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Defined Set of Cloned Termination Suppressors: In Vivo Activity of Isogenetic UAG, UAA, and UGA Suppressor tRNAs

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We have cloned an isogenetic set of UAG, UAA, and UGA suppressors. These include the Su7-UAG, Su7-UAA, and Su7-UGA suppressors derived from base substitutions in the anticodon of *Escherichia coli* $tRNA^{Trp}$ and also Su9, a UGA suppressor derived from a base substitution in the D-arm of the same tRNA. These genes are cloned on high-copy-number plasmids under *lac* promoter control. The construction of the Su7-UAG plasmid and the wild-type *trpT* plasmid have been previously described (Yarus, et al., Proc. Natl. Acad. Sci. U.S.A. 77:5092–5097, 1980). Su7-UAA (*trpT177*) is a weak suppressor which recognizes both UAA and UAG nonsense codons and probably inserts glutamine. Su7-UGA (*trpT176*) is a strong UGA suppressor which may insert tryptophan. Su9 (*trpT178*) is a moderately strong UGA suppressor which also recognizes UGG (Trp) codons, and it inserts tryptophan. The construction of these plasmids is detailed within. Data on the DNA sequences of these *trpT* alleles and on amino acid specificity of the suppressors are presented. The efficiency of the cloned suppressors at certain nonsense mutations has been measured and is discussed with respect to the context of these codons.

Termination suppressors have proven to be invaluable probes of tRNA function in vivo. The activity of such a tRNA can be indirectly assayed by measuring the amount of gene product produced from a cistron carrying a nonsense mutation (a UAA, UAG, or UGA codon created by mutation). Thus tRNAs can be studied under steady-state conditions in vivo.

In addition, the nonsense suppressors are powerful tools for the study of any gene. Nonsense mutations give definitive null phenotypes. However, in the presence of the appropriate suppressor tRNA, such a gene product of a mutant can be made, sometimes at almost normal levels. Thus the controlled expression of nonsense suppressors would allow one to obtain mutations in genes which are essential to the cell. Furthermore, the conditional synthesis of a complete product of a gene permits unambiguous identification of the polypeptide produced.

The utility of nonsense suppressors for both types of studies is enhanced by the availability of a complete, well-characterized, and convenient set of termination suppressors. We describe in this paper a cloned set of closely related *Escherichia coli* tRNA suppressor genes which fulfill these criteria. The suppressors include a very efficient amber (UAG) suppressor, an ochre (UAA) suppressor, and two opal (UGA) suppressors, all derived from $tRNA^{Trp}$. The amber and ochre suppressors and one of the opal suppressors are anticodon mutations of *trpT* and are designated *trpT175*, *trpT177*, and *trpT176*, respectively, also referred to as Su7-UAG, Su7-UAA, and Su7-UGA (41). Su7-UAA has also been called Su8 (42). The second opal suppressor, *trpT178*, is the mutation altering the D-arm of the tRNA, identified by Hirsh (19) and subsequently called either Su9 or the Hirsh suppressor. This mutation acts indirectly to stimulate the recognition of UGA codons by the UGG-reading $tRNA^{Trp}$. The position of these mutations in a standard tRNA cloverleaf configuration is shown in Fig. 1. The derivation of the plasmids carrying the suppressor alleles of *trpT* is diagrammed in Fig. 2. The cloned parental *trpT* gene, encoding $tRNA^{Trp}$ (UGG), was also included in studies of this set of cloned genes. The wild-type allele was useful as a control in some cases, and in certain genetic contexts in vivo we have demonstrated that it has substantial UGA-suppressing activity.

These isolates are each carried on a well-defined plasmid which also encodes tetracycline resistance. Each gene occurs in the same small transcription unit (Fig. 3) under the control of the *lacUV5* promoter and terminated by the *rrnC* terminator naturally occurring downstream from the *trpT* gene (51).

We have constructed these isogenetic suppressors for studies of tRNA function, and initial investigations into the context dependence of the efficiencies of the UGA suppressors are included here. In addition, these plasmids will be generally useful for *E. coli* genetics, as well as for other studies which require tRNAs whose activities are distinguishable and measurable in vivo. For these reasons we present them as a group in this report.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophage, and media. *E. coli* K-12 strains, plasmids, and bacteriophage used in this work are listed in Table 1. P1KC transductions were performed by the method of Lennox (22). Strains bearing plasmids were transformed by the $CaCl_2$ method of Dagert and Ehrlich (11).

Cultures prepared for tryptophan synthetase A and B subunit assays were grown in M9 minimal medium (25) supplemented with the appropriate amino acids. Cultures assayed for beta-galactosidase activity were grown in morpholinepropanesulfonic acid minimal medium (31) supplemented with 0.2% glucose, 1.5 mM K_2HPO_4, 1 mM isopropyl-beta-D-galactopyranoside (IPTG), 1 μg of B1 per ml, and 5 μg of tetracycline (Tet) per ml. When amino acids were added to minimal cultures, liquid cultures were made 50 μg/

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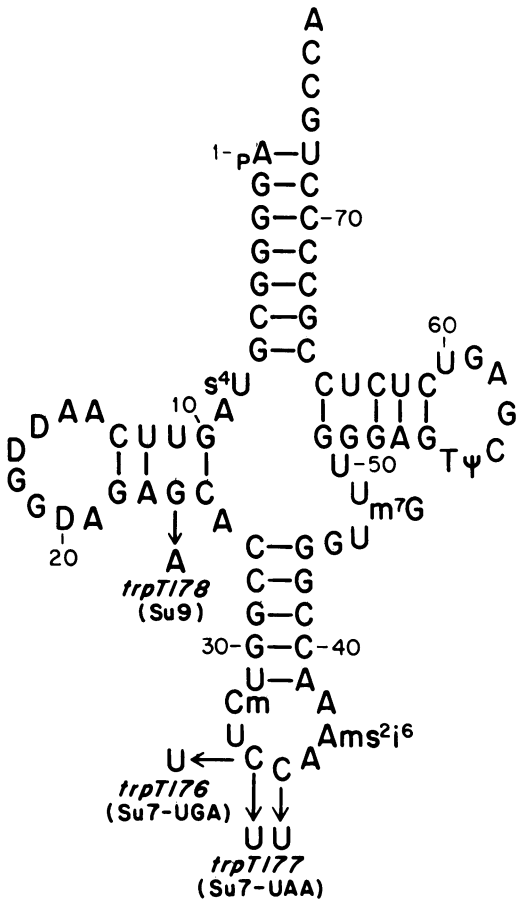


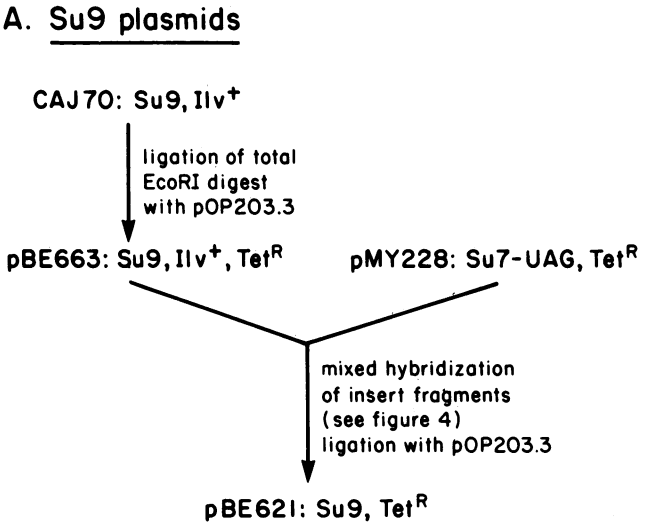
FIG. 1. Cloverleaf diagram of $tRNA^{Trp}$. Base substitutions created by each mutation are indicated. The modifications shown are from the chromosomally expressed $tRNA^{Trp}$ and have not been confirmed for these alleles in the plasmid transcription unit. However, the same modifications have been observed on suppressor tRNAs expressed from other alleles of *trpT* in the same transcription unit (M. Yarus, unpublished data).

ml and plates were made ca. 35 μg/ml, except for tryptophan, which was added at half these concentrations. Minimal plates were M63 (25) with 1.5% Bacto-Agar (Difco Laboratories). Antibiotics were added to make the following concentrations on the plates: tet, 15 μg/ml, kanamycin (Kan), 150 μg/ml, and streptomycin (Strep), 200 μg/ml.

Isolation and manipulation of DNA. Total DNA was isolated by the method of Saito and Miura (36). Unless otherwise mentioned, plasmid DNA was purified by the alkaline-sodium dodecyl sulfate procedure described by Birnboim and Doly (1). Alternate procedures were the Sarkosyl lysis method of Clewell (7) or the cleared lysate method of Clewell and Helinski (8). Analytical and preparative gel electrophoresis in a Tris-acetate buffer have been described by Yarus et al. (49). DNA fragments were extracted from polyacrylamide gels by the method of Maxam and Gilbert (24). Whole plasmid DNA was extracted from agarose gels by the glass-powder adsorption method of Vogelstein and Gillespie (45). Enzyme reactions were performed under the conditions recommended by the vendor. DNA nucleotide sequences were determined by the chemical cleavage method of Maxam and Gilbert (24).

Hybridization of heterogeneous DNA fragments. After *Hin*fI digestion of pBE663, the 84-base-pair (bp) fragment bearing the *trpT178* mutation was purified by polyacrylamide gel electrophoresis. pMY228 was *Eco*RI digested to yield the 264-bp insert, carrying the *trpT175* allele, which was isolated by polyacrylamide gel electrophoresis. The 66- and 114-bp fragments obtained by *Hin*fI digestion of this *Eco*RI fragment, carrying the *trpT175* mutation and *aspT*, respectively, were also purified by polyacrylamide gel electrophoresis. One picomole of each DNA fragment was coprecipitated and then resuspended in 10 μl of 8× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.4]). An equal volume of formamide was added, and the solution was incubated at 100°C for 3 min and then at 37°C for 30 min. The solution was then diluted 20-fold, and the DNA was ethanol precipitated. The potential products of this mixed hybridization are diagrammed in Fig. 4.

EMS mutagenesis. The strain to be mutagenized was grown to saturation in Luria broth (25). A minimal plate, supplemented to select for the desired mutations, was spread with 0.2 ml of this culture. Five microliters of ethyl methanesulfonate (EMS) was placed in a drop at the center of the



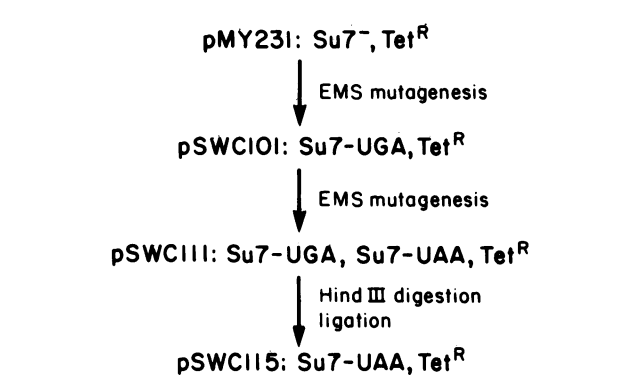


FIG. 2. Derivation of the plasmids discussed in this work.

plate, and the plate was incubated at 37°C until colonies appeared. All resulting colonies were scraped from the plate and used together to inoculate M9 minimal medium supplemented in the same way as the selective plate. Plasmid DNA was prepared from this batch culture.

Suppressor efficiency determinations. The beta-galactosidase activity assayed in these experiments was from a *lac* repressor–beta-galactosidase fusion. This protein was encoded by an in-frame fusion of the *lacI-lacZ* genes created by Δ*lac-14* characterized by Müller-Hill and Kania (30). Miller and Albertini (26) have thoroughly characterized this genetic system for use in determining suppressor efficiencies. The alleles used in this study do not appear to permit translational restarts downstream from the termination mutation (26). The nonsense mutations lie in the *lacI* portion of the hybrid gene, so that the presence of any beta-galactosidase activity must come from read-through of the termination codon.

Beta-galactosidase activity from the fused protein in permeabilized cells dropped after 2 h of incubation under the assay conditions described by Miller (25). To maintain the beta-galactosidase activity of the fusion protein throughout assay times of up to 24 h, the following modifications to this assay were made: 100 μg of bovine serum albumin per ml was added to the assay buffer of Miller (Z buffer), and 2-mercaptoethanol was added to provide a final concentration of 0.1 M during the assay. The reactions were incubated at 20°C in insulated blocks cooled by water circulating from a Lauda refrigerating water bath. Assays were repeated between 4 and 16 times.

The use of nonsense mutations in the *trpA* gene to determine transmission efficiency of a suppressor tRNA has been described by Yarus et al. (49). Tryptophan synthetase A and B subunit activities were assayed in sonicated cell extracts as described by Smith and Yanofsky (40).

Spot tests for the suppression of bacteriophage nonsense mutations were described by Yarus et al. (49).

Materials. Bacteriological culture media and Casamino Acids were obtained from Difco. L-Amino acids, antibiotics, *o*-nitrophenyl galactoside, indole, *p*-dimethylaminobenzaldehyde, and IPTG were obtained from Sigma Chemical Co. Spectrograde formamide and EMS were from Eastman Chemical Products, Inc. Bacterial alkaline phosphatase was from Worthington Diagnostics; all other enzymes were obtained from New England Biolabs.

RESULTS

Cloning *trpT178*, a non-anticodon UGA suppressor gene, on a 6.1-megadalton fragment. The *trpT178* or Su9 mutation of $tRNA^{Trp}$ is unusual because the alteration does not lie in the

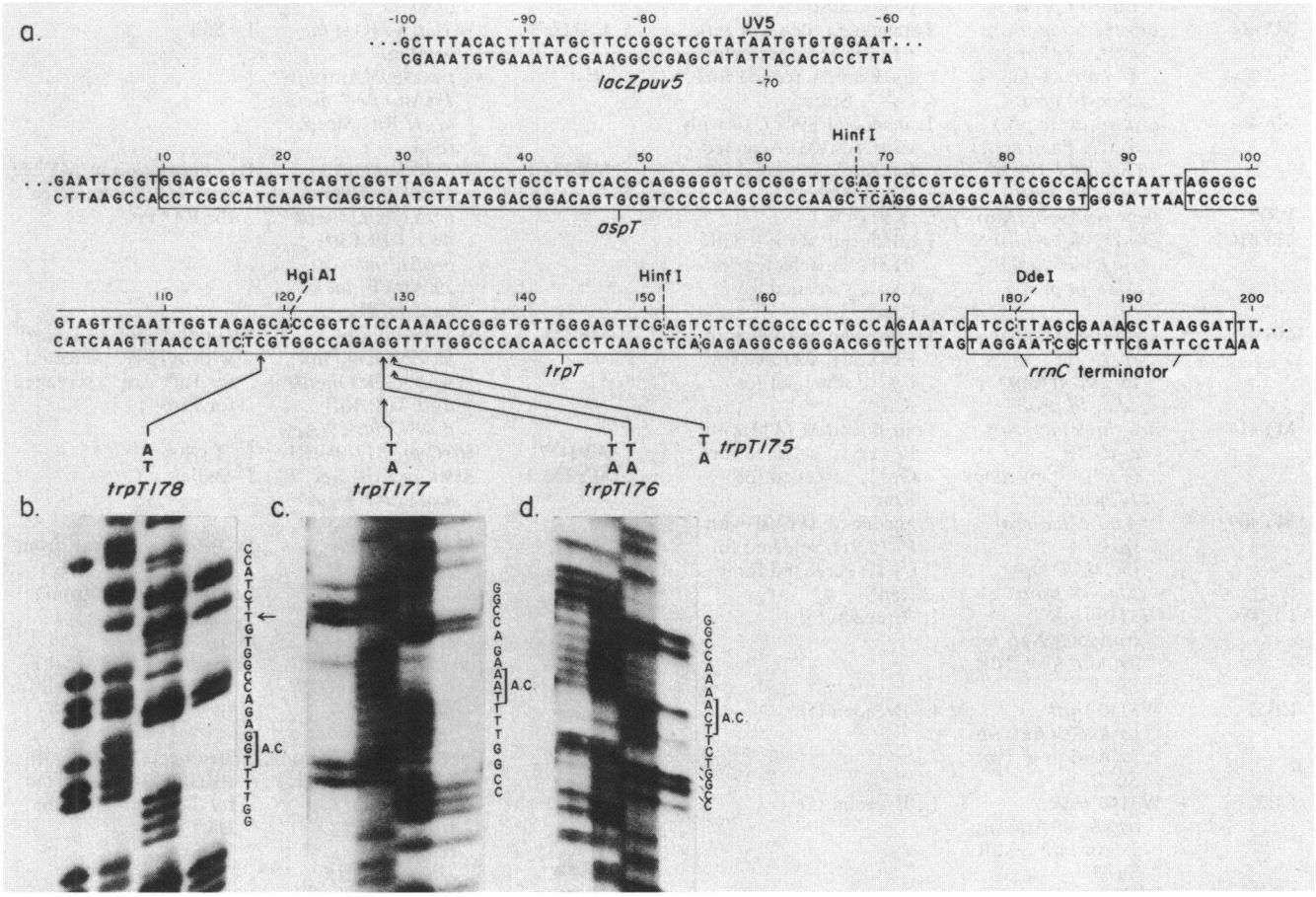


FIG. 3. (a) DNA sequence of the *Eco*RI insert containing the *trpT* alleles. The sequence of the *lacUV5* promoter and of the base change for *trpT175* are from Yarus et al. (49). All DNA sequences were obtained from the *Eco*RI-*Dde*I fragment containing *trpT*. The sequence of the fragment carrying the *trpT178* mutation was determined from the *Dde*I site through the *aspT* gene to the *Eco*RI site. Sequence determinations of the fragments carrying the *trpT176* and *trpT177* mutations did not include the entire *aspT* gene. (b, c, and d) Details from autoradiographs of 20% polyacrylamide sequencing gels (24) of DNA fragments bearing the indicated mutations. The lanes of each gel are, from left to right: G, G + A, T + C, and C.

TABLE 1. *E. coli* strains, plasmids, and bacteriophage used in this work

Strain, plasmid, or bacteriophage	Genotype or phenotype	Source or reference
Strain	*ara thi* Δ(*lac-pro*)	J. H. Miller (26)
S90C	*rpsL*/F′ *lacI*^{q} Δ*lac-14 proB*$^+$	
S90CA24	*ara thi* Δ(*lac-pro*) *rpsL*/F′ *lacI*^{q}*A24*(Am) Δ*lac-14 proB*$^+$	J. H. Miller (26)
S90CU6	*ara thi* Δ(*lac-pro*) *rpsL*/F′ *lacI*^{q}*U6*(Op) Δ*lac-14 proB*$^+$	J. H. Miller (26)
S90CU10	*ara thi* Δ(*lac-pro*) *rpsL*/F′ *lacI*^{q}*U10*(Op) Δ*lac-14 proB*$^+$	J. H. Miller (26)
CBK103	*thyA cysG*::Tn*5*	C. Berg (38)
MY398	*ara thi* Δ(*lac-pro*) *cysG*::Tn*5 rpsL*$^+$/F′ *lacI*^{q} Δ*lac-14 proB*$^+$	Transduced S90C with P1 (CBK103), selected for Kan^r, screened for Cys$^-$, Strep^s
MY399	*ara thi* Δ(*lac-pro*) *cysG*::Tn*5 rpsL*$^+$/F′ *lacI*^{q}*A24*(Am) Δ*lac-14 proB*$^+$	Transduced S90CA24 with P1 (CBK103) selected for Kan^r, screened for Cys$^-$, Strep^s
MY400	*ara thi* Δ(*lac-pro*) *cysG*::Tn*5 rpsL*$^+$/F′ *lacI*^{q}*U6*(Op) Δ*lac-14 proB*$^+$	Transduced S90CU6 with P1 (CBK103), selected for Kan^r, screened for Cys$^-$, Strep^s
MY401	*ara thi* Δ(*lac-pro*) *cysG*::Tn*5 rpsL*$^+$/F′ *lacI*^{q}*U10*(Op) Δ*lac-14 proB*$^+$	Transduced S90CU10 with P1 (CBK103), selected for Kan^r, screened for Cys$^-$, Strep^s
LS1	*trpR trpA9605*(Am)	L. Soll (42)
MY416	*ara thi* Δ(*lac-pro*) *rpsL*$^+$/F′ *lacI*^{q} Δ*lac-14 proB*$^+$	Transduced MY398 with P1 (LS1), selected for Cys$^+$, screened for Kan^s
MY415	*ara thi* Δ(*lac-pro*) *rpsL*$^+$/F′ *lacI*^{q}*A24*(Am) Δ*lac-14 proB*$^+$	Transduced MY399 with P1 (LS1), selected for Cys$^+$, screened for Kan^s
MY414	*ara thi* Δ(*lac-pro*) *rpsL*$^+$/F′ *lacI*^{q}*U6*(Op) Δ*lac-14 proB*$^+$	Transduced MY400 with P1 (LS1), selected for Cys$^+$, screened for Kan^s
MY409	*ara thi* Δ(*lac-pro*) *rpsL*$^+$/F′ *lacI*^{q}*U10*(Op) Δ*lac-14 proB*$^+$	Transduced MY401 with P1 (LS1), selected for Cys$^+$, screened for Kan^s
LB50	W3110 *trpR trpA9605*(Am) *his-29*(Am) *ilv-1* TdR Su$^+$1	L. Breeden (5)
LB51	W3110 *trpR trpA9605*(Am) *his-29*(Am) *ilv-1* TdR Su$^+$2	L. Breeden (5)
LB52	W3110 *trpR trpA9605*(Am) *his-29*(Am) *ilv-1* TdR Su$^+$3	L. Breeden (5)
MY75	W3110 *trpR trpA9605*(Am) *his-29*(Am) *ilv-1 recA56 relA*$^+$/F′ *lacI*^{q}	L. Soll (5), previously called LS286F′I^q
MY90	*trpR trpA38*(Oc)	C. Yanofsky
LB103	W3110 *lacI*^{q} *trpA9605*(Am) *his-29*(Am) TdR *ilv-1 metB proC*	L. Breeden (5), isolated from same construction as LB104
LB104	W3110 *lacI*^{q} *trpA9605*(Am) *his-29*(Am) TdR *ilv-1 metB*	L. Breeden (5)
LS270	*trpR trpA9605*(Am) *his-29*(Am) *ilv-1* TdR	L. Soll
LS653	W3110 *leu*(Op) *lacZNG659*(Op) *trpA9605*(Am) *his-29*(Am) *ilv-1 metB argH* TdR Rif^r Strep^r	L. Soll
LS690	W3110 *leu*(Op) *lacZNG659*(Op) *trpA38*(Oc) *his-29*(Am) *ilv-1 metB argH* TdR Rif^r (ϕ80h)	L. Soll
LS717	W3110 *leu*(Op) *lac-659*(Op) *trpA9605*(Am) *his-29*(Am) *ilv-1 metB argH* Rif^r Strep^r *recA*	L. Soll
MY224	W3110 *leu*(Op) *lacZNG659*(Op) *trpA38*(Oc) *metB ilv-1* TdR Rif^r *proB17 his-29*(Am)/F′ *lacI*^{q} *lacZ proB*$^+$	By conjugation of MY222 with MY202, selected for Rif^r Pro$^+$
MY222	W3110 *leu*(Op) *lacZNG659*(Op) *trpA38*(Oc) *metB ilv-1* TdR Rif^r *proB17 his-29*(Am)	By conjugation of LS690 with AT2477, selected for Rif^r Arg$^+$, screened for Pro$^-$
AT2477	*HfrC proB17 metB1*	L. Taylor
MY202	Δ(*lac-proAB*) Spc^r/F′ *lacI*^{q} *lacZ proA*$^+$ *proB*$^+$	J. Sadler
MY373	W3110 *trpR his-29*(Am) *metE*	D. Bradley, derived from LS340 (4)
CAJ70	Δ*lac* Su9$^+$ *thi*	L. Gold, derived from CAJ64
CAJ64	Δ*lac* Su9$^+$ *thi* (λ)	(37)
MY264	*lacI*^{q} *trpA9605*(Am) *his-29*(Am) *metB proC* TdR *trpT190*(Ts)	Transduced LB103 with P1 (LS874), selected for Ilv$^+$, screened for Ts
MY265	*lacI*^{q} *trpA9605*(Am) *his-29*(Am) *metB proC* TdR *trpT190*(Ts) *recA56 srlC300*::Tn*10*	Transduced MY264 with P1 (JC10240), selected for Tet^r, screened for UV^s
MY266	*lacI*^{q} *trpA9605*(Am) *his-29*(Am) *metB proC* TdR *trpT190*(Ts) *recA56* Tet^s	Selected for Tet^s on fusaric acid tet plates (23)

Continued to next page

TABLE 1—Continued

Strain, plasmid, or bacteriophage	Genotype or phenotype	Source or reference
LS874	W3110 *trpR trpA9605*(Am) *his-29*(Am) *metE trpT190*(Ts)	Soll (12)
JC10240	HfrPO45 *recA56 thr-300 ilv-318 rpsE300 srlC*::Tn*10*	(10)
Plasmids	(Fig. 2)	
pMB9	Tet^r	(35)
pOP203.3	Tet^r *lacZpUV5*	(17)
pMY231	Tet^r *trpT aspT* in pOP203.3	(49)
pMY228	Tet^r *trpT175 aspT* in pOP203.3	(49)
pMY3	Tet^r *trpT175 aspT* in pMB9	(48)
pBE621	Tet^r *trpT178* in pOP203.3	This work
pBE663	Tet^r Ilv$^+$ *trpT178* in pOP203.3	This work
pSWC101	Tet^r *trpT176* in pOP203.3	This work
pSWC111	Tet^r *trpT178 trpT176* in pOP203.3 dimer	This work
pSWC115	Tet^r *trpT177* in pOP203.3	This work
Bacteriophage		
T4 strains		
Nam52	Gene 37 amN52	W. B. Wood
Aam481	Gene 37 amA481	W. B. Wood
Cop121	Gene 37 opC121	W. B. Wood
32opC172	Gene 32 opC121	L. Gold
oc427		L. Soll (42)
Noc21	Gene rIIA ocN21	W. B. Wood
λ strains		L. Soll
PH109	*N*am7	(42)
PH119	*S*am7	(49)
PH128	*O*am29	(49)
PH129	*P*am3	(42)
PH130	*A*am32	(49)
PH131	*Q*amt57	(42)
PH145	*R*am216	(42)
PH148	*R*am6	(42)
PH151	*P*am80	(49)
PH175	*J*am27	(42)
PH176	*E*am13	(42)
PH179	*B*am10	
PH147	c1$^+$	

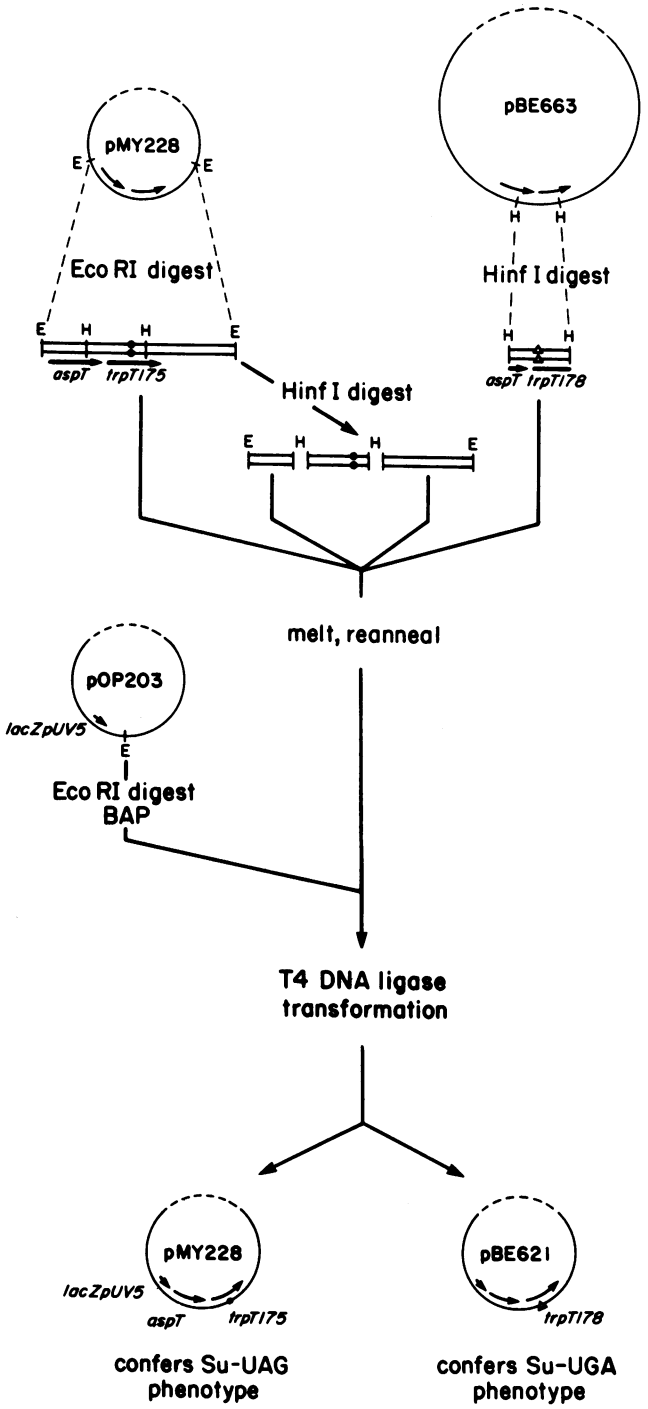


FIG. 4. Flow diagram for the construction of pBE621. BAP, Bacterial alkaline phosphatase. Symbols: ●, *trpT175* mutation; △, *trpT178* mutation.

anticodon but rather in the D-stem of the tRNA (Fig. 1; 19). This alteration permits the efficient reading of UGA codons as well as UGG codons. Previous attempts to clone this suppressor on a high-copy-number plasmid have been unsuccessful. Therefore, we adopted the following strategy to avoid an initial selection for the UGA suppressor in case its expression from a high-copy-number plasmid was lethal to the cell. We used pOP203.3 as the vector plasmid because DNA inserted into the unique *Eco*RI site would be transcribed from the *lacUV5* promoter (17) (Fig. 3) and because in strains carrying the *lacI*^{q} mutation (29), transcription of this DNA could be controlled with the gratuitous inducer IPTG. We avoided the usual initial selection for the pOP203.3-encoded tetracycline resistance in this strategy, for the gene encoding this resistance terminates with UGA (33, 44); efficient suppression of this terminator codon by a UGA suppressor tRNA might yield an inactive extended protein. Although this concern was shown to be justified (see below), strains expressing the cloned *trpT178* allele were

able to grow under the conditions we used to select for tetracycline resistance.

Because the genetically linked *ilv* operon occurs on the same *Eco*RI fragment as *trpT* (5), we first selected for plasmid clones conferring Ilv^+ upon an Ilv^- strain and then screened these isolates for the UGA suppressor phenotype. Chromosomal DNA from the Su^+ strain CAJ70 was digested with *Eco*RI and ligated with *Eco*RI-cut, dephosphorylated pOP203.3. The Ilv^- strain LB103 was transformed with the total ligation mixture. Ilv^+ transformants were selected and then screened for the Tet^r phenotype. Because this strain did not have any UGA nonsense mutations, bacteriophage T4 opal (UGA) mutants were used to screen for the presence of a UGA suppressor. Each of the four Ilv^+ Tet^r transformants supported the growth of T4 UGA mutants but not T4 UAG or UAA mutants.

Plasmid DNA was extracted from each isolate, and the ability to suppress the T4 UGA mutants was shown to be plasmid borne by using the DNA to retransform strain LB103. These plasmids contained 6.1-megadalton inserts on *Eco*RI digestion, as anticipated from the results of Breeden et al. (5). The four plasmid isolates were called pBE661 through 664.

Subcloning *trpT178* on a 264-bp fragment. Our next aim was to subclone this allele of the *trpT* gene as part of a 264-bp insert analogous to that of pMY228. The approach we took is diagrammed in Fig. 4. The 264-bp fragment derived by hybridization of fragments from pBE663 and pMY228 (Fig. 4) was ligated to *EcoRI*-cut, dephosphorylated pOP203.3. This DNA mixture was used to transform strain MY224 [*leu*(Op) and *lacZ*(Op)], and the transformed bacteria were grown on minimal lactose plates supplemented to select for Tet^r and UGA suppression. Plasmid DNA prepared from each of these isolates conferred the Tet^r, UGA-suppressing phenotype when used to retransform strain MY224. The tRNA sequence of Su9 (19) predicts that the *Hgi*AI site within the $tRNA^{Trp}$ gene is destroyed by the *trpT178* mutation. *Hgi*AI digestion of the five isolates showed that this site was indeed missing in each. One isolate, designated pBE621, was chosen for DNA sequence analysis, which verified that the cloned allele was *trpT178* (Fig. 3).

Mutating a plasmid-borne *trpT* gene to create an anticodon-based UGA suppressor gene. In addition to the point mutation which gave rise to the *trpT178* UGA suppressor Su9, $tRNA^{Trp}$ could also give rise to a UGA suppressor, Su7-UGA, by a C→T transition at the nucleotide encoding the wobble base of the tRNA anticodon (Fig. 1; 41). To generate this mutation, plasmid pMY231, bearing the wild-type *trpT* gene, was used to transform strain LS653 and mutagenized with EMS under growth conditions requiring suppression of the *lacZ*(Op) and *leu*(Op) mutations in this strain. Plasmid DNA from a batch culture of the resulting colonies was prepared by Sarkosyl lysis. This DNA was fractionated by agarose gel electrophoresis, and the fraction with the same mobility as that of supercoiled pMY231 was extracted and used to transform the *recA* strain LS717. Tetracycline-resistant transformants were scored for the Lac^+ Leu^+ phenotype resulting from suppression of UGA nonsense mutations. Of the isolates chosen for further analysis, none showed the loss of the *Hgi*AI site in the *trpT* gene, indicating that they did not bear the *trpT178* allele. One isolate was chosen for DNA sequence analysis. The sequence obtained was identical to that of the wild-type *trpT* gene except for a C→T transition at position 34 of the tRNA (Fig. 3). Thus the plasmid encoded Su7-UGA, a tRNA with an anticodon complementary to the UGA termination codon. This plasmid was designated pSWC101, and the allele was designated *trpT176*.

Mutating the plasmid-encoded anticodon-based UGA suppressor gene to create a UAA suppressor gene. Soll (41) demonstrated that Su7-UAA, which suppresses both UAA and UAG nonsense codons, can give rise to Su7-UGA by a single mutation. To generate a cloned Su7-UAA gene, we took the reverse approach and mutagenized the *trpT176*-bearing plasmid pSWC101, which encodes Su7-UGA (Fig. 2). This plasmid was used to transform strain LB104, and the resulting strain was exposed to EMS under growth conditions requiring suppression of the *trpA*(Am) and *his*(Am) mutations. Plasmid DNA was prepared from a batch culture of the colonies which arose under this selection and was used to transform strain LS690. The transformants were selected for suppression of the *his*(Am) mutation and then screened for suppression of the *trpA*(Oc), *lacZ*(Op), and *leu*(Op) nonsense mutations. To our surprise, all four nonsense mutations were suppressed in the transformant recovered.

When plasmid DNA prepared from the LS690 transformant was used to transform strain LS717 [*recA leu*(Op) *lac*(Op) *trpA*(Am) *his*(Am)], the UGA suppressor phenotype again cotransformed with the UAG suppressor phenotype. This result indicated that the unexpected phenotype did not arise from cotransformation of a parental UGA suppressor-encoding plasmid with the newly arisen UAA suppressor-encoding plasmid. Plasmid purified from these isolates had the electrophoretic mobility of a dimer and presumably carried the original *trpT176* UGA suppressor gene along with a UAA suppressor gene. This plasmid was designated pSWC111.

In confirmation of this hypothesis, cleavage of pSWC111 with *Hin*dIII to yield two linear DNA pieces of monomeric size, one carrying a UGA suppressor gene and one carrying a UAA suppressor gene, followed by ligation, permitted separation of the two suppressor phenotypes. *Hin*dIII-cut and religated pSWC111 DNA was used to transform the *recA* strain MY75 [*trpA*(Am) *his*(Am)]. Monomer plasmids extracted from amber-suppressing transformants of this strain were used to transform strain LS690 [*trpA*(Oc) *his*(Am) *lacZ*(Op) *leu*(Op)]. These plasmids conferred the ability to suppress only the UAG and UAA mutations.

One plasmid isolate, designated pSWC115, was used for subsequent DNA sequence analysis. The sequence obtained verified that this allele, *trpT177*, bore a C→T transition at position 35 of the tRNA, in addition to the T already present at position 34 in the parental allele (Fig. 3). These two alterations gave rise to the su7-UAA tRNA, with an anticodon complementary to the UAA terminator codon.

Efficiencies of the cloned suppressors. The suppressor efficiency of Su7-UAA, expressed from *trpT177* under the control of the *lacUV5* promoter of pSWC115, was measured at the *trpA38*(Oc) mutation in strain MY90 and the *trpA9605*(Am) mutation in strain MY75 (Table 2). The suppressor efficiency of Su7-UAG at *trpA9605*(Am) in strain MY75, determined by Yarus and co-workers (49), is included in this table for comparison. Su7-UAA suppressed the *trpA38* UAA codon fivefold better than the *trpA9605* UAG codon, 20 versus 3.8%, respectively.

In addition, the ability of Su7-UAA to suppress amber mutations was demonstrated by spot tests for suppression of bacteriophage λ amber mutations. The amber mutations suppressed by Su7-UAA were a subset of those suppressed by the glutamine inserter Su7-UAG (Table 3). Su7-UAA suppressed the mutations in λPH129 and λPH151, which

TABLE 2. Transmission efficiencies of suppressors expressed from *lacUV5* promoter

trpT allele on plasmid	Cognate codon	% Transmission[a]			
		lacIU6(Op)[b] (GAC UGA AGU)	*lacIU10*(Op)[c] (GAC UGA CUG)	*trpA9605*(Am)[d] (UAG)	*trpA38*(Oc)[e] (CGC UAA GAA)
trpT$^+$ (tRNA^{Trp})	UGG	4.3 ± 0.3	16 ± 0.9	ND	ND
trpT178 (Su9)	UGG/A	40 ± 3	61 ± 3	ND	ND
trpT176 (Su7-UGA)	UGA	67 ± 4	117 ± 9	ND	ND
trpT177 (Su7-UAA)	UAA/G	0.5 ± 0.1	1.1 ± 0.3	3.8 ± 0.4	20 ± 3
trpT175 (Su7-UAG)	UAG	ND	ND	94 ± 10[f]	ND

[a] Mean ± standard error of the mean. ND, Not determined.

[b] Measured as ratio of beta-galactosidase activity in strain MY414 bearing the plasmid to that in strain MY416 bearing the same plasmid. The background level of transmission through this nonsense codon was determined in the presence of pOP203.3. This level was measured as (1.4 ± 0.05)% of the activity in strain MY416. The background was subtracted from the values in the table. The sequence of context is from Miller et al. (27)

[c] As in footnote *a*, except that the nonsense mutation was in strain MY409 and the background was measured as (4.1 ± 0.2)%. The sequence of context is from Miller et al. (27).

[d] Ratio of *trpA* gene product activity to *trpB* gene product activity present in strain MY75 carrying pSWC115 was determined. This ratio was then divided by the ratio of activity present in isogenic Trp$^+$ strain MY373 to obtain the percent transmission. Precise location of *trpA9605*(Am) mutation is unknown.

[e] As in footnote *c*, except that the nonsense mutation was in strain MY90. The sequence of context is from Feinstein and Altman (16).

[f] Value from Yarus et al. (49).

were suppressed by the glutamine inserters Su2 and Su7-UAG but not by Su1 (inserts serine) or Su3 (inserts tyrosine).

The transmission efficiency of the UGA suppressors Su9 and Su7-UGA, each expressed from the *lacUV5* promoter, were measured at UGA codons in two contexts. Suppression of the *lacIU10*(Op) mutation was measured in strain MY409, and suppression of the *lacIU6*(Op) mutation was measured in strain MY414 (Table 2). Suppressor efficiency measurements in these strains are complicated by the fact that the termination codons lie in the portion of a *lacI-lacZ* gene fusion that codes for the *lac* repressor. The effects of amino acid substitutions at these positions on the expression of the tRNA genes from the *lacUV5* promoter are discussed below. The most efficient suppressor in both contexts was Su7-UGA, with efficiencies of 67% at *lacIU6*(Op) and 117% at *lacIU10*(Op). Su9 was less efficient at both codons, with 40% at *lacIU6*(Op) and 61% at *lacIU10*(Op).

The abilities of wild-type tRNA^{Trp} and su7-UAA to read through the UGA codons in these contexts were also measured. The wild-type tRNA was expected to misread UGA codons by wobble with low efficiency (20). Su7-UAA was not expected to misread UGA codons in these strains, since this would require a mismatch between the middle bases of the codon and the anticodon. Wild-type tRNA^{Trp} had efficiencies of 4.3% at *lacIU6*(Op) and 16% at *lacIU10*(Op), when expressed from a high-copy-number plasmid. A similar ratio of activities at the two UGA codons was observed in these strains in the absence of the plasmid-borne *trpT* gene, but the read-through level in each case was about fourfold lower (Table 2). Su7-UAA appeared to suppress these UGA mutations at very low levels, 0.5% above background at *lacIU6*(Op) and 1.1% at *lacIU10*(Op).

All of the suppressors whose efficiencies were measured at these mutations were lower in efficiency at the *lacIU6*(Op) mutation (GAC UAG AGU) than at the *lacIU10*(Op) mutation (GAC UGA CUG). Both of the stronger suppressors, Su9 and Su7-UGA, had efficiencies at *lacIU6*(Op) that were

TABLE 3. Growth of λ amber mutants on suppressor-bearing strains

λ genotype	Clearing on strains bearing suppressor[a]										
	Fully induced[b]							Uninduced[b]			
	Su$^+$1	Su$^+$2	Su$^+$3	Su$^-$	Su$^-$ (*trpT*$^+$)	Su$^+$7-UAG (*trpT175*)	Su$^+$7-UAA (*trpT177*)	Su$^-$	Su$^-$ (*trpT*$^+$)	Su$^+$7-UAG (*trpT175*)	Su$^+$7-UAA (*trpT177*)
*N*am7	−	+	±	−	−	+	−	−	−	−	−
*S*am7	−	−	±	−	−	−	−	−	−	−	−
*O*am29	±	+	+	−	−	+	−	−	−	−	−
*P*am3	−	+	−	−	−	+	±	−	−	+	−
*A*am32	±	+	+	−	−	+	−	−	−	−	−
*Q*amt57	−	−	±	−	−	−	−	−	−	−	−
*R*am216	−	+	−	−	−	+	−	−	−	+	−
*R*am6	+	+	+	−	−	+	+	−	−	+	−
*P*am80	−	+	−	−	−	+	+	−	−	+	−
*J*am27	−	±	±	−	−	+	−	−	−	−	−
*E*am13	+	+	−	−	−	+	−	−	−	−	−
*B*am10	+	+	+	−	−	+	−	−	−	+	−
cI$^+$	+	+	+	+	+	+	+	+	+	+	+

[a] Su1, Su2, and Su3 were in strains MY50, MY51, and MY52, respectively. All others were in strains MY75. Spot tests were performed as previously described (49). The *trpT* allele on the plasmid is within parentheses. Clearing is measured as follows: +, complete clearing overnight at 42°C; ±, partial clearing; −, no clearing.

[b] To fully induce *trpT* alleles under *lacUV5* control, the H top agar was made 1 mM IPTG.

TABLE 4. Reduced tetracycline resistance conferred upon strain MY409 by cloned UGA suppressors

trpT allele on plasmid	Radius (mm) of zone of inhibition[a]			
	375 μg		750 μg	
	−IPTG	+IPTG	−IPTG	+IPTG
None	1	1	2	2
trpT$^+$ (Su$^-$)	1	1	2	2
trpT178 (Su9)	1	2	2	3
trpT177 (Su7-UGA)	2	4	2	5

[a] The indicated amount of tetracycline is added to 2.4-cm glass-fiber filter disk as 0.15 ml of appropriate concentration tetracycline in 70% EtOH. This is placed on a lawn of 10^8 cells in F top agar. The radius of the cleared zone around the disk is measured after incubation overnight at 37°C (18).

about two-thirds of their efficiencies at *lacIU10*(Op). In contrast, wild-type tRNA^{Trp} decreased in efficiency nearly fourfold from *lacIU10(Op)* to *lacIU6*(Op).

Strains carrying the cloned UGA suppressor genes had greater sensitivity to tetracycline than did the same strains carrying the vector plasmid pOP203.3 (Table 4). Whereas IPTG-induced expression of the wild-type *trpT* allele did not appear to affect tetracycline resistance under the conditions tested, IPTG-induced expression of *trpT176* or *trpT178*, encoding the high-efficiency UGA suppressors Su7-UGA and Su9, respectively, did. In agreement with the suppressor efficiencies measured at the *lacI* nonsense mutations, Su7-UGA had a greater effect than Su9 at this natural terminator.

Su7-UGA also reads UGG in vivo. Strain MY266 carries the temperature-sensitive tRNA^{Trp} mutation *trpT190*(Ts). This mutation disrupts a bp in the aminoacyl stem of tRNA^{Trp} (12); the resulting tRNA is unstable at high temperatures (13). This strain can be rescued for growth at 42°C by using plasmid DNA that encodes a functional UGG-reading tRNA to transform it. Thus when pMY231, which carried *trpT*, was used to transform strain MY266, the transformed strain was viable at temperatures as high as 44°C in the presence of the inducer IPTG. Likewise, when pBE621 was used to transform the temperature-sensitive strain MY266, IPTG-induced expression of Su9 from *trpT178* on the plasmid permitted the transformed strain to grow at 44°C. When plasmid pSWC101, encoding Su7-UGA on *trpT176*, was used to transform the temperature-sensitive strain, IPTG-induced production of Su7-UGA permitted slow growth at 42°C in the absence of tetracycline but not at 44°C. Transformation of strain MY266 with pMY228, encoding Su7-UAG, or with a plasmid encoding a nonfunctional derivative of Su7-UAG (*trpT511*; L. Breeden et al., manuscript in preparation), did not permit growth at 42 or 44°C.

These results confirm previous observations that Su9 can translate both the nonsense codon UGA and the Trp codon UGG (20). Su7-UGA also appears to recognize UGG codons with some efficiency in vivo. The inability of Su7-UGA to rescue strain MY266 at 44°C may result from any of several effects. (i) The recognition of UGG codons by this tRNA might be restricted at the higher temperature. (ii) Su7-UGA may only recognize UGG codons very weakly, and growth at 42°C is due to the sum of the activities of this tRNA and the residual temperature-sensitive tRNA^{Trp}. (iii) Finally, Su7-UGA may be inserting an amino acid other than tryptophan, resulting in moderately temperature-sensitive proteins. The results below suggest that the last possibility is less likely than the others.

Su7-UGA does not insert glutamine. The single base change which gives rise to Su7-UAG also causes this tRNA to be misacylated with glutamine (21, 43, 47). The alteration which gives rise to Su9 does not appear to permit misacylation, for this suppressor has only been observed to insert tryptophan (20). Are Su7-UAA and Su7-UGA misacylated? Our results from the amber spot tests suggest that Su7-UAA inserts glutamine. A similar assay for UGA suppressors was not available.

The system we used to test for glutamine insertion by Su7-UGA was based upon the sensitivity of *lac* repressor function to different amino acids inserted at codon 220. This codon normally specifies a tryptophan which is important in the binding of inducer by the repressor (30). Because the cloned *trpT* alleles are expressed from the *lacUV5* promoter, the level of suppressor activity measured is sensitive to IPTG induction. Thus, using nonsense mutations at this codon in the *lacI-lacZ* fusion (UAG in strain MY415 and UGA in strain MY414), we could observe the effect of inserting a given amino acid at this position by measuring the degree of IPTG induction of suppressor activity.

Miller et al. (28) have examined the effects of amino acid substitutions at codon 220 of the *lacI* gene. Only the insertion of tryptophan at this position gave rise to a repressor with wild-type activity. In particular, the substitution of glutamine results in a repressor insensitive to IPTG, indicated by no increase in beta-galactosidase activity upon the addition of IPTG. Even though the fusion protein has only a portion of the activity of a wild-type repressor (30), we were able to detect the same effects of amino acid substitution at codon 220.

Our results (Table 5) demonstrate that the repressor activity of the fusion protein becomes insensitive to IPTG when glutamine is inserted. When the wild-type amino acid tryptophan was inserted by Su9, the suppressor activity increased fourfold upon the addition of IPTG. In contrast, very little increase in suppressor activity was observed for the glutamine-inserting Su7-UAG. Furthermore, Su7-UAG did not appear to be maximally expressed from the *lacUV5* promoter when in strain MY415, for it showed a twofold higher activity when expressed from a slightly weaker promoter on the pMB9 vector (32, 35) of pMY3. Because the expression of Su7-UAG from pMY228 is depressed under these conditions, we concluded that the repressor portion of the fusion protein is not inactivated by the insertion of glutamine.

TABLE 5. Sensitivity of IPTG induction of suppressor efficiency to amino acid inserted at *lacI* codon 220 in repressor–beta-galactosidase fusion protein

Plasmid	*trpT* allele	Amino-acylated with[a]:	% Transmission[b]	
			0 mM IPTG	1 mM IPTG
pMY228	*trpT175* (Su7-UAG)	Gln (41, 49)	24 ± 2[c]	30 ± 3
pMY3	*trpT175* (Su7-UAG)	Gln (41, 49)	69 ± 3	62 ± 3
pBE621	*trpT178* (Su9)	Trp (9)	10 ± 0.8[d]	40 ± 3
pSWC101	*trpT176* (Su7-UGA)	Unknown	11 ± 0.8	67 ± 4

[a] Numbers in parentheses are the references for aminoacylation data.

[b] Mean ± standard error of the mean.

[c] Amber suppressor efficiency was determined at *lacIA24*(Am) in strain MY415. Efficiency was determined as described in Table 2 by measuring beta-galactosidase activity. Background levels of activity were determined in the presence of pMY231 (Su$^-$) as 0.3%, both with and without IPTG.

[d] Efficiency was determined at *lacIU6*(Op) as described in Table 2. Background levels of activity were subtracted from the values in this table.

By comparing the extent of IPTG induction of Su7-UGA suppressor activity at *lacIU6*(Op) with the above observations, the possibility that Su7-UGA also inserted glutamine could be excluded. The suppressor efficiencies recorded in Table 5 show that the activity of Su7-UGA increases as dramatically as that of Su9 in the presence of IPTG. In the light of the sensitivity of repressor activity to any substitution for the tryptophan encoded at this position (28), it seems likely that Su7-UGA was inserting tryptophan.

DISCUSSION

We have presented here the details of the cloning of the genes for three nonsense suppressing tRNAs, all derived from $tRNA^{Trp}$. These three suppressor mutations, *trpT177* (Su7-UAA), *trpT176* (Su7-UGA), and *trpT178* (Su9), are isogenetic with the previously cloned *trpT175* (Su7-UAG) and *trpT* alleles (49). All were cloned into the vector pOP203.3 under the control of the *lacUV5* promoter. Within the limits of detection, all of these plasmids carrying cloned tRNA genes have the same copy number. The efficiencies of these suppressors with the promoter fully induced were measured.

Su7-UAG has been previously determined to be a high-efficiency suppressor which inserts glutamine (21, 43, 47). Su7-UAA was relatively low in efficiency and appeared to insert glutamine. The low efficiency of Su7-UAA makes Su7-UAA somewhat anomalous among the $tRNA^{Trp}$-derived suppressors but is typical for an ochre suppressor (15, 16). Su7-UGA appeared to be nearly as strong a suppressor as Su7-UAG. It did not insert glutamine; the results we report here suggest that it may insert tryptophan. Su9 was a less-efficient suppressor but was still quite strong. Previous work has shown that Su9 inserts tryptophan and recognizes UGG codons (Trp) as well as UGA codons (20). Fully induced expression of the UGA suppressors decreased the tetracycline resistance encoded by the pOP203.3 vector, presumably because they caused considerable read-through of the UGA terminator in the gene determining tetracycline resistance.

It should be noted that even wild-type $tRNA^{Trp}$ suppressed UGA codons with a low efficiency. This property has been demonstrated previously in vitro (20) and was here quantitated in vivo. This activity of $tRNA^{Trp}$ is most probably responsible for the low level of UGA terminator read-through observed in wild-type *E. coli* (14, 34, 46, 50).

Su7-UGA appears to recognize UGG codons with detectable efficiency in vivo. This ability is predicted by the wobble rules proposed by Crick (9). However, the apparently greater efficiency of Su9 at these codons conflicts with these rules. Studies of other suppressor tRNAs suggest that U-G wobble base pairing, as occurs when Su7-UGA reads UGG, is affected by the type of modification the U in the wobble position bears. We have not determined whether this nucleoside is modified in the Su7-UGA tRNA. However, recognition of UGA by Su9 must occur by an unusual C-A wobble base pairing. The unique nature of the Su9 tRNA has been emphasized by Buckingham and Kurland (6), whose work suggests that Su9 also may recognize UGU and UGC in vitro.

The efficiency of the UGA suppressors was compared in two contexts, that of the *lacIU6*(Op) mutation and that of the *lacIU10*(Op) mutation. These two mutations are among the many used by Miller and Albertini in their study of context effects upon a chromosomally encoded Su9 (26). These authors find that the context of *lacIU6*(Op) (GAC UGA AGU) is moderately good, whereas the context of *lacIU10*(Op) (GAC UGA CUG) is extremely good. Our results extended the comparison of these two contexts to include wild-type $tRNA^{Trp}$ and Su7-UGA and demonstrated the superiority of the *lacIU10*(Op) context for these suppressors as well. Furthermore, we have shown that the misreading of UGA by Su7-UAA occurs more frequently in this context also.

Even though the tRNAs we studied differed by only one or two bases in their sequences, we observed a significant difference in their responses to a change in context. The weakest of the suppressors studied, wild-type $tRNA^{Trp}$, showed a fourfold decrease in its UGA-suppressing efficiency at the *U6* mutation when compared to its efficiency at the *U10* mutation. This large decrease in efficiency was not unique to the tRNA as expressed from the cloned gene, for it was paralleled by the decrease in background levels of read-through of these UGA codons, presumably by the chromosomally-encoded $tRNA^{Trp}$. Su9 differs from the wild-type tRNA only in the D-stem. Both tRNAs must bind to the UGA codon by the same unconventional base pairing at the wobble position, yet Su9 was not as strongly affected by the context change. In contrast to $tRNA^{Trp}$, Su9 and Su7-UGA, both relatively strong suppressors, suppressed the *lacIU6*(Op) UGA codon about two-thirds as well as the *lacIU10*(Op) UGA codon, even though these suppressors differed from each other in the base present at the wobble position of the anticodon.

These results suggest that the relative effects of these two contexts depend on an aspect of tRNA structure other than the primary sequence of the anticodon loop. If the decrease in efficiency were directly dependent upon the sequence of the anticodon loops of these tRNAs, then Su9 should have behaved more like the wild-type $tRNA^{Trp}$ than like Su7-UGA. Instead, the two strong suppressors were only moderately affected by an impaired context, whereas the weak suppressor was strongly affected.

The *lacIU6*(Op) and *lacIU10*(Op) UGA codons are preceded by the same codon, GAC. Thus, the superiority of the *lacIU10*(Op) context over the *lacIU6*(Op) context probably lies in the codon 3′ to the suppressed UGA. This codon is CUG at the *U10* mutation and AGU at the *U6* mutation. Previous studies of the effects of context upon suppressor efficiency have suggested that an A 3′ to the codon being translated is superior to a C at the same position (3). The work of Miller and Albertini (26) and that of Bossi (2) suggest that A is better than C except when the C is followed by a U. Although the effect of an A on the 3′ side of the suppressed codon might be due to its ability to base pair with the U present on the 5′ side of the anticodon in all tRNAs (39), the superiority of CU on the 3′ side of the codon is not so easily explained.

Our results suggest that the relative effects of the *lacIU6*(Op) (UGA AGU) and the *lacIU10*(Op) (UGA CUG) contexts on suppressor efficiency are not dependent on the primary sequence alone of the anticodon loop of the tRNA affected. An indirect stabilizing effect upon the codon-anticodon binding due to tRNA-tRNA interactions on the ribosome or structure within the message is not excluded by these data. The use of this set of closely related suppressors, or an analogous set, should allow future studies of context to distinguish between these possibilities.

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