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Context Effects on Misreading and Suppression at UAG Codons in Human Cells

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The effect of the 3′ codon context on the efficiency of nonsense suppression in mammalian tissue culture cells has been tested. Measurements were made following the transfection of cells with a pRSVgal reporter vector that contained the classical *Escherichia coli lacZ* UAG allele YA559. The position of this mutation was mapped by virtue of its fortuitous creation of a CTAG *Mae*I restriction enzyme site. Determination of the local DNA sequence revealed a C→T mutation at codon 600 of the *lacZ* gene: CAG→TAG. Site-directed mutagenesis was used to create a series of vectors in which the base 3′ to the nonsense codon was either A, C, G, or U. Suppression of the amber-containing reporter was achieved by cotransfection with genes for human $tRNA^{Ser}$ or $tRNA^{Gln}$ UAG nonsense suppressors and by growth in the translational error-promoting aminoglycoside drug G418. Nonsense suppression was studied in the human cell lines 293 and MRC5V1 and the simian line COS-7. Overall, the rank order for the effect of changes to the base 3′ to UAG was C < G = U < A. This study confirms and extends earlier findings that in mammalian cells 3′ C supports efficient nonsense suppression while 3′ A is unsympathetic for read-through at nonsense codons. The rules for the mammalian codon context effect on nonsense suppression are therefore demonstrably different from those in *E. coli*.

Normally, nonsense or stop codons catalyze the effective cessation of protein synthesis. However, when tRNA species within the cell are able to recognize a nonsense codon, the function of the stop is suppressed. Nonsense suppression has been exploited as a means for the conditional expression of genes containing stop codons in order to study their function (23, 44). Nonsense suppression has also been used extensively in *Escherichia coli* as a tool for monitoring the efficiency of translation at an individual codon (46, 47, 51). Of particular interest is the facility that nonsense suppression affords for determining the effects of 5′ and 3′ contexts on the interaction of translation factors and aminoacyl-tRNAs with their mRNA targets (19, 39, 45, 49, 52). Recently, nonsense suppression has been harnessed in order to carry out in vitro protein engineering. In this process, chemically acylated nonsense suppressor tRNAs are used to insert nonnatural amino acid residues at specified locations by targeting a nonsense codon which interrupts the coding sequence (2, 3, 33).

There have been comparatively few reports of nonsense suppression in mammalian cells, but the transfection of sensitively detected reporters now allows this technique to be applied to cells in culture (6, 12, 13, 15, 43). In a previous report from this laboratory, we described the effects of alterations in the 3′ mRNA context on the efficiency of a human $tRNA^{Ser}$ UAG nonsense suppressor (40). The function of the suppressor was monitored by determining the efficiency of translation at a UAG codon in a β-galactosidase reporter gene which had been cotransfected into human 293 cells with a plasmid vector expressing a human UAG suppressor tRNA (25). Reading at the stop codon varied according to the 3′ context: C > G > U = A. This was a strikingly different pattern from that observed when the same UAG-containing reporters were suppressed in *E. coli* (40). It is known, however, from work with *E. coli*, that there are exceptions to the classical context rules first defined by Bossi (8) and by Miller and Albertini (37). The context effects which are observed often depend on bases other than those at the first 3′ position (7–10, 37, 48). In mammalian cells, the increased complexity of the translational apparatus might render the situation more intricate still. It is crucial, therefore, that the discovery of a different set of context rules for nonsense suppression in a mammalian background be checked at a number of different mRNA locations, to see whether these rules represent a general property of mammalian translation. In this paper we confirm that a similar pattern of 3′ context effects is obtained on the $tRNA^{Ser}$ UAG suppressor at a second location in the β-galactosidase reporter. These context effects are observed in the human cell lines 293 and MRC5V1 and in the simian cell line COS-7. We also show that a second human suppressor, $tRNA^{Gln}$ UAG, and misreading induced by the aminoglycoside antibiotic G418 (11, 35) both display broadly similar sensitivities to the 3′ codon context.

MATERIALS AND METHODS

Expression of pRSVgal reporters. Methods for the growth and transfection of human 293 cells with pRSVgal reporters and the assay of β-galactosidase using the substrate ONPG (*ortho*-nitrophenyl-β-D-galactopyranoside) have been previously described (40). In brief, adherent cells were transfected with $CaPO_4$-DNA precipitates and allowed to express for 2 days. Cells were harvested by scraping and assayed for β-galactosidase and soluble protein following the preparation of freeze-thaw lysates. Enzyme and protein assays were performed with 96-well microtiter plates and an Anthos 2001 plate reader equipped with kinetic software. All enzyme and protein determinations were performed in duplicate. The concentration of plasmid DNA for cell transfection was determined by video densitometry. Samples were digested to completion with a single cutting restriction enzyme and compared with known standards of undigested bacteriophage λ DNA on ethidium bromide-stained agarose gels. The human cell line MRC5V1 (29) was grown and transfected identically to 293 cells. COS-7 cells (22) were treated by the same methods, except that transfection was with the liposome preparation DOTAP (Boehringer; used according to manufacturer's instructions). Growth of COS-7 cells was in Dulbecco's modified Eagle medium (Gibco-BRL). Minimum essential medium (Gibco-BRL) was used for 293 and MRC5V1 cells.

Plasmids used in this study. The construction of the pRSVgal reporter plasmids used in this study is illustrated in Fig. 1. The pRSV559 series contain UAG at *lacZ* codon 600 followed by either A, C, G, or U. The *lacZ* gene in pRSVgal

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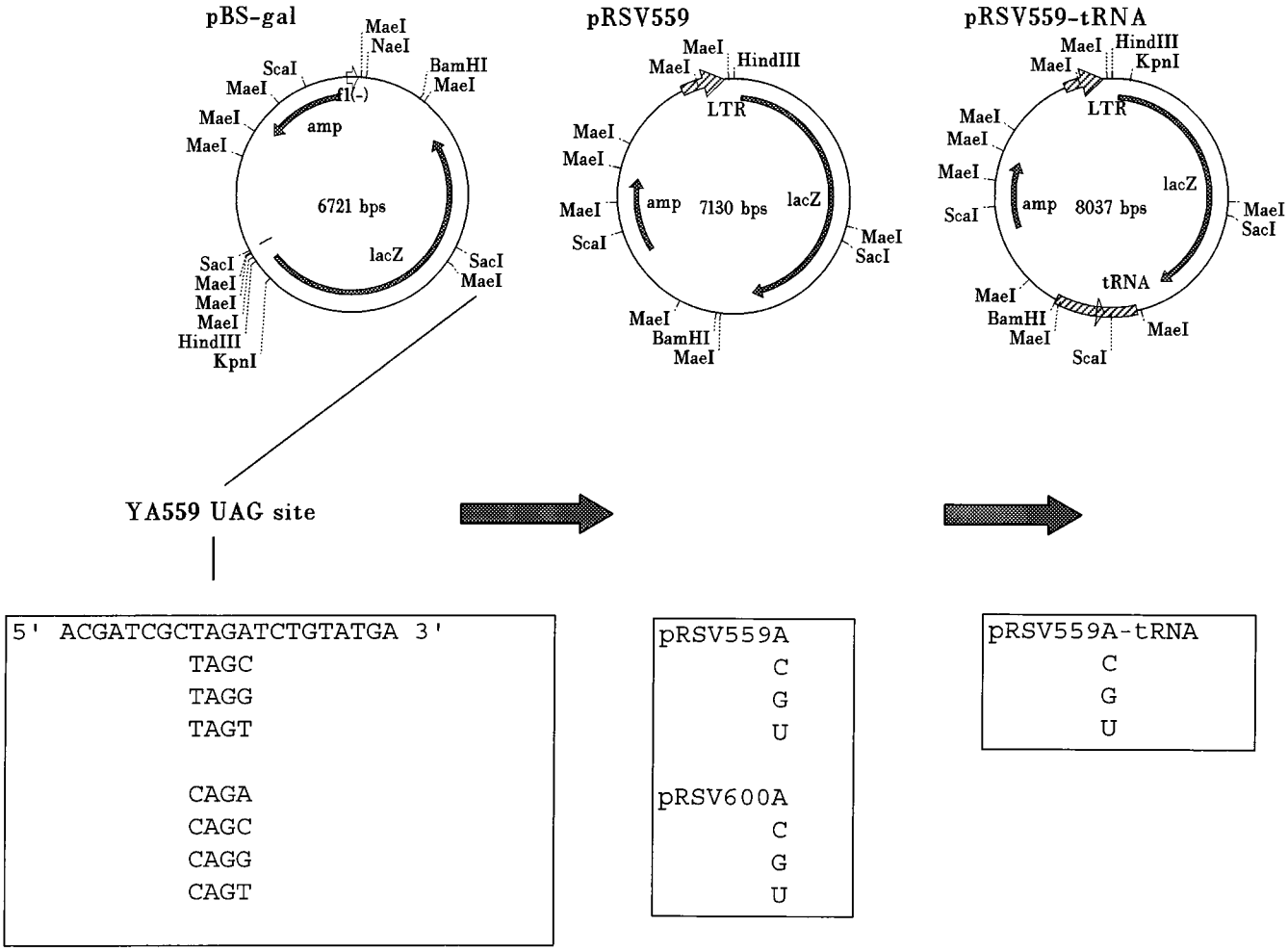

FIG. 1. Construction of plasmids used in this study. The YA559 mutation lies at the *Mae*I CTAG site within the *lacZ* coding sequence. UAG (stop) and CAG (wild-type) vectors, in each possible 3′ context, were created by site-directed mutagenesis of the pBluescript-derived vector pBS-gal by using the eight 21-mer synthetic oligonucleotides as shown. The successfully mutated *lacZ* genes were subcloned as *Hin*dIII-*Bam*HI fragments to form the pRSV559 (UAG) and the pRSV600 (wild-type) vectors for expression in human cells. The site-directed mutations were checked in pRSV559 and pRSV600 by double-stranded DNA sequencing. The creation of the alleles with a 3′ A context generates a unique *Bgl*II site (AGATCT) in pRSV559A and pRSV600A. The pRSV559 tRNA vectors were prepared by inserting a 900-bp fragment containing a human $tRNA^{Ser}$ UAG suppressor from pSV-tS-UAG into the *Bam*HI site in the pRSV559 vectors.

is an N-terminal translational fusion, *gpt-trpS-lacZ*, in which *lacZ* codon 600 is in fact codon 662 (36, 40). The pRSV600 series contain the wild-type CAG (Gln) codon, again with either an A, a C, a G, or a U as a 3′ context. The pRSV559-tRNA series was constructed by placing a 900-bp *Sau*3A fragment that contains the human $tRNA^{Ser}$ UAG gene from pSV-tS-UAG (pSV-tS-Su^+ amber) (14, 21) into the *Bam*HI site in each of the pRSV559 vectors. The identity of the correct *Sau*3A fragment was determined by dot blot hybridization using a synthetic oligonucleotide probe complementary to part of the $tRNA^{Ser}$ gene: 5′ GAGT GGTTAAGGCGATGGACT 3′. This was labelled at the 3′ end with biotin-21-dUTP by using terminal transferase and detected chemiluminescently with Gene Images reagents from United States Biochemicals (not shown). Suppression experiments demonstrated that activity of the tRNA was not dependent on the orientation of the *Sau*3A fragment (not shown).

Site-directed mutagenesis. Nucleotide changes were introduced by using synthetic oligonucleotides of 21 bases in length. The oligonucleotides containing the desired mutations are indicated in Fig. 1. These were hybridized to single-stranded templates prepared by standard methods from a phagemid vector, pBS-gal, which is based on pBluescript-SK (Stratagene) (42). The vector pBS-gal contains the *lacZ Hin*dIII-*Bam*HI fragment from pRSVgal (40). Mutated pBS-gal vectors were generated by using an oligonucleotide in vitro mutagenesis kit (version 2) supplied by Amersham. The successfully mutated pBluescript *lacZ* genes were subcloned as *Hin*dIII-*Bam*HI fragments into pRSVgal. Each construct was checked by double-stranded DNA sequencing, by using standard techniques (Sequenase; United States Biochemicals), from an oligonucleotide primer upstream of the 559 site starting at nucleotide 2010 in pRSVgal: 5′ AACGGCAACCCGTGGTCGGCTTAC 3′.

Thermolability studies. The thermolabilities of the pRSV600 3′ variant β-galactosidase proteins were investigated by using a Thermal Reactor block (Hybaid) with in-tube temperature detection. Extracts from pRSV600-transfected cells were diluted appropriately in TMD buffer (0.1 M Tris, 1 mM $MgSO_4$, 5 mM dithiothreitol [pH 7.5]), and aliquots, one for each time point, were placed into 0.5-ml microcentrifuge tubes. The tubes were placed in the block at room temperature, and the heating cycle was activated. The block was programmed to hold at a constant 55°C, and the holding temperature was attained after 30 s. Tubes were removed from the block to room temperature, and after 5 min they were subjected to centrifugation at maximum speed in a microcentrifuge to remove precipitated debris. Extracts were assayed with the standard ONPG assay at room temperature. Adjustments to protein concentration after heating were not made.

Immunological assays for β-galactosidase expression. *E. coli* β-galactosidase was assayed immunologically by a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) essentially as described elsewhere (27). Polystyrene 96-well microtiter plates (MaxiSorp; Nunc) were coated with 50 μl of a 20-μg ml^{-1} solution of an anti-β-galactosidase antibody (rabbit polyclonal; 5 Prime 3 Prime) in 0.1 M $NaHCO_3$, pH 9.6. Lysates prepared from human 293 cells transfected with the pRSV559-tRNA plasmids, or dilutions thereof, were placed into the coated wells. The second anti-β-galactosidase antibody was the mouse monoclonal antibody (clone D19-2F3-2; Boehringer). Alkaline phosphatase-conjugated, rabbit anti-mouse immunoglobulin polyclonal antibody (Dako) was used to detect the second β-galactosidase antibody by monitoring the development of nitrophenol from nitrophenol phosphate by using an Anthos 2001 microtiter plate reader equipped with kinetic software and integral plate shaking between

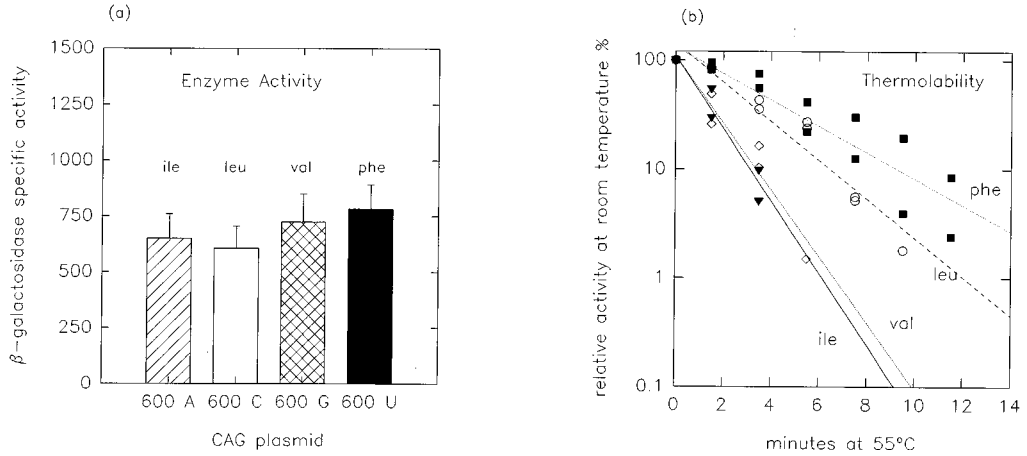

FIG. 2. Effects of changes to the 3′ codon context of Gln-600 on the activity of pRSV600 β-galactosidase. (a) The β-galactosidase specific activity, A_{414} min^{-1} mg of total protein^{-1} × 1,000, was determined for pRSV600A, -C, -G, and -U following transfection of 10 μg of plasmid DNA per 9-cm plate of human 293 cells. The data are means and standard errors for six independent experiments, including two independent plasmid preparations. (b) The stability of β-galactosidase expressed in human 293 cells from each of the pRSV600 constructs was calculated as the relative activity remaining when assayed at room temperature after a given time at 55°C. Each curve represents the mean of two independent experiments, for each of which individual datum points are represented by squares, circles, diamonds, or triangles. The total protein concentration in the extracts held at 55°C was adjusted to 4 mg ml^{-1} for each of the variants.

readings (optical filter, 414 nm; bandwidth, 10 nm). Standard curves detecting known quantities of purified *E. coli* β-galactosidase (Boehringer) were run for each experiment. Only values that fell in the linear range of a lysate dilution series were used.

RESULTS

Construction of pRSVgal reporters containing UAG in different 3′ contexts. In a previous study, UAG codons with different 3′ contexts were introduced into the RSVgal reporter at an N-terminal *Kpn*I restriction site as synthetic double-stranded 30-mer linkers (Fig. 1) (40). At this location, the amino acids inserted by this procedure had no effect on the activity of the β-galactosidase fusion protein. This construction was advantageous for studies in which nucleotides 3′ to the nonsense codon were altered to test their effect on translation through the stop codon, since changes to the flanking amino acids cannot interfere with the enzymatic signal from the β-galactosidase reporter. A disadvantage of these vectors, however, was the level of unsuppressed background caused by ribosome reinitiation (36, 40). At 2 to 4% of the wild-type level, this was sufficient to obscure measurements of nonsense suppression by a weak suppressor and misreading induced by the aminoglycoside G418. In the present study, we wanted to be able to compare the effects of the 3′ context on translation by different suppressor tRNAs and G418-induced misreading. To accomplish this, we required a site where the unsuppressed background would be sufficiently low and, moreover, a site where changes in the β-galactosidase amino acid sequence would have minimal effects on the performance of the enzymatic properties of the reporter.

In an earlier report, an RSVgal vector containing an unsequenced UAG mutation in which β-galactosidase was on the order of 0.01% of the wild-type level was described (35). The *lacZ* UAG mutation was introduced into pRSVgal via the plasmid pMLB100. This was recovered by recombinational transfer of the *lacZ* allele from the genome in *E. coli* CA7020, following irradiation of the plasmid with UV light (16, 35). The allele in CA7020 is the *lacZ* YA559 UAG, first isolated by Newton et al. (38). In order to be able to examine the effects of the 3′ context on UAG at this position, we first had to determine its exact location by DNA sequencing. Rowland et al. (41) have described a method that is often successful in identifying the site of mutations to UAG in protein-coding regions. The strategy is to use a restriction enzyme that recognizes and cuts at the site CTAG. If the codon that has mutated to TAG is preceded by C, a new CTAG restriction site will be created. Although a 4-base recognition sequence would be predicted to be rather common, in fact CTAG is rare in coding regions. Using the enzyme *Rma*I (an isoschizomer for *Mae*I), we found that there is not a single *Mae*I site in the entire wild-type *E. coli lacZ* gene but that the *lacZ* YA559 UAG allele contains a new CTAG site (Fig. 1). The YA559 mutation was therefore mapped rapidly to ±50 bp within the 3.5-kb *lacZ* gene. The surrounding nucleotides were determined by DNA sequencing from an adjacent upstream primer starting at nucleotide 1800 in pRSVgal: 5′ CCCTTCCCGGCTGTGCCGAAATGGTC (Fig. 1). The YA559 mutation occurred at codon 600 in the chromosomal *E. coli lacZ* gene, changing CAG to TAG (31).

In order to examine the effects of 3′ context on nonsense suppression, site-specific mutagenesis was used, as described in Materials and Methods, to place the wild-type CAG codon and the mutant UAG codon in each possible 3′ context. The wild-type context is 3′ U. We took the precaution to reconstruct the YA559 UAG allele from the wild-type vector. This was to guard against the possibility that other nucleotide changes might have been induced in the original YA559 allele and carried into the pRSVgal vector during subcloning (16, 35). In the event, the new and the original YA559 alleles appeared to have identical properties (not shown). The UAG-containing vectors were designated pRSV559A, -C, -G, and -U, and the corresponding wild types were pRSV600A, -C, -G, and -U (Fig. 1). These alterations to the 3′ base give rise to altered amino acid sequences. The wild-type codon is UUC (Phe). The context changes create AUC (Ile), CUC (Leu), and GUC (Val). Figure 2a shows that there was no significant effect of these alterations on the expression of β-galactosidase enzyme activity from the pRSV600 CAG-containing vectors in human 293 cells. To investigate the consequences of these single amino acid substitutions in more detail, we also determined the stability to heat denaturation of β-galactosidase expressed in human 293 cells from the four wild-type CAG constructs, pRSV600A, -C, -G, and -U. Extracts were held at 55°C for

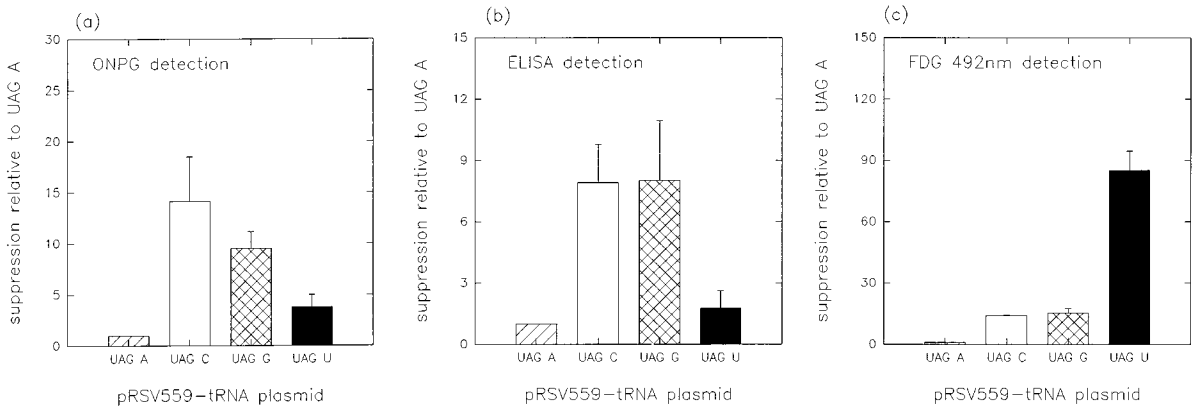

FIG. 3. Nonsense suppression in pRSV559-tRNA vectors. (a) Human 293 cells were transfected with 10 μg of each of the pRSV559-tRNA vectors which contain the reporter and the $tRNA^{Ser}$ UAG suppressor per 9-cm plate. Following expression for 2 days, cells were harvested and lysed by three freeze-thaw cycles, and extracts were assayed for β-galactosidase specific activity by using ONPG at 0.9 mg ml^{-1}. The level of suppression for pRSV559A-tRNA was assigned a value of 1.0, and the relative levels of β-galactosidase for the C, G, or U contexts were calculated. The data are means and standard errors for two independent experiments. (b) Cells were transfected as for panel a, and freeze-thaw lysates were prepared. The levels of β-galactosidase in nanograms per milligram of total protein were determined by a double-antibody sandwich ELISA as described in Materials and Methods. The quantity of β-galactosidase varied from 1.82 ng mg^{-1} ($n = 3$) for pRSV559A-tRNA to 13.89 ng mg^{-1} ($n = 3$) for pRSV559C-tRNA. The level of β-galactosidase from pRSV559A-tRNA was assigned a value of 1, and the relative levels for the 559C, -G, and -U variants were calculated. The data are means and standard errors for three independent experiments. (c) Portions of the extracts described for panel a were assayed against the substrate FDG at a concentration of 50 μM in Z buffer at pH 7.5 by methods otherwise identical to those for panel a. Fluorescein was monitored with a 10-nm-bandwidth filter centered on 492 nm. The data are means and standard errors for two independent experiments.

various lengths of time and then assayed at room temperature. The results are plotted to show the relative activity that remains after a given time at 55°C (Fig. 2b). Substitution of the 3′ amino acid to Ile or to Val leads to an enzyme that is substantially more labile at 55°C than the wild-type product. Substitution for Phe by Leu has a moderate effect on enzyme thermolability.

Effect of 3′ codon context on nonsense suppression by $tRNA^{Ser}$ UAG in human 293 cells. The effects of 3′ context on nonsense suppression at *lacZ* codon 559 UAG were compared with those from the same suppressor at a different site in an earlier study (40). This was accomplished by performing transfections with β-galactosidase reporter vectors, the pRSV559-tRNA series (Fig. 1), which also contained the human $tRNA^{Ser}$ UAG suppressor (14). These vectors were constructed by inserting a 900-bp *Sau*3A fragment carrying the human $tRNA^{Ser}$ UAG gene from pSV-tS-UAG (14) into the *Bam*HI site of pRSV559. These combined reporter and suppressor vectors permit nonsense suppression to be studied with a single plasmid. Figure 3a confirms that the pattern of suppression obtained with standard ONPG cleavage-galactosidase assays, C > G > U > A, is similar to that in the cotransfection experiments at an N-terminal site reported previously, C > G > U = A (40). Identical results were obtained with the pRSV559 vectors cotransfected with the pSV-tS-UAG suppressor (not shown).

Glutamine is the wild-type amino acid at the 559 position, since the UAG at this site was derived from a CAG (Gln) codon. It follows then that the insertion of serine by the $tRNA^{Ser}$ UAG suppressor could compromise the enzymatic activity of the reporter. This might be particularly so for the 3′ A, C, and G variants, which already carry one amino acid substitution at the next downstream codon. We therefore investigated whether the incorporation of the serine residue at the stop codon was giving a false image of the effects of 3′ context on the efficiency of the $tRNA^{Ser}$ UAG suppressor. β-Galactosidase produced from the pRSV559-tRNA vectors was assayed by using antibodies to detect the full-length protein, irrespective of its enzymatic activity. This is possible since ELISA and Western blotting (immunoblotting) studies showed that neither the polyclonal nor the monoclonal antibodies used in the present study can detect any of the N-terminal 76-kDa fragment that would be produced following termination at the 559 UAG stop (50a). This echoes the original finding that the nonsense fragment from the YA559 allele had very low "antigenicity" while nonsense fragments located either N terminally or C terminally to the 559 mutation could be detected (38). Figure 3b shows the results of ELISA detection of β-galactosidase in extracts prepared from human 293 cells transfected with the pRSV559-tRNA vectors. The pattern is virtually identical to that obtained with standard ONPG cleavage assays for enzyme activity (Fig. 3a), except that it appears that the 3′ G context may be somewhat underestimated by the ONPG assay. When the same extracts were assayed with a different substrate, fluorescein di-β-galactopyranoside (FDG), the 3′ A variant, in which Phe is replaced by Ile, had very low enzymatic activity. The 3′ C and G vectors containing Leu and Val are also severely compromised. Identical results were obtained when the wild-type p600 series vectors were assayed against FDG (not shown). It seems that substitutions at the 3′ codon alter the enzymatic activity of β-galactosidase towards some substrates. However, it is evident that the ONPG assay is a reliable reporter of translational events through the UAG codon at position 600, even when the downstream amino acid has been altered.

Effect of 3′ codon context on nonsense suppression by $tRNA^{Gln}$ UAG in human 293 cells. In our previous study of nonsense suppression context effects at an N-terminally located UAG codon (40) it was difficult to study the context sensitivity of a second suppressor, $tRNA^{Gln}$ UAG (28), because this suppressor is substantially weaker than $tRNA^{Ser}$ UAG (14, 34a). Consequently, the activity from $tRNA^{Gln}$ UAG was insufficient to enable a clear distinction to be made between suppression and translational reinitiation downstream from the N-terminal UAG (36). Very little background activity could be detected at the new site in the present study, and so, it proved possible to study 3′ codon context effects in human cells on a second suppressor tRNA. A fixed quantity of each

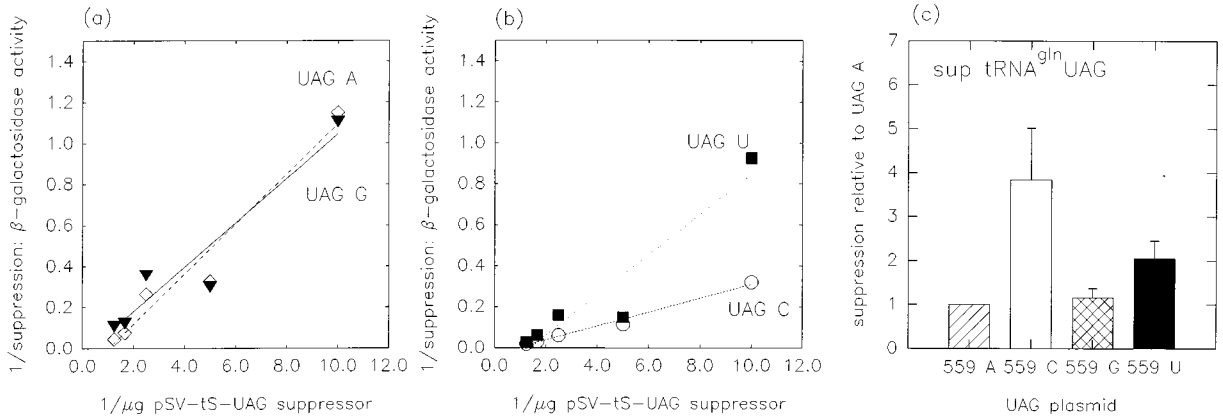

FIG. 4. Effect of 3′ context on the efficiency of suppression by the $tRNA^{Gln}$ UAG nonsense suppressor in human 293 cells. β-Galactosidase specific activity was determined in cell extracts prepared from cells cotransfected with 10 μg of pRSV559A or -G (a) or -C or -U (b) and 0, 0.1, 0.2, 0.4, 0.6, or 0.8 μg of pSV-Gln(su^+)-1 per 9-cm plate. Ten micrograms of pRSV600U was also cotransfected with the same amounts of pSV-Gln(su^+)-1 in an identical fashion. Nonspecific effects on pRSV600U, which ranged from 1.54 to 3.06, were used at each tRNA concentration to correct the values of β-galactosidase from the pRSV559 plates. The data were plotted as 1/β-galactosidase versus 1/pSV-Gln(su^+)-1. The correlation coefficients of the 1/suppression-versus-1/tRNA plots were in all cases >0.95. (c) The level of β-galactosidase expected from cotransfection with 0.5 μg of pSV-Gln(su^+)-1 was calculated from the $y = mx + c$ equation of the straight lines resulting from experiments as described for panels a and b. The level of suppression from pRSV559A was assigned a value of 1.0, and the relative levels of suppression for the pRSV559C, -G, and -U vectors were calculated. The data are means and standard errors for two independent determinations using two different pRSV559 plasmid preparations.

pRSV559 UAG reporter (10 μg) was cotransfected onto each plate of adherent 293 cells with different amounts of the $tRNA^{Gln}$ UAG vector (0.2 to 2.0 μg) as a $CaPO_4$ coprecipitate. Following transfection, expression was allowed to occur for 2 days, after which time cells were harvested and lysed and β-galactosidase specific activities were determined in the resulting cell extracts. In each experiment, a wild-type pRSV600U vector was also transfected in the same manner, to provide a control for the nonspecific stimulation of RSVgal expression that occurs upon cotransfection with the tRNA vectors (40). The results of these experiments were analyzed by double-reciprocal plots of 1/suppression versus 1/(tRNA vector), as previously described (40). Such plots are analogous to a Lineweaver-Burk transformation of Michaelis-Menten enzyme kinetic data. Typically, this analysis yields a straight line with a correlation coefficient > 0.8. The slope m, from the $y = mx + c$ equation of this line, provides a robust estimate of the efficiency of suppression, which includes data from the entire experiment. This procedure also ensures that the full extent of the competition between suppressor tRNA and eRF is examined, by allowing for the saturation effect of the suppressor for its target mRNA (51, 52). Figures 4a and b show the results of a double-reciprocal analysis. On these plots the steeper slopes indicate less efficient suppression. Figure 4c reports the mean of two independent experiments by assigning a value of 1 to the suppression observed at 0.5 μg of the tRNA plasmid with the *lacZ* UAG in the 3′ A context and comparing the effects of substituting A with C, G, or U. At this level of suppressor, suppression of pRSV559A was generally approximately 1% of the wild-type level, an increase of nearly 2 orders of magnitude above the activity from the same plasmid transfected in the absence of suppressor tRNA. Examination of Fig. 4 shows that the pattern for this suppressor is C > U > G = A. This pattern is broadly similar to that for $tRNA^{Ser}$ except for the efficiencies of the G and the U contexts. Overall, the predominant finding is that 3′ C permits efficient suppression while 3′ A restricts the efficiency of suppressor tRNAs. Thus, the pattern which has been observed at two positions in the *lacZ* gene for the efficient $tRNA^{Ser}$ UAG suppressor also holds true for the weaker $tRNA^{Gln}$ UAG species.

G418-induced misreading at UAG codons in human 293 cells. It is well known that certain aminoglycoside drugs have the ability to induce misreading of mRNA codons by endogenous cellular tRNAs (11, 24, 35). In the face of a background of translational reinitiation it was not possible to detect any G418-induced misreading at N-terminal UAG codons (36, 40). G418-mediated suppression was tested in the present study at the 559 UAG mutation in each 3′ context. Figure 5a shows that substantial suppression of the p559UAG stop codons above background is achieved. The 3′ C context is by far the most efficiently suppressed. Figure 5b shows that the level of suppression averaged at 25 and 50 μg of G418 ml^{-1}. Following correction for the effects on the corresponding pRSV600 wild-type vectors, the efficiency of suppression reaches 5% of the wild-type level. At 25 μg of G418 ml^{-1}, cell growth (as determined by the soluble protein levels in cell extracts) was 77% of that for untreated controls, and wild-type β-galactosidase-specific activity had declined to 73% of that for the no-drug plates (not shown). Substantial suppression of nonsense codons in the most sensitive contexts can therefore be achieved in this transient assay at only moderate inhibitions of cell growth. The pattern of 3′ codon context effects for G418-induced suppression, C > G = U = A, is similar to that for $tRNA^{Ser}$ UAG suppression (Fig. 3) and $tRNA^{Gln}$ UAG suppression (Fig. 4), although the aminoacyl-tRNA(s) involved in the misreading events is not known.

The 3′ codon context effect is the same in different mammalian cell lines. To establish whether the 3′ codon context effects we have demonstrated in this and previous studies are general phenomena of translation in mammalian cells or specific to the human 293 line, the pRSV559-tRNA series of reporter-suppressor vectors were transfected into two additional mammalian cell lines. Figure 6 compares the patterns of suppression in three mammalian cell lines: 293, MRC5V1, and COS-7. 293 is our standard human background, an adenovirus-transformed, embryonic kidney line (25). MRC5V1 is a simian virus 40-transformed human fetal lung fibroblast line (29). COS-7 cells are a simian virus 40-transformed African green monkey line (22). The levels of expression obtained from MRC5V1 and COS-7 cells were much lower than those from 293 cells, and

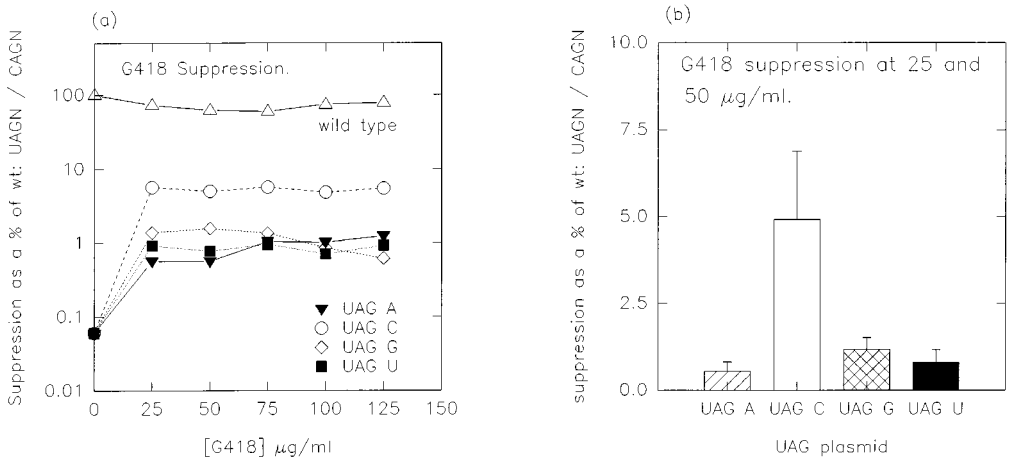

FIG. 5. Effect of 3′ context on the efficiency of G418-induced nonsense suppression. (a) Ten micrograms of the pRSV559 or pRSV600 vector was transfected onto 9-cm plates of human 293 cells. The $CaPO_4$-DNA precipitates were removed after overnight incubation and replaced with 10 ml of fresh growth medium that contained a range of G418 concentrations. Expression was permitted for 2 days. The attached cells were then harvested, and freeze-thaw extracts were prepared. β-Galactosidase specific activity was determined by standard ONPG and protein assays. At each G418 concentration the ratio of activity from a pRSV559 vector to its matched-for-context pRSV600 partner was calculated. The data are means for two independent experiments. A composite curve comprising all of the pRSV600 vectors shows the effect of G418 on wild-type specific activity with the no-drug samples assigned a value of 100%. Note the logarithmic scale. (b) To show the effects of 3′ codon context more clearly, the data from panel a at 25 and 50 μg of G418 ml^{-1} were averaged and plotted. The data are means and standard errors ($n = 4$).

consequently, the magnitude of suppression, which is determined by the level of the suppressor tRNAs, was not as great. However, the pattern of the effects of 3′ context in each of the cell lines suggests that the findings from this and previous studies on nonsense suppression in 293 cells reflect a fundamental property of the mammalian translational apparatus.

DISCUSSION

Nonsense suppression has been exploited and investigated for some time by researchers working with bacteria and yeasts (23, 44). The techniques and properties of nonsense suppressors have, however, only recently been applied to mammalian cells in culture (13, 14, 43, 50). Previously, we have communicated the finding that the base 3′ to UAG codons imparts a different pattern of context effects on the efficiency of a UAG suppressor tRNA when translation proceeds in *E. coli* compared with that which is observed when translation takes place in human cells (35, 36, 40). However, before more detailed investigations proceed along this pathway, it seems prudent to consolidate the initial discoveries, by conducting further experiments at a different mRNA location and with an additional suppressor. We have also been mindful to extend our analysis to include nonsense suppression events that occur under the influence of aminoglycoside drugs such as G418. The relationship between the pattern of nonsense suppression context effects in human cells and the observed frequencies of bases 3′ to natural and mutated stop codons has recently been discussed with regard to a possible use of nonsense suppressors as agents for gene therapy (1, 34).

Nonsense suppression was tested at a classical *lacZ* UAG codon, the YA559 allele in a pRSVgal reporter. The position of the mutation was mapped and sequenced, and a series of plasmids were produced with either A, C, G, or U as the immediate 3′ context. Immunological assays specific for the full-length β-galactosidase confirmed that ONPG assays were a good indicator of translation through this nonsense codon, although there was evidence that the ONPG assay slightly

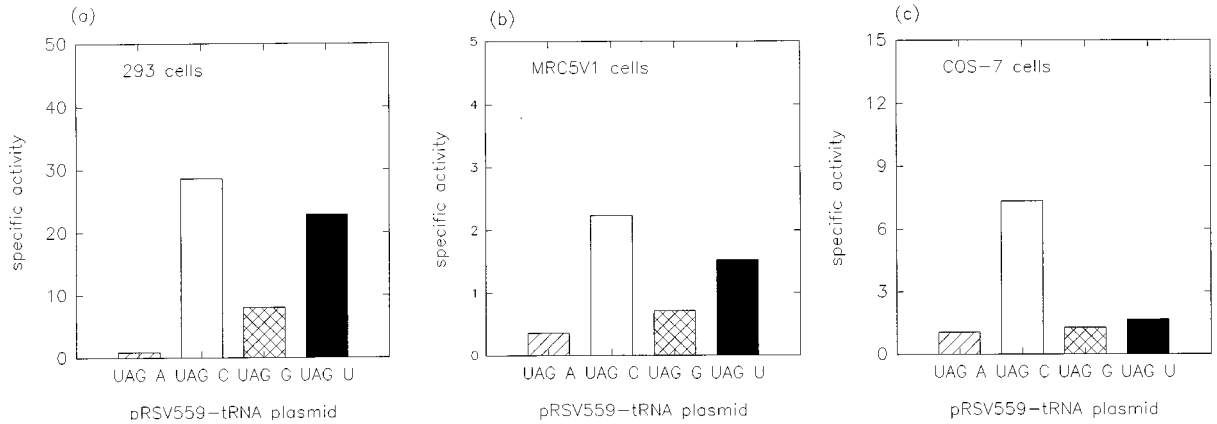

FIG. 6. 3′ codon context effects on nonsense suppression in different mammalian cells. Ten micrograms of the pRSV559-tRNA series containing the reporter and the $tRNA^{Ser}$ UAG suppressor was transfected onto 9-cm plates of 293 (a) ($n = 1$) or MRC5V1 cells (b) ($n = 3$) by using $CaPO_4$ and COS-7 cells (c) ($n = 2$) by using DOTAP. The mean β-galactosidase specific activity (against ONPG) was determined as described for Fig. 3 and in Materials and Methods.

underestimates the levels of the reporter with 3′ G. The efficiency of the $tRNA^{Ser}$ UAG suppressor displayed a C > G = U > A pattern according to 3′ context. This is slightly different from the pattern found for the same suppressor at another location in the N-terminal portion of the *lacZ* gene in pRSVgal (40). At this site suppression varied as C > G > U = A. Inspection of the bases surrounding these sites, CGC **UAG** NUC for YA559 and GUG **UAG** NGC for the N-terminal site, reveals that there are differences at the first 2 bases of the 5′ codon and the next (2nd) base of the 3′ codon. Influences on the codon context effect have been reported at both of these locations in *E. coli* (7, 48). The effects of codon context were also tested on different species of tRNA. When a $tRNA^{Gln}$ UAG suppressor was tested at the YA559 site, the pattern C > U > G = A was observed. In these experiments we have also been able to monitor the effect of 3′ context on nonsense suppression induced at the YA559 site by the error-promoting drug G418. The 3′ codon context effect on G418-induced read-through, C > G = U = A, bears a close resemblance to that observed for the $tRNA^{Ser}$ and the $tRNA^{Gln}$ suppressors. A general trend can now be identified. 3′ C is always the most efficiently suppressed context, and 3′ A is the least efficiently suppressed context. The relative efficiencies of 3′ G and 3′ U vary, though, from site to site and between the tRNA suppressors. This suggests that the codon context effect is largely a property of the translational machinery rather than an effect which is specific to the $tRNA^{Ser}$ suppressor or its posttranscriptional modifications (40). It appears likely that this pattern of context effects is an intrinsic characteristic of the mammalian protein synthetic apparatus. Similar patterns were observed in 293 cells; a human line, MRC5V1; and the simian line COS-7. The stimulation of read-through by 3′ C has also been observed for the leaky UGA codon in Sindbis virus transcripts translated in rabbit reticulocyte lysates (32).

In recent years it has become apparent that the level of an mRNA transcript can be severely reduced by the location of a premature translational termination codon (20). In our studies we have not undertaken an examination of the effect of untranslated nonsense codons on the abundance of *lacZ* mRNA. If translation through a termination codon relieves an effect on mRNA degradation, the action that a suppressor tRNA has on the level of expression from a nonsense codon-containing reporter will be a compound effect, comprising increased ribosome transits and increased numbers of mRNA templates. Nevertheless, as long as the 3′ codon context does not affect mRNA stability through a completely different mechanism, the pattern of context effects we have identified will remain an attribute of the mammalian translational machinery. While it might be expected that the absence of ribosomes from the downstream portion of a message punctuated by a termination codon would render it sensitive to endonucleolytic attack, investigations have indicated that nuclear abundance and the processing of introns can also be altered (4, 5, 17). In this regard we note that the pRSVgal construct used in our studies contains no introns but does contain a poly(A) addition site (26). The mechanism by which intranuclear control could be mediated is unclear, but one hypothesis suggests that at least a fraction of an mRNA population is subjected to translational "scanning" as it exits the nucleus (17).

This study has used site-directed mutagenesis to alter the 3′ context of a nonsense codon at amino acid position 600 in the β-galactosidase gene. Naturally, these changes introduce alterations in the downstream amino acid. While no effects on the overt enzymatic activity with the substrate ONPG were detected, evidence that the three-dimensional structure of the β-galactosidase is nevertheless affected was provided by the dramatically increased thermolability that accompanies the substitution of Phe by Ile and Val and, to a lesser extent, Leu. More interestingly, from the standpoint of the general utility of this approach to explore the effects of codon context on translational decoding, the variant β-galactosidases displayed substantially reduced catalytic efficiencies for the alternative enzyme substrate, FDG. This finding is not without precedence among site-directed variants of β-galactosidase. A number of missense substitutions at positions 461 (Glu) and 503 (Tyr) displayed differential activities with the substrates ONPG and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (18). We were able to confirm that our routine ONPG assays gave a realistic picture of the level of ribosome transits through the stop codon by validating the ONPG assay with an ELISA which was specific for the full-length β-galactosidase. Recently, the three-dimensional structure of *E. coli* β-galactosidase has been reported (30). Codon 600, at which we placed a UAG stop, is unconserved among homologous enzymes from other species and does not form part of the substrate binding site. Significantly, we showed by suppression that serine could replace glutamine without losing substantial amounts of enzyme activity. The 3′ context changes which were the focus of this study did, however, alter the next amino acid specified by codon 601. This position is an invariant Phe in six species of β-galactosidase, and the three-dimensional structure indicates that this residue forms part of the substrate binding pocket (30). It is unsurprising therefore that substitutions at this position changed the enzyme's activity towards one substrate (FDG) but not another (ONPG).

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