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Aminoglycoside antibiotics mediate context-dependent suppression of termination codons in a mammalian translation system

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ABSTRACT

The translation machinery recognizes codons that enter the ribosomal A site with remarkable accuracy to ensure that polypeptide synthesis proceeds with a minimum of errors. When a termination codon enters the A site of a eukaryotic ribosome, it is recognized by the release factor eRF1. It has been suggested that the recognition of translation termination signals in these organisms is not limited to a simple trinucleotide codon, but is instead recognized by an extended tetranucleotide termination signal comprised of the stop codon and the first nucleotide that follows. Interestingly, pharmacological agents such as aminoglycoside antibiotics can reduce the efficiency of translation termination by a mechanism that alters this ribosomal proofreading process. This leads to the misincorporation of an amino acid through the pairing of a near-cognate aminoacyl tRNA with the stop codon. To determine whether the sequence context surrounding a stop codon can influence aminoglycoside-mediated suppression of translation termination signals, we developed a series of readthrough constructs that contained different tetranucleotide termination signals, as well as differences in the three bases upstream and downstream of the stop codon. Our results demonstrate that the sequences surrounding a stop codon can play an important role in determining its susceptibility to suppression by aminoglycosides. Furthermore, these distal sequences were found to influence the level of suppression in remarkably distinct ways. These results suggest that the mRNA context influences the suppression of stop codons in response to subtle differences in the conformation of the ribosomal decoding site that result from aminoglycoside binding.

Keywords: aminoglycosides; mammals; readthrough; suppression; termination; translation

INTRODUCTION

In bacteria, translation termination occurs when a polypeptide chain release factor recognizes a termination codon located in the ribosomal A site and facilitates the release of the nascent polypeptide chain from the tRNA located in the ribosomal P site. Release factor 1 (RF1) facilitates polypeptide chain release at amber (UAG) and ochre (UAA) stop codons, whereas release factor 2 (RF2) mediates release at opal (UGA) and ochre (UAA) stop codons (Scolnick et al., 1968; Nakamura et al., 1996; Buckingham et al., 1997). A third release factor, RF3, is a GTPase that stimulates the activity of RF1 and RF2 (Milman et al., 1969; Freistroffer et al., 1997). In eukaryotes, the release factor eRF1 facilitates polypeptide chain release at all three translation termination codons, and the GTPase eRF3 modulates eRF1 function (Frolova et al., 1994; Stansfield et al., 1995; Zhouravleva et al., 1995).

Translation termination is normally a highly efficient process. Although the misincorporation of an amino acid at a stop codon (a process termed suppression) normally occurs at a frequency of only 10^{-4} in intact cells, the overall efficiency of this process can be influenced by a number of variables. Not only do different stop codons appear to facilitate the termination process with different efficiencies, but the efficiency of translation termination can also be influenced by the local sequence context surrounding the stop codon. A statistical analysis of the context surrounding the stop codons at the end of genes in several organisms revealed a bias toward certain nucleotides on either side of the termination codon. In all species examined, the strongest bias was found at the base immediately following

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the stop codon. This led to the proposal that the actual translation termination signal may consist of a tetranucleotide sequence (Brown et al., 1990, 1993; Poole et al., 1995). The importance of nucleotides immediately upstream and downstream of the stop codon in determining the efficiency of translation termination is clearly established in prokaryotes (Feinstein & Altman, 1977; Bossi & Roth, 1980; Pedersen & Curran, 1991; Kopelowitz et al., 1992; Moffat et al., 1994; Mottagui-Tabar et al., 1994). Studies of the context influence on the efficiency of translation termination in yeast cells indicated that upstream and downstream sequences act together to determine the overall efficiency of translation termination. In particular, the fourth base was found to be an important determinant for the efficiency of termination in yeast, but this effect varied as a function of the stop codon that was present (Fearon et al., 1994; Bonetti et al., 1995).

The importance of the local sequence context in determining the efficiency of translation termination in higher eukaryotes is less well understood. Much of our current knowledge of translation termination in mammalian cells was obtained from the study of the programmed suppression of stop codons in viral systems. For example, Maloney murine leukemia virus contains an in-frame UAG codon that mediates the extent of viral RNA polymerase production (Yoshinaka et al., 1985; Wills et al., 1991; Feng et al., 1992). In general, these viral systems provide examples of programmed readthrough, as they are facilitated by secondary structures within the mRNA that compete with the termination process at a single termination codon (Wills et al., 1991; Gesteland et al., 1992). In contrast, pharmacological agents such as aminoglycoside antibiotics can reduce the efficiency of translation termination by a more general mechanism that alters ribosomal proofreading. Recent studies have shown that premature stop mutations in the *CFTR* gene can be suppressed when human cells are treated with aminoglycoside antibiotics such as G418 or gentamicin, resulting in the restoration of CFTR expression and function (Howard et al., 1996; Bedwell et al., 1997). Another study found that gentamicin can restore the expression of full-length dystrophin in the *mdx* mouse, which carries a premature stop mutation in the dystrophin gene (Barton-Davis et al., 1999). Taken together, these studies provide compelling evidence that a pharmacological approach aimed at suppressing premature stop mutations may be applicable to common genetic diseases such as cystic fibrosis and muscular dystrophy.

Little is currently known about how the sequence context influences the susceptibility of stop codons to aminoglycoside-mediated suppression in mammalian cells. To address this question, we developed a reporter system that allowed us to examine the susceptibility of different termination codons in different contexts to suppression by aminoglycosides in a rabbit reticulocyte translation system. Our results demonstrate that the sequence context surrounding a stop codon plays an important role in determining its susceptibility to aminoglycoside-mediated suppression. This suggests that the sequence context within the mRNA influences how the translation apparatus responds to subtle changes mediated by aminoglycosides within the ribosomal decoding site.

RESULTS

We previously demonstrated that the aminoglycosides G418 and gentamicin can suppress premature stop mutations in the *CFTR* gene (Howard et al., 1996; Bedwell et al., 1997). These findings raised the possibility that this pharmacological approach could be used to treat many human diseases caused by premature stop mutations. However, because the sequence context has been shown to influence the efficiency of translation termination in various species, we wanted to determine whether the sequence context can also influence the ability of aminoglycosides to suppress stop codons in mammals. To do this, we constructed a reporter system that allowed us to accurately monitor the efficiency of translation termination in different sequence contexts using a rabbit reticulocyte translation system (Fig. 1). Translation of these constructs produced a polypeptide of 25 kDa if translation terminated at the stop codon located within the interchangeable "readthrough cassette." However, a longer product of 35 kDa was produced if the stop codon within the readthrough cassette was suppressed, allowing translation to continue to the end of the reporter gene. Quantitation of the relative abundance of the 25-kDa and 35-kDa products allowed us to accurately determine the efficiency of translation termination mediated by the stop codon and the flanking context contained within each readthrough cassette. Because these two products represent both the properly terminated and readthrough products, this system allowed us to accurately determine the absolute level of readthrough that occurred (see Materials and methods for further details).

Relative termination efficiencies as a function of the tetranucleotide termination signal

One of the problems encountered in past attempts to investigate the relative efficiencies of translation termination at different termination signals has been the difficulty encountered in quantitating the extremely low basal level of readthrough at stop codons. As an initial test of the sensitivity of the mammalian readthrough assay system we developed, we first asked whether we could detect differences in the efficiency of termination at each of the 12 possible tetranucleotide termination sequences using a standard coupled

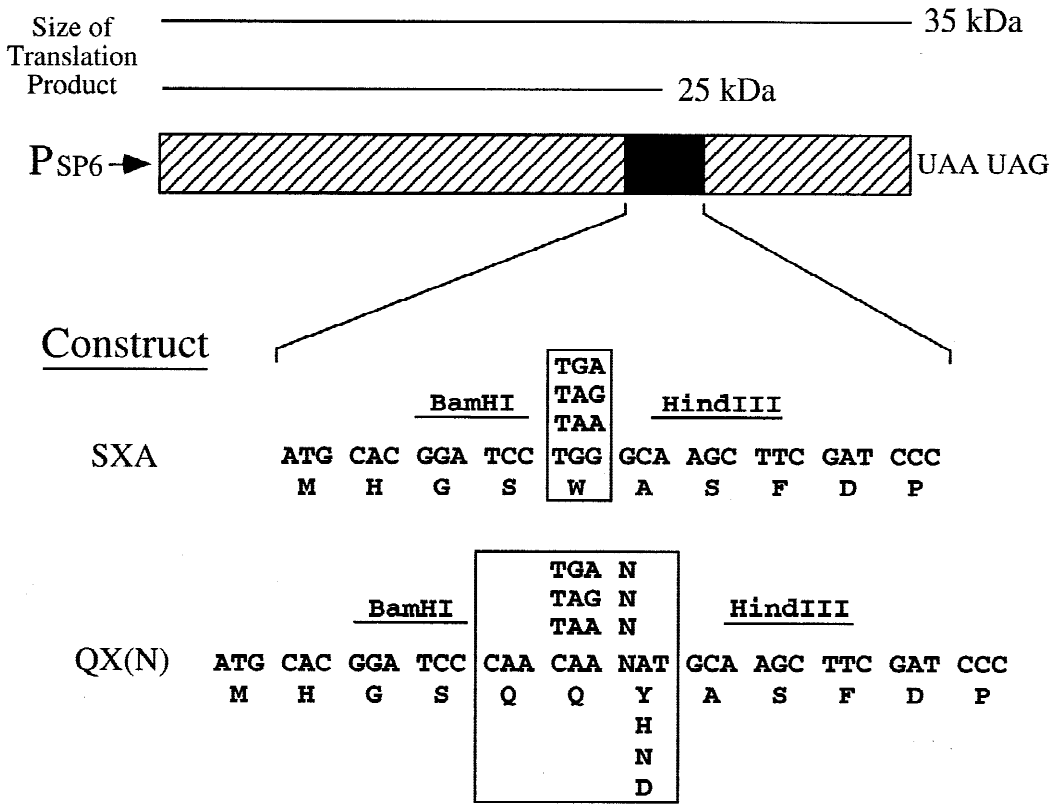

FIGURE 1. Readthrough constructs used in this study. The sequences that differ between the SXA and QX(N) constructs are boxed.

transcription-translation system. Although the absolute efficiency of termination varied as a function of the lot of reticulocyte lysate used and its concentration in the translation reaction, the relative efficiency of termination observed with different tetranucleotide sequences was generally reproducible (Fig. 2). We found that the tetranucleotide UGAC consistently mediated the least efficient termination, with readthrough occurring at a frequency of 3–4%. The tetranucleotide UAGU was the second least efficient tetranucleotide sequence, yielding readthrough at a frequency of 1–2%. The other UGA(N) and UAG(N) tetranucleotide sequences routinely exhibited a similar termination efficiency (~1% readthrough). Finally, the UAA(N) tetranucleotide sequences reproducibly mediated the most efficient termination, with ≤0.5% readthrough observed. These results suggest that the identity of the stop codon plays an important role in determining the efficiency of translation termination, with the rank order of efficiency being UAA > UAG ≥ UGA. The fourth position of the tetranucleotide also plays a role in determining the efficiency of termination, but its effect appears to be influenced to a significant extent by the stop codon present. For example, the UGAC termination signal routinely exhibited a three- to sixfold higher level of readthrough than the other UGA(N) signals. However, readthrough of the UAGC and UAAC signals was not significantly higher than the readthrough observed at the other UAG(N) and UAA(N) signals, respectively.

Dose-response curve for G418-mediated readthrough of a translation termination codon

Previous studies have shown that some aminoglycosides can suppress stop codons in cultured mammalian cells (Martin et al., 1989; Phillips-Jones et al., 1995; Howard et al., 1996; Bedwell et al., 1997). In those studies, it was necessary to expose the intact cells to a relatively high concentration of aminoglycoside (50–200 μg/mL) to facilitate a significant level of readthrough. However, due to the poor permeability of these charged compounds across the plasma membrane, the actual intracellular concentration required to facilitate readthrough during translation is unclear. To gain a better estimate of the intracellular concentration of G418 required to induce readthrough, we next determined how translation termination was affected in our readthrough assay system as increasing concentrations of the aminoglycoside antibiotic G418 were introduced into the translation reaction (Fig. 3). The reporter construct used for this initial titration was a member of the QX(N) set of constructs containing the tetranucleotide termination signal UGAC in the readthrough cassette. We

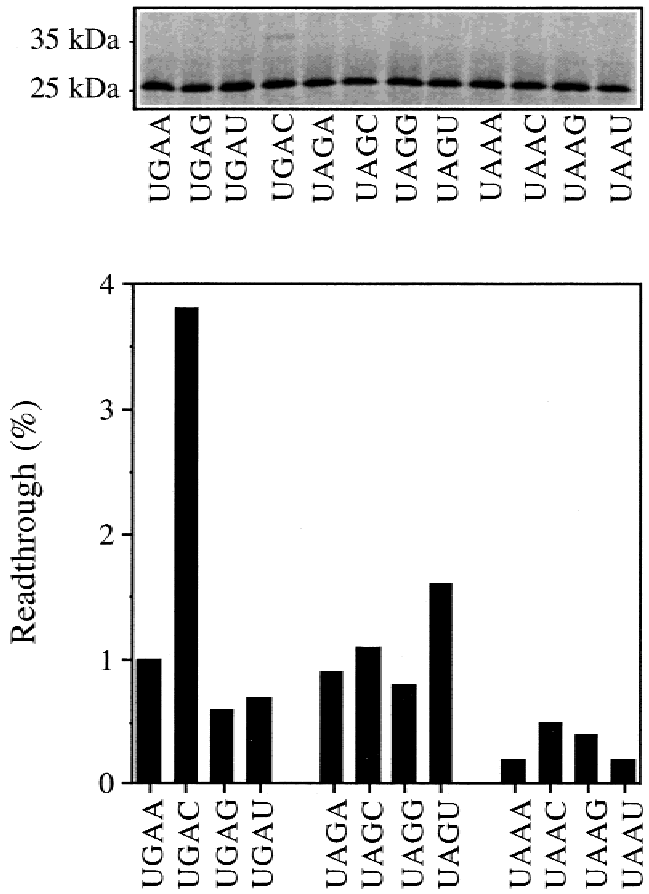

FIGURE 2. Basal suppression of tetranucleotide termination signals in the QX(N) context.

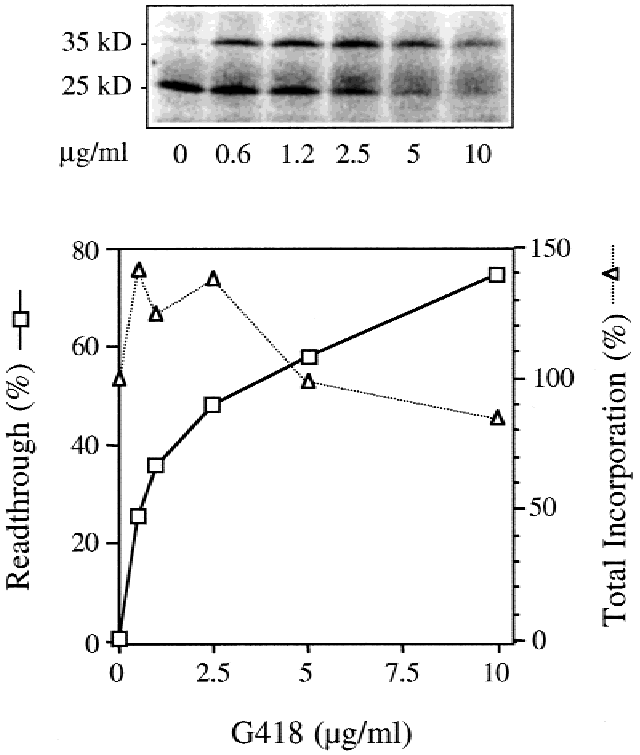

FIGURE 3. Titration of the G418-induced suppression of the UGAC tetranucleotide termination signal in the QX(N) context.

found that the total protein synthesis rate increased somewhat when low G418 concentrations were added (up to 2.5 μg/mL), and then decreased as the G418 concentration was increased further. The addition of G418 also caused a striking increase in the level of readthrough of the UGAC termination signal. At a concentration of 1.2 μg/mL G418, the frequency of readthrough was already at a level of 35–40%. Additional increases in the G418 concentration yielded less dramatic increases in readthrough. When the G418 concentration reached 10 μg/mL, 70–80% readthrough was observed. These results demonstrate that very low concentrations of G418 can produce a robust level of suppression of the UGAC tetranucleotide stop codon.

Influence of the tetranucleotide termination signal on G418-mediated suppression

We next carried out an analysis of G418-mediated suppression at each of the 12 tetranucleotide termination sequences in the QX(N) series of reporter constructs. Based on our initial titration of the UGAC construct with G418, we chose to examine the level of readthrough obtained with these reporter constructs in the presence of 0, 1.2, and 2.5 μg/mL G418. These antibiotic concentrations yielded near-maximal levels of readthrough while still allowing a minimal inhibition of protein synthesis. We found that these concentrations of G418 suppressed the UGA(N) tetranucleotide constructs at an efficiency of 25–60% (Fig. 4A). The relative order of susceptibility to readthrough as a function of the fourth base was C $>$ A, G $>$ U.

Readthrough levels for the UAG(N) tetranucleotide termination signals were generally less than was observed for the UGA(N) tetranucleotide signals (Fig. 4B). We observed readthrough at a level of 25–50% for these constructs, resulting in a rank order of suppression of U $>$ C, G $>$ A as a function of the fourth position of the UAG(N) tetranucleotide signals. This order was different than was observed for the UGA(N) constructs, indicating that the influence of the fourth base in determining the level of readthrough depends upon the stop codon within the tetranucleotide signal. Finally, examination of the UAA(N) tetranucleotide termination sequences revealed that these signals were the least susceptible to suppression by G418, with a maximal readthrough of only 6–18% (Fig. 4C). The relative order of susceptibility of the UAA(N) tetranucleotide signals as a function of the fourth position was C $>$ U $>$ G $>$ A. Overall, these results indicate that the fourth position of these tetranucleotide signals are only able

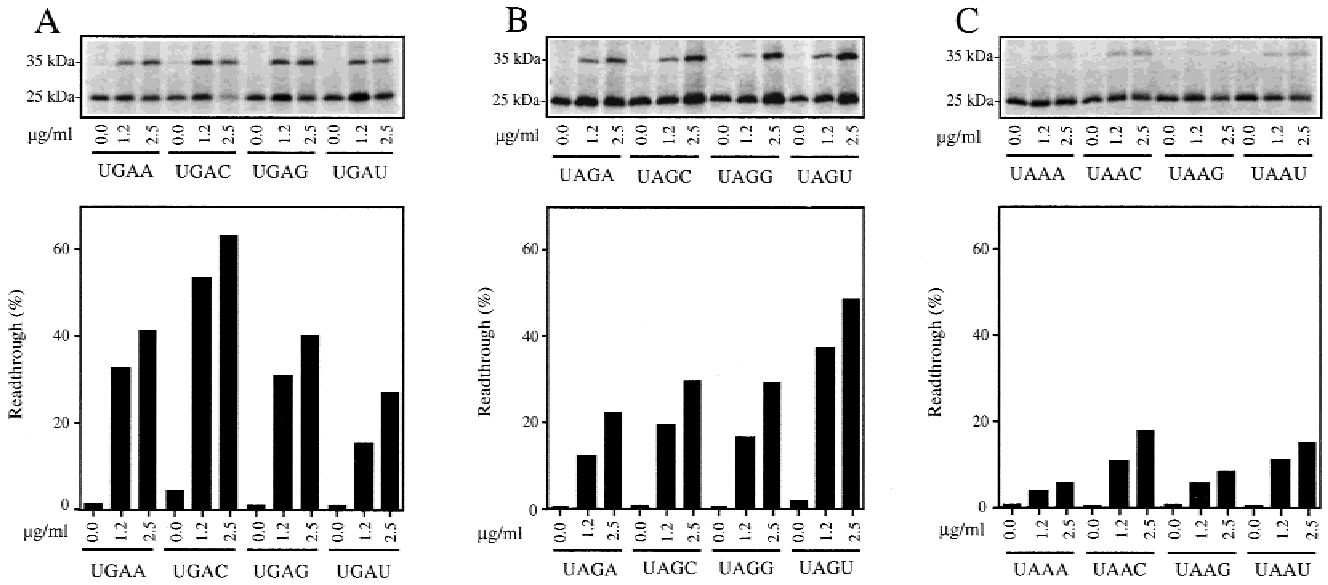

FIGURE 4. G418-induced suppression of tetranucleotide termination signals in the QX(N) context. Suppression was examined with the (**A**) UGA(N); (**B**) UAG(N); and (**C**) UAA(N) tetranucleotide termination signals.

to influence the level of readthrough by two- to threefold in this mammalian translation system.

The highest readthrough induced by G418 occurred with the UGAC and UAGU constructs, whereas G418-mediated readthrough increased the least with the UAAN constructs. This pattern of suppression was identical to the trends observed in the absence of G418, suggesting that the level of readthrough induced by G418 may simply be superimposed on the basal level of readthrough allowed by each construct. Overall, the susceptibility to G418-mediated readthrough ranged from 6% (UAAA) to 63% (UGAC). This 10-fold difference clearly demonstrates that the identity of the tetranucleotide termination signal plays a significant role in determining the overall susceptibility to G418-mediated readthrough.

We next wanted to determine whether residues beyond the tetranucleotide termination signal also influenced the efficiency of G418-mediated suppression. To do this, we examined the ability of G418 to mediate suppression in another set of reporter constructs. The design of the SXA constructs was very similar to the QX(N) constructs, except the upstream CAA codon and the downstream (N)AT codon have been removed (see Fig. 1). This moved a TCC (serine) codon into position directly upstream and a GCA (alanine) codon immediately downstream of the stop codon. Thus, a G was located in the fourth position of each of the three possible tetranucleotide termination signals in these constructs. When G418 was introduced into translation reactions programmed with these SXA templates, we found that readthrough of the UAAG and UAGG termination sequences was 1.4- to 3-fold less than was observed with the same tetranucleotide signals in the QX(N) constructs (Fig. 5). In contrast, the UGAG signal in the SXA context yielded 1.2-fold more readthrough than was observed with the same tetranucleotide signal in the QX(N) vector. These results indicate that the sequence context beyond the tetranucleotide termination signal can also play a significant role in determining the susceptibility of mammalian termination signals to G418-induced suppression.

Analysis of the ability of other aminoglycosides to suppress translation termination signals

Aminoglycosides are commonly used to treat bacterial infections, and a large number of natural and synthetic members of this family have been developed over the past few decades. Because we were interested in determining whether different aminoglycosides can induce distinct levels of readthrough in these sequence contexts, we next examined several other aminoglycosides in our readthrough assay. There are two predominant classes of aminoglycosides: those with a 4,6 disubstituted deoxystreptamine ring (Fig. 6A) and those with a 4,5 disubstituted deoxystreptamine ring (Fig. 6B). G418 is a member of the 4,6 disubstituted class. A close relative of G418 within this class is gentamicin, which is commercially available as a mixture of three forms (gentamicin C1a, gentamicin C1, and gentamicin C2) that differ from each other by differences in constituent groups attached to carbon 6 on ring 1. We found that gentamicin promoted a significant level of readthrough with each of the QX(N) constructs, although at a level that was 2–14-fold lower than the level mediated by G418 (Table 1). In spite of the lower

maximal level of readthrough induced by gentamicin, the overall pattern of suppression was also similar to the pattern observed with G418 (Table 2).

We also measured the readthrough induced by gentamicin in the SXA constructs. Surprisingly, gentamicin did not increase the level of readthrough significantly above background in the UAGG or UAAG constructs, whereas it increased the basal level of readthrough by 13-fold in the UGAG construct. These results are strikingly different from the results obtained in the QX(N) constructs. Because the SXA and the corresponding QX(N) constructs differ only by the three bases upstream and the two bases downstream of the tetranucleotide termination signal, our results indicate that these flanking residues play a critical role in determining the susceptibility of each of the termination signals to suppression by these structurally similar aminoglycosides.

We also examined sisomycin, another member of the 4,6 disubstituted class. We found that a significantly higher concentration of this compound (80 μg/mL) was required to inhibit the translation system, suggesting that it may bind to the ribosome less efficiently than G418 or gentamicin. When tested at a concentration of 40 μg/mL with the QX(N) constructs, sisomycin induced two- to sixfold less readthrough than gentamicin in the UGAN and UAGN groups, and stimulated little or no readthrough in the UAAN group.

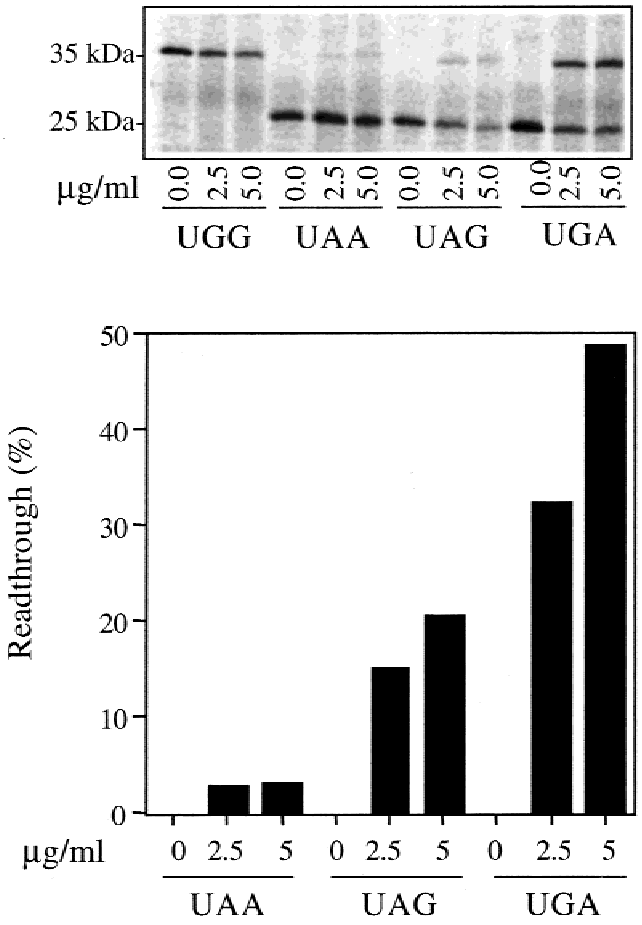

FIGURE 5. G418-induced suppression of tetranucleotide termination signals in the SXA context.

Finally, we found that four other compounds in the 4,6 disubstituted class (kanamycin A, kanamycin B, tobramycin, and amikacin) were unable to induce a significant level of readthrough in the readthrough constructs tested. Interestingly, these four compounds share a common ring 3 that is not found on any other members of the 4,6 class that were tested. This suggests that some feature of the ring 3 present in these compounds may prevent them from inducing readthrough in the mammalian translation system.

Members of the 4,5 disubstituted class of aminoglycosides differ from the 4,6 disubstituted class in two ways. First, they contain a distinct linkage between ring 2 (2-deoxystreptamine) and ring 3. They also frequently consist of four (or more) rings, rather than the three rings found in members of the 4,6 disubstituted class. To determine how these differences affect mammalian translation termination, we next examined the readthrough mediated by these compounds in our reporter constructs. After determining that ~40 μg/mL was the maximum concentration of the compounds of this class that could be used without inhibiting the translation system, we examined the effect of paromomycin on translation termination. When paromomycin was introduced into translation reactions containing the QX(N) series of constructs, we found that this compound generally induced readthrough somewhat less efficiently than gentamicin (Table 1). The relative order of susceptibility to readthrough as a function of the fourth base of the UGAN tetranucleotide sequences was also similar to gentamicin: C > A ≥ G, U.

When we examined paromomycin-induced readthrough with the UAGN group of the QX(N) series, we found that readthrough at the UAGA and UAGC tetranucleotide signals was again similar to that observed with gentamicin, but the UAGG and UAGU tetranucleotide termination signals were both suppressed approximately twofold less efficiently. Finally, the suppression of the UAAN tetranucleotide signals by paromomycin was very low, with maximal levels of readthrough only 2–10-fold above background. The rank order of suppression induced by paromomycin with both the UAGN and UAAN constructs as a function of the fourth base was significantly different than was observed with gentamicin (Table 2). In contrast, we found that paromomycin induced a much higher level of readthrough than gentamicin at all three termination signals in the SXA reporter constructs, but these levels were still two- to threefold less than the readthrough induced by G418. These results indicate that this member of the 4,5 disubstituted class of aminoglycosides can induce a significantly different pattern of suppression than was

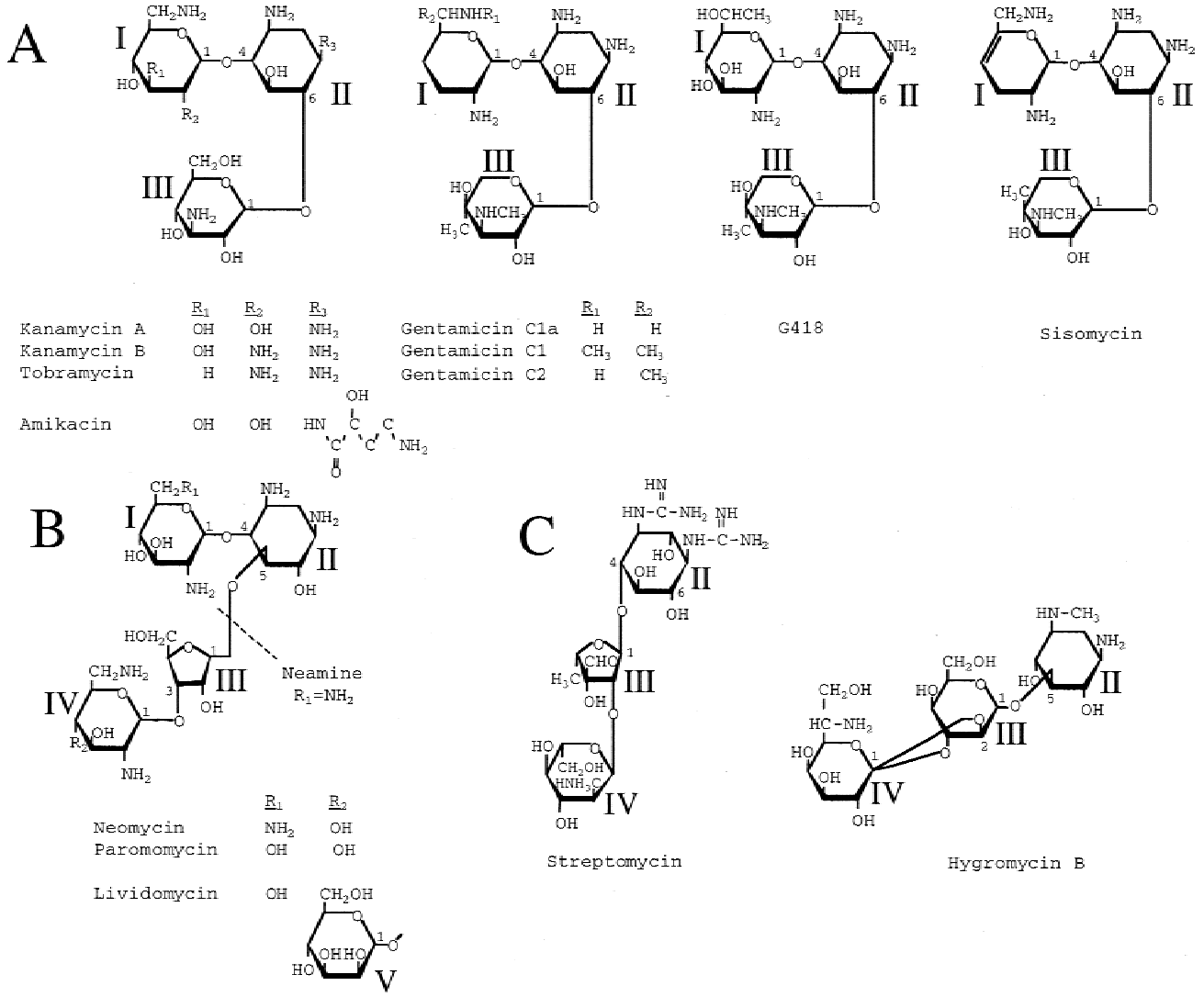

FIGURE 6. Structures of the aminoglycosides used in this study. For consistency, the 2-deoxystreptamine ring is labeled ring II in each structure. **A**: 4,6 disubstituted class. **B**: 4,5 disubstituted class. **C**: other aminoglycosides.

observed with members of the 4,6 disubstituted class, and these differences occur in a context-dependent manner.

Another member of the 4,5 disubstituted class of aminoglycosides is neomycin (Fig. 6B). The levels of readthrough mediated by neomycin at the UGAN and UAGN signals of the QX(N) constructs were generally less than those observed with paromomycin, although readthrough of the UAGG signal was found to be ~2.5-fold higher. Readthrough of all of the UAAN constructs was very low, with only the UAAC and UAAU constructs providing more readthrough than was observed in the absence of drug. Overall, neomycin was one of the least effective compounds at mediating suppression (other than the kanamycin group).

The third member of the 4,5 disubstituted class of aminoglycosides tested was lividomycin. This compound, while identical to paromomycin in rings 1–4, contains a fifth ring that extends the structure more than most other members of this class. When lividomycin was introduced into translation reactions programmed with the QX(N) constructs, we found that the levels of readthrough in most cases were similar to paromomycin. However, the presence of the additional ring was found to produce a significant increase in the overall level of readthrough with certain tetranucleotide signals, particularly the UAGN constructs in the QX(N) series. We also examined the ability of lividomycin to induce readthrough in the SXA constructs, and found the effects of this compound to be generally similar to those observed with paromomycin.

Finally, we also tested three more distant members of the aminoglycoside family. Neamine, which consists solely of rings 1 and 2 of neomycin (Fig. 6B), was unable to induce a significant level of readthrough of the UGAC signal from the QX(N) constructs (the only con-

TABLE 1. Comparison of readthrough induced by aminoglycosides.

			Percent readthrough (fold-stimulation above background)						
				4,6-Disubstituted class			4,5-Disubstituted class		
Template	Construct	Termination signal	No drug	G418[a]	Gentamicin[a]	Sisomycin[b]	Paromomycin[b]	Lividomycin[b]	Neomycin[b]
pDB589	SXA	UGAG	0.3	48.8 (163×)	3.9 (13×)	ND[c]	14.2 (47×)	9.2 (31×)	ND
pDB588	SXA	UAGG	0.2	20.6 (103×)	0.3 (1.5×)	ND	7.2 (36×)	9.9 (50×)	ND
PDB587	SXA	UAAG	<0.2	3.1 (16×)	0.2 (1×)	ND	1.9 (9.5×)	0.4 (2×)	ND
pDB602	QX(N)	UGAA	1.0	41 (41×)	16.8 (17×)	6.8 (6.8×)	12.2 (12×)	15.4 (15×)	10.6 (11×)
pDB603	QX(N)	UGAC	3.8	63.2 (17×)	29.8 (7.8×)	14.2 (3.7×)	28.5 (7.5×)	28.3 (7.4×)	19 (5×)
pDB604	QX(N)	UGAG	0.6	40.1 (67×)	17.2 (29×)	2.8 (4.7×)	10.1 (17×)	15.4 (26×)	9.6 (16×)
pDB605	QX(N)	UGAU	0.7	27 (39×)	9.2 (13×)	1.9 (2.7×)	9.5 (13.6×)	18.6 (27×)	11.4 (16×)
pDB598	QX(N)	UAGA	0.9	22.1 (25×)	4 (4.4×)	1.5 (1.7×)	4.2 (4.7×)	17.4 (19×)	2.3 (2.6×)
pDB599	QX(N)	UAGC	1.1	29.5 (27×)	8.1 (7.4×)	2 (1.8×)	7.3 (6.6×)	20 (18×)	3.2 (2.9×)
pDB600	QX(N)	UAGG	0.8	29.4 (37×)	7.4 (9.3×)	1.7 (2.1×)	3.9 (4.9×)	28.6 (36×)	9.6 (12×)
pDB601	QX(N)	UAGU	1.6	48.6 (30×)	17.6 (11×)	8.1 (5×)	3.7 (2.3×)	10.9 (6.8×)	3.5 (2.2×)
pDB594	QX(N)	UAAA	0.2	5.9 (30×)	1 (5×)	0.2 (1×)	0.8 (4×)	4.5 (23×)	<0.2 (1×)
pDB595	QX(N)	UAAC	0.5	18.1 (36×)	3.2 (6.4×)	<0.2 (<1×)	1.2 (2.4×)	3.3 (6.6×)	1.2 (2.4×)
pDB596	QX(N)	UAAG	0.4	8.5 (21×)	1.9 (4.8×)	<0.2 (<1×)	2.1 (5.3×)	1.8 (4.5×)	<0.2 (<1×)
pDB597	QX(N)	UAAU	0.2	15.1 (76×)	1.1 (5.5×)	1 (5×)	2 (10×)	3.7 (19×)	2.1 (11×)

[a]Measured at 5 μg/mL final concentration.
[b]Measured at 40 μg/mL final concentration.
[c]ND: not determined.

struct tested). Finally, both streptomycin and hygromycin B lack the equivalent of ring 1 found in the 4,5 and 4,6 disubstituted compounds (Fig. 6C). Streptomycin was able to increase readthrough only slightly in the UGAC signal of the QX(N) constructs when present at a concentration of 80 μg/mL. Hygromycin B inhibited the overall level of translation at much lower concentrations than any other aminoglycosides tested, with complete inhibition occurring at concentrations of 0.1–0.5 μg/mL. Under the highest concentrations that still supported translation, little or no readthrough could be detected. These results indicate that rings 1 and 2, as well as additional structural elements, are required to mediate the suppression of stop codons in this mammalian translation system.

DISCUSSION

The results of this study are most easily interpreted in the context of the function of the ribosomal decoding site. In *Escherichia coli*, a small portion of helix 44 of the 16S rRNA carries out critical functions associated with aminoacyl-tRNA recognition and proofreading at the ribosomal A site (Fig. 7). Recent kinetic studies of the initial selection and proofreading of aminoacyl tRNA on the ribosome suggest that a cognate codon-anticodon interaction induces a conformational change in the decoding site of the 16S rRNA much more efficiently than a near-cognate codon (Pape et al., 1999). It was also suggested that near-cognate codon recognition in the presence of aminoglycosides promotes the

TABLE 2. Effects of fourth base on suppression.

	UGAN		UAGN		UAAN	
Condition	Relative suppression	Range	Relative suppression	Range	Relative suppression	Range
No drug	C > A > G, U	Sixfold	U > A, C, G	Twofold	C, G > A, U	Twofold
G418	C > A, G > U	Twofold	U > C, G > A	Twofold	C > U > G > A	Threefold
Gentamicin	C > A, G > U	Threefold	U > C, G > A	Fourfold	C > G > A, U	Threefold
Sisomycin	C > A > G > U	Sevenfold	U > C > A, G	Fivefold	U > A, C, G	Fivefold
Paromomycin	C > A ≥ G, U	Threefold	C > A, G, U	Twofold	G, U > A, C	Twofold
Neomycin	C > A, G, U	Twofold	G > C, U > A	Fourfold	U > C > A, G	Tenfold
Lividomycin	C > U > A, G	Twofold	G > C > A > U	Threefold	A > U > C > G	Threefold

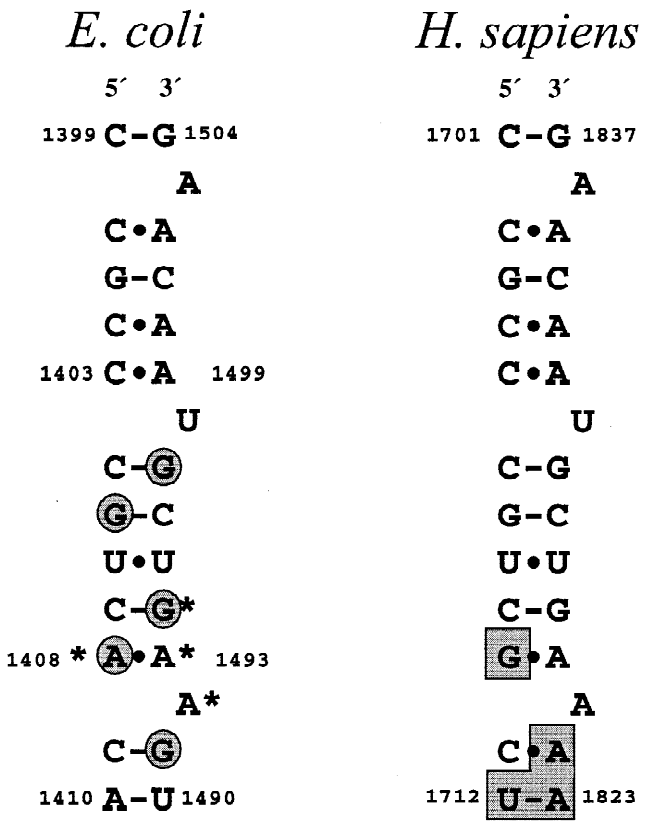

FIGURE 7. Comparison of the decoding sites within the *E. coli* 16S rRNA and the human 18S rRNA. Residues that differ between these two organisms are boxed, and residues in a model *E. coli* decoding stem RNA protected from DMS modification by paromomycin are circled (Yoshizawa et al., 1998). Residues in the *E. coli* structure protected from DMS modification by mRNA in a model decoding structure and also protected by an mRNA-dependent interaction of tRNA with the A site of ribosomes are marked by asterisks (Purohit & Stern, 1994).

transition of the 16S rRNA decoding center from a tRNA binding conformation to a productive state that could increase the rate of readthrough at a stop codon (Pape et al., 2000). The decoding center on the ribosome includes not only the decoding region on helix 44, but also other rRNA segments such as helix 27 of the 16S rRNA (Lodmell & Dahlberg, 1997). These distant segments of the 16S rRNA are in close proximity in the overall three-dimensional structure of the ribosome (Cate et al., 1999).

Recent studies have provided solution structures of both gentamicin C1a and paromomycin in complex with a model RNA derived from the bacterial decoding site (Fourmy et al., 1996; Yoshizawa et al., 1998). These structures reveal both similarities and differences in how these two compounds bind to the decoding site. In both structures, the decoding site RNA is composed of two continuous A-form helical stems that are separated by an asymmetric, internal loop caused by a distortion resulting from an unpaired nucleotide (A1492) and the noncanonical A1408–A1493 pair (Fig. 7). Both paromomycin and gentamicin were found to bind in the major groove within a pocket formed by this internal loop. The decoding stem within the corresponding region of the mammalian 18S rRNA is surprisingly similar to the bacterial decoding stem, with the exception of a critical A1408 → G substitution that lowers the affinity of aminoglycoside binding to the mammalian structure (Recht et al., 1996). In a recent study, the normal *E. coli* A1408 16S rRNA was replaced by a 16S rRNA carrying an A1408 → G substitution (Recht et al., 1999). This single-nucleotide change led to a reduced affinity for aminoglycoside binding, suggesting that this difference in the decoding site plays a key role in determining the prokaryotic specificity for aminoglycoside binding, as well as the predominantly antibacterial mode of action of these compounds. It was found that the G1408 change confers the greatest resistance to aminoglycosides with an amino group on carbon 6 of ring 1 and a hydroxyl group on carbon 2 of ring 1 (such as kanamycin A and amikacin). In particular, it was suggested that the amino group on carbon 6 of ring 1 functions as a key determinant for resistance in the context of the G1408 substitution. Consistent with this conclusion, we found that kanamycin A and amikacin were unable to promote readthrough in any of the constructs used in our mammalian assay system. We also found that kanamycin B and tobramycin (which have amino groups on carbons 2 and 6 of ring 1) were also unable to promote readthrough in our system. However, this lack of activity associated with aminoglycosides that contain an amino group on position 6 of ring 1 is not absolute, as both sisomycin and neomycin could induce readthrough in constructs containing the UGA(N) and UAG(N) termination signals. Because the four inactive compounds described above share a common ring 3 that is not found in any of the active compounds, a feature of this ring (such as the presence of carbon 6 and/or the attached hydroxyl group) may also inhibit aminoglycoside binding and/or function.

One of the most surprising findings of our study was the complex effects observed in the relative level of readthrough when only the fourth base of the tetranucleotide termination signal was changed (Table 2). In assays with the UGAN series of constructs, we found that C in position 4 always led to the most readthrough. This trend was observed in the basal readthrough of the in vitro translation system, and in readthrough induced by members of the 4,6 or 4,5 disubstituted classes of aminoglycosides. In most cases, readthrough was lowest when U was present in the fourth position of the UGAN constructs. The only exception to this trend was with lividomycin, where A and G in position 4 allowed the lowest level of readthrough. When assays were done with the UAGN constructs, we found that the 4,6 disubstituted class of aminoglycosides was again similar to the uninduced level of readthrough, with U me-

diating the most readthrough followed by C. Nucleotides A or G induced the least readthrough with these compounds. However, this trend was not maintained when we examined the level of readthrough induced by the 4,5 disubstituted classes of aminoglycosides in the UAGN constructs. Finally, a distinct trend in the rank order of suppression was mediated by almost every aminoglycoside in the UAAN constructs. These results again indicate that different aminoglycosides influence the decoding site (and consequently, the proofreading step that discriminates between cognate and near-cognate aminoacyl tRNAs within the A site) in subtly different but important ways. We conclude from these results that a simple pattern of suppression that can be attributed solely to the fourth position of the tetranucleotide signal does not exist.

Our results also demonstrate that the sequence context beyond the tetranucleotide termination signal can influence the level of readthrough induced by several aminoglycosides. Because both gentamicin and paromomycin interact specifically with the decoding site RNA, this suggests that these flanking sequences influence how the mRNA interacts with one or more components of the termination apparatus. This could include an interaction between the mRNA and the ribosome. This model is consistent with the recent finding that residues A1492 and A1493 of the *E. coli* decoding stem make a direct contact with the mRNA backbone that may mediate a role in discriminating codon-anticodon interactions (Yoshizawa et al., 1999). Alternatively, the sequence context could mediate its effect through an interaction between the mRNA and eRF1. This possibility is consistent with the recent finding that the recognition of a UGA stop codon by bacterial RF2 can be influenced by the 3 nt distal to the stop codon (Poole et al., 1998). Overall, our study indicates that the aminoglycoside bound to the decoding stem, the tetranucleotide termination signal, and the sequence context within the mRNA beyond the tetranucleotide signal all play important roles in determining the susceptibility of a stop codon to suppression.

We also compared our findings to results previously obtained using a defined polypeptide chain release assay (McCaughan et al., 1995). That study used synthetic tetranucleotide RNA molecules to program release of a model peptide (N-formylmethionine), thus avoiding the effects of more distal sequences within the mRNA. As we observed in our complete translation system, their study found that the tetranucleotide termination signals UGAC and UAGU were relatively inefficient at mediating peptide release, whereas the UAAA and UAAG signals promoted efficient peptide release. However, their study made the more general conclusion that a pyrimidine in the fourth position of the tetranucleotide signal consistently resulted in less efficient termination than a purine residue, which is not consistent with the findings of our study. However, there were several differences in these studies that could account for these different conclusions. For example, the release assay measured the rate at which the nascent chain is released in the absence of any possible competition between eRF1 and near-cognate aminoacyl-tRNAs for occupancy of the A site. In contrast, the intact translation system used in our study illustrates the balance that is normally maintained between polypeptide chain release and the suppression of the termination signal. In addition, our study introduced two additional levels of complexity that were not examined in the study using the release assay. First, our assays used constructs that encoded more distal flanking sequences within the mRNA. It is clear from our results that changes in the sequence context beyond the tetranucleotide termination signal can greatly influence the susceptibility of a signal to suppression. Second, our study also examined how different aminoglycosides can influence the suppression of termination signals. Our results indicate that structurally similar aminoglycosides can produce very distinct patterns of suppression as a function of the last nucleotide of the tetranucleotide termination signal. Finally, it is important to note that other translation factors present in the complete translation system may also influence the overall efficiency of translation termination. For example, one or more components of the surveillance complex (which includes Upf1p, Upf2p, and Upf3p) may mediate important effects on termination, because the loss of any of these proteins results in a nonsense suppressor phenotype (Czaplinski et al., 1999). Consistent with this possibility, Upf1p has been shown to interact directly with both eRF1 and eRF3 (Czaplinski et al., 1998).

Premature stop mutations were first considered as potential therapeutic targets when it became apparent that the molecular defects associated with many human diseases included this class of mutation (Atkinson & Martin, 1994). The first demonstration that aminoglycosides could suppress premature stop mutations in a defective human gene was carried out with a cystic fibrosis model system. It was shown that four premature stop mutations found in the *CFTR* gene (G542X, R553X, R1162X, and W1282X) could be suppressed by G418 and gentamicin, as measured by the appearance of full-length, functional CFTR (Howard et al., 1996; Bedwell et al., 1997). These four CFTR premature stop mutations were UGAG or UGAA tetranucleotide termination signals, which were both found to be moderately responsive to suppression by G418 (~40%) and gentamicin (17%) in the current study.

More recently, it was shown that gentamicin administration could also restore dystrophin expression in a mouse model that contained a premature stop mutation (with the UAAA tetranucleotide) in the *mdx* gene (Barton-Davis et al., 1999). The *mdx* mouse serves as a model for Duchenne muscular dystrophy in humans.

The finding that expression of the dystrophin gene could be restored by gentamicin was somewhat surprising, as this animal contained a UAAA tetranucleotide that is very poorly responsive in our in vitro translation system (only ~1% maximal readthrough). This indicates that much more remains to be learned about the susceptibility of stop mutations to aminoglycoside-mediated suppression in mammalian cells.

MATERIALS AND METHODS

Construction of DNA templates for readthrough assays

The plasmid-based reporter constructs used in this study contain three basic elements under SP6 promoter control. The first is an open reading frame (ORF) derived from the 5′ end of the *PGM* cDNA encoding rat phosphoglucomutase (Rivera et al., 1993). This partial cDNA encodes a 25-kDa polypeptide. This sequence is followed by an interchangeable readthrough cassette that contains different stop codons and surrounding sequences that were designed to allow the systematic analysis of the influence of sequence context on translation termination. A similar series of cassettes was previously used to examine the effects of various sequence contexts on the efficiency of translation termination in yeast (Fearon et al., 1994; Bonetti et al., 1995). Finally, another small ORF (encoding the 10-kDa alpha complementation region of β-galactosidase) follows the readthrough cassette. This last ORF was terminated by tandem, in-frame stop codons (UAA UAG). The context and stop codon present in each of the readthrough cassettes used in this study is described in Figure 1 and Table 1.

The first set of constructs, called the SXA constructs, contained one of the three stop codons (TAG, TGA, or TAA) or a tryptophan codon (TGG) at position five of the readthrough cassette. Flanking these codons was an upstream TCC (serine) codon and a downstream GCA (alanine) codon. The second set of constructs, called the QX(N) constructs, was designed to test how each of the possible tetranucleotide termination sequences facilitated translation termination. These constructs contained one of the three stop codons or a CAA (glutamine) codon at position six of the cassette. The flanking upstream codon in the QX(N) constructs is also a CAA (glutamine) codon, and the downstream sequence was (N)AT (where N is A, C, G, or T). As a result, the downstream codon alternately encoded tyrosine, histidine, asparagine, or aspartate. This configuration allowed us to examine every possible tetranucleotide context in the readthrough assay system. Other than these differences in a single codon upstream and downstream of the stop codon, the SXA and QX(N) constructs were identical. The plasmid numbers of each of the SXA and QX(N) constructs that contained stop codons in the readthrough cassette are listed in Table 1. The control SXA plasmid containing the TGG sense codon was pDB606, and the control QX(N) plasmids containing the CAA sense codons in the readthrough cassette were pDB590 (CAAA); pDB591 (CAAC); pDB593 (CAAG); and pDB592 (CAAT).

In vitro translation reactions and quantitation of readthrough

A DNA-dependent, SP6 transcription/rabbit reticulocyte translation system was used for protein synthesis. Each translation reaction (12.63 μL, total volume) contained 6.25 μL nuclease-treated reticulocyte lysate (Promega); 0.63 μL 20× reaction buffer; 2.5 μL rNTPs; 0.25 μL amino acids (minus methionine); 1.0 μL [^{35}S]-methionine/cysteine (NEN Life Sciences); 0.25 μL RNasin (Promega); 0.25 μL SP6 RNA polymerase (Promega); 0.5 μL water or aminoglycoside; and 1.0 μL DNA template (from a 1 mg/mL stock). The reaction was initiated by adding a mixture containing all other components to a tube containing the DNA template. The reaction was then incubated at 30 °C for 2 h. The [^{35}S]-labeled polypeptides resulting from these reactions were separated by electrophoresis using 12.5% SDS-PAGE. The radioactivity in each band was then quantitated by PhosphorImager analysis (Molecular Dynamics). The background counts were deducted from each band prior to calculations. The value "percent readthrough" was determined as follows: the quantity of 35-kDa product divided by the total of both products (25 kDa + 35 kDa) × 100. Because all methionine and cysteine codons are located upstream of the readthrough cassette, the 25-kDa and 35-kDa products contain the same number of labeled amino acids and correction for differences in the number of labeled amino acids in each translation product was not required.

NOTE ADDED IN PROOF

More recent experiments suggest that at least one member of the kanamycin class (tobramycin) can induce readthrough in certain contexts in the mammalian translation system.

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