

Antisuppressor Mutation in *Escherichia coli* Defective in Biosynthesis of 5-Methylaminomethyl-2-Thiouridine

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Mutations in three *Escherichia coli* K-12 genes were isolated that reduce the efficiency of the lysine-inserting nonsense suppressor *supL*. These antisuppressor mutations *asuD*, *asuE*, and *asuF* map at 61.9, 25.3, and 76.3 min, respectively, on the *E. coli* chromosome. Biochemical and genetic analysis of the mutant strains revealed the reason for the antisuppressor phenotype for two of these genes. The activity of lysyl-tRNA synthetase was reduced in strains with *asuD* mutations. The modification of 5-methylaminomethyl-2-thiouridine, the wobble base of tRNA^{Lys}, was impaired in *asuE* mutant strains, presumably at the 2-thiolation step.

The modified nucleosides in tRNA are of particular importance when localized in or adjacent to the anticodon. Over 100 sequenced tRNAs have been shown to contain a modified wobble base at the 5' end of the anticodon and over 150 have a modified purine adjacent to the 3' end of the anticodon (40). Many of these modifications have been reported to increase the specificity and efficiency of codon translation (31). Any changes in coding specificity are of obvious interest as mechanisms of relating codon usage to gene expression (15).

Inosine, a modification of adenosine with increased coding alternatives (13), replaces adenosine whenever it occupies the wobble position. Queosine, a complex derivative of guanosine, replaces guanosine in tRNA^{His}, tRNA^{Asp}, tRNA^{Asn}, and tRNA^{Tyr} in many organisms and has a greater affinity for U than for C (16). Similarly, when uridine is present in the first position of the anticodon it is almost always modified, frequently to a 2-thiouridine derivative (40). Sekiya et al. (37) first suggested that the 2-thio moiety restricted base pairing with G. However, Sen and Ghosh (39) showed that although chemical removal of the 2-thio modification from yeast tRNA^{Lys} substantially reduced the recognition of both AAG and AAA, the undermodified tRNA still retained a marked preference for AAA.

Mutants with altered nucleoside modification at the wobble position would offer useful tools to examine the role of these modifications in tRNA-codon interaction and the consequences of undermodification on gene expression. Two classes of mutations have been isolated in *Escherichia coli* which affect modification of uridine in the wobble position. Two groups (6, 23) have isolated mutations in a locus (*trmC*) which result in a block in a methylation in the biosynthesis of 5-methylaminomethyl-2-thiouridine (mnm⁵s²U), but did not observe any phenotype associated with the mutation. Colby et al. (11) isolated a mutant, designated JF3, which restricted the functioning of the UAA/G (ochre)-suppressing form of bacteriophage T4 tRNA^{Gln} but did not affect the UAG (amber)-suppressing form of the tRNA. They were able to demonstrate altered modification of the wobble base of T4 tRNA^{Gln}, a derivative of uridine whose precise structure is not known but is believed to be a 2-thiouridine derivative

(12). However, this mutation was extremely pleiotropic and further analysis has not been reported.

In the accompanying paper (41), we described a selection procedure based upon the behavior of suppressed *lacI* nonsense mutations for isolating and manipulating antisuppressor mutations. In this report, we describe the isolation of mutations which act as antisuppressors against *supL*, a UAA/G suppressor derived from tRNA^{Lys} (9, 33). One class of mutations results in a defect in the biosynthesis of mnm⁵s²U and genetically can be shown to affect several other nonsense suppressors that contain a modified uridine in the wobble position.

MATERIALS AND METHODS

Bacterial and phage strains. All of the strains used in this work were derivatives of *E. coli* K-12 and most are listed in Table 1. Additional strains used for three-point crosses were constructed by transduction from strains listed in Table 1. The bacteriophage strains used are described in Table 2.

Media. Media for routine growth and manipulation of antisuppressor mutations have been described (26). Scoring of markers in transductional crosses was as follows: *icd*, glutamate auxotrophy; *fabD*, ability to grow at 42°C; *pit*, growth on arsenate medium (44); *tre*, color on modified eosin methylene blue-trehalose plates (4). When necessary, kanamycin was included at 50 µg/ml and colicin E1 (a gift of C. Bulawa and C. Raetz) was used at a concentration sufficient to prevent the growth of sensitive cells.

Genetic techniques. Procedures for the isolation and characterization of antisuppressor mutations and linked Tn10 insertions are described in the accompanying paper (41). Tn5 insertion mutagenesis of p124 was performed by infecting 10⁸ cells of strain JFC124 with 5 × 10⁸ λ *kan* phage for 30 min at 30°C. L broth (1 ml) was added to the infected cells, which were then incubated at 30°C for 3 h. The cells were then diluted into 1 liter of L broth containing kanamycin and grown overnight at 37°C. Plasmid DNA prepared from this culture was used to transform strains JFC117 and JFC205 to kanamycin resistance. Insertions in *purB* or *asuE* were identified as Ade⁻ or LacI⁻ transformants, respectively. The precise location of unique Tn5 insertions was determined by restriction mapping (19).

DNA manipulations. Restriction enzyme digestions were performed in 50 mM Tris-hydrochloride-100 mM NaCl-10 mM MgCl₂ (pH 7.4) for all enzymes except for *ClaI*. *ClaI* digestions were performed in 6 mM Tris-hydrochloride-50

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TABLE 1. Bacterial strains

Strain	Sex/episomes	Genotype	Source or reference
AB2300	Hfr(AB312)	<i>ilvD188 argH1 PurF1 xyl-7 supL2 supE44</i>	CGSC ^a
CSH57L	F ⁻	<i>ara leu lacY purE gal trp his argG malA rpsL xyl mtl ilv metB thi lysA</i>	J. Davies
CSH70	Hfr P4X	<i>metB argE (UAG)thi</i>	J. Miller
EB106	F ⁻	<i>icd-11 dadR1 trpE61 trpA62 tna-5</i>	CGSC
GM19	F ⁻	<i>thr-1 leuB6 proA2 hisG4 trmC2 thyA12 metB1 thi-1 deoB16 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 tsx-33 supE44</i>	CGSC
Hfr3000	HfrH	<i>thi-1 relA1 lacZ118</i>	CGSC
IQ1	F' <i>lacI (UAG)2 lacPL8 proAB⁺</i>	$\Delta(lac-pro) supE$	J. Miller
IQ5	F' <i>lacI (UAG) 228 lacPL8 proAB⁺</i>	$\Delta(lac-pro) supE$	J. Miller
IQ11	F' <i>lacI (UAA) 031 lacPLproAB⁺</i>	$\Delta(lac-pro) supE$	J. Miller
JA200(pLC3-2)	F ⁺ , pLC3-2 (<i>purB⁺ asuE⁺</i>)	<i>thr-1 leuB6 trpE63 recA56 thi-1 ara-14 lacY1 galK2 galT22 xyl-5 mtl-1 supE44</i>	CGSC
JC10240	Hfr KL16	<i>thr-300 ilv-318 rpsE300 recA56 srlC300::Tn10</i>	CGSC
JFC117	F ⁻	PCO254, <i>srlC300::Tn10 recA56</i>	This work
JFC121	F ⁻	L48, <i>lct⁺</i>	This work
JFC124	F'IQ1, p124	MK83L, <i>zce-464::Tn10 purB51</i>	This work
JFC143	F'IQ11	MK78L, <i>lysA</i>	This work
JFC152	F'	MK60, <i>zie-530::Tn5 ilvD188 (UAA)</i>	This work
JFC201	F'IQ1	MK60L, <i>zce-464::Tn10</i>	This work
JFC202	F'IQ1	MK60L, <i>zcf-465::Tn10</i>	This work
JFC203	F'IQ1	MK60L, <i>zcf-469::Tn10</i>	This work
JFC205	F'IQ1	MK83L, <i>srlC-300::Tn10 recA56</i>	This work
JFC206	F'IQ5	MK60L, <i>zhg-525::Tn10 pit-10</i>	This work
JFC207	F'IQ5	MK60L, <i>zhg-526::Tn10 pit-10</i>	This work
JFC208	F'IQ5	MK60L, <i>zhg-458::Tn10 pit-10</i>	This work
JFC209	F'IQ5	MK60L, <i>pit::Tn10</i>	This work
JK268	F ⁻	<i>dadR1 trpE61 trpA62 tna-5 purB58</i>	CGSC
K10	HfrC	<i>relA1 pit-10 tonA22 spoT1</i>	CGSC
KL228	Hfr	<i>thi-1 leuB6 gal-6 lacY1 supE44</i>	CGSC
KL96	Hfr	<i>thi-1</i>	J. Adler
L48	F ⁻	<i>gltA5 fabD89 lct-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL20 tsx-57 tfr-5 supE44</i>	CGSC
LCB107	F ⁻	<i>tre-1 trpA43 metB1 lacY1 malA1 rpsL134 supE44</i>	CGSC
MK60	F ⁻	$\Delta(tonB-trpAB) his argE (UAG) gyrA \Delta(lac-pro)$	41
MK60L	F ⁻	MK60, <i>supL2</i>	41
MK60L/IQ1	F'IQ1	MK60L	This work
MK76L/IQ1	F'IQ11	MK60L, <i>asuD6</i>	This work
MK78L/IQ11	F'IQ11	MK60L, <i>zgb-456::Tn10 asuD6</i>	This work
MK81L/IQ1	F'IQ1	MK60L, <i>asuE8</i>	This work
MK83L/IQ1	F'IQ1	MK60L, <i>asuE9</i>	This work
MK84L/IQ1	F'IQ1	MK60L, <i>zcf-457::Tn10 asuE9</i>	This work
MK89L/IQ5	F'IQ5	MK60L, <i>asuF12</i>	This work
MK91L/IQ5	F'IQ5	MK60L, <i>zhg-458::Tn10 asuF12</i>	This work
PCO254	F ⁻	<i>purB51 trp-45 his-68 tyrA2 thi-1 lacY1 gal-6 malA1 xyl-7 supE44 mtl-2 rpsL125 tonA2 tsx-70</i>	CGSC
PK191	Hfr	<i>thi-1 \Delta(lac-pro)</i>	J. Adler
U482	HfrH	<i>asd-1 thi-1 relA1 spoT1</i>	CGSC
W3421	F ⁻	<i>argA21 galT23</i>	CGSC

^a CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

mM NaCl–6 mM MgCl₂ (pH 7.9). Plasmid p124 was derived from pLC3-2 by in vitro deletion of two *Hind*III fragments. DNA preparation, ligation, and transformation procedures have all been described previously (22).

Isolation of labeled tRNA. tRNA labeled with L-[methyl-¹⁴C]methionine was obtained by growing cells overnight in minimal medium supplemented with 0.1% Casamino Acids, with adenine and uracil at 40 µg/ml each. The cultures were diluted 100-fold into 5 ml of fresh medium containing 5 µCi of L-[methyl-¹⁴C]methionine and grown for 5 h at 37°C. tRNA was isolated by phenol extraction and DEAE-cellulose chromatography (41).

tRNA^{Glu} labeled with ³²P was isolated from uniformly labeled tRNA (41) by hybridization with complementary

single-stranded DNA from strain M13mp2rrn20. M13mp2rrn20 DNA (100 µg) was fixed to a 1.5-cm-diameter nitrocellulose filter and hybridized with ³²P-labeled tRNA. Hybridizations were performed in 50 µl of 50% formamide–5× SSPE (SSPE:10 mM NaH₂PO₄, 180 mM NaCl, 1 mM EDTA [pH 7.4]) at 42°C for 36 h. The filter was washed three times with hybridization solution at 42°C and twice in 2× SSPE at room temperature for 20 and 5 min, respectively. The hybrid selected tRNA^{Glu} was eluted in 300 µl of water by incubation at 80°C for 5 min. After addition of 10 µg of unlabeled tRNA^{Met} (Oak Ridge), the tRNA was precipitated with ethanol. The tRNA was purified on a 20% polyacrylamide–7 M urea gel run in 50 mM Tris-borate–1 mM EDTA (pH 8.3). The labeled tRNA was located by

TABLE 2. Bacteriophage strains

Bacteriophage	Source	Comments
P1 <i>vir</i>	L. Csonka	
λNK55 (b221 c1857 Oam29 cIII-167::Tn10)	W. Reznikoff	Source of Tn10s
λKan (b221 c1857 Oam29 Pam80 <i>rex</i> ::Tn5)	W. Reznikoff	Source of Tn5s
T4D	W. McClain	
T4 <i>psuloc</i> (<i>psu</i> ₁ ⁺ <i>oc</i> eL1)	W. McClain	Suppressor anticodon: NUA
T4 <i>psulam</i> (<i>psu</i> ₁ ⁺ <i>am</i> eL1a)	W. McClain	Suppressor anticodon: CUA (24)
T4 <i>psu2oc</i> (<i>psu</i> ₂ ⁺ <i>oc</i> eL3aΔ27)	W. McClain	Suppressor anticodon: NUA (36)
T4 <i>psu2am</i> (<i>psu</i> ₂ ⁺ <i>am</i> L3Δ27)	W. McClain	Suppressor anticodon: CUA (12)
T4 <i>psu4op</i> (<i>psu</i> ₄ ⁺ <i>op</i> eL1G)	W. McClain	Suppressor anticodon: NCA (20)
M13mp2rrn20	E. Lund	Contains 2-kilobase <i>Eco</i> RI fragment from <i>rrnB</i> complementary to tRNA ^{Glu} ₂

autoradiography, extracted, and prepared for sequence analysis as described previously (41).

Modified nucleotide analysis. Labeled tRNA was digested with a mixture of RNase T1, RNase T2, and RNase A (41) and subjected to two-dimensional thin-layer chromatography. The solvents used were solvent I (isobutyric acid–0.5 M NH₄OH [5:3, vol/vol]), solvent II (isopropanol–HCl–water [70:15:15, vol/vol/vol]), and solvent III (0.1 M sodium phosphate [pH 6.8]–ammonium sulfate–*n*-propanol [100:60:2, vol/vol/vol]). ³²P-labeled nucleotides were visualized by autoradiography, and ¹⁴C-labeled nucleotides were detected by autoradiography (34).

Assay of aminoacyl-tRNA synthetases. Cell extracts were prepared by a modification of the method of Morgan et al. (28). Cells were grown in supplemented minimal medium to late exponential phase and harvested by centrifugation. The cells were washed with 0.5% NaCl–0.5% KCl and frozen at –70°C. The frozen cells (1 g) were ground with 2 g of alumina, the paste was mixed with 5 ml of extract buffer (10 mM Tris-hydrochloride [pH 7.5], 10 mM NH₄Cl, 10 mM β-mercaptoethanol, 10% glycerol), and cell debris was removed by low-speed centrifugation. Ribosomes were removed by centrifugation at 198,000 × *g* for 2 h, and the supernatant was dialyzed overnight against extract buffer. Aliquots of the extract were frozen at –70°C and thawed immediately before use. Relative protein concentrations were determined spectrophotometrically (8).

Crude *E. coli* tRNA to be used as substrate was deacylated by incubation at 37°C for 2 h in 0.2 M Tris-hydrochloride (pH 9.0) followed by precipitation with ethanol. Deacylated tRNA (500 μg) was suspended in 0.1 ml of aminoacylation buffer, 10 mM NH₄[piperazine-N, N'-bis-(2-ethanesulfonic acid)] (NH₄PIPES; pH 7.0)–1.0 mM ATP–0.4 mM CTP–10 mM MgCl₂–60 mM KCl–0.5 mM EDTA, containing [¹⁴C]lysine (50 Ci/mol) at a concentration of 50 μM. The mixture was heated to 55°C for 5 min and cooled to 37°C. Cell extract (2.5 μl) was added, and the mixture was incubated at 37°C. Aliquots were removed and spotted onto Whatman 3MM disks, followed immediately by the addition of 100 μl of 0.05 M cetyltrimethylammonium bromide in 10% acetic acid. The disks were washed with 1% acetic acid three times for 5 min and three times for 10 min each and were dried and counted by liquid scintillation. The activity of the synthetase was determined from the initial rate of aminoacylation.

Determination of T4 plating efficiencies. MK60/IQ1 derivatives containing either *asuE9* or *asuF12* were constructed by transducing strain MK60/IQ1 with a P1 lysate grown on MK84L/IQ1 or MK91L/IQ5, respectively, selecting for Tet^r. The presence of the *asu* mutation was confirmed by backcrosses to strain MK60L/IQ1 with P1 lysates grown on potential

candidates. Serial dilutions of T4 phage were absorbed to 100 μl of overnight L broth cultures for 10 min at 25°C and plated on EHA plates with EHA top agarose at 37°C. EHA plates contain (per liter) 13 g of tryptone, 8 g of NaCl, 2 g of sodium citrate, 1.3 g of glucose, and 10 g of agar. EHA top agarose contains (per liter) 13 g of tryptone, 8 g of NaCl, 2 g of sodium citrate, 3 g of glucose, and 6.5 g of agarose (type I; Sigma Chemical Co.).

In vitro methylation of tRNA. The ability of tRNA to be methylated in vitro was tested by using an MK60L/IQ1 cell extract and [³H]S-adenosylmethionine (5 Ci/mmol; Amersham Corp.) as described previously (23).

RESULTS

Isolation of antisuppressor mutations. Encouraged by the successful isolation of antisuppressor mutations against *supE* (41), we wanted to isolate additional mutations by using a suppressor tRNA containing different modified nucleotides. We chose to use *supL*, a UAA/G suppressor derived from tRNA^{Lys} which had recently been sequenced and shown to contain mnm⁵s²U in the wobble position of the anticodon (33). To use *lacI* nonsense mutations as an indicator of suppression efficiency, we needed to modify our original selection to compensate for the low efficiency of suppression shown by UAA/G suppressors. We therefore included the *lacI*^r mutation to increase the amount of repressor made (39). Strains containing weak UAA/G suppressors and a *lacI* nonsense mutation appear LacI⁺ on 5-bromo-4-chloro-3-indolyl-β-D-galactoside indicator plates only if the *lacI*^r mutation is present (25; unpublished results).

LacI[–] mutants were isolated from strains containing *supL* and a variety of *lacI* UAA and UAG mutations after ethyl methane sulfonate mutagenesis and growth on phenyl-β-galactoside. The LacI[–] mutants were screened as previously described (41) for mutations unlinked to *lac* or *supL*. The results of these isolations are summarized in Table 3. From 829 LacI[–] colonies, we obtained eight strains which appeared to harbor antisuppressor mutations. The number of classes of mutations represented was determined by isolat-

TABLE 3. Summary of antisuppressor selections

Mutagenized strain	No. of LacI [–] colonies	No. of mutants in <i>lac</i>	No. of mutants in <i>supL</i>	Anti-suppressor mutants
MK60L/IQ7	150	143	7	None
MK60L/IQ8	104	89	14	<i>asu-5</i>
MK60L/IQ9	180	162	17	<i>asu-6</i>
MK60L/IQ1	195	184	8	<i>asu-7,8,9</i>
MK60L/IQ5	200	189	8	<i>asu-10,11,12</i>

ing linked *Tn10* insertions and conducting allelism tests (41). On this basis, we could distinguish three classes of mutations: *asuD* (*asu*-6,7,10), *asuE* (*asu*-5,8,9,11) and *asuF* (*asu*-12). Subsequent analysis of the mutants was consistent with the existence of only three classes.

Genetic mapping of *asu* mutations. Preliminary mapping of *asu* mutations was accomplished by checking for cotransduction between any of the markers in the multiply marked strain CSH57L and the *asu*-linked *Tn10*s. Only *zgb*-457::*Tn10* (linked to *asuD*) gave a positive result, showing linkage to *lysA*. Additional three-point crosses with *thyA* and *argA* (crosses 1 to 6, Table 4) established the map order shown in Fig. 1A.

The *Tn10* insertions linked to *asuE* and *asuF* (*zcf*-457::*Tn10* and *zhg*-458::*Tn10*, respectively) showed no linkage by transduction to any of the markers in strain CSH57L. Therefore, approximate locations were determined by introducing the *Tn10* insertions into Hfr strains, followed by conjugation to strain CSH57L (data not shown). These experiments indicated that *zhg*-458::*Tn10* (linked to *asuF*) mapped between *ilv* and *argG*. Transduction were performed with additional markers in this region, revealing that *asd* and *pit* gave 12 and 77% cotransduction, respectively, with this *Tn10* insertion. Attempts to perform three-point crosses involving *asd* were unsuccessful, and so additional *Tn10* insertions linked to *asuF* were isolated and used in two- and three-point crosses with the *asd*, *asuF*12, and *pit* markers. The results of these crosses (Table 4) allowed us to place the *asuF* locus at 76.3 min, as indicated in Fig. 1B.

The *asuE*-linked *Tn10* insertion, *zcf*-457::*Tn10*, was mapped in a similar fashion to the region between *gal* and *trp*. Transductions between this *Tn10* and other markers in this area revealed 97% cotransduction with *purB*. Additional *Tn10* insertions linked to *asuE* were isolated and used in the three-point crosses listed in Table 4. The map location

determined from these experiments is shown in Fig. 1C. We observed substantial non-reciprocity in crosses in the *purB*-*fabD* region, in particular those involving *zcf*-464::*Tn10*. This anomaly may be mediated by the *el4* element, known to be located in this region (3, 14, 38).

Because of the close linkage of *purB* and *asuE*, it is possible that these two genes are part of the same transcriptional unit. To answer this question, we obtained a plasmid, pLC3-2, which complemented the *purB* and *asuE* mutations (10). To simplify the plasmid, two *Hind* III fragments were deleted from pLC3-2 to yield plasmid p124, which retained the ability to complement *purB* and *asuE* mutations. *Tn5* insertions into the *purB* and *asuE* genes on the plasmid were isolated and tested for polar effects on the other locus (5). We did not detect any insertions which were simultaneously *Ade*⁻ *Asu*⁻. The location of the *Tn5* insertions was determined by restriction mapping and allowed us to precisely locate the *purB* and *asuE* genes in p124. The results are summarized in Fig. 2.

Effect of *asu* mutations on other nonsense suppressors. The ability of the antisuppressor mutations to affect suppressors other than *supL* was tested by using linked *Tn10*s to transduce the antisuppressor mutations into JFC152 derivatives containing different suppressors. The ability of these suppressor-antisuppressor combinations to suppress *lacI*^q (UAG), *lacI*^r (UAA), and *argE* (UAG), and *ilvD* (UAA) mutations is shown in Fig. 3. The *asuD* mutations affected only *supL*; the *asuE* and *asuF* mutations both affected all UAA/G suppressors tested. None of the antisuppressor mutations affect the efficient UAG suppressors *supD*, *supE*, *supF*, or *supP* (data not shown). Using a variety of *lacI*^q (UAG) and *lacI*^r (UAA) alleles, we have not detected any evidence of context effects (7, 27).

We also tested whether *Asu*⁻ strains could support the growth of bacteriophage T4 derivatives that required a

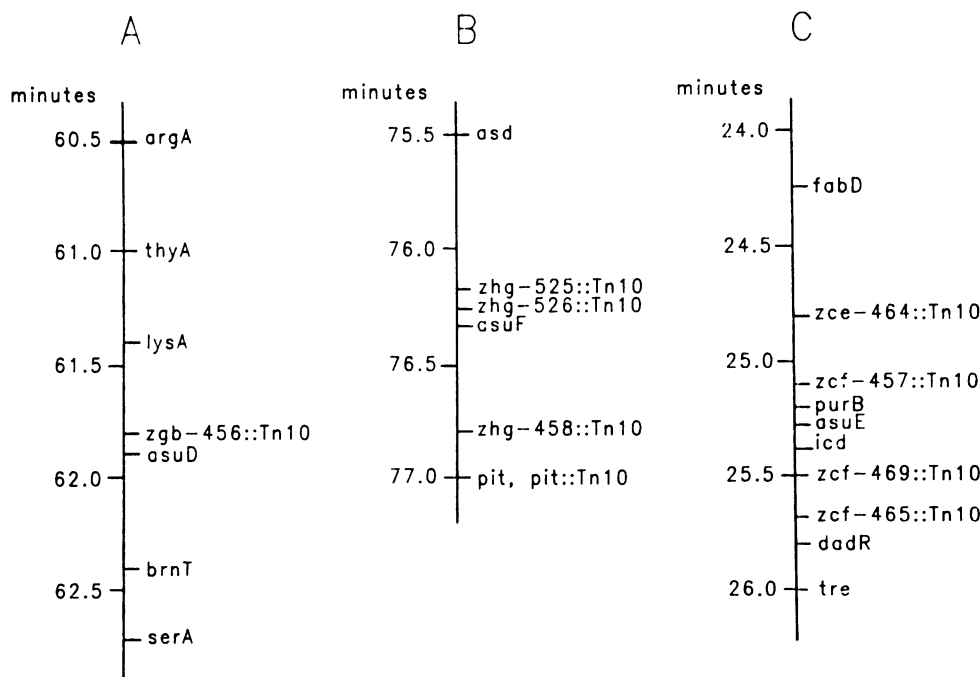


FIG. 1. Linkage map for three *supL*-derived antisuppressors. Three-point crosses (Table 4) were used to determine the order of the markers. The relative distance between them was deduced from cotransduction frequencies (45). The map location of previously mapped genes is from Bachmann (3). Shown are the regions around (A) *asuD*, (B) *asuF*, and (C) *asuE*.

TABLE 4. Summary of three-point crosses used to map *asuD*, *asuE*, and *asuF*^a

Cross no.	Donor	Recipient	Selected marker	Total no. of transductants	Distribution of unselected markers				Implied gene order
1	JFC143	GM19	Thy ⁺	296	Lys ⁺ Tet ^r (36)	Lys ⁺ Tet ^s (55)	Lys ⁻ Tet ^r (113)	Lys ⁻ Tet ^s (92)	<i>thyA-lysA-(zgb-456::Tn10)</i>
2	JFC143	GM19	Tet ^r	198	Thy ⁺ Lys ⁺ (5)	Thy ⁺ Lys ⁻ (72)	Thy ⁻ Lys ⁺ (92)	Thy ⁻ Lys ⁻ (29)	<i>thyA-lysA-(zgb-456::Tn10)</i>
3	CSH57L, <i>zga-456::Tn10</i> <i>asuD6</i>	MK60L/IQ1	Tet ^r	713	Asu ⁺ Lys ⁺ (17)	Asu ⁺ Lys ⁻ (23)	Asu ⁻ Lys ⁺ (306)	Asu ⁻ Lys ⁻ (367)	<i>lysA-(zgb-456::Tn10)-asuD</i>
4	JFC143	W3421	Tet ^r	270	Arg ⁺ Lys ⁺ (2)	Arg ⁺ Lys ⁻ (6)	Arg ⁻ Lys ⁺ (130)	Arg ⁻ Lys ⁻ (132)	<i>argA-(zgb-456::Tn10)-lysA</i>
5	CSH57L, <i>zga-456::Tn10</i>	PC1523	Tet ^r	348	Lys ⁺ Ser ⁺ (33)	Lys ⁺ Ser ⁻ (187)	Lys ⁻ Ser ⁺ (26)	Lys ⁻ Ser ⁻ (102)	<i>lysA-zgb-456::Tn10-serA</i>
6	PC1523, <i>zga-456::Tn10</i> <i>lysA</i>	MK76L/IQ1	Tet ^r	359	Asu ⁺ Ser ⁺ (270)	Asu ⁺ Ser ⁻ (63)	Asu ⁻ Ser ⁺ (26)	Asu ⁻ Ser ⁻ (0)	<i>zgb-456::Tn10-asuD-serA</i>
7	JFC208	MK89L/IQ5	Tet ^r	93	Asu ⁺ Pit ⁺ (21)	Asu ⁺ Pit ⁻ (14)	Asu ⁻ Pit ⁺ (6)	Asu ⁻ Pit ⁻ (52)	<i>asuF-(zhg-458::Tn10)-pit</i>
8	JFC206	MK89L/IQ5	Tet ^r	75	Asu ⁺ Pit ⁺ (44)	Asu ⁺ Pit ⁻ (20)	Asu ⁻ Pit ⁺ (11)	Asu ⁻ Pit ⁻ (0)	<i>(zhg-525::Tn10)-asuF-pit</i>
9	JFC207	MK89L/IQ5	Tet ^r	90	Asu ⁺ Pit ⁺ (66)	Asu ⁺ Pit ⁻ (20)	Asu ⁻ Pit ⁺ (4)	Asu ⁻ Pit ⁻ (0)	<i>(zhg-526::Tn10)-asuF-pit</i>
10	MK83L/IQ1	JFC202, <i>purB51</i>	Ade ⁺	171	Asu ⁺ Tet ^r (5)	Asu ⁺ Tet ^s (0)	Asu ⁻ Tet ^r (26)	Asu ⁻ Tet ^s (140)	<i>purB-asuE-(zcf-465::Tn10)</i>
11	MK83L/IQ1	JFC203, <i>purB51</i>	Ade ⁺	90	Asu ⁺ Tet ^r (1)	Asu ⁺ Tet ^s (0)	Asu ⁻ Tet ^r (7)	Asu ⁻ Tet ^s (82)	<i>purB-asuE-(zcf-469::Tn10)</i>
12	MK83L/IQ1	JFC201, <i>purB51</i>	Ade ⁺	55	Asu ⁺ Tet ^r (0)	Asu ⁺ Tet ^s (2)	Asu ⁻ Tet ^r (2)	Asu ⁻ Tet ^s (51)	<i>(zce-464::Tn10)-purB-asuE</i>
13	MK83L/IQ1	MK84L/IQ1, <i>purB51</i>	Ade ⁺	92	Asu ⁺ Tet ^r (0)	Asu ⁺ Tet ^s (5)	Asu ⁻ Tet ^r (3)	Asu ⁻ Tet ^s (84)	<i>(zce-457::Tn10)-purB-asuE</i>
14	JFC201	JK268	Tet ^r	100	Ade ⁺ DadR ⁺ (6)	Ade ⁺ DadR ⁻ (61)	Ade ⁻ DadR ⁺ (0)	Ade ⁻ DadR ⁻ (33)	<i>(zce-464::Tn10)-purB-dadR</i>
15	JFC202	JK268	Tet ^r	118	Ade ⁺ DadR ⁺ (12)	Ade ⁺ DadR ⁻ (49)	Ade ⁻ DadR ⁺ (18)	Ade ⁻ DadR ⁻ (39)	<i>purB-(zcf-465::Tn10)-dadR</i>
16	MK84L/IQ1	JK268	Tet ^r	100	Ade ⁺ DadR ⁺ (13)	Ade ⁺ DadR ⁻ (74)	Ade ⁻ DadR ⁺ (0)	Ade ⁻ DadR ⁻ (13)	<i>(zcf-457::Tn10)-purB-dadR</i>
17	JFC202	JK268	Ade ⁺	90	DadR ⁺ Tet ^r (10)	DadR ⁺ Tet ^s (0)	DadR ⁻ Tet ^r (6)	DadR ⁻ Tet ^s (74)	<i>purB-(zcf-456::zcf-456::Tn10)-dadR</i>
18	JK268, <i>zcf-465::Tn10</i>	MK83L/IQ1	Tet ^r	129	Asu ⁺ DadR ⁺ (24)	Asu ⁺ DadR ⁻ (0)	Asu ⁻ DadR ⁺ (89)	Asu ⁻ DadR ⁻ (16)	<i>asuE-(zcf-456::zcf-456::Tn10)-dadR</i>
19	LCB107, <i>zcf-469::Tn10</i>	PCO254	Tet ^r	80	Ade ⁺ Tre ⁺ (28)	Ade ⁺ Tre ⁻ (4)	Ade ⁻ Tre ⁺ (0)	Ade ⁻ Tre ⁻ (48)	<i>purB-(zcf-469::Tn10)-tre</i>
20	JFC201, <i>purB51</i>	JFC121	FabD ⁺	202	Ade ⁺ Tet ^r (19)	Ade ⁺ Tet ^s (160)	Ade ⁻ Tet ^r (0)	Ade ⁻ Tet ^s (23)	?
21	JFC201	JFC121	Tet ^r	150	Ade ⁺ FabD ⁺ (46)	Ade ⁺ FabD ⁻ (60)	Ade ⁻ FabD ⁺ (5)	Ade ⁻ FabD ⁻ (39)	<i>fabD-(zce-464::Tn10)-purB</i>
22	PCO254, <i>zce-464::Tn10</i>	EB106	Tet ^r	56	Ade ⁺ Icd ⁺ (0)	Ade ⁺ Icd ⁻ (17)	Ade ⁻ Icd ⁺ (36)	Ade ⁻ Icd ⁻ (3)	<i>(zce-464::Tn10)-purB-icd</i>
23	JFC202	EB106	Tet ^r	189	Icd ⁺ DadR ⁺ (8)	Icd ⁺ DadR ⁻ (15)	Icd ⁻ DadR ⁺ (166)	Icd ⁻ DadR ⁻ (0)	<i>icd-(zcf-465::Tn10)-dadR</i>
24	JFC203	EB106	Tet ^r	189	Icd ⁺ DadR ⁺ (11)	Icd ⁺ DadR ⁻ (37)	Icd ⁻ DadR ⁺ (141)	Icd ⁻ DadR ⁻ (0)	<i>icd-(zcf-469::Tn10)-dadR</i>

^a Crosses were performed by P1 vir transduction. A P1 vir lysate grown on the donor was used to transduce the recipient, selecting for the indicated marker. Unselected markers were scored as described in the text. The implied gene order was deduced from examination of the unselected marker distribution. Those recombinant classes requiring four crossovers were expected at low frequency (45).

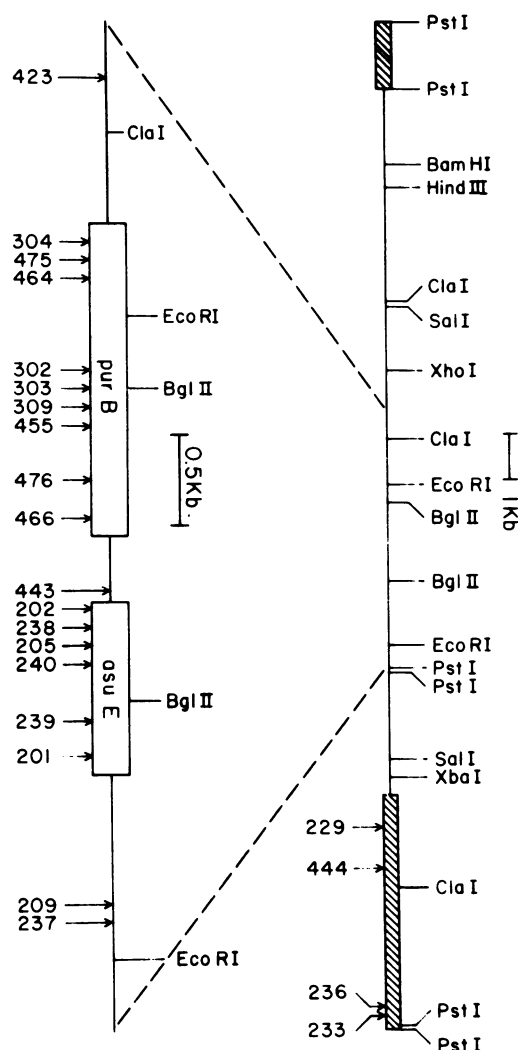


FIG. 2. Fine-structure map of *purB* and *asuE*. The restriction map of plasmid p124 is shown across the top (arbitrarily opened at the double *Pst*I sites in the vector). The thin line indicates the chromosomal insert; the hatched line, the ColE1 vector. This plasmid was derived from pLC 3-2 by in vitro deletion of two *Hind*III fragments at the remaining *Hind*III site. Arrows indicate the location and isolation numbers of Tn5 insertions. The phenotypes of the p124:Tn5 plasmids in JFC117 (*purB*), JFC205 (*asuE9*), and JFC210 (*asuE8*) strains delineated the locations of the *purB* and *asuE* genes. The map is drawn so that clockwise on the *E. coli* chromosomal map is from left to right.

phage-encoded suppressor for growth. The plating efficiencies of phages T4D, T4 *psu1oc*, T4 *psulam*, T4 *psu2oc*, T4 *psu2am*, and T4 *psu4op* were determined on derivatives of strain MK60/IQ1 (see above). These host cells lack *supL* and thus eliminate the complications of *supL* suppression of the phage nonsense mutations. In all cases, the plating efficiencies were equal on *Asu*⁺ and *Asu*⁻ strains, except for the plating of T4 *psu2oc* on *asuE* and *asuF* strains, in which case the plating efficiency was <1% that of the *Asu*⁺ control. Since the suppressor tRNA^{Gln} in the T4 *psu2oc* and T4 *psu2am* phages differ only by the base present in the wobble position, the fact that the *asuE* and *asuF* mutations reduce the plating efficiency of T4 *psu2oc* implies that these mutations affect the modifications of the wobble base in the T4 *psu2oc* suppressor tRNA^{Gln}.

Biochemical analysis of *asu* mutations. Based upon the observation that *asuD* mutations affected only *supL*, we assayed strains containing *asuD* mutations for levels of tRNA^{Lys} and the activity of lysyl-tRNA synthetase. The mutant strains showed normal amounts of tRNA^{Lys} (data not shown) but possessed only 43% of the lysyl-tRNA synthetase activity present in the wild-type strain. We have not analyzed the mutants in greater detail but believe that the reduced synthetase activity probably lowers the levels of aminoacylated tRNA^{Lys} in the cell, resulting in less efficient suppression.

We next analyzed the levels of methylated nucleotides present in crude tRNA from strains containing *asuE* and *asuF* mutations because the majority of modified nucleotides in *E. coli* contain methyl groups. In particular, the wobble base of tRNA^{Lys}, mnm⁵s²U, should be readily detected by this procedure. The *asuF* methylated nucleotide composition was indistinguishable from the wild type, although *asuE* tRNA demonstrated a reproducible difference (Fig. 4).

The tRNA isolated from a strain containing a mutation in *asuE* is missing two nucleotides (N1 and N3) present in the *Asu*⁺ control and contains an increased amount of a component (N2) that is barely detectable in the wild-type strain. Spot N3 comigrates with authentic mnm⁵s²U (provided by S. Nishimura). These results suggest that the *asuE* mutations result in a defect in mnm⁵s²U biosynthesis, with a concomitant increase in a component that is a putative precursor to mnm⁵s²U.

To establish that the altered nucleotides were related to mnm⁵s²U, we purified tRNA^{Glu} from strains MK60L/IQ1 (*asuE*⁺) and MK81L/IQ1 (*asuE8*) by RNA-DNA hybridization. tRNA^{Glu} contains mnm⁵s²U in the wobble position (1, 32). The minor nucleotide analysis of *asuE*⁺ and *asuE8* tRNA^{Glu} is shown in Fig. 5. Wild-type tRNA^{Glu} contains two nucleotides, N1 and N3, whose molar yields (estimated by their relative intensities on the autoradiogram) appear to be fractional by comparison with the intensity of the ribothymidine phosphate. N3 has the same mobility as authentic mnm⁵s²U. A ³⁵S-labeled nucleotide with a chromatographic mobility similar to N1 was observed in tRNA^{Glu} and was tentatively identified as 5-aminomethyl-2-thiouridine (1). The possibility also exists that N1 might be carboxymethylamino-methyl-2-thiouridine, the modified wobble base found in *Bacillus subtilis* tRNA^{Lys} (46). The *asuE8* tRNA^{Glu} has undetectable levels of mnm⁵s²U and N1 but has a novel nucleotide N2 instead.

The mutation *trmC* affects the modification of mnm⁵s²U, yielding a precursor that can be methylated in vitro (6, 23). The results of in vitro methylation reactions with [methyl-³H]S-adenosylmethionine indicated that MK81L/IQ1 (*asuE8*) tRNA could not be methylated, whereas tRNA from GM19 (*trmC*) could (data not shown).

DISCUSSION

Three classes of antisuppressor mutations have been isolated from *supL*-containing strains by a modification of the *lacI*-selective scheme described in the accompanying paper (41). Mutations in the *asuD* locus affect only *supL* and reduce the activity of lysyl-tRNA synthetase present in cell extracts. This locus mapped at 61.9 min, which is distinct from the map positions of the lysyl-tRNA synthetase genes, *lysS* and *lysU*, 6 and 93 min, respectively (17, 43). This suggests that the *asuD* locus may code for a synthetase regulatory gene (29) or a protein responsible for post-translational modification of the synthetase (17). The reduced activity of the synthetase presumably lowers the amount of

Suppressor:	<i>supB</i>	<i>supC</i>	<i>supL</i>	<i>supN</i>
	C G	G C	G C	
	C G	C G	U A	
	G C	A U	U A	
	G C	G C	G C	
	U A	U ψ	A ψ	
Anticodon stem and loop ^a :	Um ψ	C A	C A	
	U m ² A	U A*	U t ⁶ A	?
	N A	U* A	M A	
	U	U	U	
tRNA:	<i>glnI</i>	<i>tyr</i>	<i>lys</i>	?
<i>asu</i> ⁺ <i>lacI</i> (UAG)	+	+	+	+
<i>lacI</i> (UAA)	+	+	+	+
<i>argE</i> (UAG)	+	+	+	+
<i>ilvD</i> (UAA)	+	+	+	+
<i>asuD6</i> <i>lacI</i> (UAG)	+	+	-	+
<i>lacI</i> (UAA)	+	+	-	+
<i>argE</i> (UAG)	+	+	-	+
<i>ilvD</i> (UAA)	+	+	-	+
<i>asuE9</i> <i>lacI</i> (UAG)	-	-	-	-
<i>lacI</i> (UAA)	-	-	-	-
<i>argE</i> (UAG)	-	+	-/+	+
<i>ilvD</i> (UAA)	-	-	-	-
<i>asuF12</i> <i>lacI</i> (UAG)	-	-	-	-
<i>lacI</i> (UAA)	-	-	-	-
<i>argE</i> (UAG)	-	+	-	+
<i>ilvD</i> (UAA)	-	-	-	-

FIG. 3. Effect of *supL* antisuppressors on other *E. coli* ochre suppressors. N, Uncharacterized s²U derivative (47); U*, modified uridine potentially not containing a 2-thiolation (2); A*, 2-methylthio-6-isopentenyladenosine; M, mnm⁵s²U. *lacI* (UAG) corresponds to the two alleles *lacI*^a (UAG)2 and *lacI*^a (UAG)228, and *lacI* (UAA) corresponds to the two alleles *lacI*^a (UAA)026 and *lacI*^a (UAA)031; these were carried on Fs IQ1, IQ5, IQ9, and IQ11 respectively (25, 41). +, Phenotype was wild type (i.e., Lac⁺, Arg⁺, or Ilv⁺) and indicates suppression of the nonsense mutations; -, antisuppression is occurring (i.e., a Lac⁻, Arg⁻, or Ilv⁻ phenotype). All phenotypes were determined in JFC152 derivatives after 24 h at 37°C.

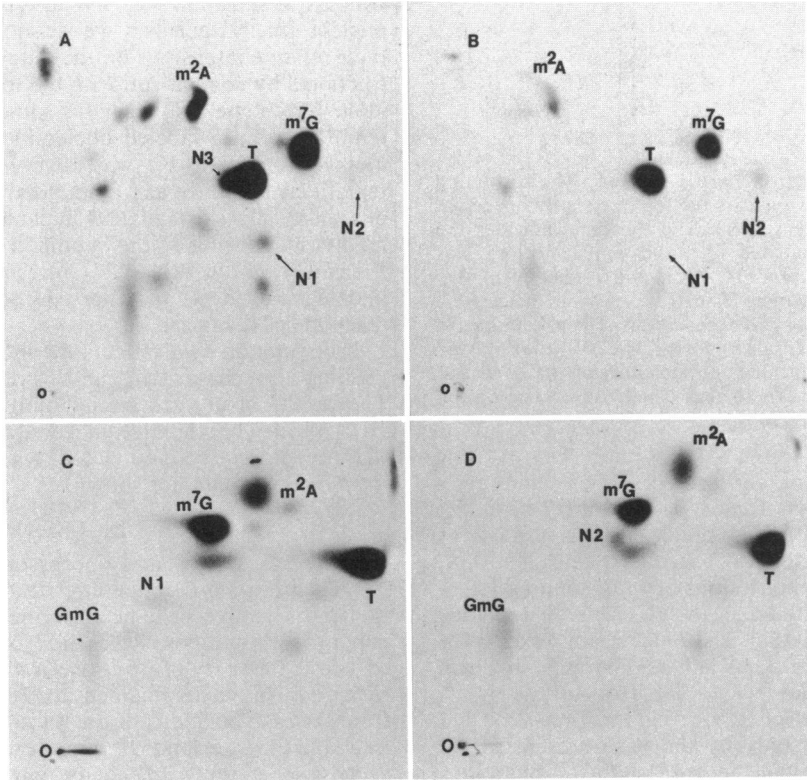


FIG. 4. Autoradiography of in vivo [methyl-¹⁴C]-labeled mononucleotides from (A,C) strain MK60L/IQ1 (wild type) and (B,D) strain MK81L/IQ1 (*asuE8*) tRNA. The labeled mononucleotides were prepared as described in the text. They were separated by cellulose thin-layer chromatography in solvent I (vertical dimension) and either solvent III (A,B) or solvent II (C,D) horizontal dimension.

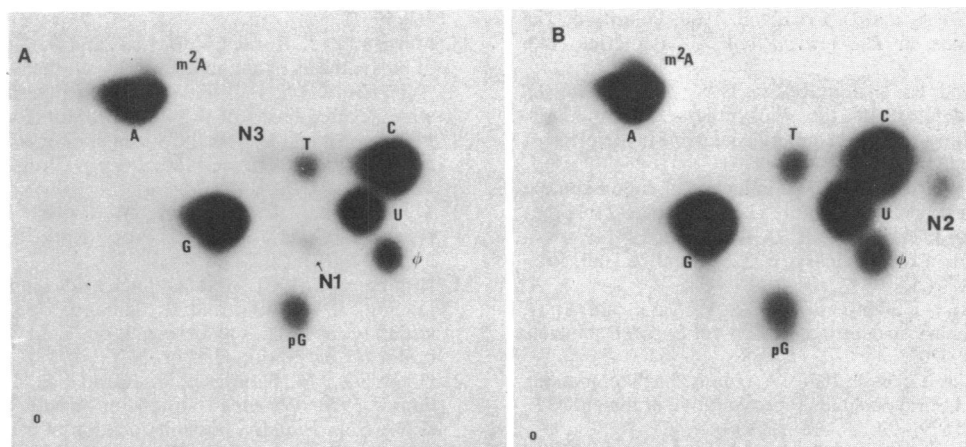


FIG. 5. Autoradiography of tRNA^{Glu} [³²P]-labeled mononucleotides from (A) strain MK60L/IQ1 and (B) strain MK81L/IQ1. The [³²P]-labeled mononucleotides from hybrid-selected tRNA^{Glu} were separated by cellulose thin-layer chromatography in solvent I (vertical dimension) and solvent III (horizontal dimension). N3 has the same mobility as mnm⁵s²U.

charged suppressor tRNA in the cell, resulting in less efficient suppression.

The experiments described in this paper did not allow us to determine the nature of the *asuF12* mutation. Formally, antisuppressors are mutations that increase the probability that peptide release will be favored over peptide elongation via the suppressor tRNA. This competition can be shifted either by impairing the function of the suppressor tRNA or by increasing the activity of the release factor. The *asuF12* mutation has no discernible effect on tRNA amounts or structure and so may be a release factor mutation. The *strM* mutation maps near *asuF* and may be allelic with it because the phenotype of both are consistent with increased translation accuracy in mutant strains (35).

Strains containing *asuE* mutations have been shown to restrict the growth of T4 *psu2oc* but not T4 *psu2am*. This result delineates the wobble base as the site of *asuE* action because the suppressor tRNA in these two T4 derivatives differ only at the wobble position. The absence of the modified uridine in the T4 *psu2oc* tRNA^{Glu} apparently inhibits its suppressor activity. Similar results were found for the *E. coli* UAA/G suppressors *supB*, *supC*, *supL*, and *supN*, which also contain modified uridines in the wobble position.

The tRNA^{Glu} composition analysis demonstrated that the *asuE* mutation affected a step in the modification of mnm⁵s²U, the wobble base in this tRNA. An uncharacterized nucleotide, N2, accumulates in strain MK81L/IQ1 (*asuE8*) at the expense of mnm⁵s²U. The biosynthesis of mnm⁵s²U is obviously a multistep pathway. The fact that a modified nucleotide accumulates in the *asuE*-containing strain indicates that *asuE* affects a step other than the first in this biosynthetic pathway.

The mutation *trmC* also affects the modification of mnm⁵s²U. tRNA isolated from *trmC*-containing strains can be methylated in vitro to yield mature mnm⁵s²U, which suggests that *trmC* defines a gene responsible for the terminal methylation of this base (23). The facts that *asuE* tRNA cannot be methylated in vitro and that *asuE* mutations map at a locus distinct from *trmC* imply that *asuE* affects a step other than the methylation of 5-aminomethyl-2-thiouridine.

An explanation consistent with the present data is that *asuE* mutations prevent the thiolation at the two position of uridine. This interpretation is in agreement with the observations of Sen and Ghosh (39) that removal of the 2-thiour-

idine moiety of yeast tRNA^{Glu} resulted in a dramatic decrease in the ability of the tRNA to bind to AAA and AAG codons in vitro. We observe an analogous effect in vivo when neither UAA nor UAG codons are suppressed in the presence of *asuE* mutations.

The unknown nucleotide, N2, has a chromatographic mobility distinct from ribothymidine. This and the fact that N2 is labeled in vivo by [methyl-¹⁴C]methionine suggests that N2 may be 5-methylamino methyl uridine if the *asuE* mutation indeed prevents the 2-thiolation. Experiments to determine the structure of N2 and the elucidation of the mnm⁵s²U biosynthetic pathway are in progress.

The isolation of the gene coding for a tRNA modification enzyme will make possible a number of interesting experiments. In particular, the study of the regulation of the enzymes biosynthesis and the consequences of under- or over-expression of the gene can be examined. The most interesting results may come from reversion analysis of *asuE* mutations. It may be possible to isolate mutations within the suppressor tRNA or within ribosomal components which relieve the requirement for wobble modification. Finally, if *asuE* mutations do result in loss of 2-thiolation of uridine, it should be possible to determine the precise role of 2-thio modification on tRNA codon interactions.

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