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Introduction of UAG, UAA, and UGA Nonsense Mutations at a Specific Site in the *Escherichia coli* Chloramphenicol Acetyltransferase Gene: Use in Measurement of Amber, Ochre, and Opal Suppression in Mammalian Cells

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We have used oligonucleotide-directed site-specific mutagenesis to convert serine codon 27 of the *Escherichia coli* chloramphenicol acetyltransferase (*cat*) gene to UAG, UAA, and UGA nonsense codons. The mutant *cat* genes, under transcriptional control of the Rous sarcoma virus long terminal repeat, were then introduced into mammalian cells by DNA transfection along with UAG, UAA, and UGA suppressor tRNA genes derived from a human serine tRNA. Assay for CAT enzymatic activity in extracts from such cells allowed us to detect and quantitate nonsense suppression in monkey CV-1 cells and mouse NIH3T3 cells. Using such an assay, we provide the first direct evidence that an opal suppressor tRNA gene is functional in mammalian cells. The pattern of suppression of the three *cat* nonsense mutations in bacteria suggests that the serine at position 27 of CAT can be replaced by a wide variety of amino acids without loss of enzymatic activity. Thus, these mutant *cat* genes should be generally useful for the quantitation of suppressor activity of suppressor tRNA genes introduced into cells and possibly for the detection of naturally occurring nonsense suppressors.

Nonsense mutations, which cause premature termination of protein synthesis, can be suppressed by tRNAs which recognize termination codons. The availability of a wide variety of nonsense suppressor tRNA genes has played a crucial role in the isolation and study of nonsense mutations in procaryotes and lower eucaryotes (7). In mammalian cells, it has so far not been possible to isolate cell lines carrying nonsense suppressors by using classic genetic selections; consequently, nonsense mutations have been identified in only a few nonviral genes (8, 21).

Recently, we demonstrated that both a *Xenopus laevis* $tRNA^{Tyr}$ gene and a human $tRNA^{Ser}$ gene could be altered by oligonucleotide-directed site-specific mutagenesis to recognize and suppress amber (UAG) and ochre (UAA) codons in vivo (5, 19, 20). An opal (UGA) suppressor tRNA gene derived from a human $tRNA^{Ser}$ gene was also constructed (5). However, since the opal suppressor tRNA gene could not be propagated in mammalian cells as part of a replicating simian virus 40 (SV40) recombinant, it was not possible to demonstrate its functional expression. In addition, the *X. laevis* $tRNA^{Tyr}$ amber and ochre suppressors were also used to establish permanent Su^+ mammalian cell lines (16, 19), although the level of nonsense suppression in these cell lines was low (36).

It is likely that many other suppressor tRNA genes which are active in mammalian cells will be constructed and used to isolate mammalian cell lines containing functional suppressors. A knowledge of the levels of suppression in these various cell lines will aid significantly in their eventual use for the isolation and characterization of nonsense mutations in cellular and viral genes. It is therefore important to have a rapid, quantitative, and sensitive method to assay for the level of suppression of nonsense mutations in different cell types. This paper reports on the development of such a method.

We have used site-specific mutagenesis to introduce UAG, UAA, and UGA nonsense mutations at a defined position in the *Escherichia coli* chloramphenicol acetyltransferase (*cat*) gene. The mutant *cat* genes have been used in conjunction with a rapid and sensitive high-pressure liquid chromatography (HPLC) assay to detect and quantitate levels of suppression in different mammalian cells transfected with suppressor tRNA genes. The activity of a human $tRNA^{Ser}$ gene mutated to recognize an opal nonsense codon was assayed in this fashion and provided the first direct evidence that an opal suppressor tRNA gene is functional in mammalian cells. Finally, we show that a wide variety of amino acids can be inserted at the site of mutation in chloramphenicol acetyltransferase (CAT) without loss of enzymatic activity. Thus, the mutant *cat* genes can be used to assay many different suppressor tRNA genes which may be introduced into cells and possibly for the detection of naturally occurring nonsense suppressors.

MATERIALS AND METHODS

***E. coli* strains and plasmids.** The *E. coli* strains used were CDJ64 [F^- Val^r Δ(*lac-proB*) *nal rif thi sup-9*, Xac [F^- *ara* Δ(*lac-pro*)XIII *argE*(Am) *metB nalA thi* Rif^r], and GW5180. This last strain, derived from *E. coli* JM103, carries *recA* as an additional marker, is *Eco*PI^-, and was obtained from Lorraine Marsh and Graham Walker of this department.

The SV40-based plasmids pSV-tS Su^-, pSV-tS Su^+(Am), pSV-tS Su^+(Och), and pSV-tS Su^+(Opal), which contain a wild-type human serine tRNA gene or the amber, ochre, and opal suppressor derivatives of this gene, respectively, have been described previously (5). pUCtS Su^-, pUCtS

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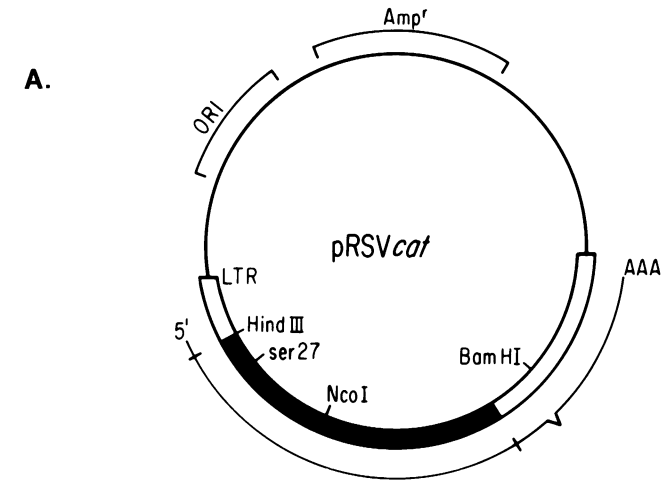

B. *CAT GENE SEQUENCE:*

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                    25                  30
          F   E   A   F   Q   S   V   A   Q   C   T
5'---- TTT GAG GCA TTT CAG TCA GTT GCT CAA TGT ACC----3'
3'---- AAA CTC CGT AAA GTC AGT CAA CGA GTT ACA TGG----5'
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MUTAGENIC PRIMERS:

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5' TTTCAGTAAGTTGCT      PRIMER A (OCHRE)
5' TTTCAGTAGGTTGCTC     PRIMER B (AMBER)
5' CATTTCAGTGAGTTGCTCA  PRIMER C (OPAL)
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FIG. 1. Oligonucleotide-directed mutagenesis of the *cat* gene. (A) Structure and relevant restriction sites of pRSV*cat* (11, 12). LTR, Long terminal repeat. (B) Nucleotide and amino acid sequence surrounding serine 27. The transcribed DNA strand was used as the template for site-directed mutagenesis. Oligonucleotide primers synthesized to replace the serine codon (underlined) with nonsense codons are shown. Primer A changes the serine TCA codon to TAA; primer B changes this codon to TAG; primer C changes this codon to TGA.

Su^+(Am), pUCtS Su^+(Och), and pUCtS Su^+(Opal) were constructed by cloning the 900-base-pair (bp) *Sau*3A fragment containing the wild-type serine tRNA gene or the suppressor derivatives into the *Bam*HI site of pUC12 (32). pRSV*cat* (Fig. 1) contains the gene for CAT from transposon Tn*9* under the control of the Rous sarcoma virus long terminal repeat (11).

Oligonucleotides. The oligonucleotides 5'-TTTCAGTA AGTTGCT, 5'-CATTTCAGTGAGTTGCTCA, and 5'-TTTCAGTAGGTTGCTC were synthesized on an Applied Biosystems 380A DNA synthesizer, purified by polyacrylamide gel electrophoresis (22), and characterized by sequencing (29).

Oligonucleotide-directed site-specific mutagenesis. pRSV*cat* DNA was digested with *Hin*dIII and *Bam*HI, and the small fragment, which contains the *cat* gene, was purified and cloned into similarly digested bacteriophage vector M13mp8 replicative-form (RF) DNA. Single-stranded virion DNA, designated M13*cat* and containing the transcribed strand of the *cat* gene, was prepared and purified by alkaline sucrose gradient centrifugation (18). This DNA was used as a template for site-directed mutagenesis to introduce amber and ochre nonsense mutations into the *cat* gene. Mutagenesis was carried out as described previously except that the S1 nuclease step to remove incompletely elongated DNA molecules was omitted (5). Covalently closed circular DNA was recovered from low-melting-point agarose gels and used to transform *E. coli* JM103.

We were unable to obtain a *cat* gene derivative containing a UGA mutation by using the above method; thus, mutagenesis by a modified gapped duplex procedure was used (17). Briefly, 1.5 μg of the large *Hin*dIII-*Nco*I fragment from M13*cat* RF DNA was mixed with 0.5 μg of M13*cat* virion DNA in a 45-μl reaction mixture containing 200 mM KCl and 12.5 mM Tris hydrochloride, pH 7.5. The DNA was denatured by heating at 100°C for 3 min and allowed to reanneal at 65°C for 5 min, followed by incubation at room temperature for a further 10 min. Of this gapped duplex mixture, 15 μl was mixed with 100 pmol of phosphorylated primer C (Fig. 1) and incubated at 65°C for 5 min, followed by slow cooling to room temperature over a period of 1 h. The reaction mixture was then adjusted to 100 mM KCl, 30 mM Tris hydrochloride, pH 7.5, 15 mM $MgCl_2$, 2 mM dithiothreitol, 0.5 mM ATP, 0.2 mM each of the four deoxynucleotide triphosphates; 3 U of the large fragment of DNA polymerase, and 5 U of T4 DNA ligase were added, and the mixture was incubated at room temperature for 2 h, followed by incubation at 14°C for 12 h. An appropriate dilution was then used to transform *E. coli* GW5180 (*recA*).

In all cases, individual phage plaques were screened for the desired mutation by hybridization with the appropriate 5'-end ^{32}P-labeled mutagenic oligonucleotide under conditions of increasing stringency (33, 37). Phage showing strong hybridization at the discriminating temperatures were plaque purified, and the presence of the desired mutation(s) was confirmed by DNA sequencing (26).

Insert DNA from each mutant type was excised from RF DNA by digestion with *Hin*dIII and *Nco*I. The gel-isolated fragments were recloned into *Hin*dIII-*Nco*I-digested pRSV*cat* to yield the expression plasmids pRSV*cat*(*am27*), pRSV*cat*(*oc27*), and pRSV*cat*(*op27*).

DNA transfections of mammalian cells and assay for CAT. Transfections were carried out with a total of 20 μg of DNA per 100-mm plate either by the DEAE-dextran-dimethyl sulfoxide shock procedure (23) or by the calcium phosphate coprecipitation method (13, 34). Cell extracts were prepared 48 h posttransfection in 100 μl of 0.25 M Tris hydrochloride, pH 7.8, and CAT activity was determined with [^{14}C]chloramphenicol (46 mCi/mmol, 5 nmol per assay) as described previously (12). Briefly, 25 μl of the standard cell extract was incubated at 37°C for 1 h in a final volume of 150 μl containing 500 mM Tris hydrochloride, pH 7.8, 0.5 mM acetyl coenzyme A (P-L Biochemicals), and 5 nmol of [^{14}C]chloramphenicol. The reaction was terminated by extraction with 1 ml of ethyl acetate, and the reaction products were analyzed by ascending chromatography of the ethyl acetate extract on silica gel plates with chloroform-methanol (95:5) as the solvent. Following autoradiography, radioactive spots were excised from the plate, and radioactivity was quantitated by scintillation counting.

Alternatively, CAT activity was determined by HPLC with unlabeled chloramphenicol (14). Reactions were carried out as above except that the amount of acetyl coenzyme A was increased to 1 mM and the chloramphenicol (Sigma Chemical Co.) used was unlabeled and at a final concentration of 50 to 100 μM. The residue remaining after drying the ethyl acetate extract was reconstituted in a small volume (25 μl) of ethyl acetate. Unreacted chloramphenicol and its acetylated derivatives were separated on a Hewlett Packard 1090 HPLC, equipped with a 1-μl flow cell, by using a Zorbax (Du Pont Co.) silica gel column and isocratic elution with chloroform-methanol (95:5). The eluent was monitored at 280 nm, and peaks were integrated with a Hewlett Packard 3392A integrator. CAT activities are given as a percentage of the 3-acetylchloramphenicol formed (27). Standard curves were generated for both the radiochemical and HPLC assay by using commercially available CAT (P-L Biochemicals) to ensure that all determinations were within the linear range of the assay (0 to 30% conversion of chloramphenicol to 3-acetylchloramphenicol; the extracts were diluted, when necessary, to ensure that the amount of chloramphenicol converted to acetylated derivative did not exceed this range). Under saturating substrate concentrations, the HPLC assay was found to be linear with respect to time of incubation (up to 3 h at 37°C) and with respect to the amount of extract used in the assay (2 to 50 μl of the standard 100-μl extract from a 100-mm plate). Protein concentrations of cell extracts were determined by using a commercially available kit (BioRad Laboratories).

RESULTS

Introduction of amber, ochre, and opal nonsense mutations at codon 27 of the *cat* gene by oligonucleotide-directed site-specific mutagenesis. pRSV*cat* is a mammalian expression vector which contains the *E. coli* Tn*9 cat* gene under control of the Rous sarcoma virus long terminal repeat (11) (Fig. 1A). This gene is expressed in a wide variety of mammalian cells, and the enzymatic assay to quantitate its activity is simple and sensitive (12). Thus, the availability of *cat* genes with defined nonsense mutations would provide a simple and convenient assay of suppressor tRNA activity in any cell type in which the *cat* gene can be expressed.

We previously constructed amber, ochre, and opal suppressor tRNA genes by site-directed mutagenesis of a human serine tRNA gene (5). Therefore, in attempts to develop an assay for measurement of suppressor tRNA activity, a serine codon in the *cat* gene was selected for mutagenesis since the homologous amino acid would be inserted at the site of mutation by the serine suppressor tRNAs. Of the 10 serine codons in the *cat* gene (1, 28) the TCA codon for serine at position 27 was chosen for the following reasons. (i) This codon is close to the amino terminus of the gene; thus, translation termination at this site would result in the synthesis of a short polypeptide having no enzymatic activity. (ii) Mutagenesis involves a single base change to introduce opal or ochre termination codon at this site. (iii) This TCA is followed by a G. It is known, at least in bacteria, that termination codons that are followed by a purine are in a better "context" for readthrough by suppressor tRNAs (2, 24).

The synthetic oligonucleotides used to introduce the desired base changes in the *cat* gene are shown in Fig. 1B. Primer A contains a single-base mismatch which would convert the TCA serine codon at position 27 to the ochre termination codon TAA. Primer B contains a 2-base mismatch which would change this codon to the amber codon TAG, and primer C contains a single-base mismatch to generate the opal termination codon TGA.

Site-directed mutagenesis to introduce amber and ochre mutations into the *cat* gene was carried out with an M13mp8 recombinant clone which carried the small *Hin*dIII-*Bam*HI fragment from pRSV*cat*. The potential mutants were screened by hybridization with the mutagenic primers under conditions of increasing stringency, and positives were fully characterized by sequence analysis of the M13 recombinant virion DNAs. With primer A, the frequency of mutagenesis was 8%, whereas with primer B, which contains two mismatches, 1 positive was obtained among 72 phage isolates screened.

We were unable to isolate the corresponding UGA mutation at this site on the *cat* gene under identical mutagenic conditions by using the mutagenic primer C. To obtain the UGA mutant, mutagenesis was carried out with the gapped duplex approach to reduce nonspecific priming and enhance the recovery of covalently closed circular duplex DNA (17). In addition, *E. coli* GW5180 (*recA*) was used as the host strain. With this approach, opal mutations at position 27 of the *cat* gene were obtained at a frequency of 10%. One of these was plaque purified and shown to contain the expected C→G change by sequencing.

Each of the mutant *cat* genes was excised from the respective M13 RF DNA by digestion with *Hin*dIII and *Nco*I and cloned back into the pRSV expression vector. These plasmids are designated pRSV*cat*(*am27*), pRSV*cat*(*oc27*), and pRSV*cat*(*op27*).

Use of the pRSV*cat* DNAs carrying UAG, UAA, and UGA nonsense mutations to assay for nonsense suppression in mammalian cells transfected with suppressor tRNA genes. (i) CV-1 cells. To test for Su$^+$ tRNA-mediated suppression of the various *cat* nonsense mutations in mammalian cells, cells were cotransfected with the different pRSV*cat* DNAs and the various serine suppressor tRNA genes which we have previously constructed (5). In general, transfections were carried out with 5 to 10 μg of indicator plasmid DNA (pRSV*cat* or the nonsense mutants) along with 10 μg of plasmid DNA carrying either the Su$^-$ or the different Su$^+$ tRNA^{Ser} genes.

When either pRSV*cat*(*am27*), pRSV*cat*(*oc27*), or

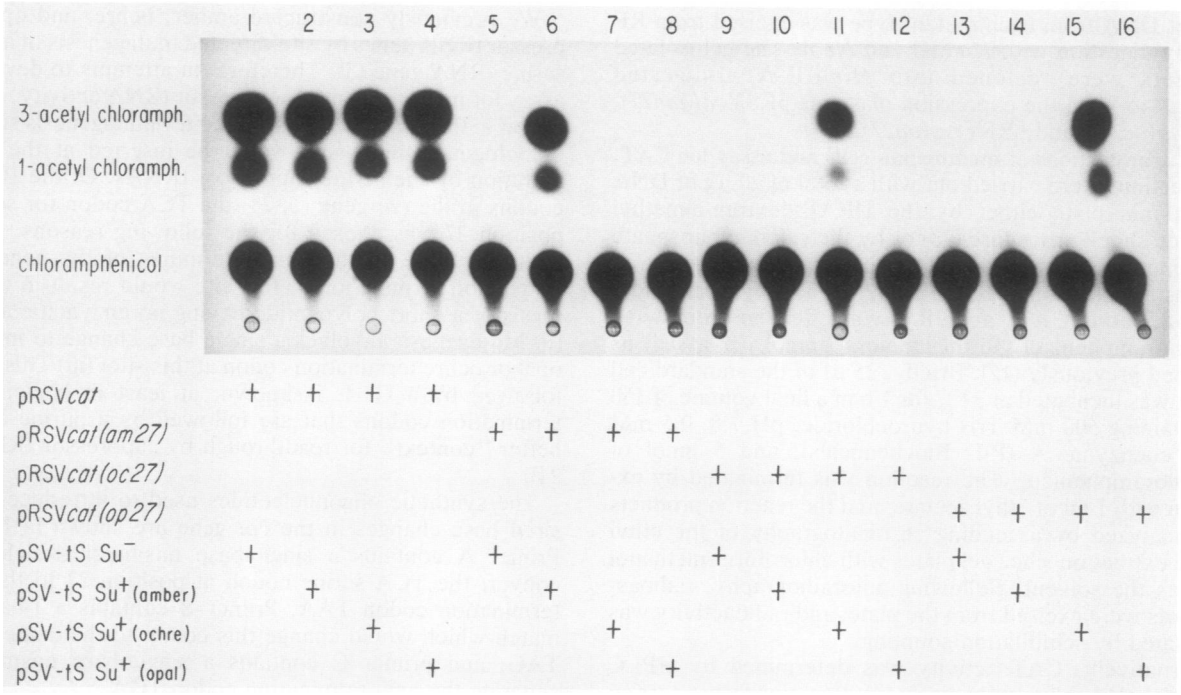

FIG. 2. CAT activity in CV-1 cells cotransfected with the various pRSV*cat* DNAs and Su^- or Su^+ tRNA genes. CV-1 cells were cotransfected with wild-type pRSV*cat* or the different *cat* nonsense mutations along with Su^- or the different Su^+ tRNA gene-containing plasmids as indicated in the figure. Assays for CAT activity were performed on equivalent amounts of cell extract from each transfection with [^{14}C]chloramphenicol as described in Materials and Methods, and the products were analyzed by TLC. The positions of the monoacetylated derivatives and unreacted chloramphenicol are indicated.

pRSV*cat*(*op27*) DNA was transfected into CV-1 cells along with pSV-tS Su^- (plasmid containing the wild-type serine tRNA gene), no CAT activity above background levels (cells transfected with an equivalent amount of pBR322) was detected (Fig. 2, lanes 5, 9, and 13). When the various pRSV*cat* plasmid DNAs were cotransfected into CV-1 cells along with plasmid DNAs carrying the corresponding Su^+ tRNA gene, CAT activity was restored for each mutant (Fig. 2, lanes 6, 11, and 16). Readthrough of the different termination codons was dependent on cotransfection with the appropriate suppressor tRNA gene. Thus, in cells transfected with pRSV*cat*(*am27*), enzymatic activity was detected in the cell extract if the cells were cotransfected with the amber suppressor tRNA gene, but not with the ochre or opal suppressor tRNA genes (Fig. 2, compare lane 6 with lanes 7 and 8). Similarly, in cells transfected with pRSV*cat*(*oc27*) or pRSV*cat*(*op27*), enzymatic activity was detected only if such cells were also cotransfected with DNA carrying the ochre or opal suppressor tRNA gene, respectively.

These results confirm previous observations that in mammalian cells, ochre and amber suppressor tRNAs are specific for UAA and UAG codons, respectively (5, 19). This is in contrast to bacteria, in which UAA suppressor tRNAs can also read through UAG codons (3).

More significantly, these results provide the first direct evidence that an opal Su^+ tRNA gene can be functionally expressed in mammalian cells. We had previously not been able to test for activity of the opal suppressor tRNA gene, as we were unable to propagate SV40 recombinants carrying the opal suppressor tRNA gene (5).

The data in Fig. 2 also show that in mammalian cells, as in bacteria and yeasts, opal suppressor tRNAs are specific for UGA termination codons.

To quantitate the suppression efficiency of the different Su^+ tRNA genes, CAT enzymatic activity present in the cell extracts was determined by HPLC. The HPLC assay is sensitive and quantitative and has the added advantage that it is faster and less expensive than the radioisotope assay. Less than 10 pmol of chloramphenicol or its acetylated derivatives can be detected. The HPLC system resolved the two monoacetylated forms of chloramphenicol into distinct peaks (Fig. 3b). To identify the material in these peaks, a reaction in which [^{14}C]chloramphenicol was incubated with commercially available CAT was fractionated by HPLC, and the different peaks were collected and analyzed by thin-layer chromatography (TLC). As shown in Fig. 3e, peak 1, which had a retention time of 3.74 min, corresponds to 3-acetylchloramphenicol. The peak which had a retention time of 4.03 min is 1-acetylchloramphenicol, while the peak with a retention time of 5.77 min corresponds to unreacted chloramphenicol. 1,3-Diacetylchloramphenicol can also be resolved, and when present elutes before 3-acetylchloramphenicol.

The suppression efficiency of the different Su^+ tRNA genes was evaluated by comparing the amount of 3-acetylchloramphenicol produced by extracts from cells cotransfected with the various mutant pRSV*cat* DNAs and DNAs containing the different Su^+ tRNA genes with that from cells cotransfected with wild-type pRSV*cat* plasmid DNA and the same Su^+ tRNA gene. Values for CAT activity were quantitatively similar whether the activity was determined by HPLC or by the radiochemical assay (data not shown). Fig. 3 shows a representative HPLC assay for CAT activity from CV-1 cells cotransfected with pRSV*cat*(*am27*) DNA and either the Su^- tRNA gene (panel c) or the Su^+ tRNA gene (panel d). The results averaged from three separate transfection experiments are shown in Fig. 4. CV-1

cells cotransfected with pRSV*cat*(*am27*) and pSV-tS Su^+(Am) produced 40% of the enzymatic activity observed in cells cotransfected with wild-type pRSV*cat* and the amber suppressor tRNA gene. Similarly, pSV-tS Su^+(Och) was capable of restoring the activity of pRSV*cat*(*oc27*) to 18% of wild-type levels, while pSV-tS Su^+(Opal) restored the activity of pRSV*cat*(*op27*) up to 50% of wild-type levels. The activities of the ochre and amber suppressor tRNA genes as measured by the CAT assay are similar to those observed when adenovirus or influenza virus was used to estimate the readthrough efficiency of these tRNA genes (5).

The above studies were carried out with suppressor tRNA genes carried on SV40 recombinant plasmids which were able to replicate in CV-1 cells, and therefore the copy number of the tRNA genes was high. When similar experiments were carried out with the suppressor tRNA genes cloned into bacterial plasmids devoid of SV40 sequences (pUC12 series of plasmids), the pattern of suppression observed was the same for each suppressor tRNA gene; however, as expected, the overall levels of suppression were reduced in each case. In the example shown in Fig. 4, ptS-Su^+(Am) restored the activity of pRSV*cat*(*am27*) to approximately 10% of wild-type levels in CV-1 cells, compared with 40% suppression when the amber tRNA gene cloned into a replicating SV40 vector was used.

(ii) NIH3T3 cells. Similar transfection experiments were carried out in mouse NIH3T3 cells. As in CV-1 cells, CAT enzymatic activity in NIH3T3 cells transfected with the various pRSV*cat* plasmids was detected only when the cells were cotransfected with plasmid DNAs which contained the appropriate suppressor tRNA gene. The levels of suppression by the amber, ochre, and opal Su^+ tRNA genes was 30, 10, and 33%, respectively (Fig. 4). With NIH3T3 cells, little difference in suppressor activity was observed whether the tRNA genes were in SV40-based vectors or in pUC12. This is as expected, since the SV40 vectors do not replicate in NIH3T3 cells and therefore the effective tRNA gene dose should be the same whether the tRNA genes are carried on an SV40 vector or on a bacterial plasmid. Comparing transfections with nonreplicating plasmid DNAs, the levels of suppression were higher in NIH3T3 cells than in CV-1 cells [Fig. 4, compare CV-1 cells and NIH3T3 cells cotransfected with pRSV*cat*(*am27*) and pUCtS Su^+(Am)]. This is probably related to differences in transfection efficiencies of the two cell types, the efficiency being higher in NIH3T3 cells.

Serine at position 27 of CAT can be replaced with a variety of amino acids without loss of enzymatic activity. There are now available several suppressor tRNA genes which are known to function in mammalian cells. These include the human serine suppressor tRNA genes (described in this report; see also reference 5) and the amber and ochre suppressors derived from *X. laevis* $tRNA^{Tyr}$ (19, 20). In addition, suppressors derived from $tRNA^{Lys}$ and $tRNA^{Gln}$ have been described, although their in vivo function has yet to be established (31).

For the pRSV*cat* nonsense mutations to be of general use in assaying for different nonsense suppressors, it is necessary to demonstrate that insertion of amino acids other than serine at position 27 of CAT is compatible with enzymatic activity. Since the pRSV*cat* construct retains sequences adequate for expression of CAT in *E. coli* (11), we established a suppression spectrum for the three different *cat* nonsense mutants by using *E. coli* strains bearing various nonsense suppressor tRNA genes.

E. coli strains were transformed with the different pRSV*cat* DNAs, and their ability to grow on LB plates in the presence of 20 μg of chloramphenicol per ml was assessed. The results of these experiments are summarized in Table 1. Wild-type pRSV*cat* conferred resistance to chloramphenicol in all strains tested. Strains carrying the *supD* (serine inserting), *supE* (glutamine inserting), *supF* (tyrosine inserting), or

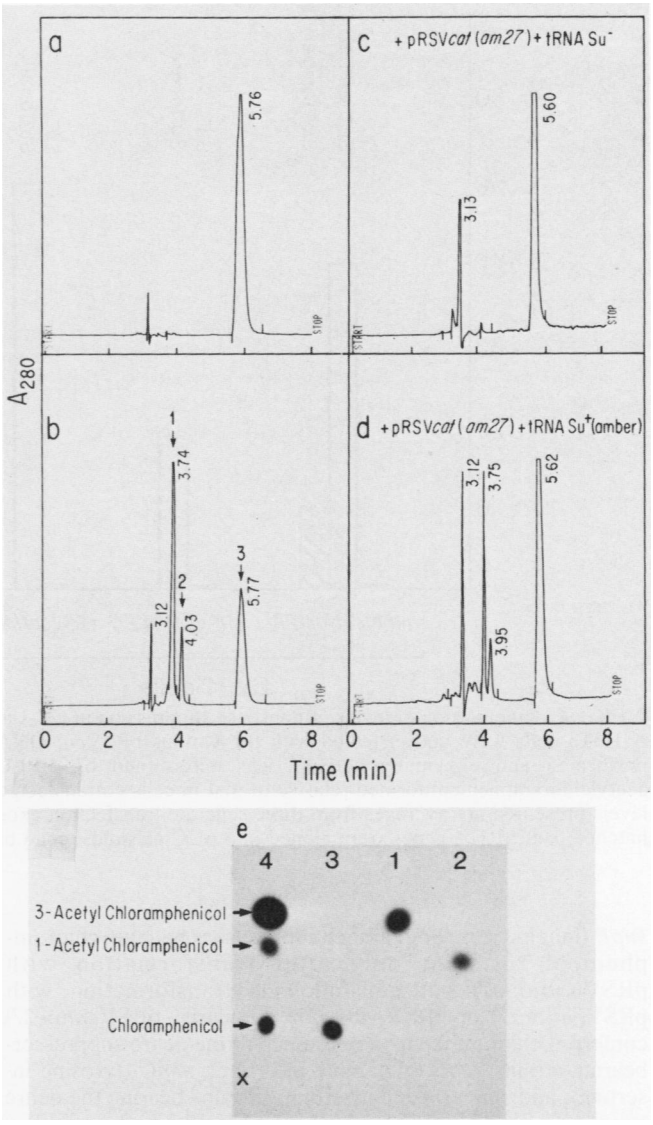

FIG. 3. Determination of CAT activity by HPLC. (a and b) CAT assays were performed with nonradioactive chloramphenicol (15 nmol per reaction) in the absence (a) or presence (b) of 0.5 U of CAT. The reaction mixtures were incubated at 37°C for 10 min and extracted with ethyl acetate. Portions (1 μl of 100 μl) were analyzed by HPLC. For the case shown in panel b, the reaction was supplemented with 0.1 μCi of [^{14}C]chloramphenicol (2 nmol). The peaks labeled a, 2, and 3 in panel b were collected and analyzed by TLC, as shown in panel e. Peak 1 corresponds to 3-acetylchloramphenicol, peak 2 to 1-acetylchloramphenicol, and peak 3 to unreacted chloramphenicol. Panel e, lane 4, represents the products of reaction prior to HPLC fractionation. The peak eluting at 3.12 min in the HPLC scans represents the solvent peak. (c and d) CAT activity in CV-1 cells cotransfected with pRSV*cat*(*am27*) and pSV-tS Su^- (c) or pRSV*cat*(*am27*) and pSV-tS Su^+(Am) (d). Reactions were carried out for 1 h with 50 μM chloramphenicol and 25 μl of cell extract in each case. Five percent of the reaction products were analyzed by HPLC. X, Origin.

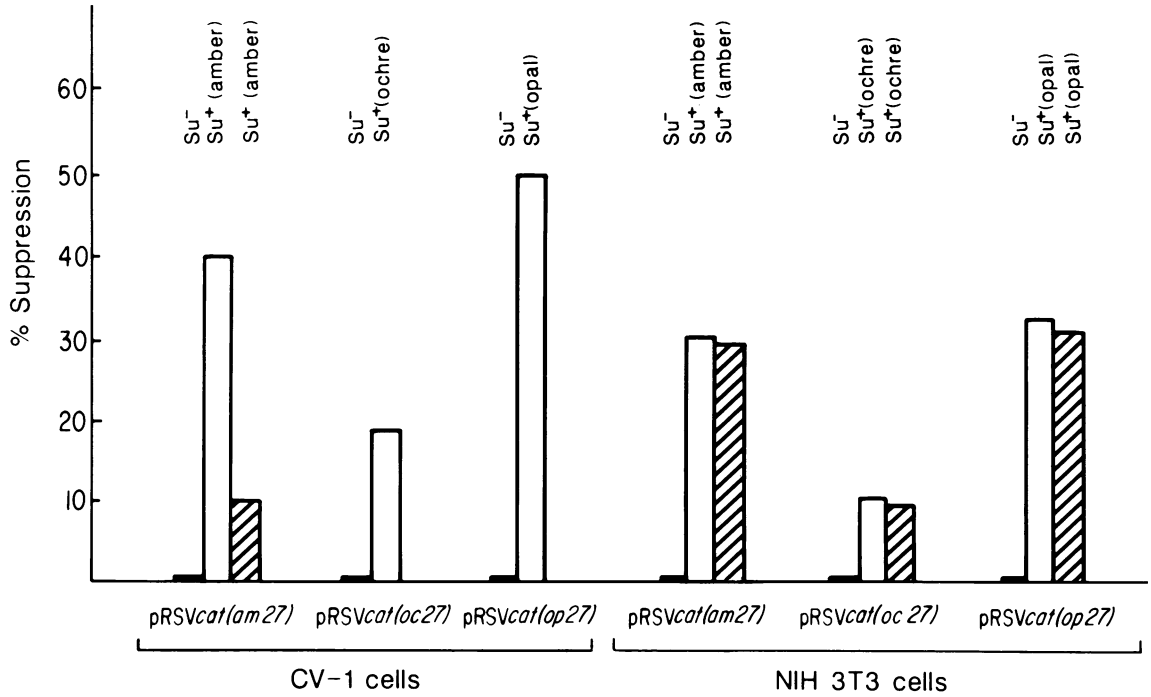

FIG. 4. Quantitation of levels of nonsense suppression in CV-1 and NIH3T3 cells transfected with suppressor tRNA genes. CV-1 cells or NIH3T3 cells were cotransfected with the various pRSV*cat* DNA vectors and suppressor tRNA genes as indicated. CAT assays were performed and enzymatic activity was determined by HPLC. Levels of suppression were determined as the amount of 3-acetylchloramphenicol formed relative to that in cells cotransfected with the wild-type pRSV*cat* DNA and the same Su^+ tRNA gene. The levels presented are averages from three separate transfection experiments. Open bars, tRNA genes were carried on SV40-based vectors; hatched bars, tRNA genes were cloned into pUC plasmids; solid bars, Su^- tRNA genes.

supP (leucine inserting) amber suppressors became chloramphenicol resistant only after transformation with pRSV*cat*(*am27*) but not following transformation with pRSV*cat*(*oc27*) or pRSV*cat*(*op27*). Similarly, pRSV*cat*(*oc27*) conferred chloramphenicol resistance to the ochre suppressor-bearing strains *supB* (glutamine insertine), *supC* (tyrosine inserting), and *supG* (lysine inserting). Strains bearing the ochre suppressors were also chloramphenicol resistant after transformation with pRSV*cat*(*am27*). This is consistent with the observation that in bacteria ochre suppressors are capable of reading UAG as well as UAA termination codons, while amber suppressors are UAG specific. All strains transformed with pRSV*cat*(*op27*) were able to grow to some extent in the presence of chloramphenicol. This most likely reflects the known leakiness of UGA nonsense mutatons in *E. coli* (24). However, only in strain CDJ64, which carries an efficient UGA suppressor, was pRSV*cat*(*op27*) able to confer growth properties similar to those with wild-type pRSV*cat*.

The efficiency of suppression of the different *cat* nonsense mutations in the presence of the various *E. coli* tRNA suppressors was examined by the filter clearing method, using filters impregnated with 5 mg of chloramphenicol. The results obtained reflect what is generally known about the different *E. coli* nonsense suppressors (30). *supD*, *supE*, *supF*, and *supP*, which are known to be strong amber suppressors, were capable of restoring CAT activity in cells transformed with pRSV*cat*(*am27*) to levels approaching that present in cells transformed with the wild-type plasmid. This was also true with the strain carrying *sup-9*, an efficient opal suppressor, transformed with pRSV*cat*(*op27*). The *E. coli* ochre suppressors *supB*, *supC*, and *supG*, which are known to be weaker than the amber suppressors, suppressed pRSV*cat*(*oc27*) less efficiently than the amber suppressors suppressed pRSV*cat*(*am27*).

These results indicate that amino acids other than serine (i.e., Gln, Trp, Tyr, Lys, and Leu) can be introduced into position 27 of CAT without loss of enzymatic activity. Thus, the pRSV*cat* mutants which we have generated should be of general use for the detection and quantitation of nonsense mutation suppression mediated by a variety of suppressor tRNA genes.

DISCUSSION

In the last few years, several tRNA genes have been altered by site-directed mutagenesis to function as nonsense suppressors in mammalian cells (5, 19, 20, 31). As more suppressor tRNA genes become available, it is important to have a general and reliable assay system to measure the in vivo suppressor activity of the different Su^+ tRNA genes. Such an assay should be sensitive and allow rapid and quantitative estimates of levels of nonsense suppression regardless of the specificity of suppressor tRNA gene or cell type. The mutant pRSV*cat* DNAs described in this report have allowed us to develop such an assay system. Site-specific mutagenesis was used to introduce amber, ochre, and opal mutations in place of a serine codon at position 27 of the *E. coli cat* gene. Cotransfection of CV-1 and NIH3T3 cells with the mutant pRSV*cat* DNAs and DNAs carrying different Su^+ tRNA genes allowed quantitation of nonsense suppression in these cell lines by assaying for CAT activity in cell extracts. Using such an assay, we show that an opal suppressor tRNA gene can be functionally expressed in mammalian cells. We also show that an ochre suppressor is

TABLE 1. Suppression spectrum of *cat* nonsense mutations in *E. coli*

Strain	Nonsense codon suppressed	Amino acid inserted	Chloramphenicol sensitivity and growth[a]							
			pRSV*cat* (wild type)		pRSV*cat(am27)*		pRSV*cat(oc27)*		pRSV*cat(op27)*	
			a	b	a	b	a	b	a	b
Xac (*supO*)			1.9	+ +	3.4–3.6	−	3.4–3.6	−	3.0	+
Xac (*supB*)	UAA, UAG	Gln	1.9	+ +	3.1	+	2.4	+ +	3.0	+
Xac (*supC*)	UAA, UAG	Tyr	1.9	+ +	3.2	+	2.4	+ +	3.0	+
Xac (*supD*)	UAG	Ser	1.9	+ +	2.0	+ +	3.4–3.6	−	3.0	+
Xac (*supE*)	UAG	Gln	1.9	+ +	2.0	+ +	3.4–3.6	−	3.0	+
Xac (*supF*)	UAG	Tyr	1.9	+ +	2.1	+ +	3.4–3.6	−	3.0	+
Xac (*supG*)	UAA, UAG	Lys	1.9	+ +	2.6	+ +	2.4	+ +	3.0	+
Xac (*supP*)	UAG	Leu	1.9	+ +	2.0	+ +	3.4–3.6	+ +	3.0	+
CDJ64 (*sup-9*)	UGA	Trp	1.9	+ +	3.4–3.6	−	3.4–3.6	−	2.4	+ +

[a] Zone of growth inhibition around 0.5-in. (ca. 1.5-cm) disks containing 5 mg of chloramphenicol. The data are given as diameter of clearing (in centimeters). b, Growth on LB plates containing 20 μg of chloramphenicol per ml. Symbols: + +, indistinguishable from cells transformed with wild-type plasmid; +, weaker but definite growth (isolated colonies are 20 to 50% of wild-type size); −, no growth.

a weaker suppressor than either the amber or opal suppressor derived from the same tRNA gene. The suppression pattern of the pRSV*cat* mutants in *E. coli* suggests that replacement of the serine at position 27 by a number of amino acids has little effect on CAT enzymatic activity.

One of the key features of the mutant pRSV*cat* DNAs is that all three types of nonsense mutations are in the same position and thus in an identical context. Therefore, direct comparisons of the levels of suppression attained from suppressors of different codon specificities can be made (2, 24). Previously it was not possible to make such a direct comparison of the relative efficiencies of the serine amber and ochre suppressor tRNAs because the UAA codon in adenovirus used to measure ochre suppression was in a different context from that of the UAG codon of influenza virus used to measure amber suppression (5).

When pRSV*cat(am27)*, pRSV*cat(oc27)*, or pRSV*cat(op27)* DNA was transfected into mammalian cells along with plasmid DNAs carrying the wild-type (Su^-) serine tRNA gene, no CAT activity above background levels was detected, indicating that the nonsense mutations introduced into the CAT genes are tight (Fig. 2, lanes 5, 9, and 13). CAT activity was restored only when the pRSV*cat* mutant DNAs were cotransfected along with the appropriate Su^+ tRNA gene. In CV-1 cells transfected with pRSV*cat(am27)* and pSV-tS Su^+(Am), enzymatic activity was restored to 40% of wild-type levels (Fig. 4). When pRSV*cat(oc27)* DNA was cotransfected with pSV-tS Su^+(Och), CAT activity in the cell extract was restored to only 18% of wild-type levels. Thus, it appears that in mammalian cells, as in bacteria, the ochre suppressor is a weaker suppressor than the corresponding amber or opal (described below) suppressors derived from the same tRNA gene. Previously, the ochre suppressor derived from *X. laevis* $tRNA^{Tyr}$ was shown to be as efficient as the corresponding amber suppressor in mammalian cells (19). However, the ochre and the amber codons in the influenza virus mRNA used for measuring suppression were in different contexts, and the possibility that the ochre codon was in a more favorable reading context than the amber codon could not be ruled out.

The lower activity of the ochre suppressor cannot be attributed to differences in the amount of suppressor tRNA made in vivo, since the ochre and amber suppressor tRNA genes are transcribed and processed to similar levels in CV-1 cells and the mature tRNAs carry the same base modifications (5). Another possibility is that the ochre suppressor $tRNA^{Ser}$ is aminoacylated to a lower extent than the corresponding amber suppressor tRNA. This is unlikely, since the ochre suppressor differs from wild-type $tRNA^{Ser}$ in one position, while the amber suppressor differs in two positions. Therefore it is improbable that the seryl-tRNA synthetase aminoacylates the amber suppressor tRNA better than the ochre suppressor. It could be argued that the lower activity of the ochre suppressor is due to readthrough of the natural UAA termination codon in the *cat* gene by the ochre suppressor tRNA, leading to the synthesis of an inactive *cat* gene product. This is probably not the case, since levels of enzymatic activity in cells transfected with wild-type pRSV*cat* DNA and the ochre suppressor tRNA gene were the same as in cells transfected with the wild-type pRSV*cat* DNA and the wild-type serine tRNA gene (Fig. 2, lanes 1 and 3). Thus, the most likely explanation for the relatively lower efficiency of ochre suppression is that the ochre suppressor tRNA competes less efficiently with polypeptide chain termination factors than the corresponding amber and opal suppressor tRNAs (6).

The serine opal suppressor tRNA gene restored CAT activity in CV-1 cells transfected with pRSV*cat(op27)* DNA to 50% of the level in cells transfected with wild-type pRSV*cat* DNA (Fig. 4). This is the first demonstration that an opal suppressor tRNA gene can be functionally expressed in mammalian cells. Previously it was not possible to assay for activity of the opal suppressor tRNA because we were unable to propagate recombinant SV40 virus stocks which retained the opal Su^+ tRNA gene. When CV-1 cells were cotransfected with an SV40 vector containing the opal suppressor gene along with defective helper virus DNA, only wild-type SV40, resulting from recombination between the two DNA species, was obtained following cell lysis (5). We attributed this result to the possibility that overproduction of the opal suppressor was lethal to the cells or interfered with SV40 propagation. Interestingly, propagation of SV40 recombinants containing either the amber or ochre $tRNA^{Ser}$ suppressors was not restricted in this fashion. The results obtained with pRSV*cat(op27)* demonstrate that in short-term transfection experiments, the Su^+ opal $tRNA^{Ser}$ gene can be expressed in mammalian cells and that the tRNA is in fact a very strong and specific suppressor of UGA nonsense codons (Fig. 2, compare lane 16 with lanes 8 and 12).

Mutant pRSV*cat* DNAs can also be used to measure suppressor activity in NIH3T3 cells. The levels of suppression observed were 30, 10, and 33% for the amber, ochre, and opal suppressor tRNA genes, respectively (Fig. 4).

These levels were similar whether the Su^+ tRNA genes were carried on an SV40 vector or cloned into bacterial plasmids not containing SV40 DNA. In the case of CV-1 cells, the activity of the Su^+ tRNA genes was higher when they were cloned into SV40-based vectors, because these vectors contain the T antigen gene and viral origin of replication and are thus capable of replicating in monkey cells. Recently, Burke and Mogg (4) described the isolation of a *cat* gene which contains an amber termination codon in place of a glutamine codon at position 39. They used this *cat* gene to measure the suppressor activity of a transfected *X. laevis* $tRNA^{Tyr}$ amber suppressor tRNA gene (20) in monkey as well as human cells. Thus, *cat* genes carrying nonsense mutations can be used to analyze nonsense suppression in monkey, mouse, and human cell lines.

The suppression spectrum in *E. coli* of the three *cat* nonsense mutations (Table 1) suggests that insertion of amino acids other than serine at position 27 does not interfere with CAT enzymatic activity. In fact, a wide variety of amino acids can be inserted at this position with little effect on CAT enzymatic activity. This important finding means that the mutant pRSV*cat* plasmid DNAs can be used to assay the activity of almost any suppressor tRNA. The results with the suppression spectrum also reemphasize the specificity of mammalian nonsense suppressors (5, 20) in that whereas *E. coli* ochre suppressors (*supB*, *supC*, and *supG*) are also able to suppress the *cat* amber nonsense mutation, the mammalian ochre suppressor is specific for UAA (Table 1; Fig. 2, compare lane 11 with lanes 7 and 15). In this respect, mammalian cells are similar to *Saccharomyces cerevisiae* in that each tRNA suppressor is specific to a particular nonsense codon (7). Opal suppressor tRNAs are specific for UGA codons in both mammalian cells and bacterial cells.

The sensitivity of the CAT assay also suggests the use of the mutant pRSV*cat* DNAs in measuring the naturally occurring nonsense suppression which may occur in certain biological systems. For instance, a UGA suppressor activity has been detected in rabbit reticulocytes (10), and some serine tRNAs from bovine liver and chicken liver can read UGA codons in vitro (9, 15). It is not known, however, whether these tRNA species function as UGA suppressors in vivo. Particularly interesting is the observation that in murine leukemia virus (MLV)-infected cells, low-level suppression of a UAG is required for the synthesis of a viral protease and reverse transcriptase (25). The amino acid inserted in response to this UAG stop codon is glutamine (35), but it is not known whether suppression of the UAG is mediated by misreading by a normal glutamine tRNA or by a minor amber suppressor tRNA species. In the experiments shown in Fig. 4, extracts from NIH3T3 cells transfected with pRSV*cat*(*am27*) and the Su^- tRNA gene showed no CAT activity over background. This would suggest that NIH3T3 cells, used for MLV infection, do not have measurable levels of amber suppressor activity. We cannot rule out the possibility that the UAG codon which separates the *gag-pol* coding sequence in MLV RNA is in an exceptionally favorable context for suppression, so that this UAG is suppressed under conditions in which the UAG at position 27 of the mutant *cat* gene is not. Another possibility is that infection of NIH3T3 cells by MLV leads to the induction of synthesis of a glutamine amber suppressor tRNA or creates conditions allowing low-level readthrough of UAG codons by an endogenous suppressor tRNA. Clearly, the mutant pRSV*cat*(*am27*) that we have reported here will be useful in examining these possibilities.

Finally, the availability of a general method for quantitative measurement of suppressor tRNA activity provides a good biological assay in mammalian cells for a gene that is transcribed by RNA polymerase III. It is hoped that this assay will also prove useful in studies of *cis*- or *trans*-acting elements that regulate activity of genes transcribed by RNA polymerase III in vivo in mammalian cells.

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