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Differential utilization of leucyl-tRNAs by *Escherichia coli*

(tRNA utilization/protein synthesis *in vitro*/ribosomes/suppressor tRNA)

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ABSTRACT **The utilization of the isoaccepting species of leucyl-tRNA in protein synthesis has been examined in *E. coli*. Two minor leucyl-tRNA species, isoacceptors $tRNA_3^{Leu}$ and $tRNA_4^{Leu}$, are the predominant species found bound to ribosomes during exponential growth in minimal medium. In rich medium, an increased proportion of $tRNA_1^{Leu}$ is found bound to ribosomes. One species, $tRNA_5^{Leu}$, is always absent from ribosomes. In a mutant strain in which normal $tRNA_3^{Leu}$ and $tRNA_5^{Leu}$ are reduced or absent, $tRNA_1^{Leu}$ is a major ribosome-bound species in minimal medium. Protein synthesis *in vitro* with RNA from *E. coli* further suggests that $tRNA_5^{Leu}$ is rarely used for total protein synthesis; however, this species is active with RNA from MS2 phage. We propose that $tRNA_1^{Leu}$ can substitute for $tRNA_3^{Leu}$ under rapid growth conditions, and that $tRNA_5^{Leu}$ is used minimally in total protein synthesis.**

Because of the degeneracy of the genetic code, it is understandable that the incorporation of individual amino acids into protein would require the use of more than one species of tRNA. Nevertheless, the degeneracy of the genetic code cannot in all cases explain the multiplicity of tRNA species for a single amino acid (1). This is especially true in view of the tenets of Crick's wobble hypothesis (2). Many authors have, therefore, suggested other physiological roles for some tRNA species in addition to their presumed primary role in protein synthesis. Regulatory roles for several tRNAs have been clearly established. For example, it has been shown that minor base alterations of histidyl-, leucyl-, and isoleucyl-tRNAs affect the regulation of their cognate biosynthetic pathways (3–5). In addition to these specific effects, it is also clear that tRNA is involved in a more general control of gene expression by eliciting guanosine 5′-diphosphate-3′-diphosphate formation and thereby affecting the stringent response in *E. coli* (6). These ubiquitous roles of tRNA in regulation of gene expression raise the question whether all tRNA species can participate in regulation with equal efficacy or whether only certain isoaccepting species for a given amino acid are used for a regulatory function. If, indeed, certain isoaccepting species of tRNA are preferentially utilized in the regulation of gene expression, is this use then accompanied by a decreased participation in protein synthesis? Alternatively, could differential utilization of tRNA isoaccepting species play a role in the regulation of protein synthesis itself? In this communication, we examine and compare the participation in protein synthesis of the five isoaccepting leucyl-tRNAs in the hope of gaining insight into the reasons for such an abundance of isoaccepting species.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *Escherichia coli* strain K-12 was grown in either minimal media or rich media (L-Broth) to the mid-logarithmic-growth phase (7). *E. coli* strain S-26 ($Su6^+$) (8) was obtained from the Yale type culture collection. The presence of an amber suppressor locus in this strain was verified by plaque assay utilizing a T4 amber DNA polymerase mutant kindly provided by P. Geiduschek.

Aminoacylation of tRNA. Leucyl-tRNA synthetase was partially purified using DEAE-cellulose essentially as described by Muench and Berg (9). tRNA was aminoacylated as outlined (10).

Preparation of tRNA from Various Cell Fractions. One liter cultures of *E. coli* (2×10^8 cells per ml) were chilled quickly with buffered ice (0.01 M Tris·HCl, pH 7.5, containing 0.014 M $MgCl_2$ and 5 mM 2-mercaptoethanol), and centrifuged at 4° for 10 min at $16,000 \times g$. The cells were resuspended in 5 ml of this buffer and treated for 30 min at 4° with 10 μg/ml of lysozyme (Worthington). The cells were disrupted in the French press, and cell debris was removed by centrifugation at $10,000 \times g$ for 1 hr at 4°, and then by centrifugation of the supernatant fluid at $30,000 \times g$ in the same rotor for 30 min. The entire preparation was layered over a 50 ml linear sucrose gradient (10–30%, wt/vol) with a 5 ml cushion of 60% sucrose and centrifuged at 15,000 rpm for 15 hr in a Beckman SW25 swinging-bucket rotor. Fractions containing supernatant fluid, 70S monosomes, and polysomes were pooled. Each pool was concentrated by ethanol precipitation and resuspended in 0.05 M Tris·HCl buffer, pH 8.5, containing 0.1 mM $MgCl_2$ and 5 mM 2-mercaptoethanol. Each preparation was mixed with an equal volume of phenol (saturated with the same buffer), and mixed for 15 min at room temperature. The aqueous phase was removed and incubated for 30 min at 37°, which is sufficient to completely deacylate the leucyl-tRNAs. Each preparation was passed through a Sephadex G-25 column equilibrated with H_2O adjusted to pH 8.0 with dilute NaOH, and contained 10 mM Mg Cl_2 and 5 mM 2-mercaptoethanol. Samples were aminoacylated as outlined above and then prepared for RPC-5 chromatography as follows: the reaction was stopped by the addition of 0.2 volumes of 2 M Na acetate, pH 5.0 at 4°. An equal volume of phenol saturated with 0.05 M Tris·HCl buffer, pH 7.0, was added. The aqueous phase was removed and passed through a Sephadex G-25 column equilibrated with 0.01 M acetate buffer, pH 4.5, containing 1 mM EDTA, 0.01 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 0.5 M NaCl. Samples were chromatographed through an RPC-5 column as described (10).

Preparation of *E. coli* mRNA. One liter of *E. coli* K12 was grown to 2 to 4×10^8 cells per ml in either minimal media or broth, poured on ice, and harvested. Cells were lysed either by the Godson procedure for making polysomes (11) or by addition of 1% sodium dodecyl sulfate and three freeze-thawings in a Dry Ice/ethanol bath. The lysate (3 ml) was treated with 50 μg of DNase at 0° for 15 min. Five drops of diethylpyrocarbonate (Baycovin) were added in the cold to eliminate nuclease activity, followed quickly by ethanol precipitation of RNA (to remove Baycovin); RNA was then extracted with phenol/chloroform (1/1, vol/vol), according to standard procedures (12).

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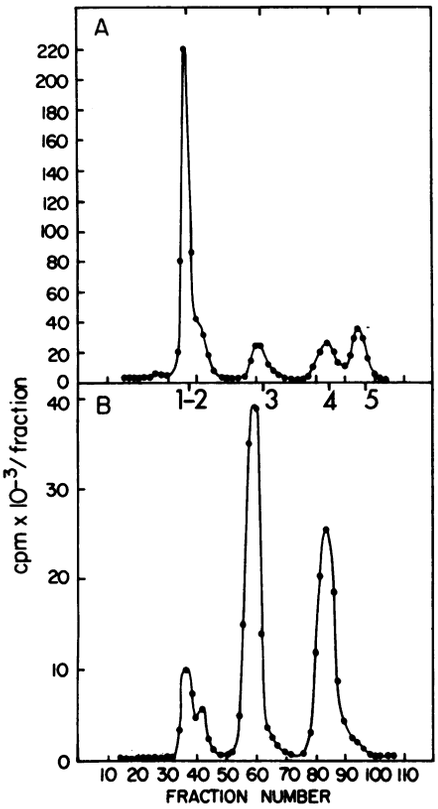

FIG. 1. RPC-5 chromatographic profiles of ribosome- and nonribosome-bound [^{3}H]leucyl-tRNA isolated from cells grown in minimal glucose medium. Separations were performed at 25° by using a linear 250 ml NaCl gradient (0.5–0.8 M). Elution positions of leucyl-tRNA isoaccepting species 1 through 5 are indicated by their respective numbers in this and subsequent figures. (*A*) Profile of nonribosomal-bound [^{3}H]leucyl-tRNA. (*B*) Profile of ribosome bound [^{3}H]leucyl-tRNA.

Protein Synthesis with Leucyl-tRNA *In Vitro*. Each purified, individual isoaccepting species (10) was first acylated with [^{3}H]leucine (47 Ci/mmol New England Nuclear) and then used at a final concentration of 250–500 pmol/ml (0.01–0.02 mg/ml). Only about 20% of each isoaccepting species was acylated and, therefore, the final concentration of acylated [^{3}H]leucine was 50–100 pmol/ml. The same concentration of cpm/ml (1 to 2 × 10^6) for each isoaccepting species was used in parallel samples; the overall RNA concentration of each isoacceptor was also equivalent in parallel samples.

E. coli crude extract (S30) was prepared as previously described (12) except that a French press was used to disrupt the cells. Protein synthesis *in vitro* was also carried out as described (12), except that reactions contained polyethylene glycol (molecular weight, 6000), 30 mg/ml and 1 mM leucine.

RESULTS

To determine the *in vivo* utilization in protein synthesis of the five leucyl-isoaccepting species tRNAs, we performed the following experiment. *E. coli* cells were grown in minimal media, lysed, and centrifuged through a sucrose gradient. Those portions of the gradient containing ribosomes (polysomes and monosomes) were pooled; the top of the gradient was pooled separately. RNA was extracted from both pools, and tRNA was deacylated, reacylated with [^{3}H]leucine, and then chromatographed through an RPC-5 column. The results of such an experiment are shown in Fig. 1. A different pattern of tRNA isoaccepting species is found on ribosomes (Fig. 1*B*) compared to ribosome-free cell supernatants (Fig. 1*A*).

Table 1. Distribution of leucyl-tRNA isoaccepting species from *E. coli* grown in rich and minimal media*

Fraction (media)	Species			
	$tRNA_{1,2}^{Leu}$	$tRNA_3^{Leu}$	$tRNA_4^{Leu}$	$tRNA_5^{Leu}$
Ribosome bound				
K-12 (rich)	39	34	26	1
K-12 (minimal)	13	47	39	1
Su6$^+$ (minimal)	53.4	11.6	35	0
Nonribosome bound				
K-12 (rich)	77	1.5	6	16.5
K-12 (minimal)	64	7	12	17
Su6$^+$ (minimal)	85	2.5	13.5	1
Total cell				
K-12 (rich)	65	12.8	11.3	10.9

* Expressed as the sum of radioactivity recovered from pooled chromatographic fractions; for each peak, times 100, divided by the sum of total radioactivity recovered from all five peaks in each chromatogram.

The actual percentage of each isoaccepting species is calculated in Table 1. We have combined the values for $tRNA_2^{Leu}$ with $tRNA_1^{Leu}$ in Table 1, because it was not possible to clearly separate these species on an RPC-5 column. The small amounts of $tRNA_2^{Leu}$ present were quite variable in different experiments, and on some occasions, no $tRNA_2^{Leu}$ was observed at all. $tRNA_3^{Leu}$ and $tRNA_4^{Leu}$ account for 86% of total ribosome-bound leucyl-transfer RNA in cells grown in minimal media. Little if any acylated $tRNA_5^{Leu}$ is found on ribosomes. These results are strikingly different from the relative tRNA percentages obtained from whole cell tRNA (Table 1), obtained from cells grown on either rich or minimal media. Note the difference in the percentage of $tRNA_1^{Leu}$, which comprises approximately 60% of the leucyl-tRNA from total cell extracts (Table 1), but is present on ribosomes in much lower proportions. Isoaccepting species, $tRNA_1^{Leu}$, is found instead largely in the ribosome-free cell supernatant (Fig. 1*A*; Table 1) in minimal media.

When the growth rate of *E. coli* is increased by cultivation in rich media, different relative proportions of tRNAs are observed (Fig. 2). In this experiment, $tRNA_1^{Leu}$ is now found in substantial amounts on ribosomes (Table 1). Isoaccepting species $tRNA_3^{Leu}$ and $tRNA_4^{Leu}$ are also still substantially present, and $tRNA_5^{Leu}$, as seen before, is largely absent, on ribosomes. Furthermore, the relative amounts of $tRNA_3^{Leu}$ and $tRNA_4^{Leu}$ present in the cell supernatant is reduced, which might be expected due to their preponderant utilization on ribosomes.

The partitioning of the total leucyl-tRNA acceptor activity between ribosomes and supernatant was determined. Average values from several experiments suggest that there is an increase in the ratio of ribosome- to nonribosome-bound tRNA as the growth rate is increased (data not shown). Under our conditions of isolation, some peptidyl-tRNA would be lost and thus not considered in these calculations. To determine whether the

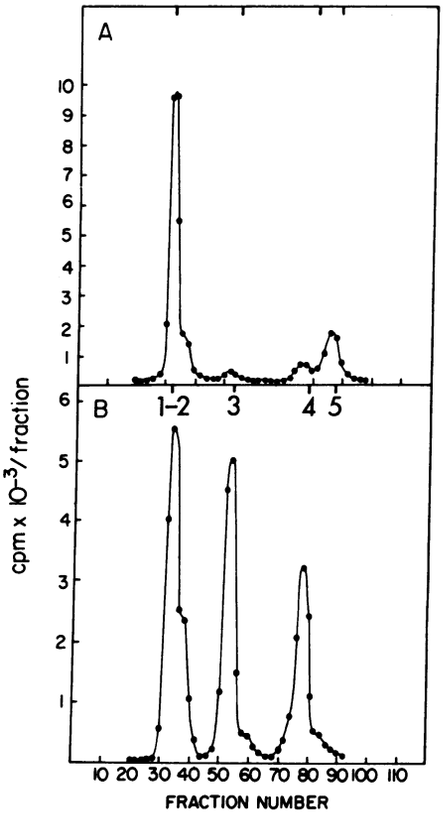

FIG. 2. RPC-5 chromatographic profiles of ribosome- and nonribosome-bound [^{3}H]leucyl-tRNA isolated from cells grown in L-broth. Separations were performed at 25° using a linear 250 ml NaCl gradient (0.5–0.8 M). (*A*) Profile of nonribosome-bound [^{3}H]leucyl-tRNA. (*B*) Profile of ribosome-bound [^{3}H]leucyl-tRNA.

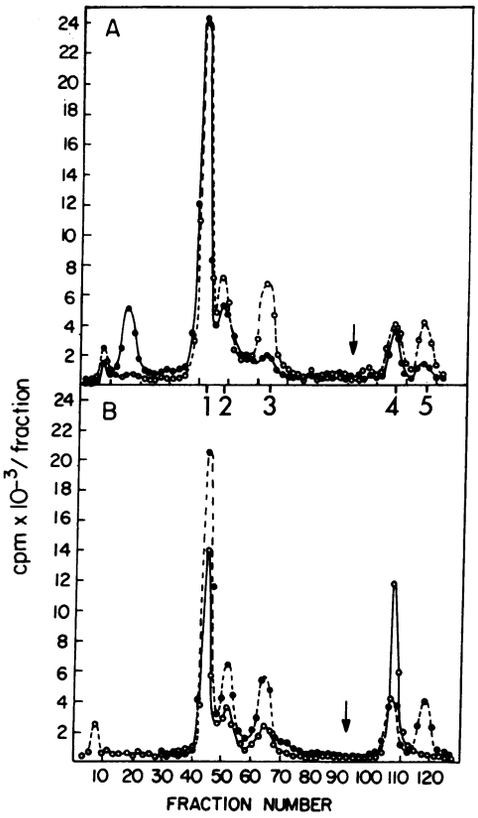

FIG. 3. RPC-5 chromatographic profiles of ribosome- and nonribosome-bound [^{3}H]leucyl-tRNA isolated from *E. coli* strain Su6$^+$ grown in minimal media. Separations were performed at 37° using a linear 250 ml NaCl gradient (0.5–0.8 M) followed by a linear 100 ml NaCl gradient (0.8–1.0 M). Arrows indicate where the second gradient began. (*A*) Profile of nonribosomal-bound [^{3}H]leucyl-tRNA. (O - - - O) total *E. coli* [^{14}C]leucyl-tRNA; (●—●) Su6$^+$ [^{3}H]leucyl-tRNA. (*B*) Profile of ribosomal-bound [^{3}H]leucyl-tRNA. (● - - - ●) Total *E. coli* [^{14}C]leucyl-tRNA; (O—O) Su6$^+$ [^{3}H]leucyl-tRNA.

tRNA utilization pattern observed in Fig. 2 also applied to peptidyl-tRNA, that we had not recovered in the initial experiments, we extracted and purified peptidyl-tRNA from ribosome pellets according to published procedures (13), stripped and acylated the tRNA with [^{3}H]leucine, and again used RPC-5 column chromatography to determine their relative utilization. The results were essentially the same as those obtained in Fig. 2*B* (data not shown), and indicated that patterns of utilization observed for ribosome bound tRNA^{Leu} also applied to peptidyl-tRNA^{Leu}.

The *E. coli* strain Su6$^+$ contains a mutation (8) which leads to the appearance of two amber suppressor tRNAs that can be separated on BD-cellulose (14); these two suppressors were shown to insert leucine into proteins *in vivo* (8) and *in vitro* (14) in response to the amber codon. An analysis of leucyl-tRNA in this strain, using RPC-5 chromatography, suggests that tRNA$_3^{Leu}$ and tRNA$_5^{Leu}$ species are altered (Fig. 3*A*; Table 1), because only small amounts of material from this strain coelute with wild-type markers for tRNA$_3^{Leu}$ and tRNA$_5^{Leu}$. A new peak appears eluting early in the gradient (Fig. 3*A*) and another new peak can also be obtained by elution with higher salt (not shown). In contrast to the results found with wild-type *E. coli* in Figs. 1 and 2, the RPC-5 chromatogram of ribosome-bound tRNA of Su6$^+$ shows that tRNA$_1^{Leu}$ is now the major tRNA species present.

To directly assess the protein synthesis capabilities of the leucyl-tRNA isoaccepting species, we developed a protein synthesizing system *in vitro*. A reaction mixture consisting of an *E. coli* crude extract (S30) and all the other reagents for protein synthesis *in vitro*, was distributed to individual tubes, with or without exogenous mRNA; each tube contained a single purified isoaccepting species of leucyl-tRNA acylated with [^{3}H]leucine, as described in *Materials and Methods*. The S30 extracts themselves contained a total of 120–160 pmol/ml of endogenous, acylatable leucyl-tRNA, of which some 20–40 pmol/ml were actually found to be acylated under our protein synthesis conditions (by using only the endogenous tRNA synthetase). Hence, we were concerned that some of our [^{3}H]leucine might be transferred from the exogenously charged isoaccepting species under test to a different endogenous leucyl-tRNA isoaccepting species during the incubation. We took the precaution of adding a high concentration of nonradioactive leucine in the reaction mixture, to dilute the specific activity of any [^{3}H]leucine obtained by deacylation. In a control experiment, the amount of [^{3}H]leucine incorporated from acylated tRNA was independent of the nonradioactive leucine concentration. We further performed the following control experiment: to a standard protein synthesis reaction (containing unlabeled leucine), we added 50 pmol/ml of acylated [^{3}H]-leucyl-tRNA$_3^{Leu}$; in one sample, we also added 1250 pmol/ml of nonacylated tRNA$_1^{Leu}$, and in another sample, 50 nmol/ml of total *E. coli* tRNA. No exogenous mRNA was added. After incubation at 37° for 15 min, all RNA was extracted, and both samples were chromatographed on RPC-5, along with a [^{14}C]leucyl-tRNA marker. The results of this experiment demonstrate the virtual absence of transfer of [^{3}H]leucine from [^{3}H]leucyl-tRNA$_3^{Leu}$ to tRNA$_1^{Leu}$ or any other leucyl-isoac-

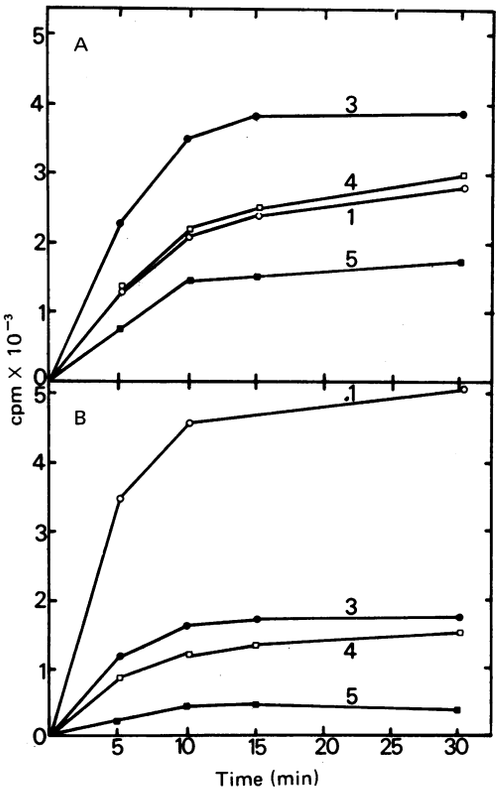

FIG. 4. (*A*) MS2 RNA. (*B*) *E. coli* mRNA. Kinetics of incorporation of [^{3}H]leucine into protein *in vitro* from acylated [^{3}H]leucyl-tRNA isoaccepting species. Reactions (0.1 ml) contained either MS2 RNA (0.5 mg/ml) or *E. coli* mRNA (1.3 mg/ml; obtained from cells grown in broth), and a single isoaccepting species of [^{3}H]leucyl-tRNA (0.02 mg/ml), as indicated by the numbers in the figure. At various times, 25-μl aliquots were removed and the amount of radioactivity in protein was determined. Background incorporation in the absence of added mRNA was subtracted from the results, and were as follows for 5, 10, 15, and 30 min points, in sequence. $tRNA_1^{Leu}$: 979, 1589, 1938, 2687. $tRNA_3^{Leu}$: 371, 361, 568, 676. $tRNA_4^{Leu}$: 346, 391, 498, 619. $tRNA_5^{Leu}$: 525, 796, 1069, 1758.

cepting species under our experimental conditions (data not shown).

A further concern was that different isoaccepting species might have different rates of deacylation in our reactions, which could lead to erroneous conclusions regarding their relative utilization. However, we found that the kinetics of deacylation for $tRNA_{1,3,4, \text{ and } 5}^{Leu}$ were all comparable, with a half-life of about 18 min (data not shown).

In some of these experiments, we encountered fairly high levels of background incorporation of [^{3}H]leucine into alkali-resistant trichloroacetic acid-precipitable material in the absence of exogenous mRNA. Because it is known that leucine is added directly to the NH_2-termini of certain intact ribosomal proteins (15), we thought it possible that at least a part of these background values were a consequence of this phenomenon. To determine what portion of the experimental backgrounds were due to the presence of endogenous mRNA in the extract, we incubated protein synthesis reactions without exogenous mRNA in the presence and absence of chloramphenicol (0.2 mg/ml), which is sufficient to inhibit messenger-mediated protein synthesis by 99%. For all the isoaccepting species, most if not all of the background incorporation proved to be insensitive to chloramphenicol inhibition (data not shown); hence, these background values do not reflect message mediated protein synthesis.

Table 2. Protein synthesis with acylated [^{3}H]leucyl-tRNA *in vitro*

Source of mRNA	Exp. no.	Radioactivity/reaction (cpm)			
		$tRNA_1^{Leu}$	$tRNA_3^{Leu}$	$tRNA_4^{Leu}$	$tRNA_5^{Leu}$
E. coli	1	3636	1506	1851	511
	2	2718	1496	1927	303
MS2	3	2526	6490	2886	1545

Reactions (25 μl for Exps. 1 and 2; 50 μl for Exp. 3) contained either MS2 RNA (0.5 mg/ml) or *E. coli* RNA (0.9 mg/ml for Exp. 1; 1.3 mg/ml for Exp. 2), and a single isoaccepting species of [^{3}H]leucyl-tRNA (0.02 mg/ml). Incubations were for either 10 min (Exp. 1) or 30 min (Exps. 2 and 3) at 37°. *E. coli* RNA was prepared from both cells grown in minimal media (Exp. 1) and cells grown in broth (Exp. 2). Background incorporation in the absence of added mRNA was subtracted from the results, and had these values. Exp. 1: $tRNA_1^{Leu}$, 544; $tRNA_3^{Leu}$, 162; $tRNA_4^{Leu}$, 376; $tRNA_5^{Leu}$, 192. Exp. 2: $tRNA_1^{Leu}$, 499; $tRNA_3^{Leu}$, 115; $tRNA_4^{Leu}$, 361; $tRNA_5^{Leu}$, 215. Exp. 3: $tRNA_1^{Leu}$, 2463; $tRNA_3^{Leu}$, 685; $tRNA_4^{Leu}$, 801; $tRNA_5^{Leu}$, 1355.

mRNAs tested included mRNA from *E. coli*, which was used at saturating levels, and RNA from bacteriophage MS2, which was used at one-half or greater saturation levels (16). The results of a number of experiments are shown in Table 2 and Fig. 4. $tRNA_5^{Leu}$ is poorly used for protein synthesis with *E. coli* mRNA, which is consistent with its observed absence from ribosomes *in vivo* (Figs. 1*B* and 2*B*). However, this species of tRNA is utilized with MS2 RNA message *in vitro*. The difference between MS2 and *E. coli* RNA in stimulating utilization of this species is especially evident from the rates of incorporation seen in the kinetics experiment of Fig. 4. $tRNA_1^{Leu}$ is the most active species with *E. coli* message (isolated from both minimal and rich media grown cells), in contrast to its diminished proportion on some ribosomes (Fig. 1*B*); however, $tRNA_1^{Leu}$ is seen to have a somewhat lower activity with MS2 RNA *in vitro*. Isoaccepting species $tRNA_3^{Leu}$ and $tRNA_4^{Leu}$ are competent with both messages, with $tRNA_3^{Leu}$ being the most active species for MS2 RNA.

DISCUSSION

In this report, we have examined the utilization of *E. coli* leucyl-tRNA isoaccepting species in protein synthesis. We find that two minor species of leucyl-tRNA, $tRNA_3^{Leu}$ and $tRNA_4^{Leu}$, are the predominant leucyl-tRNA species bound to ribosomes (and presumably participating in protein synthesis) during exponential growth in minimal medium. Little if any $tRNA_5^{Leu}$ is found on ribosomes in these experiments. The major leucyl-tRNA isoaccepting species in the cell, $tRNA_1^{Leu}$, is found in much lower proportions on ribosomes. Therefore, utilization of these tRNAs does not reflect their relative total proportion in the cell. Previous workers have also noted that $tRNA_1^{Leu}$ appeared to be present in reduced amounts on polysomes, compared to the expected values from its proportion in the cell (17). Unpublished experiments have shown that there is a species of normal *E. coli* leucyl-tRNA present in total cell tRNA which is a nicked form of $tRNA_1^{Leu}$ (Brian R. Reid, personal communication); this species may account for some of the material in our small variable $tRNA_2^{Leu}$ peaks in these experiments.

When utilization of these same tRNAs are examined during exponential growth in rich media, different distributions are observed. Isoaccepting species $tRNA_1^{Leu}$ is now present on ribosomes in a relatively higher proportion. Furthermore, $tRNA_4^{Leu}$ and especially $tRNA_3^{Leu}$ are depleted from the

ribosome-free cell supernatant. Again, little if any $tRNA_5^{Leu}$ is observed on these ribosomes.

These data can be interpreted in the following manner. Under conditions of rapid growth (rich media), the total cellular complement of $tRNA_3^{Leu}$ is utilized in protein synthesis. This potentially rate-limiting condition is relieved by increased utilization of $tRNA_1^{Leu}$ in addition to $tRNA_3^{Leu}$. Such a mechanism could allow the cell to have a flexible response due to increased demands for tRNA in protein synthesis. In minimal media, $tRNA_3^{Leu}$ is present in adequate amounts to support the reduced demands on the protein synthesis apparatus; hence, $tRNA_1^{Leu}$ is not called upon to supplement $tRNA_3^{Leu}$, and is therefore present in lower proportions on the ribosomes. Finally, on the basis of these experiments, $tRNA_5^{Leu}$ appears not to be required for total protein synthesis.

To test these interpretations, we examined the tRNA utilization patterns of the mutant *E. coli* $Su6^+$, which is deficient in or lacking normal $tRNA_3^{Leu}$ and $tRNA_5^{Leu}$ (Fig. 3, Table 1). These two isoaccepting species are probably unnecessary for protein synthesis because $Su6^+$ grows without them. $tRNA_1^{Leu}$ can indeed substitute for $tRNA_3^{Leu}$, as shown by the fact that this strain utilizes $tRNA_1^{Leu}$ and $tRNA_4^{Leu}$ for protein synthesis in minimal medium, while under the same growth conditions, the wild-type cell utilizes primarily $tRNA_3^{Leu}$ and $tRNA_4^{Leu}$.

The reduction of normal $tRNA_5^{Leu}$ in $Su6^+$, combined with the failure to detect this tRNA on ribosomes, favors our suggestion that this species is not required for total protein synthesis in *E. coli*. A regulatory role for this isoaccepting species has been suggested by experiments of Jegede and Brenchley (18).

Although $tRNA_5^{Leu}$ appears to be minimally involved in protein synthesis in *E. coli in vivo*, and is also poorly active for protein synthesis *in vitro* with total *E. coli* message, we have shown, nevertheless, that it is indeed competent for protein synthesis *in vitro* by using bacteriophage MS2 RNA message. The presumed major difference between phage and *E. coli* mRNAs is that phage RNA appears to have a more elaborate secondary/tertiary structure (19); it is possible, therefore, that $tRNA_5^{Leu}$ is used in protein synthesis with stable messages only. Alternatively, a rare codon response with phage RNA could account for this data. Experiments designed to test these possibilities are in progress.

Obviously, codon responses and wobble phenomena figure prominently in attempts to understand these experiments. How else could a substitution of $tRNA_1^{Leu}$ for $tRNA_3^{Leu}$ be rationalized? Recently, Di Natale and Eilat (20) examined and summarized previously reported codon responses of the leucyl-tRNA isoaccepting species. Identifying the isoaccepting species by their positions of elution from RPC-5, they reported the following codon assignments. $tRNA_1^{Leu}$, CUG; $tRNA_2^{Leu}$, CUG and CUA; $tRNA_3^{Leu}$, CUU and CUC; $tRNA_4^{Leu}$, UUG; $tRNA_5^{Leu}$, UUG and UUA. Furthermore, sequence data is available on two of these isoaccepting species; the anticodon of $tRNA_1^{Leu}$ indeed corresponds to a CUG codon (21), and the anticodon of $tRNA_3^{Leu}$ corresponds to CUC codon (22). The latter tRNA was originally isolated from BD-cellulose, and called $tRNA_2^{Leu}$, but has since been correlated with the third peak eluting from RPC-5 columns, $tRNA_3^{Leu}$ (W. M. Holmes and G. W. Hatfield, unpublished results). Clearly these assignments alone, with only classic wobble permitted, cannot explain the data presented here, because the experiments with $Su6^+$ imply that (*i*) $tRNA_5^{Leu}$ is unnecessary for protein synthesis, and (*ii*) $tRNA_1^{Leu}$ substitutes for $tRNA_3^{Leu}$ which indicates that $tRNA_1^{Leu}$ responds to CUC codons with a third position C-C wobble which is not permitted in classic wobble theory. Therefore, more rigorous methods of testing of codon and wobble responses, by examining the ability of pure tRNAs to insert their amino acids into growing polypeptide chains coded for by naturally sequenced messages, are currently in progress in this laboratory.

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