (*b*) Isoleucyl-$tRNA^{Ile}$. The sample contained 300 μg of wild-type tRNA charged with [^{3}H]isoleucine and 1450 μg of *hisT* tRNA charged with [^{14}C]isoleucine. Peak tubes of ^{14}C and ^{3}H had 1215 and 681 cpm, respectively. Recoveries were about 90% for ^{14}C and 70% for ^{3}H. An experiment with the labels reversed gave a similar pattern. (*c*) Valyl-$tRNA^{Val}$. The sample contained 315 μg of wild-type tRNA charged with [^{14}C]valine and 120 μg of *hisT* tRNA charged with [^{3}H]valine. Peak tubes of ^{14}C and ^{3}H had 1859 and 778 cpm, respectively. Recoveries were about 58% for ^{14}C and 71% for ^{3}H.

Preparation and Aminoacylation of tRNA. Strains TA265 and TA253 were grown on minimal medium E (0.5% in glucose) (14) and the tRNA extracted as described (ref. 15, $tRNA^{Leu}$ and $tRNA^{Val}$; ref. 5, $tRNA^{Ile}$). The tRNA was aminoacylated (16) with the following modifications. The appropriate labeled amino acid (Fig. 1) replaced [^{3}H]histidine, and the aminoacyl-tRNA synthetases, prepared as described (17), replaced histidyl-tRNA synthetase. The concentration of tRNA used was adjusted to give about 100,000 cpm of aminoacylated tRNA. The reaction was stopped after 15 min at 37° with 2 volumes of 1 M magnesium acetate and 15 volumes of 75% ethanol at −20°. The tRNA was eluted into 1 ml of the RPC-5 buffer containing 0.6 M sodium chloride.

Column Chromatography. The RPC-5 column was packed with adsorbent (prepared by method C) and operated as described (18), except that we used a pressure of 50 lb/in^2, giving a flow rate of 0.5 ml/min. Fractions of 1 ml were collected, mixed with 10 ml of Bray's scintillation fluid, and counted in a Nuclear Chicago Mark I liquid scintillation counter. Efficiency of counting was 13% for ^{3}H and 25% for ^{14}C.

Enzyme Assays. Cells were grown as indicated, harvested by centrifugation, washed once, and resuspended in 8 volumes of buffer (0.05 M potassium phosphate, pH 7.5). The cells were disrupted (Branson 125W sonifier) and the debris was removed by centrifugation at 1° (Sorvall RC-2, SS-1 rotor, 15,000 rpm for 20 min). Enzyme assays were performed immediately. The assay methods were as described: threonine deaminase [L-Threonine hydro-lyase(deaminating), EC 4.2.1.-16] (19), dihydroxyacid dehydratase (2,3-Dihydroxyacid hydro-lyase, EC 4.2.1.9) (10), acetohydroxy acid synthase (Acetolactate synthase, EC 4.1.3.18) (20), 2-isopropylmalate dehydratase (3-hydroxy-4-methyl-3-carboxyvalerate hydro-lyase, EC 4.2.1.33) (21); 3-isopropylmalate dehydrogenase (2-hydroxy-4-methyl-3-carboxyvalerate: NAD^+ oxidoreductase, EC 1.1.1.85) (22), and protein (23).

RESULTS

Chromatographic Behavior of the hisT Mutant tRNA. Our previous studies had shown that mutation of the *hisT* gene caused loss of the enzyme (4) that converts uridine to pseudouridine in the anticodon loop of $tRNA^{His}$ (3). The unmodified $tRNA^{His}$ could be distinguished chromatographically from the wild-type $tRNA^{His}$ as it was retarded on an RPC-3 column (3, 15). This retardation of tRNA from a *hisT* mutant was also seen with $tRNA^{Tyr}$ and the major species of $tRNA^{Leu}$ (3). As both of these species were known to contain pseudouridine in the anticodon region, this suggested that the *hisT* mutation was pleiotropic, preventing all modification of uridine in the anticodon region of tRNA to pseudouridine (3, 4). This was at least partially confirmed when $tRNA_1^{Leu}$ from a *hisT* mutant was shown to lack pseudouridine in the anticodon loop (11).

Co-chromatography of leucyl-$tRNA^{Leu}$ from the *hisT* mutant and the wild type on the newer RPC-5 column showed (Fig. 1*a*) that all seven $tRNA^{Leu}$ species separated are altered in the *hisT* mutant.

The major species of $tRNA^{Ile}$ in *E. coli* has no pseudouridine in the anticodon region (24). Co-chromatography of isoleucyl $tRNA^{Ile}$ from the *hisT* mutant and the wild type (Fig. 1*b*) confirmed that the major species is unaltered in the mutant. Harada and Nishimura (25) have recently found that a minor isoleucine-accepting species, which recognizes the AUA codon,

contains a pseudouridine at the 3′ side of the anticodon loop. It appears from Fig. 1b that species 1 may be this minor species. We have consistently seen that the *hisT* tRNA has considerable extra $tRNA^{Ile}$, perhaps two species, appearing after the main component.

Co-chromatography of valyl $tRNA^{Val}$ from the *hisT* mutant and the wild type (Fig. 1c) showed that the valyl tRNAs are identical in the two strains. This is as expected since $tRNA^{Val}$ has no pseudouridine except in the TΨCG sequence (26), which is unaffected in the *hisT* mutant (3).

Resistance of hisT Mutants to Amino-Acid Analogues. The greater resistance of *hisT* mutants than of wild type to a variety of amino-acid analogues (3) indicates that the unmodified tRNA resulting from the *hisT* mutation affects other amino-acid pathways in addition to histidine. Table 1 shows that *hisT* mutants were more resistant than an isogenic wild-type strain to the leucine analogues trifluoroleucine and β-hydroxyleucine, and to norleucine. This difference in resistance was enhanced by growth on a poor carbon source, while the nitrogen source had no effect.

The relative resistance of the *hisT* mutants is not the result of a reduced rate of uptake of the analogues, since resistance is observed to norleucine dipeptides or tripeptides which enter the cell through the dipeptide and tripeptide permeases (27). The wild type and *hisT* strains were equally sensitive to trilysine, indicating that the oligopeptide permease is functional in the *hisT* mutant.

An alteration in the regulation of the leucine operon by the unmodified tRNA could also account for the altered analogue resistance in the *hisT* mutant, as is the case with the histidine operon (3). Evidence for an effect on leucine regulation is presented in the following section.

The Effect of hisT Mutations on the Repression of the Branched Chain Amino-Acid Biosynthetic Enzymes. Since trifluoroleucine resistance in *S. typhimurium* is usually accompanied by derepression of the enzymes that form branched-chain amino acids (8), it was of interest to compare the levels of these enzymes in *hisT* and *hisT*$^+$ strains of *S. typhimurium*. Accordingly, the same strains examined in the analogue sensitivity tests (Table 1) were grown in the presence and absence of repressing levels of the three branched-chain amino acids, as well as in L-broth. The activities of two leucine biosynthetic enzymes, 2-isopropylmalate dehydratase and 3-isopropylmalate dehydrogenase, were determined. These enzymes are normally repressed when cells grow in the presence of excess leucine. We also measured three isoleucine and valine biosynthetic enzyme activities which are multivalently repressed when all three branched-chain amino acids are present in the medium.

Table 2 shows that the isogenic wild-type strain, TA265, exhibits the normal extent of repression of all five enzymes when excess isoleucine, valine, and leucine are incorporated into the minimal medium. Growth in L- broth resulted in the strong repression normally exerted by rich media over these enzymes. This repression has been observed before and may be due to a process different from that causing the "specific" repression exerted by the branched-chain amino acids.

In contrast, in the two *hisT* strains (TA253, TA1309), all five enzymes were refractory to the repression usually exerted by the branched-chain amino acids. For both the *hisT* missense and the *hisT* amber strain, the activity of threonine deaminase is about 20% lower in the presence of excess branched-chain amino acids than in their absence. This small difference in activity may not be due to repression; but, if it is, the repression appears to be markedly reduced owing to the *hisT* lesion. In both *hisT* mutants, the much stronger "repression" caused by growth in L-broth is about like that observed in the *hisT*$^+$ parental strain. Thus, the *hisT* lesion appears to have interfered with the "specific" repression exerted by the three branched-chain amino acids, but not with the "non-specific" repression occurring in L-broth.

TABLE 1. *Inhibition of growth by amino-acid analogues*

Carbon/ nitrogen source	Analogue	Inhibition zone diameter (mm) Wild type (TA265)	*hisT1504* (TA253)	*hisT2890* (TA1309)
Glucose/ NH_3	5,5,5-Trifluoroleucine	14	<6	<6
	β-Hydroxy-leucine	25	15	15
	Norleucine	<6	<6	<6
	Gly-gly-norleucine	13	<6	<6
	Norleucyl-norleucine	20	10	10
	Trilysine	16	16	16
Glucose/ proline	5,5,5-Trifluoroleucine	13	<6	<6
Citrate/ NH_3	5,5,5-Trifluoroleucine	33	<6	<6
	Norleucine	24	<6	<6
	Norleucyl-norleucine	25	<6	<6
Citrate/ proline	5,5,5-Trifluoroleucine	34	10	10

Bacteria (0.1 ml of a nutrient broth culture) were added to the top agar of a pour plate with minimal-salts medium with carbon (0.4%) and nitrogen (10 mM) sources as indicated. A filter paper disc (6 mm) containing 2 μmoles with respect to the L-isomer (or 0.8 for gly-gly-norleucine) of the analogue was then placed in the center of the plate. Incubation was at 37° overnight. The inhibition of the trifluoroleucine and β-hydroxy-leucine is completely reversed by adding L-leucine (10 μmoles) to the plate. Although the norleucine is a methionine analogue and the inhibition is reversed by methionine, we have also found that the inhibition of norleucine and the norleucine peptides is partially reversed by L-leucine (analyzed as free of methionine).

The Effect of hisT Mutations on Derepression of the Isoleucine and Valine Biosynthetic Enzymes. Whether the *hisT1504* mutation also interferes with the "derepress" signal generated when one of the branched-chain amino acids is limiting can only be determined with a strain containing an *ilv* mutation along with the *hisT1504* mutation. Table 3 shows that a "normal" derepression pattern results from limiting isoleucine or limiting valine in the isogenic *hisT*$^+$ strain. The two isoleucine and valine biosynthetic enzymes were derepressed upon limiting either amino acid [with limiting valine, it is assumed that both acetohydroxyacid synthases were derepressed (28) and, with limiting isoleucine, that only acetohydroxy acid synthase II (29) was derepressed]. As expected, the leucine biosynthetic enzyme was not derepressed by restricting the supply of either amino acid. Essentially the same pattern of derepression was observed with the strain carrying the *hisT1504* mutation; however, the extent of derepression was not as great. Growth in the presence of an excess of all the branched-chain amino

TABLE 2. *The effect of the hisT mutation on repression of enzymes of the leu and ilv operons*

Strain	Medium*	Specific activity (μmol/min per mg of protein)				
		Threonine deaminase	Dihydroxyacid dehydratase	Aceto-hydroxy acid synthase	2-Isopropyl-malate dehydra-tase	3-Isopropyl-malate dehydro-genase
TA265 (*hisT*+)	Minimal	0.21	0.050	0.041	0.011	0.055
	Minimal + ile, val, leu	0.07	0.017	0.009	0.003	0.020
	L-broth	0.03	0.010	0.002		0.006
TA253 (*hisT1504*)	Minimal	0.31	0.063	0.049	0.012	0.100
	Minimal + ile, val, leu	0.24	0.060	0.044	0.014	0.085
	L-broth	0.06	0.014	0.003		0.030
TA1309 (*hisT2890*)	Minimal	0.31	0.050	0.044	0.015	0.114
	Minimal + ile, val, leu	0.25	0.045	0.038	0.015	0.115
	L-broth	0.06	0.012	0.004		0.040

* Amino-acid supplements were in excess: isoleucine and leucine (500 μM); valine (800 μM). Cells in all cases were harvested during late log phase.

acids did not repress the levels of the three enzymes, confirming the effect of the *hisT* mutation shown in Table 2. Thus, while the *hisT* mutation leads to a loss of repressibility of the *ilv* gene cluster, its effect on the derepression signal generated by limited valine or isoleucine is not striking. Indeed, in view of the significant fact that the *hisT* lesion is pleiotropic, the limited derepression of the isoleucine and valine biosynthetic enzymes may be the result of a relatively nonspecific, rather-than a specific, interference with the derepression signal.

DISCUSSION

The present work makes much more definitive the involvement of the leucine and isoleucine tRNAs in the regulation of the leucine, isoleucine, and valine biosynthetic pathways. The involvement of tRNA had been suggested by a variety of work on these operons (7, 9, 10, 30–32) including the isolation of regulatory mutants with lowered tRNA synthetase activities (6, 33, 34). It was always possible, however, that the tRNA synthetases alone were involved, an objection that is removed by showing that an alteration in the tRNA itself is associated with altered regulation. This involvement of tRNA

TABLE 3. *The effect of a hisT lesion on derepression of isoleucine and valine biosynthetic enzymes*

Strain	Medium*	Specific activity (μmol/min per mg of protein)		
		Threo-nine deami-nase	Aceto-hydroxy acid synthase	3-Isopropyl-malate dehydro-genase
TA3003 (*hisT*+, *ilv-542*)	Ile, val, leu	0.08	0.009	0.025
	Ile, val, leu	1.85	0.110	0.029
	Ile, *val*, leu	0.82	0.187	0.025
TA3002 (*hisT1504*, *ilv-542*)	Ile, val, leu	0.55	0.050	0.047
	Ile, val, leu	1.04	0.095	0.043
	Ile, *val*, leu	0.69	0.155	0.040

* Amino acids were added in excess (as in Table 2), except where italicized, in which case it was limiting: *ile* = 80 μM ile (cells harvested 1 hr after end of growth); *val* = 80 μM glycyl-valine (cells harvested at a titer of 6 × 10^8/ml).

may be similar to that with histidine regulation where charged $tRNA^{His}$ in its pseudouridylated form is necessary for repression (5) and the five classes of regulatory mutants (*hisR*, *hisT*, *hisS*, *hisU*, and *hisW*) show alterations in the synthesis, maturation, or aminoacylation of $tRNA^{His}$ (3, 5, 35, 36).

Various models of the repression of the *ilv* operon have suggested a role for branched-chain aminoacyl tRNAs (7, 30, 37, 38). Our finding of the alteration of all of the leucine species and of a minor isoleucine species, as well as alterations in the amounts of two isoleucine species and a leucine species in a mutant defective in the regulation of the operon, leaves unclear which of these various tRNA species actually are involved in the control.

It is interesting that the lack of pseudouridine in the anticodon loop of many tRNA species does not lead to any major alteration in the cell. In fact, the *hisT* gene product is dispensible, as has been shown by Chang, Roth, and Ames (2). The growth rates of *hisT* mutants are almost normal (2, 5) (the reduction of 15% might be due to the drain of energy caused by higher levels of many biosynthetic enzymes). This implies that tRNA lacking pesudouridine in the anticodon region interacts normally with the protein synthesizing machinery of the cell. Furthermore, it was been shown that $tRNA^{His}$ from *hisT* mutants behaves like wild-type $tRNA^{His}$ in the aminoacylation reaction (15, 16)—the rate, extent of the reaction, and the affinity for the enzyme are identical. For $tRNA^{Leu}$ and $tRNA^{Ile}$, careful kinetic measurements have not been done, but the rate and extent of the reaction are identical for the mutant and the wild type.

The correspondence between the lack of pseudouridine in tRNA and the alteration of regulation is maintained at least for three biosynthetic pathways: leucine, isoleucine and valine, and histidine. This leads to the hypothesis that there is a general feature of tRNA structure necessary for the interaction at the level at which the regulation of many operons occurs. The presence of pseudouridine seems to be necessary for the maintenance of such structure. This conclusion is strengthened by the observation that in every case tested the lack of pseudouridine leads to a similar effect on the chromatographic behavior of tRNA on an RPC-5 column—a retardation in the elution. This could be related to a similar structural change that would strengthen the interaction between the tRNA and the absorbant. The alteration in regula-

tion is also always in the same direction—inability to repress. Since it is not known how tRNA acts as a regulatory molecule, it is difficult to correlate the structural and the functional alteration produced by the lack of pseudouridine. A possible model might follow from the hypothesis that aminoacylation induces some conformational change in tRNA (39, 40). Since the amino acylated form of tRNA is required for regulation of the histidine operon (5), one might think that the conformational change that occurs upon aminoacylation is a requisite for the regulatory function. The lack of pseudouridine might prevent the conformational change from occurring, "freezing" the tRNA in the unacylated conformation even after aminoacylation. As a consequence there would be no signal to the cell that sufficient amino acid was present. The effect of the *hisT* mutation on other amino-acid pathways and tRNAs is under further study.

These considerations all point to a precise function for pseudouridine in the anticodon region that may be related to regulation of the biosynthesis of some enzymes. If the cell had the capacity to regulate the pseudouridylation in the anticodon region, it would be possible to change the level of the enzymes independently of the level of aminoacyl-tRNA. This would confer more flexibility to the "regulatory systems." Such a possibility is currently under investigation.

Attempts to understand the function of modified bases in tRNA have been directed at revealing a role in the protein synthesis process, such as interactions with ribosomes (41) and codon-anticodon interaction (42). These studies led to important discoveries. Our study with *hisT* suggests that for some of the modified bases the role might not be related to protein synthesis *per se* but to the function of tRNA as a regulatory molecule. This function might be performed at the level of transcription or translation, or both, but it is not necessarily related to the "adapter" function of tRNA in protein synthesis.

tRNA has been thought to play a role in many biological phenomena including differentiation, development, and cancer. It is possible that the increase in the frequency of modified bases in tRNA as one goes from mycoplasm to mammals might be related to the increasing importance of tRNA as a regulatory molecule during evolution§.

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§ A comparison of the frequency of modified bases in the tRNAs of *E. coli* and the liver of a higher organism based on the data collected in the *CRC Handbook of Biochemistry* (2nd edition) shows that there are roughly nine modified bases per tRNA molecule in *E. coli* and fourteen in the liver. Pseudouridine in particular is twice as frequent (four in liver and two in *E. coli*). In tRNA from rat liver there are 19 modified bases in 84 residues.