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Presence of the hypermodified nucleotide N^6-(Δ^2-isopentenyl)-2-methylthioadenosine prevents codon misreading by *Escherichia coli* phenylalanyl-transfer RNA

(tRNA genes/wobble hypothesis/*in vitro* translation)

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ABSTRACT **The overall structure of transfer RNA is optimized for its various functions by a series of unique post-transcriptional nucleotide modifications. Since many of these modifications are conserved from prokaryotes through higher eukaryotes, it has been proposed that most modified nucleotides serve to optimize the ability of the tRNA to accurately interact with other components of the protein synthesizing machinery. When a cloned synthetic *Escherichia coli* $tRNA^{Phe}$ gene was transfected into a bacterial host that carried a defective phenylalanine tRNA-synthetase gene, $tRNA^{Phe}$ was overexpressed by 11-fold. As a result of this overexpression, an undermodified $tRNA^{Phe}$ species was produced that lacked only N^6-(Δ^2-isopentenyl)-2-methylthioadenosine (ms^2i^6A), a hypermodified nucleotide found immediately 3′ to the anticodon of all major *E. coli* tRNAs that read UNN codons. To investigate the role of ms^2i^6A in *E. coli* tRNA, we compared the aminoacylation kinetics and *in vitro* codon-reading properties of the ms^2i^6A-lacking and normal fully modified $tRNA^{Phe}$ species. The results of these experiments indicate that while ms^2i^6A is not required for normal aminoacylation of $tRNA^{Phe}$, its presence stabilizes codon–anticodon interaction and thereby prevents misreading of the genetic code.**

In 1973, Jukes (1) proposed that a hypermodification of the nucleotide immediately 3′ to the anticodon in transfer RNA serves to ensure the fidelity of protein synthesis by preventing wobble base-pairing interactions between the third anticodon and first codon position nucleotides. This hypothesis was based on the observation that all major tRNAs that read codons beginning in A or U contain, with the exception of *Escherichia coli* $tRNA^{fMet}$, a hypermodified purine nucleotide following the anticodon sequence (see ref. 2). Accordingly, it was suggested that this anticodon-adjacent hypermodification stabilizes the relatively weak A·U/U·A base pairings and thereby prevents misreading of the genetic code (2). The classic exception to this pattern, *E. coli* $tRNA^{fMet}$, contains an unmodified adenine immediately 3′ to its anticodon and is able to read the codons GUG (valine) and UUG (leucine) by a third anticodon position wobble mechanism, as well as the AUG (methionine) codon by Watson–Crick base pairing (1, 3, 4). The proposed restricted codon reading by the hypermodified nucleotide 3′ to the anticodon was further supported upon examining the three-dimensional crystal structure of yeast $tRNA^{Phe}$ (5). Here, it was determined that the three anticodon and two distal nucleotides were stacked in a continuous helix on one side of the anticodon loop, with the first position or wobble nucleotide (6) at the bottom or 5′ end of the stack (5, 7). These observations suggested that the hypermodified purine immediately 3′ to the anticodon stabilizes the helical stacking interaction and thus prevents the third anticodon position nucleotide from participating in wobble interactions with the first nucleotide of the codon (2, 8). In contrast, analysis of the three-dimensional crystalline structure of *E. coli* $tRNA^{fMet}$ showed that the lack of a hypermodified nucleotide immediately 3′ to the anticodon results in an altered stacking pattern of the loop nucleotides and possibly a conformation that allows third anticodon position wobble (9).

The pattern of anticodon-adjacent nucleotide hypermodification also is seen in the tRNAs from higher vertebrate mitochondria, since mitochondrial tRNAs that read codons starting with A or U contain N^6-(Δ^2-isopentenyl)-2-methylthioadenosine (ms^2i^6A) or *N*-(9-β-D-ribofuranosylpurin-6-ylcarbamoyl)threonine (t6A) immediately 3′ to their anticodons (10, 11). A single exception to this pattern has been observed in the bovine mitochondrial $tRNA^{Leu}$ (UUR), which contains an unmodified adenine immediately following its anticodon sequence (12). Since $tRNA^{Leu}$ (UUR) would read the alternative CUR leucine codons via a third anticodon position wobble mechanism, the lack of hypermodification of its anticodon-adjacent purine does not result in misincorporation through codon misreading.

Previous studies of hypermodified nucleotide function in tRNA have included detailed investigations of the role of ms^2i^6A in *E. coli* $tRNA^{Phe}$ and $tRNA^{Tyr}$ in the aminoacyl tRNA-synthetase reaction and *in vitro* polymer-dependent protein synthesis (8, 13–15). These studies suggested that the lack of ms^2i^6A did not decrease the ability of the tRNA to be charged with its cognate amino acid but did alter the efficiency with which the tRNA bound to the ribosome (13). In addition, physical–biochemical studies have demonstrated that the presence of various hypermodified nucleotides alters the rigidity of dinucleotides in solution (8). These results are consistent with the hypothesis that one major function of ms^2i^6A (or t^6A, for that matter) is to stabilize the stacking interactions of the anticodon loop helix and thereby prevent codon misreading. Furthermore, *E. coli* and *Salmonella typhimurium* mia^- mutants whose tRNAs are deficient in ms^2i^6A have been observed to grow significantly slower than wild-type strains (16, 17) and produce proteins with a lower error frequency (18–21). Thus, it has been proposed that tRNA species that possess the proper nucleotide modification immediately 3′ to the anticodon may be preferentially used over the undermodified species in protein synthesis (22, 23).

In this present work, we obtained *in vivo* overexpression of a cloned *E. coli* $tRNA^{Phe}$ gene and as a result could purify large amounts of both the normal fully modified $tRNA^{Phe}$ species and a second $tRNA^{Phe}$ species that lacked the

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Abbreviation: ms^2i^6A, N^6-(Δ^2-isopentenyl)-2-methylthioadenosine.

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hypermodified nucleotide ms^2i^6A and contained an unmodified adenine immediately 3′ to the anticodon sequence but was otherwise fully modified. After determining that both homogenic $tRNA^{Phe}$ species had identical aminoacylation kinetics, we used a coupled *in vitro* transcription and translation system and radiolabeled protein sequencing to obtain direct evidence that the presence of ms^2i^6A in *E. coli* $tRNA^{Phe}$ prevents deleterious wobble base pairings between the third anticodon and first codon position nucleotides during the course of protein synthesis.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The isolation and nucleotide sequence of the *E. coli pheU* gene has been reported (24). The *pheU* gene-containing plasmid pRK3 has been redesignated pRKU. The *E. coli* hosts, Hb-101 and NP-37, also have been described (24, 25). The pAEPhe plasmid contains a 200-base-pair synthetic *pheU* gene, constructed from six overlapping oligonucleotides cloned into the *Eco*RI and *Hin*dIII sites of pAT153. All oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer using the modified cyanoethyl chemistry (26, 27).

Preparation and Fractionation of tRNA. Crude *E. coli* tRNA was prepared by phenol extraction from late logarithmic-phase cultures (24). The tRNA was enriched for $tRNA^{Phe}$ by passage over DEAE-cellulose (DE-52, Whatman) and successively fractionated on an RPC-5 column (50 × 0.8 cm) at pH 7.6, an Aminex-28 column (30 × 0.2 cm) at pH 4.5, and a second RPC-5 column (30 × 0.2 cm) at pH 4.5 (28–30). This method yielded $tRNA^{Phe}$ with an average purity of at least 1.2 nmol/A_{260}.

Aminoacylation of $tRNA^{Phe}$. Column fractions were assayed for phenylalanine acceptor activity using a crude *E. coli* aminoacyl tRNA-synthetase preparation (12 mg of protein per ml) and [U-^{14}C]phenylalanine (50 μCi/mmol; 1 Ci = 37 GBq) (28, 31). Kinetic parameters of aminoacylation were determined by using purified $tRNA^{Phe}$ as described (32).

tRNA Base Composition and Sequence Analysis. Base analysis of the purified $tRNA^{Phe}$ species was performed by the postlabeling method of Randerath *et al.* (33). ^{3}H-labeled nucleoside diols were resolved by two-dimensional chromatography on cellulose sheets in acetonitrile/ammonia/water and Organic II/formic acid/water, respectively. Sequence analysis of wild type and ms^2i^6A-lacking $tRNA^{Phe}$ species was performed on 5′ ^{32}P-end-labeled tRNA by the method of Gupta and co-workers (34). RPC-5 column-purified $tRNA^{Phe}$ (10–15 μg) was electrophoresed on a 7 M urea/15% polyacrylamide gel and visualized by staining with 0.2% methylene blue (pH 5.5). Full-length tRNA bands were eluted from the gel, ethanol precipitated, and partially hydrolyzed in deionized formamide at 96°C for 7 min. The tRNA fragments then were end-labeled, resolved on 7 M urea/15% polyacrylamide gels, and transferred to polyethyleneimine (PEI)-cellulose thin-layer chromatography (TLC) sheets for one-dimensional ascending chromatography in 0.55 M ammonium sulfate. The resulting chromatograms were autoradiographed overnight at −70°C to visualize the tRNA sequence. Modified nucleotides were eluted from the PEI-cellulose TLC sheets and further analyzed by two-dimensional TLC on Avicel plates in isobutyric acid/ammonia/water and isopropanol/HCl/water, respectively, with unlabeled nucleotide standards (33).

***In Vitro* Translation and Radiosequencing.** *In vitro* protein synthesis was performed using a previously described (35, 36), commercially available prokaryote DNA-dependent translation system (Amersham) supplemented with pUC19 DNA, [*ring*-2,3,4,5,6-^{3}H]phenylalanine (101 Ci/mmol), [U-^{14}C]leucine (348 mCi/mmol), the 18 other unlabeled amino acids, and either purified $tRNA^{Phe}$ species at a 20-fold excess over the endogenous $tRNA^{Phe}$ contained in the translation system supplement (37). Radiosequencing of protein produced in the presence of a 20-fold excess of wild-type *E. coli* $tRNA^{Phe}$ showed no significant misincorporation of phenylalanine (see Fig. 2*b*). Alternatively, the system was supplemented with [U-^{14}C]phenylalanine (460 mCi/mmol) and [3,4,5(N)-^{3}H]leucine (140 Ci/mmol) to minimize isotopic effects. The translation products initially were analyzed on 0.1% $NaDodSO_4$/12.5% polyacrylamide gels. Subsequently, the reaction products were purified to radiochemical homogeneity by S-200 chromatography and subjected to 20 cycles of automated Edman degradation on a Beckman 890C sequenator using the dilute quadrol program (38). The radioactivity in each sequenator cycle fraction then was measured to determine phenylalanine and leucine incorporation at each position.

RESULTS

Cloning and Expression of *E. coli* $tRNA^{Phe}$ Genes. A 3.6-kilobase-pair *Sal* I fragment containing the *E. coli pheU* gene was excised from the pID2 plasmid (24), subcloned in pBR322, and designated pRKU (previously designated pRK3 in ref. 24). A smaller version of the *pheU* operon was constructed from six overlapping synthetic oligonucleotides subcloned in pAT153 and designated pAEPhe. The construction strategy of this synthetic tRNA gene was similar to that described by Normanly *et al.* (39) for a synthetic leucine amber suppressor gene. However, the natural 5′ and 3′ *pheU* flanking sequences were included so that transcription would begin and terminate at the normal sites. Since we sought to gain a greatly elevated level of expression of the cloned $tRNA^{Phe}$ genes, we used the NP-37 host strain, which carries a temperature-sensitive lesion in its phenylalanine tRNA-synthetase (*pheS*) gene. When either version of the cloned, $tRNA^{Phe}$ gene was introduced into the NP-37 strain, the synthetase mutation was overcome and the host grew at the nonpermissive temperature. As shown in Table 1, temperature resistance in NP-37 transformants was accompanied by a large increase in the expression of $tRNA^{Phe}$. Although the precise mechanism behind the increased $tRNA^{Phe}$ production is not understood, it has been speculated that increased levels of $tRNA^{Phe}$ can compensate for an altered K_m in the mutant phenylalanine tRNA-synthetase produced by the *E. coli* NP-37 host (27, 41).

Purification of Two $tRNA^{Phe}$ Species. To determine whether the greatly increased expression of the cloned *E. coli* $tRNA^{Phe}$ genes resulted in the accumulation of both mature and undermodified species, total tRNA was prepared from NP-37 transformants and partially fractionated by RPC-5 chromatography (31). As shown in Fig. 1, NP-37/pAEPhe transformants produced an elevated level of $tRNA^{Phe}$, which eluted at a position identical to the mature fully modified

Table 1. Comparison of *in vitro* amino acid acceptor levels

Strain (plasmid)	pmol/A_{260} Methionine (50 μCi/mmol)	pmol/A_{260} Phenylalanine (50 μCi/mmol)	Phenylalanine overexpression
E. coli Hb-101	45.1 ± 4.1	40.1 ± 6.1	—
Hb-101 (pRKU)	45.0 ± 4.0	68.2 ± 6.9	1.7
NP-37 (pRKU)	44.8 ± 4.0	461.2 ± 20.2	11.5
NP-37 (pRKW) (40)	44.9 ± 4.0	693.7 ± 15.5	17.3
NP-37 (pAEPhe)	44.7 ± 4.0	441.1 ± 15.5	11.0

Values represent an average of two independent assays in which acceptor levels were determined in triplicate. Background (no tRNA) was determined for each assay and subtracted to give corrected values. Phenylalanine overexpression was calculated by dividing the phenylalanine acceptor levels obtained by the value for *E. coli* Hb-101.

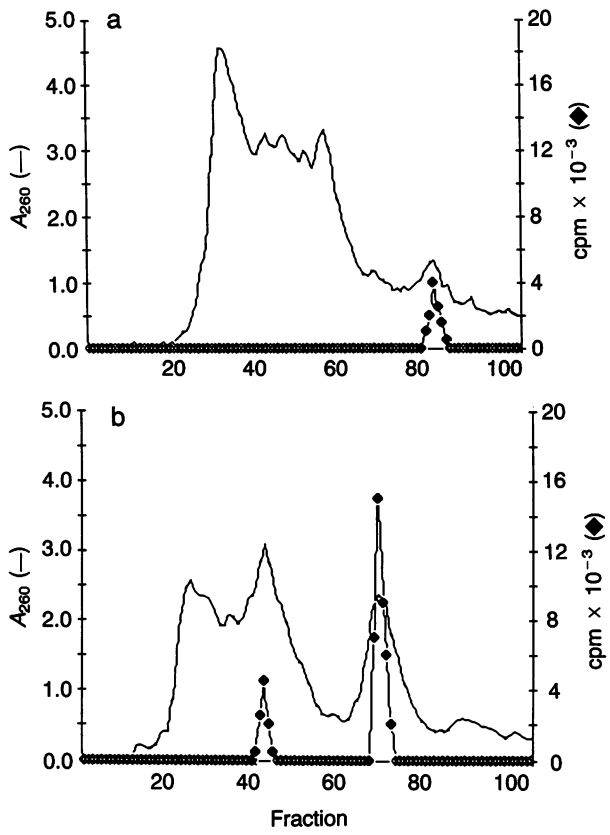

FIG. 1. Fractionation of total tRNA from *E. coli* Hb-101 (*a*) and *E. coli* NP-37(pAEPhe) (*b*) on RPC-5. Elution of tRNA was monitored by measuring the absorbance at 260 nm. Phenylalanine acceptor activity was determined by using crude *E. coli* aminoacyl tRNA-synthetase as described in *Materials and Methods*.

$tRNA^{Phe}$ as well as a second earlier eluting species. An identical chromatographic pattern was observed with tRNA from NP-37/pRKU transformants (data not shown). Since ms^2i^6A contains a large hydrophobic side chain that increases the retention time of *E. coli* $tRNA^{Phe}$ on RPC-5, it was suspected that the earlier eluting $tRNA^{Phe}$ species produced by NP-37 transformants might contain an alternatively or totally unmodified adenine nucleotide immediately 3′ to the anticodon. This suspicion was confirmed by postlabeling nucleotide analysis and complete tRNA sequence determination of both $tRNA^{Phe}$ species (data not shown). These sequencing studies demonstrated that the earlier eluting $tRNA^{Phe}$ differed from the later eluting species only in that it contained an unmodified adenine instead of a ms^2i^6A immediately 3′ to its anticodon. These experiments also showed that both $tRNA^{Phe}$ species produced by NP-37 transformants had identical primary nucleotide sequences and contained their full compliment of all other modified nucleotides.

Aminoacylation Kinetics of ms^2i^6A-Lacking $tRNA^{Phe}$. To test whether both ms^2i^6A-lacking and ms^2i^6A-containing *E. coli* $tRNA^{Phe}$ species could be properly recognized by phenylalanine tRNA-synthetase and subsequently charged with their cognate amino acid, their aminoacylation kinetics were determined. The apparent K_m and V_{max} of aminoacylation for the ms^2i^6A-lacking and ms^2i^6A-containing *E. coli* $tRNA^{Phe}$ species were 0.90×10^{-7} M and 0.86×10^{-7} M, and 0.54 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ and 0.48 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, respectively. These results directly confirm the earlier conclusions of Yanofsky and Soll (42) and Goddard and Lowden (43) and are consistent with the previous suggestion that the nucleotide immediately 3′ to the anticodon is not one of the primary sites of phenylalanine tRNA-synthetase recognition in $tRNA^{Phe}$ (32).

***In Vitro* Codon Reading Properties of ms^2i^6A-Lacking $tRNA^{Phe}$.** To investigate the codon reading properties of both the wild-type and ms^2i^6A-lacking *E. coli* $tRNA^{Phe}$ species, we used an *E. coli* cell-free coupled transcription/translation system primed with pUC19 plasmid DNA (35–37). Using this approach, only the β-lactamase gene carried on the pUC19 plasmid was transcribed and translated when a 20-fold excess of either normal or ms^2i^6A-lacking $tRNA^{Phe}$ and [^{14}C]phenylalanine was incubated with an S30 extract of *E. coli* K-12, and the products were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (data not shown). To compare the codon reading properties of the normal and ms^2i^6A-lacking *E. coli* $tRNA^{Phe}$ species, β-lactamase was synthesized in the presence of either $tRNA^{Phe}$ species with [^{3}H]phenylalanine and [^{14}C]leucine. The translation products were purified to radiochemical homogeneity by Sephacryl S-200 chromatography and subjected to 20 cycles of automated Edman degradation. By measuring the radioactivity present in each of the first 20 sequenator cycle fractions, we determined the positional incorporation of phenylalanine and leucine in the protein. As shown in Fig. 2*a*, the β-lactamase protein contains phenylalanine residues at positions 6, 13, 14, and 17 and leucine residues at positions 10 and 19 (44). The results of a typical radiosequencing experiment, shown in Fig. 2*b*, demonstrated that the normal *E. coli* $tRNA^{Phe}$ only incorporated phenylalanine in response to UUC and UUU phenylalanine codons. In contrast, the results shown in Fig. 2*c* demonstrate that the ms^2i^6A-lacking $tRNA^{Phe}$ species incorporated phenylalanine at the positions that correspond to the leucine codons (CUU) as well as the normal phenylalanine codons. Similar results were obtained when the labeled amino acids were reversed to [^{14}C]phenylalanine and [^{3}H]-leucine (Fig. 2*d*). Since no misincorporation of phenylalanine was observed when the wild-type $tRNA^{Phe}$ was used at a 20-fold excess over the endogenous $tRNA^{Phe}$ contained in the translation system, it was concluded that the "double-wobble" codon reading resulted primarily from the lack of ms^2i^6A. Interestingly, none of the radiosequencing data showed phenylalanine incorporation at codon 12, corresponding to proline (CCC), suggesting that there was no second position wobble.

DISCUSSION

Through these experiments, we have obtained direct evidence that the presence of the hypermodified nucleotide ms^2i^6A in the position immediately 3′ to the anticodon in *E. coli* $tRNA^{Phe}$ restricts codon reading *in vitro* by preventing a wobble base pairing with the first position codon nucleotide. Furthermore, when an unmodified adenine is present immediately following the anticodon, recognition of the CUU codons by $tRNA^{Phe}$ involves a double-wobble base pairing mechanism because both the first and third nucleotides of the codon are read by non-Watson–Crick base pairing interactions. As previously discussed, earlier reports indicated that the presence of the hypermodified nucleotide ms^2i^6A in $tRNA^{Phe}$ and $tRNA^{Tyr}$ optimizes the interaction between the tRNA and ribosomes, with the undermodified species being less efficient in translating the proper codons (reviewed in ref. 45). Although our positional label analysis experiments showed that ms^2i^6A-lacking $tRNA^{Phe}$ can incorporate phenylalanine in response to CUU (and presumably CUC) codons, we also observed that the total phenylalanine incorporation was lower when the *in vitro* transcription/translation system was supplemented with the undermodified $tRNA^{Phe}$ rather than the normal fully modified $tRNA^{Phe}$ species (unpublished observation). This is consistent with earlier reports (8, 13–15). It also should be noted that an aberrant protein produced *in vivo* by incorporating an undesirable amino acid via the first position wobble mechanism, such as may occur in ms^2i^6A-deficient strains, may be rapidly degraded by the extremely active

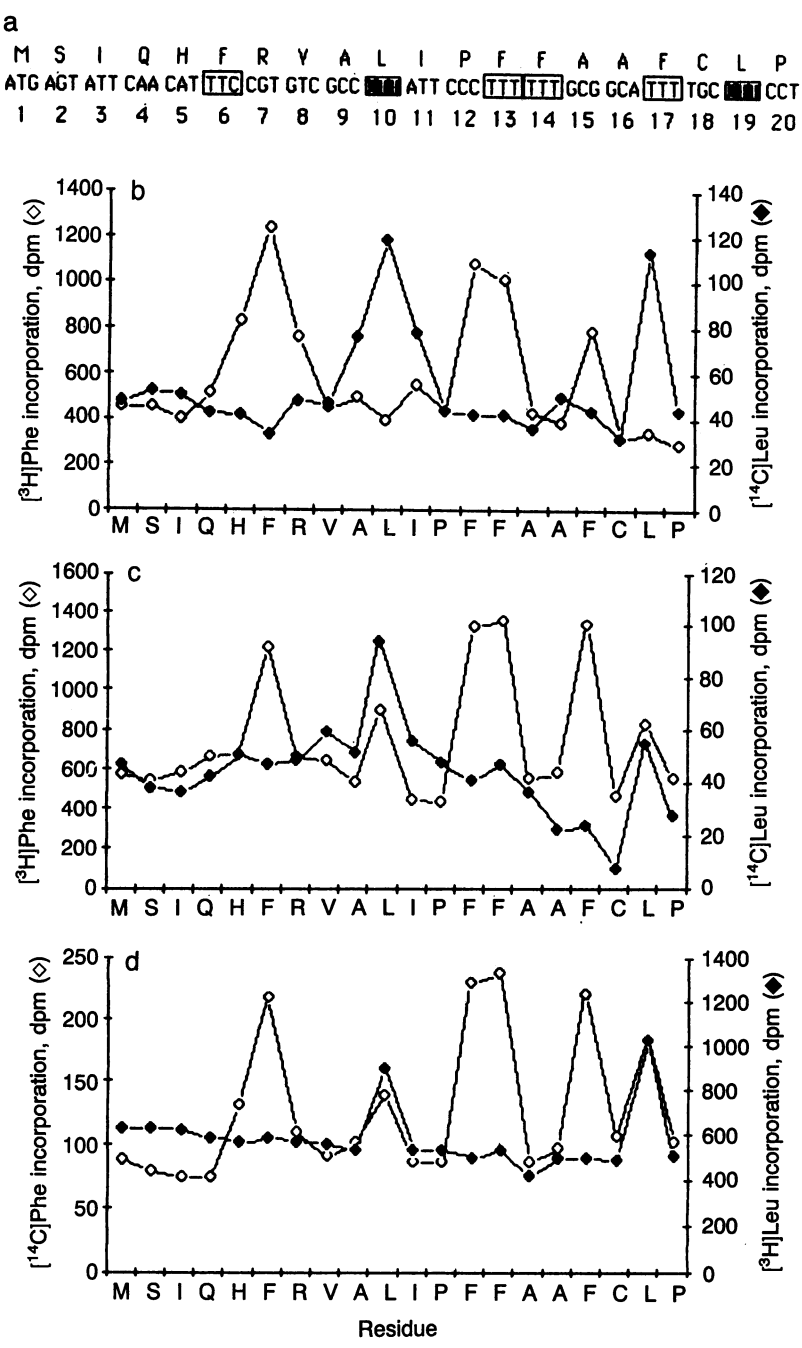

FIG. 2. (*a*) Nucleotide and amino acid sequences of the pUC19-encoded amino terminus of β-lactamase (44). Phenylalanine and leucine codons are highlighted. Results of radiosequencing experiments: β-lactamase was produced in the presence of normal fully modified $tRNA^{Phe}$ (*b*) and ms^2i^6A-lacking $tRNA^{Phe}$ (*c* and *d*). Raw dpm of sequenator cycle fractions are plotted versus the amino-terminal sequence of β-lactamase. *In vitro* transcription/translation reactions were performed as described in *Materials and Methods* using [^{3}H]phenylalanine and [^{14}C]leucine (*b* and *c*) or [^{14}C]phenylalanine and [^{3}H]leucine (*d*). Amino acids are designated by the single-letter code.

bacterial protein degradation pathway (reviewed in ref. 46). Since such a degradative mechanism most likely is not active in the *in vitro* system used here, it is likely that the decreased level of phenylalanine incorporation is the result of a lower *in vitro* translation rate due to less efficient codon–anticodon interactions of the ms^2i^6A-lacking $tRNA^{Phe}$ (8, 13).

If we assume that our experiments have given insight into one of the functions of nucleotide modifications immediately 3′ to the anticodon, we must consider the naturally occurring absence of modification in this position in at least two tRNAs, the *E. coli* $tRNA^{Ser}$ species with GGA and CGA anticodons (47). Since both of these tRNAs contain an unmodified adenine immediately 3′ to their anticodons rather than the expected ms^2i^6A, we can speculate that their G+C-rich anticodon sequence is such that the anticodon loop and stem more readily assume a tertiary structure that mimics the effect of ms^2i^6A and thereby prevents first codon position wobble. This speculation is supported by anticodon-anticodon hybridization experiments (referenced in ref. 46). Interestingly the analogous eukaryote $tRNA^{Ser}$ species contain a hypermodified nucleotide, N^6-isopentenyladenosine (i^6A), immediately 3′ to their anticodons (2).

In an elegant series of competition experiments, Smith and co-workers (22, 23) reported on the effect of hypomodification of the 3′ anticodon adjacent nucleotide in mammalian $tRNA^{Phe}$ on altering the codon preference of the tRNA in an *in vitro* hemoglobin protein synthesis system (22, 23). Here, they observed that the presence of m^1G in place of the Y base at the position immediately 3′ to the anticodon in $tRNA^{Phe}$ resulted in the preferential translation of the second of two adjacent phenylalanine codons. In contrast, the fully modified $tRNA^{Phe}$ species containing Y base preferentially translated the first of the consecutive phenylalanine codons. There was, however, no indication that the hypomodified mammalian $tRNA^{Phe}$ species could read CUN codons by a third anticodon position wobble mechanism. These observations, coupled with our results, confirm the hypothesis that a major role of the modified nucleotide immediately 3′ to the anticodon in tRNA is to modulate the tRNA's decoding properties. Our work extends this hypothesis to the conclusion that the complete lack of

modification of the 3′ anticodon adjacent nucleotide allows for misreading of the genetic code, a catastrophic event, which is prevented from occurring in tumor cells by the presence of a monomethylated guanosine in the hypomodified Y base-lacking mammalian $tRNA^{Phe}$ (23).

The results of this present work also must be considered in light of the earlier reports of the slower growth rate, slower polypeptide step time, and reduced error frequency observed both *in vivo* and *in vitro* for the *E. coli* and *S. typhimurium* mia^- mutants (16–21). As discussed above, our results are consistent with the conclusion from these reports that the lack of ms^2i^6A lowers the efficiency of cognate codon–anticodon interactions and thus causes a reduced *in vivo* growth rate through a slower polypeptide step time. However, Diaz *et al.* (20) reported that the ms^2i^6A-lacking $tRNA_4^{Leu}$ species less efficiently misincorporates leucine in response to the phenylalanine UUU codon *in vitro*, suggesting that the ms^2i^6A-lacking $tRNA_4^{Leu}$ species is proofread more efficiently than the ms^2i^6A-containing $tRNA_4^{Leu}$ wild-type species. Although these results seemingly conflict with our present studies, it should be pointed out that our results were obtained when the *in vitro* protein synthesis system was supplemented with either a 20-fold excess of the highly purified ms^2i^6A-lacking or ms^2i^6A-containing $tRNA^{Phe}$ species. Since our results and those reported by others (42, 43) demonstrated that the aminoacylation kinetics for both $tRNA^{Phe}$ species were identical, it can be assumed that the ratio of ms^2i^6A-lacking/ms^2i^6A-containing $tRNA^{Phe}$ species is 20:1 in the *in vitro* transcription/translation system. From the experimental results shown in Fig. 2, it can be concluded that phenylalanine is incorporated at both phenylalanine and leucine codons in the presence of a 20-fold excess of the ms^2i^6A-lacking $tRNA^{Phe}$ species, while no misincorporation is observed in the presence of a 20-fold excess of the ms^2i^6A-containing $tRNA^{Phe}$ species. Furthermore, misincorporation of phenylalanine in response to the leucine codons occurs to an extent approximating 50% of the level of leucine incorporation by the endogenous $tRNA^{Leu}$ species. Thus, although significantly less efficient, the ms^2i^6A-lacking $tRNA^{Phe}$ species indeed will respond to the leucine codon and misincorporate phenylalanine in these positions. Therefore, we hypothesize that the increased proofreading of a mia^- tRNA at noncognate codons is made negligible by dramatically increasing the absolute amount of ms^2i^6A-lacking tRNA in the *in vitro* assay. This is supported by the fact that we observed misincorporation of phenylalanine in response to the leucine codon at approximately half of the level of leucine incorporation at the same leucine codons when the ms^2i^6A-lacking $tRNA^{Phe}$ was in 20-fold excess over either ms^2i^6A-containing $tRNA^{Phe}$ or $tRNA^{Leu}$. Our observation that no phenylalanine misincorporation into leucine codons occurred when the ms^2i^6A-containing $tRNA^{Phe}$ was in $\approx$20-fold excess over endogenous $tRNA^{Leu}$ could be consistent with this hypothesis if the anticodon loop stacking interactions are enhanced by the presence of ms^2i^6A, and misincorporation due to first position wobble is thereby reduced below a measurable level.

Therefore, our present results support the notion that ms^2i^6A in *E. coli* $tRNA^{Phe}$ plays a major role in preventing misreading of the genetic code by restricting first codon position wobble and enhancing cognate codon–anticodon interactions. The generalized presence of hypermodified nucleotides immediately 3′ to a tRNA's anticodon therefore may provide a more efficient mechanism for preventing protein synthesis errors and thus endow the cell with an evolutionary selective advantage. Furthermore, as originally suggested by Jukes (1), the appearance of anticodon-adjacent hypermodification may have coincided with the expansion of the genetic code from 10 to 18 amino acids, thus increasing the potential for diversity among proteins.

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1. Jukes, T. H. (1973) *Nature (London)* **246,** 22–26.
2. Nishimura, S. (1980) in *Transfer RNA: Structure, Properties and Recognition*, eds. Shimmel, P. R., Soll, D. & Abelson, J. N. (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp. 59–79.
3. Miller, J. H. (1974) *Cell* **1,** 73–76.
4. Steitz, J. A. (1979) in *Biological Regulation and Development: Gene Expression*, ed. Goldberger, R. F. (Plenum, New York), Vol. 1, pp. 349–399.
5. Kim, S.-H. (1978) *Adv. Enzymol.* **46,** 277–314.
6. Crick, F. H. C. (1966) *J. Mol. Biol.* **19,** 548–555.
7. Kim, S.-H. (1980) in *Transfer RNA: Structure, Properties, and Recognition*, eds. Shimmel, P. R., Soll, D. & Abelson, J. N. (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp. 83–100.
8. Watts, M. T. & Tinoco, I., Jr. (1978) *Biochemistry* **17,** 2455–2463.
9. Woo, N. H., Roe, B. A. & Rich, A. (1980) *Nature (London)* **286,** 546–550.
10. Roe, B. A., Wong, J. F. H., Chen, E. Y., Armstrong, P. A., Stankiewicz, A., Ma, D.-P. & McDonough, J. (1980) in *Mitochondrial Genes*, eds. Slonimski, P., Borst, P. & Attardi, G. (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp. 45–49.
11. Barrell, B. G., Anderson, S., Bankier, A. T., de Bruijn, M. H. L., Chen, E., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R. & Young, I. G. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 3164–3166.
12. Wong, J. F. H. (1981) Ph.D. Thesis (Kent State Univ., Kent, OH).
13. Gefter, M. L. & Russell, R. L. (1969) *J. Mol. Biol.* **39,** 145–157.
14. Hoburg, A., Aschhoff, H. J., Kersten, H., Manderschied, U. & Gassen, H. G. (1979) *J. Bacteriol.* **140,** 408–414.
15. Thiebe, R. & Zachau, H. G. (1968) *Eur. J. Biochem.* **5,** 546–555.
16. Janner, F., Vogeli, G. & Fluri, R. (1980) *J. Mol. Biol.* **139,** 207–219.
17. Eisenberg, S. P., Soll, L. & Yarus, M. (1979) *J. Mol. Biol.* **135,** 111–126.
18. Bohman, K. T., Srichaiye, T., Isaksson, L. A. & Bjork, G. (1986) *J. Bacteriol.* **166,** 1022–1027.
19. Ericson, J. & Bjork, G. (1986) *J. Bacteriol.* **166,** 1013–1021.
20. Diaz, I., Ehrenberg, M. & Kurland, C. G. (1986) *Mol. Gen. Genet.* **202,** 207–211.
21. Diaz, I., Pedersen, S. & Kurland, C. G. (1987) *Mol. Gen. Genet.* **208,** 373–376.
22. Smith, D. W. E., McNamara, A. L., Mushinski, J. F. & Hatfield, D. L. (1985) *J. Biol. Chem.* **260,** 147–151.
23. Smith, D. W. E. & Hatfield, D. L. (1986) *J. Mol. Biol.* **189,** 663–671.
24. Schwartz, I., Klotsky, R. A., Elseviers, D., Gallagher, P. J., Krauskopf, M., Siddiqui, M. A. Q., Wong, J. F. H. & Roe, B. A. (1983) *Nucleic Acids Res.* **11,** 4379–4389.
25. Bolivar, F. & Bachman, K. (1979) *Methods Enzymol.* **68,** 245–267.
26. Beaucage, S. L. & Caruthers, M. H. (1981) *Tetrahedron Lett.* **22,** 1859–1862.
27. Sinha, N. D., Biernat, J., McManus, J. & Koster, H. (1984) *Nucleic Acids Res.* **12,** 4539–4557.
28. Roe, B. A. (1975) *Nucleic Acids Res.* **3,** 21–42.
29. Pearson, R. L., Weiss, J. F. & Kelmers, A. D. (1971) *Biochim. Biophys. Acta* **288,** 770–774.
30. Gilliam, L., Millerard, S., Blew, D., Tigerstrom, M., Wimmer, E. & Tener, G. M. (1967) *Biochemistry* **6,** 3043–3050.
31. Roe, B. A., Marcu, K. & Dudock, B. (1973) *Biochim. Biophys. Acta* **319,** 25–36.
32. Roe, B. A., Sirover, M. & Dudock, B. (1973) *Biochemistry* **12,** 4146–4154.
33. Randerath, K., Gupta, R. C. & Randerath, E. (1980) *Methods Enzymol.* **65,** 638–679.
34. Gupta, R. C., Roe, B. A. & Randerath, K. (1979) *Nucleic Acids Res.* **7,** 959-970.
35. Collins, J. (1979) *Gene* **6,** 29–42.
36. Pratt, J. M., Boulnois, G. J., Darby, V., Orr, E., Wahle, E. & Holland, I. B. (1981) *Nucleic Acids Res.* **9,** 4459–4474.
37. Goldman, E. & Hatfield, G. W. (1979) *Methods Enzymol.* **59,** 292–309.
38. Brauer, A. W., Margolis, M. N. & Haber, A. (1975) *Biochemistry* **14,** 3029–3035.
39. Normanly, J., Ogden, R. C., Horvath, S. J. & Abelson, J. (1986) *Nature (London)* **321,** 213–219.
40. Wilson, R. K., Brown, T. & Roe, B. A. (1986) *Nucleic Acids Res.* **14,** 5937.
41. Caillet, J., Plumbridge, J. A. & Springer, M. (1985) *Nucleic Acids Res.* **13,** 3699–3710.
42. Yanofsky, C. & Soll, L. (1977) *J. Mol. Biol.* **113,** 663–667.
43. Goddard, J. P. & Lowden, M. (1981) *FEBS Lett.* **130,** 221–222.
44. Sutcliffe, J. G. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 3737–3741.
45. Bjork, G. R. (1984) in *RNA Processing*, ed. Apirion, D. (CRC, Boca Raton, FL), pp. 314–316.
46. Hershko, A. & Ciechanover, A. (1982) *Annu. Rev. Biochem.* **51,** 335–364.
47. Grosjean, H., Nicoghosian, K., Haumont, E., Soll, D. & Cedegren, R. (1985) *Nucleic Acids Res.* **13,** 5697–5706.