JOURNAL OF BACTERIOLOGY, Jan. 1985, p. 377–384
0021-9193/85/010377-08$02.00/0

Vol. 161, No. 1

Isolation and Characterization of Antisuppressor Mutations in *Escherichia coli*

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Received 19 July 1984/Accepted 10 October 1984

Nonsense mutations in *lacI* have been shown to be useful as indicators of the efficiency of nonsense suppression. From strains containing *supE* and a *lacI* nonsense mutation, selection for LacI⁻ mutants has resulted in the isolation of four antisuppressor mutations. Tn*10* insertions linked to these mutations were isolated and used to group the four mutations into three loci. The *asuA1* and *asuA2* mutations are linked to *trp*, reduce suppression by *supE* approximately twofold, and affect a variety of suppressors. The *asuB3* mutation was mapped by P1 cotransduction to *rpsL* but does not confer resistance to streptomycin. The *asuC4* mutation reduced suppression by *supE* by 95% and was shown biochemically to result in the loss of two pseudouridine modifications from the 3′ side of the anticodon stem and loop of $tRNA_2^{Gln}$. This mutation is linked to *purF*, suggesting that it is a new allele of *hisT*.

One of the most distinguishing features of tRNA is the presence of a high percentage of modified nucleosides. Despite extensive studies on the structure of these bases (20), relatively little is known of their precise function in tRNA. The ability to isolate mutations which result in the loss of modified nucleosides would be a valuable aid in understanding their role in tRNA function.

Two general approaches have been used to isolate modification-defective mutants. The first involves random screening of mutagenized cultures biochemically for loss of specific modified nucleosides. Several groups (3, 18, 25) have successfully isolated modification-defective mutants in this manner. However, this approach can only be used to isolate mutations affecting easily assayable modified nucleosides, and subsequent genetic analysis is tedious. Furthermore, the effect of these mutations on tRNA function cannot be easily determined.

An alternative approach is to use a readily monitored function of tRNA and isolate mutations which alter this function. These mutants can then be analyzed and strains lacking modified nucleosides can be identified. A convenient indicator of tRNA function is nonsense suppression, in which a tRNA is altered at a single nucleotide, usually in the anticodon (26), so that it can recognize and insert an amino acid at a nonsense codon. This property provides a means for monitoring the effect of particular mutations on tRNA function. Mutations which reduce the efficiency of nonsense suppression, or antisuppressor mutations, have proven very useful in elucidating the steps in tRNA processing (28, 29) and in the study of tRNA structure (31). Using selections for antisuppressors, only one modification-defective mutant has been isolated (7), and this mutation was extremely pleiotropic and difficult to analyze. The failure to isolate additional modification mutants in previous studies can probably be attributed to the fact that all of the selective schemes required either a complete loss of suppression or a temperature-sensitive phenotype for identification. These restrictions precluded the isolation of mutations which reduce but do not abolish suppression or are not in an essential gene. Since no modified nucleosides have been shown to be essential for tRNA function (24), it might be expected that many modification-defective mutants may have been overlooked in these studies.

To efficiently explore the role of modified nucleosides in tRNA function, we have devised a genetic selection which allows the isolation and manipulation of mutations which reduce the efficiency of nonsense suppression in *Escherichia coli*. Mutations affecting the biosynthesis of any modified nucleosides which are required for efficient tRNA utilization should be found among these antisuppressor mutations. The selection is based upon observations made by Coulondre and Miller (9) in their study of *lacI*, the gene coding for the repressor of the *lac* operon. A strain which harbors a nonsense mutation in *lacI* is constitutive for β-galactosidase synthesis because of the absence of repressor. The introduction of a nonsense suppressor which efficiently suppresses the *lacI* nonsense mutation restores synthesis of the repressor, which acts to halt expression of *lacZ*, resulting in the LacI⁺ phenotype. LacI⁻ mutants can be directly selected by requiring growth on phenyl-D-galactoside (32). Selection for lactose-constitutive mutants from a strain containing a *lacI* nonsense mutation and a nonsense suppressor is expected to yield mutations which reduce the efficiency of nonsense suppression, as well as any other mutations which result in constitutive expression of *lacZ*.

We have tested this selective scheme by isolating antisuppressor mutations of *supE*, a UAG (amber) suppressor derived from $tRNA_2^{Gln}$ (13). The isolation of Tn*10* insertions linked to the antisuppressor loci has allowed us to genetically characterize these mutations, and biochemical analysis has shown that one mutation results in the undermodification of the suppressor tRNA.

(A preliminary account of this work was presented at the Annual Meeting of the American Society of Biological Chemists, St. Louis, Mo., June 1981.)

MATERIALS AND METHODS

Bacterial and phage strains. All strains used in this study were derived from *E. coli* K-12, and most are listed in Table 1. Additional strains were derived from those listed in Table

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TABLE 1. Bacterial strains

Strain	Episome	Genotype of chromosome	Source, reference, or construction
CSH50	F⁻	*ara* Δ(*lac-pro*) *rpsL*	J. Miller
CSH57L	F⁻	*ara leu lacY purE gal trp his argG mal xyl mtl ilv metA* or *B rpsL lysA*	J. Davies
CSH70	Hfr P4X	*metB argE* (UAG)	J. Miller
E7086	F′ *lacPL8 proAB*⁺	Δ(*lac-pro*) *supE*	W. Reznikoff
E7091	F′ *lacZ200 proAB*⁺	Δ(*lac-pro*) *supE*	W. Reznikoff
E7093	F′ *lacZ624 proAB*⁺	Δ(*lac-pro*) *supE*	W. Reznikoff
UQ6	F⁻	Δ(*lac-pro*) *ara rpsE hisD*	D. MacNeil
CA165	Hfr H	*relA1 lacI22 lacZ13 supB69*	B. Bachmann
CA167	Hfr H	*relA1 lacI22 lacZ13 supC70*	B. Bachmann
FTP205	F⁻	Δ(*tonB-trpAB*) *supD82*	M. Murgola
AB1157	F⁻	*thr leu argE3 his proA2 lacY galK2 mtl xyl ara rpsL tsx supE44 thi*	B. Bachmann
Ymel	F⁻	*supF*	W. Reznikoff
AB2300	Hfr (AB312)	*ilvD188 argH1 purF1 xyl supL2 supE44*	B. Bachmann
JRC151	F⁻	Δ(*tonB-trpAB*) *his*	M. Murgola
IQ1	F′ *lacI*^{q} (UAG2)*PL8 proAB*	Δ(*lac-pro*) *supE*	J. Miller
MK20	F′ *lacPL8 proAB*⁺	Δ(*lac-pro*) *ara rpsL*	E7086 × CSH50
MK21	F′ *lacI3113PL8 proAB*⁺	Δ(*lac-pro*) *ara rpsL*	EMS mutagenesis of MK20
MS22	F′ *lacI3116PL8 proAB*⁺	Δ(*lac-pro*) *ara rpsL*	EMS mutagenesis of MK20
MK25	F′ *lacI3119PL8 proAB*⁺	Δ(*lac-pro*) *ara rpsL*	EMS mutagenesis of MK20
MK27	F′ *lacI3121PL8 proAB*⁺	Δ(*lac-pro*) *ara rpsL*	EMS mutagenesis of MK20
MK29	F′ *lacI3123PL8 proAB*⁺	Δ(*lac-pro*) *ara rpsL*	EMS mutagenesis of MK20
MK31	F′ *lacI3125PL8 proAB*⁺	Δ(*lac-pro*) *ara rpsL*	EMS mutagenesis of MK20
MK38	F′ *lacI3132PL8 proAB*⁺	Δ(*lac-pro*) *ara rpsL*	EMS mutagenesis of MK20
MK50	Hfr P4X	*met argE* (UAG) rpoB	Spontaneous Rif^r from CHS70
MK56	F⁻	Δ(*tonB trpAB*) *his gyrA*	Spontaneous Nal^r from JRC151
MK57	F⁻	Δ(*tonB trpAB*) *his met argE* (UAG) *rpoB gyrA*	MK50 × MK56
MK58	F⁻	Δ(*tonB trpAB*) *his argE* (UAG) *gyrA*	P1(CSH50), select Met⁺
MK59	F⁻	Δ(*tonB trpAB*) *his argE* (UAG) *gyrA proA* or *B*::Tn*10*	NK55, select Tet^r
MK60	F⁻	Δ(*tonB trpAB*) *his argE* (UAG) *gyrA* Δ(*lac-pro*)	Penicillin selection, Tet^s, Lac⁻
MK60B	F⁻	MK60, *supB69*	P1(CA165) select Arg⁺
MK60C	F⁻	MK60, *supC70*	P1(CA167) select Arg⁺
MK60D	F⁻	MK60, *supD82*	P1(FTP205) select Arg⁺
MK60E	F⁻	MK60, *supE44*	P1(AB1157) select Arg⁺
MK60F	F⁻	MK60, *supF*	P1(Ymel) select Arg⁺
MK60L	F⁻	MK60, *supL2*	P1(AB2300) select Arg⁺
MK60E/Z200	F′ *lacZ200 proAB*⁺	MK60E	E7091 × MK60E
MK60E/624	F′ *lacZ624 proAB*⁺	MK60E	E7093 × MK60E
MK61E	F⁻	MK60E, *rpsE*	P1(UQ6) select Spc^r
MK60E/I3115	F′ *lacI3115PL8 proAB*⁺	MK60E	MK21 × MK60E
MK60E/I3116	F′ *lacI3116PL8 proAB*⁺	MK60E	MK22 × MK60E
MK60E/IQ1	F′ *lacI*^{q} (UAG2)*PL8 proAB*⁺	MK60L	IQ1 × MK60L
MK62E/I3116	F′ *lacI3116PL8 proAB*⁺	MK60E, *asuA1*	This work
MK63E/I3116	F′ *lacI3116PL8 proAB*⁺	MK60E, *zch-453*::Tn*10 asuA*⁺	This work
MK64E/I3116	F′ *lacI3116PL8 proAB*⁺	MK60E, *zch-453*::Tn*10 asuA1*	This work
MK65E/I3116	F′ *lacI3116PL8 proAB*⁺	MK60E, *asuA2*	This work
MK66E/I3116	F′ *lacI3116PL8 proAB*⁺	MK60E, *zch-453*::Tn*10 asuA2*	This work
MK67E/I3116	F′ *lacI3116PL8 proAB*⁺	MK60E, *asuB3*	This work
MK68E/I3116	F′ *lacI3116PL8 proAB*⁺	MK60E, *zhc-454*::Tn*10 asuB*⁺	This work
MK69E/I3116	F′ *lacI3116PL8 proAB*⁺	MK60E, *zhc-454*::Tn*10 asuB3*	This work
MK70E/I3116	F′ *lacI3116PL8 proAB*⁺	MK60E, *asuC4*	This work
MK71E/I3116	F′ *lacI3116PL8 proAB*⁺	MK60E, *zfa-455*::Tn*10 asuC*⁺	This work
MK72E/I3116	F′ *lacI3116PL8 proAB*⁺	MK60E, *zfa-455*::Tn*10 asuC4*	This work
MK100/I3116	F′ *lacI3116PL8 proAB*⁺	MK60, *zbf-451*::Tn*10*	This work
MK100E/I3116	F′ *lacI3116PL8 proAB*⁺	MK60, *zbf-451*::Tn*10 supE44*	This work

1 by episome transfer, and the precise genotype can be readily determined from the strain number. For example, strain MK60D/I3116 is strain MK60D containing the episome from strain MK22 bearing the *lacI3116* mutation. P1 *vir* was obtained from L. Csonka, λ *psu*2 (13) was obtained from H. Ozeki, and λ NK55 (15) was provided by W. Reznikoff.

Media. Routine growth of bacteria was done in L broth or on L plates or in minimal A medium (22) supplemented with 0.1% vitamin-free Casamino Acids in addition to required nutrients. Selection for tetracycline resistance was done on L plates containing 15 μg of tetracycline per ml and 2.5 mM sodium pyrophosphate (TP plates). Determination of the LacI phenotype was accomplished by streaking or replica plating onto supplemented minimal medium containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XGal) per ml. Low-phosphate medium (17) was used for the preparation of

^{32}P-labeled tRNA. NZ medium contained (per liter) 10 g of NZ amine, type A (Humko Sheffield, Ltd.), 0.5 g of yeast extract, 5 g of NaCl, 10 mM $MgCl_2$, and 0.2% maltose.

Genetic procedures. Routine genetic procedures including bacterial matings, mutagenesis, penicillin selection, and the isolation of antibiotic-resistant mutants have been described (22). P1 transductions for auxotrophic markers were performed as described by Miller (22), except that the infected cells were spread directly onto selective plates. Transduction for tetracycline resistance was accomplished by growing the recipient to early stationary phase in L broth plus 5 mM $CaCl_2$. P1 lysate (0.01 to 0.15 ml) was mixed with 0.15 ml of cells and incubated at 37°C for 60 min. The mixture was then spread onto TP plates and incubated at 37°C for 12 h. When other drug resistance markers were transduced, the mixtures were spread onto L plates containing 0.015 M sodium pyrophosphate, incubated at 37°C overnight, and replica plated onto L plates containing the appropriate antibiotic.

Insertions of the drug resistance transposon Tn*10* were obtained by growing a culture in NZ medium to a density of ca. 5×10^8 cells per ml. Phage λNK55 was added to give a multiplicity of infection of 1 to 2. The mixture was incubated at room temperature for 30 min, and then at 37°C for an additional 30 min. Portions were spread onto TP plates and incubated at 42°C overnight. A random pool of Tn*10* insertions was obtained by eluting several plates containing 2,000 to 3,000 Tet^r colonies with 0.1 M sodium phosphate (pH 7.0). The cells were washed twice, suspended in 40% glycerol, and stored at −20°C. This stock culture was diluted into L broth containing 15 μg of tetracycline per ml, grown to exponential phase, and used to prepare a P1 lysate.

Isolation of linked Tn*10* insertions. A Tn*10* insertion linked to *supE* was obtained by using a P1 lysate prepared on the pool of Tn*10* insertions to transduce strain MK60E/I3116 to Tet^r. Rare LacI$^-$ transductants, which arose by cotransduction of the *supE*$^+$ allele with a nearby Tn*10* insertion, were identified by replica plating to medium containing XGal. P1 lysates were prepared on several Tet^r LacI$^-$ isolates and used to again transduce strain MK609E/I3116 to Tet^r. Transductants that remained LacI$^+$ contained a Tn*10* insertion linked to the Sup$^+$ allele of *supE*. One isolate yielded a lysate which gave 30% cotransduction of Tet^r and LacI$^+$. This Tn*10* insertion was designated *zbf-451*::Tn*10*.

Tn*10* insertions linked to antisuppressor mutations and their wild-type alleles were isolated in a similar manner, except that derivatives of strain MK60E/I3116 containing *asu* mutations were used as recipients in the transductions and rare LacI$^+$ transductants were isolated. Subsequent transductions into Asu$^-$ mutants yielded strains carrying Tn*10* insertions linked to either an *asu* mutation or its wild-type allele. P1 lysates prepared on these derivatives could be used to transfer the mutant or wild-type alleles of the antisuppressor loci into additional strains for genetic or biochemical analysis.

Measurement of the efficiency of nonsense suppression. Quantitation of the reduction in the efficiency of nonsense suppression by the antisuppressor mutations was carried out as described by Zengel and Lindahl (38). Derivatives of strains MK60E/Z200 and MK60E/Z624 containing the antisuppressor mutations were constructed by transduction with the Tn*10* insertions linked to the *asu* mutations. Asu$^+$ and Asu$^-$ transductants were identified by back transduction of MK60E/I3116 to Tet^r. Asu$^+$ and Asu$^-$ derivatives of each strain were grown in supplemented minimal medium to a density of ca. 2×10^8 cells per ml. Isopropyl-β-D-thiogalactoside was added to a final concentration of 5×10^{-4} M to induce the *lac* operon. Aliquots were withdrawn at 5-min intervals and placed on ice. The samples were then assayed for β-galactosidase activity (22), and the rate of β-galactosidase synthesis in Asu$^+$ and Asu$^-$ strains was taken to measure the effect of the antisuppressor mutation on the efficiency of suppression by *supE* at the particular nonsense codon.

Isolation of ^{32}P-labeled $tRNA_2^{Gln}$. Two methods were used to isolate ^{32}P-labeled $tRNA_2^{Gln}$ from the antisuppressor mutants. The first method was essentially as described by Inokuchi et al. (13). Strains were grown to a density of ca. 2×10^8 cells per ml in low-phosphate medium with 0.2% maltose in place of glucose. The cells were concentrated by centrifugation, suspended in 0.1 M $MgSO_4$, and irradiated with UV light. The cells were infected with λ *psu*2 at a multiplicity of infection of 10, diluted into prewarmed medium, and labeled with 200 μCi of ^{32}P-labeled phosphate per ml for 3 h in the presence of 50 μg of chloramphenicol per ml. The culture was then extracted with phenol and precipitated with ethanol in the presence of 0.3 M potassium acetate (pH 4.5).

The precipitated RNA was suspended in 0.01 M Tris-hydrochloride (pH 7.5)–0.01 M NaCl and applied to a 0.5-ml DEAE-cellulose column. The column was washed with 0.01 M Tris-hydrochloride (pH 7.5)–0.25 M NaCl, and the RNA was eluted with 0.01 M Tris-hydrochloride (pH 7.5)–1.0 M NaCl–7.0 M urea. The eluted RNA was precipitated with 2 volumes of ethanol and dissolved in 20 μl of water, and an equal volume of 10 M urea–0.025% xylene cyanole–0.025% bromphenol blue was added. The sample was applied to a 15% polyacrylamide gel (acrylamide-methylenebis[acrylamide] [19:1, wt/wt]). Electrophoresis was carried out at 20 V/cm for 6 h in 50 mM Tris borate–1 mM EDTA (pH 8.3), and the tRNA was visualized by autoradiography. Gel slices containing the desired species of RNA were cut out with a scalpel, homogenized by passage through a 1-ml syringe, and eluted as described previously (19). After addition of 50 μg of carrier tRNA, the labeled RNA was precipitated with 2 volumes of ethanol.

The second method used to isolate labeled $tRNA_2^{Gln}$ was by hybridization of ^{32}P-labeled cellular RNA to λ psu_2 DNA immobilized on nitrocellulose filters. Labeled RNA was obtained by growing cells in low-phosphate medium to a density of 2×10^8 cells per ml, followed by addition of ^{32}P-labeled phosphate (200 μCi/ml) and incubation for 60 min at 37°C. Total tRNA was isolated by phenol extraction and DEAE-cellulose chromatography as described above. The tRNA was precipitated with ethanol and suspended in 50 to 200 μl of 50% formamide–6× SSPE (SSPE: 0.18 M NaCl, 0.01 M sodium phosphate, 0.001 M EDTA [pH 7.0])–0.3% sodium dodecyl sulfate. Phage λ psu_2 DNA was isolated as described by Davis et al. (10). The DNA (50 μg) was fixed to nitrocellulose filters and hybridized to total tRNA as described by Weiner (37), except that the hybridization buffer contained 5× SSPE. After hybridization, the filters were washed six times with 2× SSPE–0.3% sodium dodecyl sulfate and once with ice-cold water, and the RNA was eluted by incubation at 80°C for 5 min in water. Potassium acetate was added to a final concentration of 0.1 M, and the RNA was precipitated with ethanol. The RNA was dissolved in 20 μl of water and subjected to electrophoresis as described above.

tRNA sequence analysis. ^{32}P-labeled tRNA eluted from a polyacrylamide gel was dissolved in 0.2 ml of 0.2 M potassium acetate (pH 5.0) and precipitated with ethanol. The

RNA was pelleted, dried, suspended in 50 μl of water, and taken to dryness on a piece of Parafilm. Procedures for RNase T1 fingerprinting and the elution of oligonucleotides from DEAE-cellulose paper were as described by Barrell (2). The nucleotide composition of intact tRNAs or oligonucleotides was determined by two-dimensional thin-layer chromatography after digestion to nucleotides by RNase T2 (23). For intact tRNAs, the samples were digested with a solution containing 10 μl of 50 U of RNase T2 per ml, 0.25 mg of RNase T1 per ml, and 0.25 mg of RNase A per ml in 0.05 M ammonium acetate (pH 4.5) at 37°C for 4 to 8 h. Oligonucleotides were digested for 2 h at 37°C with a solution containing 10 μl of 10 U of RNase T2 per ml, 0.05 mg of RNase T1 per ml, and 0.05 mg of RNase A per ml in 0.05 M ammonium acetate (pH 4.5).

The mixtures were applied to a cellulose thin-layer chromatography plate (20 by 20 cm or 10 by 10 cm; Brinkmann Instruments, Inc.). Chromatography in the first dimension was done in isobutyric acid–0.5 M NH_3 (5:3, vol/vol), and after air drying for 12 h, the second dimension was developed in isopropanol-concentrated HCl-water (70:15:15, vol/vol/vol). After air drying for 12 h, the plates were subjected to autoradiography with intensifying screens (Ilford). The amount of each nucleotide present was quantitated by removing each spot (30) and counting the amount of label in a scintillation counter.

RESULTS

The nonsense suppressor *supE*, which is derived from $tRNA_2^{Gln}$, was chosen for the initial isolation of antisuppressor mutations because a transducing phage carrying *supE* had been isolated (13) which would facilitate biochemical analysis. For the antisuppressor selection to work efficiently, we required a *lacI* nonsense mutation which yielded a $LacI^+$ phenotype when suppressed by *supE* (white or very pale blue on XGal indicator medium) and which failed to grow on medium containing phenyl-D-galactoside as the sole carbon source. To increase the sensitivity of our detection system, all of the strains carried the *lacPL8* mutation in the *lacZ* promoter. This mutation decreased the amount of β-galactosidase synthesized ca. 10-fold relative to a $lacP^+$ strain. This served to decrease the sensitivity of the XGal indicator medium so that small changes in the amount of repressor made could be detected (9).

Several *lacI* nonsense mutations were isolated and transferred to strain MK60E, and the levels of β-galactosidase and the extent of growth on phenyl-D-galactoside were measured. The results are shown in Table 2. Strains which possess levels of β-galactosidase of ca. 5 U or higher develop a significant color on indicator plates and show substantial growth on phenyl-D-galactoside. Therefore two easily suppressed nonsense mutations, *lacI3115* and *lacI3116*, were chosen for selection of antisuppressor mutations.

TABLE 2. Characteristics of *lacI* nonsense mutations suppressed by *supE*

Strain	β-Galactosidase activity[a]	Growth in phenyl-D-galactosidase (OD_{600})[b]
MK60E/I^+	0.5	0.05
MK60E/I3115	0.7	0.06
MK60E/I3116	2.3	0.19
MK60E/I3119	5.3	0.31
MK60E/I3121	5.3	0.33
MK60E/I3123	15.3	0.58
MK60E/I3125	2.1	0.17
MK60E/I3132	198	1.10

[a] The strains were grown to mid-exponential phase in supplemented minimal medium and assayed for β-galactosidase activity

[b] An overnight culture was diluted 100-fold into minimal A medium with phenyl-D-galactoside as the sole carbon source. After 24 h of growth at 37°C, the absorbance of the culture at 600 nm was measured.

TABLE 3. Summary of the antisuppressor selections

Strain	No. of $LacI^-$ colonies	No. of mutations in *lac*	No. of mutations in *supE*	Antisuppressor mutations isolated
MK60E/13115	104	86	18	—
MK60E/13116	208	106	98	*asu-1*, *asu-2*, *asu-3*, and *asu-4*

Strains MK60E/I3115 and MK60E/I3116 were mutagenized with ethyl methane sulfonate, outgrown, and then diluted 100-fold into phenyl-D-galactoside medium and grown for 24 h at 37°C. The cultures were diluted and plated onto medium containing XGal, and $LacI^-$ colonies were isolated. To distinguish additional *lacI* mutations from antisuppressor mutations, the F′ *lac-pro* episomes were mated to strain MK61E, and Spc^r Pro^+ transconjugants were selected. Mutations mapping in the *lac* operon yielded $LacI^-$ transconjugants and were discarded.

Mutations affecting the structural gene of the suppressor tRNA were identified by transducing the remaining mutants to Tet^r with P1 grown on strain MK100E/I3116, which contains *supE* linked to a Tn*10* insertion. $LacI^-$ mutants resulting from mutations tightly linked to the suppressor locus will yield ca. 30% $LacI^+$ Tet^r transductants. However, if the mutation causing the $LacI^-$ phenotype is unlinked to *supE*, then 100% of the Tet^r transductants will be Lac^-. The results of the two selections are displayed in Table 3. Although no Asu^- mutants were obtained from strain MK60E/I3115, four mutants were isolated from strain MK60E/I3116, which contained mutations unlinked to *lac* or *supE*. These were designated *asu* mutations.

Genetic analysis of *asu* mutations. Genetic analysis of the antisuppressor mutations required the ability to transfer the mutations to other strains. The ability to grow on phenyl-D-galactoside was unsuitable as a selective marker because of substantial background growth and the possibility of secondary mutations occurring at each selection. To circumvent these difficulties, we used the LacI phenotype as a nonselective visual indicator of the efficiency of suppression to isolate Tn*10* insertions linked to the antisuppressor mutations (see above). These insertions allowed transfer of the *asu* mutations to a variety of strains by simply transducing the Tet^r character and observing the antisuppressor phenotype on indicator plates.

The number of loci represented by the *asu* mutations was determined by conducting allelism tests with the linked Tn*10* insertions. A diagram of this procedure is shown in Fig. 1. The four mutations were found to represent three loci, designated *asuA*, *asuB*, and *asuC*. Although it is possible that the *asuA1* and *asuA2* mutations are in separate genes located the same distance away but on opposite sides of the Tn*10* insertion, subsequent analysis suggested that these two mutations are in fact the same gene.

Effect of antisuppressor mutations at other nonsense codons and nonsense suppressors. Each antisuppressor mutation was

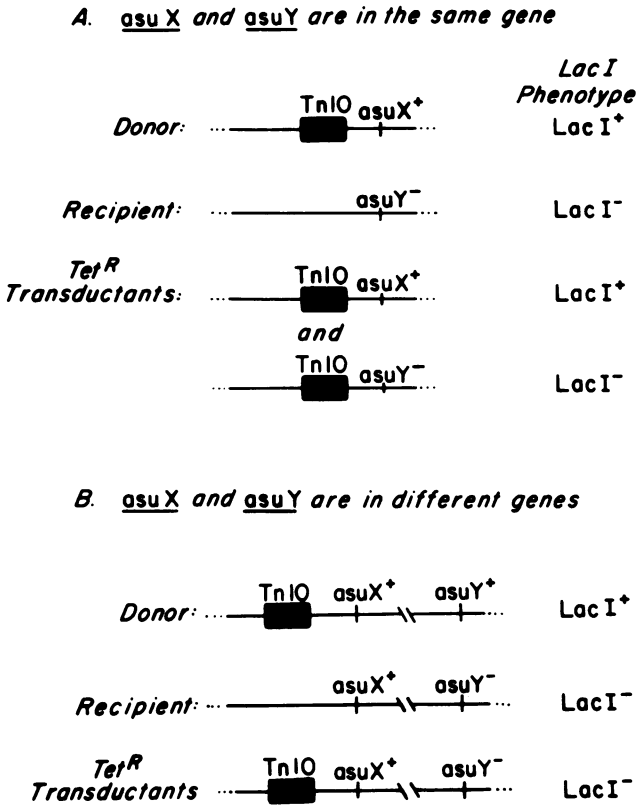

FIG. 1. Allelism tests of antisuppressor mutations were conducted by using P1 grown on strains containing Tn*10* insertions linked to *asu*$^+$ alleles and transducing Asu$^-$ strains to Tet^r. If the two antisuppressor mutations are in the same gene (A), then both LacI$^+$ and LacI$^-$ transductants are obtained. If the two mutations are in different genes (B), then only LacI$^-$ transductants are found.

transduced into strains containing several *lacI* nonsense mutations and either *supE* or a different nonsense suppressor, and the resulting LacI phenotype was determined. The results are summarized in Table 4. The three classes of mutations can be readily distinguished by their effects on other nonsense suppressors, but there does not appear to be any evidence of context effects (4, 11), at least within the sensitivity of this method.

Quantitation of the reduction in suppression efficiency. The antisuppressor mutations were further characterized by measuring their effect on the rate of β-galactosidase induction in strains containing *supE* and a *lacZ* nonsense mutation (38). The ratio of the rate of β-galactosidase synthesis in the presence of an *asu* mutation relative to the rate of synthesis in its absence should reflect the reduction in the efficiency of suppression. The results are shown in Table 5. The *asuA1* and *asuA2* mutations reduce suppression by ca. 40 to 50%, the *asuB3* by ca. 60%, and the *asuC4* by ca. 95%. These results show that mutations with widely differing effects on suppression can be detected by using the *lacI* system for monitoring suppression.

TABLE 5. Quantitation of the reduction in the efficiency of suppression of *supE* by antisuppressor mutations

Antisuppressor[a] mutation	Expt no.	Relative rate of β-galactosidase induction[b]	
		lacZ200	*lacZ624*
asu$^+$	1	1.00	1.00
	2	1.00	1.00
asuA1	1	0.68	0.70
	2	0.65	0.68
asuA2	1	0.37	0.38
	2	0.38	0.42
asuB3	1	0.42	0.47
	2	0.47	0.48
asuC4	1	0.06[c]	0.07
	2	0.05[c]	0.06

[a] Antisuppressor mutations were introduced into strains MK60E/Z200 and MK60E/Z624 by contransduction with a linked Tn*10* insertion.
[b] Ratio of the rate of β-galactosidase synthesis after induction by isopropyl-β-D-thiogalactopyranoside in strains containing the antisuppressor mutation relative to an Asu$^+$ transductant.
[c] These values were obtained from steady-state levels of induced β-galactosidase because the rate of induction was too low to be accurately measured.

Genetic mapping. The presence of a drug resistance transposon near an antisuppressor locus allows the genetic mapping of the *asu* mutations by simply mapping the locations of the transposon. Transduction of strain CSH57L to Tet^r with P1 grown on the appropriate donors revealed that the *asuA* locus was linked to *trp* and that the *asuB* locus was linked to *rpsL*. Further transductions (data not shown) demonstrated 100% linkage of the *asuB3* mutation to *rpsL*. This suggests that this mutation is an allele of *rpsL*, which restricts suppression (34). However, the *asuB3* mutation does not confer resistance to streptomycin (unpublished observations). Mapping of the *asuC4* mutation is described below.

Effect of antisuppressor mutations on tRNA$_2^{Gln}$. If any of the antisuppressor mutations affected tRNA modification, they would most likely be limited to those modifications found in the suppressor tRNA. Therefore, ^{32}P-labeled tRNA$_2^{Gln}$ was isolated from strains containing *asu* mutations either by hybridization of uniformly labeled tRNA to λ *psu*2 DNA or after UV irradiation and infection with λ *psu*2 (13). The

TABLE 4. Effect of *asu* mutations on different *lacI* nonsense mutations and on several nonsense suppressors

Anti-suppressor mutation	LacI phenotype								
	supE[a] *lacI3115*	*supE* *lacI3116*	*supE* *lacI3119*	*supE* *lacI3125*	*supD* *lacI3116*	*supF* *lacI3116*	*supB* *lacI*^{q} (UAG)2[b]	*supC* *lacI*^{q} (UAG)2[b]	*supL* *lacI*^{q} (UAG)2[b]
asuA1[c]	+[d]	−	−	−	±	±	−	−	−
asuA2	±	−	−	−	−	−	−	−	−
ausB3	−	−	−	−	+	+	+	+	+
asuC4	−	−	−	−	+	+	+	+	+

[a] Relevant genotype of the recipient strains. All recipients are derivatives of strain MK60 containing the indicated suppressor and *lacI* allele.
[b] The *lacI*^{q} mutation was required to increase the amount of repressor made to compensate for the low efficiency of suppression by UAA/G suppressors. In the absence of an antisuppressor mutation, these strains have a LacI$^+$ phenotype.
[c] The antisuppressor mutations were introduced into the recipient strains by cotransduction with a linked Tn*10* insertion.
[d] Symbols: +, LacI$^+$ phenotype, indicating efficient suppression; −, LacI$^-$ phenotype, indicating that antisuppression is occurring; ±, partial LacI$^-$ phenotype.

tRNA was purified by gel electrophoresis (Fig. 2), and the modified nucleotide content was determined. Autoradiograms of the modified nucleotide analysis of $tRNA_2^{Gln}$ isolated by hybridization to λ *psu*2 DNA with tRNA isolated from a wild-type strain and from the *asuC4* mutant are shown in Fig. 3. The amount of pseudouridine is reduced ca. 60% in $tRNA_2^{Gln}$ isolated from the mutant.

Analysis of the oligonucleotides generated by RNase T1 revealed that pseudouridine was present in normal amounts in the TψCG fragment of $tRNA_2^{Gln}$ but that the two pseudouridines normally present in the oligonucleotide making up the anticodon stem and loop were completely missing (data not shown). Similar results were obtained with RNA isolated after infection of UV-irradiated cells with λ *psu*2.

These results suggested that the *asuC4* mutation was a new allele of *hisT*, which has been shown to cause a loss of pseudouridine in a number of tRNAs in both *E. coli* and *Salmonella typhimurium* (8). The *hisT* mutation has been mapped to 49.5 min in *E. coli* (6) and is linked to *purF*. To determine whether the *asuC4* mutation mapped in this region, strain AB2300 (*purF*) was transduced to Tet^r with a P1 lysate grown on a strain containing the Tn*10* insertion linked to *asuC4*. Of the Tet^r transductants, 55% were Pur^+, supporting the idea that the *asuC4* mutation is an allele of *hisT*. Bossi and Roth (4) have also described the antisuppressing properties of *hisT* mutations of *supE* in *Salmonella* strains.

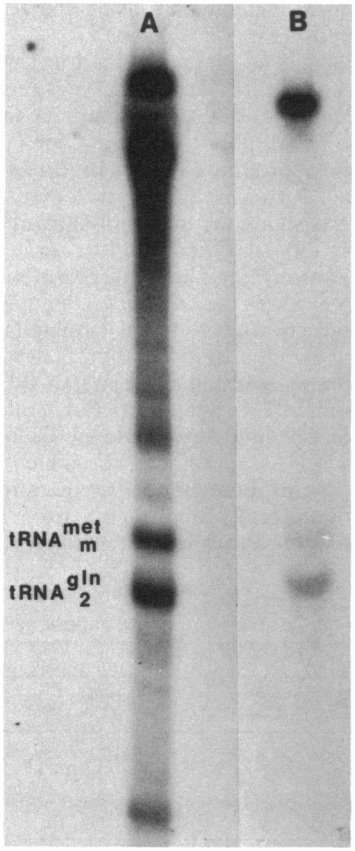

FIG. 2. Polyacrylamide gel electrophoresis of ^{32}P-labeled tRNA synthesized after infection of UV-irradiated cells with λ *psu*2 (lane A) or labeled tRNA purified by hybridization to λ *psu*2 DNA (lane B). The identity of the tRNAs was confirmed by RNase T1 fingerprinting.

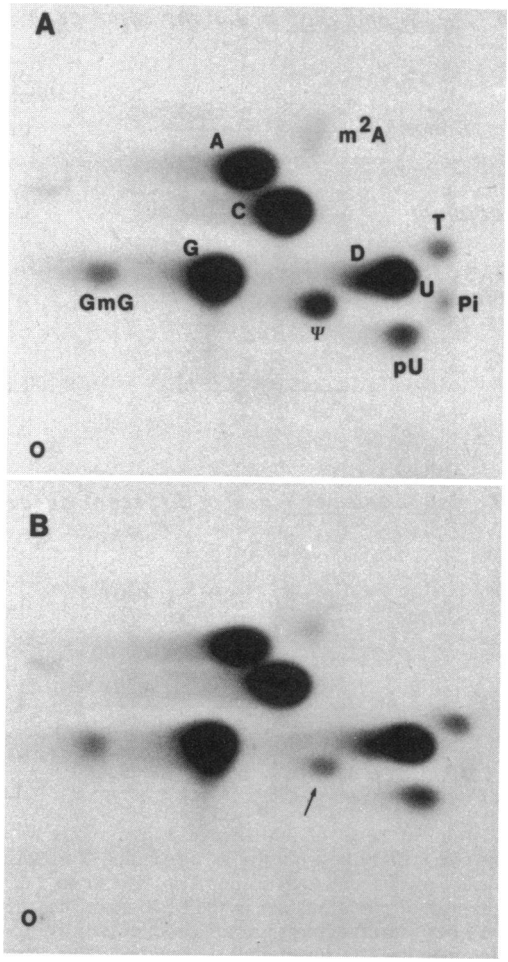

FIG. 3. Autoradiograms of minor nucleotide analyses of $tRNA_2^{Gln}$ isolated by hybridization to λ *psu*2 DNA from a wild-type strain (panel A) and from a strain containing the *asuC4* mutation (panel B). ^{32}P-labeled $tRNA_2^{Gln}$ was digested with RNase T2 and subjected to two-dimensional thin-layer chromatography as described in the text. Chromatography in the first dimension was from bottom to top, and the second dimension was developed from left to right. The origin is indicated by an O in the lower left corner. The arrow indicates the reduced amount of pseudouridine found in tRNA from an *asuC4*-containing strain.

We have not been able to detect any alterations in the amounts of modified nucleotides in total tRNA or $tRNA_2^{Gln}$ from *asuA*-containing strains, although deficiencies in 4-thiouridine or 2′-*O*-methyluridine would not have been detected by the methods used in this study. However, the two previously identified genes involved in 4-thiouridine biosynthesis do not map near *trp* (1), and 2′-*O*-methyluridine is not found in most of the suppressor tRNAs (12). Measurements of the amounts of glutamine-accepting tRNA and the activity of glutamine aminoacyl-tRNA synthetase showed wild-type levels in *asuA*-containing strains (unpublished results).

DISCUSSION

The use of *lacI* nonsense mutations to monitor the efficiency of suppression has permitted the development of genetic procedures for isolating and characterizing mutations which reduce the efficiency of nonsense suppression.

By applying techniques first developed for the study of the *lac* operon, antisuppressor mutations can be easily isolated. Since these mutants appear blue on indicator plates containing XGal, the mutations can be readily detected during genetic manipulations. This property, in combination with the techniques for transposon manipulation developed by Kleckner et al. (16), provides a powerful tool for the study of tRNA function.

Mutations in three loci have been isolated from a strain containing *supE*. The defects in two of these classes have been identified: one has been shown to result in a defect in tRNA modification and the other maps in the gene for ribosomal protein S12. Strains containing mutations in the *asuA* locus have no detectable alterations in modified nucleotide composition, and genetic analysis indicates that this class of mutations affects a variety of nonsense suppressors, suggesting a general defect in tRNA utilization. This locus maps near *trp*, at ca. 27 min on the *E. coli* linkage map (1). There are two known genes in this region which might, if mutated, cause the observed phenotype.

Mutations in the gene coding for DNA topoisomerase I have been shown to increase the expression of some promoters, presumably resulting from excessive supercoiling of the chromosome (33). Mutations which increase the expression of the *lacPL8* promoter would have a phenotype similar to an antisuppressor mutation. However, suppression of *lacZ* nonsense mutations is also reduced by *asuA* mutations. It does not seem likely that alterations in the amount of supercoiling could increase expression from the *lacPL8* promoter and decrease expression from the wild-type promoter.

A second gene in this region, *pth*, codes for peptidyl-tRNA-hydrolase, an enzyme believed to be involved in the release of the nascent peptide from the ribosome or in the metabolism of aberrantly produced peptidyl-tRNAs (21). Our attempts to assay this enzyme have been unsuccessful, and so it remains possible that alterations in *pth* could produce an antisuppressor phenotype. Further work should clarify this point.

The *asuB3* mutation has been shown genetically to be an allele of *rpsL*, the gene coding for ribosomal protein S12. This protein is altered in streptomycin-resistant strains and has been shown to be involved in the fidelity of protein synthesis and to restrict the efficiency of some nonsense suppressors (34); *supE* is particularly sensitive to alterations in *rpsL* (5). Since the *asuB3* mutation does not confer resistance to streptomycin, it is presumably an alteration of *rpsL* which affects only the functions involved in the fidelity of protein synthesis and does not interfere with the action of the antibiotic.

The *asuC4* mutation has been shown to be defective in the conversion of two adjacent uridines to pseudouridine in the anticodon stem and loop of $tRNA_2^{Gln}$. Genetic mapping has placed this mutation near *purF* at 50 min (6), which supports the idea that the *asuC4* mutation is a new allele of *hisT*. The *hisT* locus has been shown to code for a pseudouridylate synthase which can convert uridine to pseudourine in tRNA (8).

An interesting feature of this mutation is that it exerts a detectable antisuppressing effect only on *supE*. Turnbough et al. (36) have observed that a large number of tRNAs have altered chromatographic mobilities as a result of the *hisT* mutation and that this mobility shift is correlated with the presence of a pseudouridine in the 3′ side of the anticodon stem and loop of the tRNA from *hisT*$^+$ strains. Several nonsense suppressors, including *supB*, *supF*, and *supL*, contain a single pseudouridine in this region (12) but none appears to be affected by the *asuC4* mutation.

We have observed that $tRNA^{Phe}$ isolated from a *hisT* mutant lacks the modification of pseudouridine at position 39 and shows measurably different physical characteristics compared with wild-type $tRNA^{Phe}$. Interestingly, the undermodified $tRNA^{Phe}$ promotes in vitro polyphenylalanine biosynthesis at 112% of the rate of wild-type $tRNA^{Phe}$ (J. Cannon and R. M. Bock, unpublished results).

These results suggest that the *hisT* enzyme may be responsible for all of the pseudouridine modifications in the 3′ side of the anticodon stem and loop region of tRNA, but only when two adjacent pseudouridines occur is their presence required for efficient tRNA function. To date, only two tRNAs in *E. coli*, $tRNA_2^{Gln}$ and $tRNA^{His}$, have been found to contain two adjacent pseudouridine modifications in this position (12). The efficiency of $tRNA_2^{Gln}$ utilization is reduced substantially when these pseudouridines are absent, and a similar effect of $tRNA^{His}$ is inferred from studies on the regulation of the *his* operon of *S. typhimurium* (14).

The genetic selection we have devised exploits the fact that the efficiency of suppression is a measure of the competition between normal tRNA-codon interaction and chain termination. The effects of mutations which slightly alter tRNA utilization during normal translation are amplified when nonsense suppression is examined. This is observed in the case of *hisT* (or *asuC4*) mutations, which have been found to reduce the elongation rate of protein synthesis by 20 to 30% (27) but reduce the efficiency of suppression by *supE* by ca. 95%. This property allows the isolation of mutations which do not have a large effect on tRNA function during normal protein synthesis but can be detected by their effect on nonsense suppression.

The extreme sensitivity of this system is illustrated by the isolation of mutations which reduce the efficiency of suppression ca. twofold (*asuA* mutations). This is in contrast to previous selections for loss of suppression (29, 31), in which nearly complete loss of suppression was required. The use of *lacI*^{q} UAA or UAG alleles compensates for the low efficiency of suppression by UAA/G suppressors and allows the isolation and characterization of mutations which affect very weak suppressors. We have used this strategy to isolate antisuppressor mutations against the lysine inserting UAA/G suppressor *supL* and have found one class of mutations which affects the modification of the wobble base of several tRNAs (35). This genetic system for the analysis of tRNA function should prove valuable in establishing the role of modified nucleosides in tRNA function.

ACKNOWLEDGMENTS

We thank Frances Webb for excellent technical assistance and John Cannon for critical review of the manuscript. We thank Barbara Bachmann, William Reznikoff, Jeffrey Miller, and Emmanuel Murgola for providing bacterial strains.

This work was supported by the University of Wisconsin Graduate School, and M.A.S. was supported by predoctoral training grant 5-T32-GM07215-04 from the National Institutes of Health.

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