

Therapies of Nonsense-Associated Diseases

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Abstract

A large number of diseases are caused by premature stop mutations that often lead to a complete loss of protein function and a severe reduction in mRNA levels due to nonsense-mediated mRNA decay (NMD). Two main approaches to develop treatments for diseases caused by premature stop mutations have been investigated. The first is to reduce the efficiency of translation termination through the use of pharmacological agents or by the expression of suppressor tRNAs. The second approach is to replace the premature stop mutation with wild-type sequence using gene repair techniques. Although each of these approaches have been demonstrated using in vitro studies or mouse models, currently only strategies using pharmacological agents to suppress stop mutations have reached preliminary clinical trials. The future of suppression therapy will require finding ways to increase the efficacy of current compounds to suppress premature stop mutations without side effects, or to design or discover safe new compounds that suppress premature stop mutations with increased efficiency. In addition, combined therapies that simultaneously suppress a premature stop mutation and inhibit NMD of the nonsense-containing mRNA may be the most effective way to increase the efficiency of suppression therapy.

Introduction

A large number of diseases including cystic fibrosis, Duchenne muscular dystrophy, β -thalassemia, and many types of cancers are caused by the presence of premature stop mutations in mRNAs. Premature stop mutations can arise as a result of mutations within germline or somatic DNA, inaccurate or inefficient pre-mRNA splicing, or improper RNA editing. According to the Human Gene Mutation Database, 12% of all mutations reported are single point mutations that result in a premature stop codon.¹ If mutations that alter the translational reading frame such as deletions, insertions, and splicing mutations are also considered, premature stop mutations may be responsible for as many as one-third of all inherited genetic disorders or cancers.² Furthermore, the disease phenotypes caused by premature stop mutations are frequently more severe than those that result from missense mutations, since premature stop mutations often result in a complete loss of protein function.

Suppression of Premature Stop Mutations

One approach to treat diseases that result from in-frame premature stop mutations is to reduce the efficiency of translation termination so production of some full-length, functional protein is restored. Translation termination in eukaryotic cells occurs when one of the three stop codons, UAA (ochre), UAG (amber), or UGA (opal), enters the ribosomal A site. Stop codon recognition is not carried out by codon-anticodon interactions since no tRNA anticodons are

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complementary to any of the stop codons. Rather, stop codon recognition is mediated by a protein known as eukaryotic release factor 1 (eRF1). eRF1 recognizes each of the three stop codons in the ribosomal A site,^{3,4} and RNA cross-linking studies suggest that this interaction is direct.⁵ Upon recognition of a stop codon, eRF1 transmits a signal to the ribosomal peptidyl transferase center that leads to release of the nascent polypeptide from the peptidyl-tRNA located in the ribosomal P site.⁶ Another eukaryotic release factor, eRF3, is a GTPase that binds eRF1⁷ and facilitates the efficiency and accuracy of stop codon recognition.⁸

Under normal conditions, translation termination is a very efficient process with an estimated error rate of approximately 0.1%.⁹⁻¹² However, eRF1 and near-cognate aminoacyl-tRNAs (aminoacyl-tRNAs with an anticodon complementary to two of the three nucleotides of the stop codon) normally compete for A site binding. Under certain conditions, the rate at which near-cognate aminoacyl-tRNAs successfully compete with eRF1 at a stop codon can be increased, resulting in incorporation of an amino acid carried by a near-cognate aminoacyl-tRNA into the nascent polypeptide.¹³ This process is termed "termination suppression" or "readthrough". In the case of a premature stop mutation, readthrough normally results in the continued elongation of the polypeptide chain in the correct reading frame and the production of full-length protein.

Obvious perturbations to the efficiency of translation termination include mutations in the translational machinery such as ribosomal proteins,¹⁴⁻¹⁹ ribosomal RNAs (rRNAs),²⁰⁻²³ termination factors,²⁴⁻³¹ and aminoacyl-tRNAs.³²⁻³⁵ Interestingly, the identity of the stop codon itself also affects termination efficiency. Generally, termination is most efficient at UAA stop codons, followed by the UAG and UGA stop codons. In addition, the sequence context both upstream and downstream of the stop codon also influences termination efficiency.^{12,36-42} In particular, the first nucleotide downstream of the stop codon plays an important role in determining the efficiency of translation termination, and sequence analysis of natural stop codons has revealed a strong bias at that position in many species (including humans). This observation led to the proposal that eRF1 may normally recognize a tetranucleotide termination signal.⁴² RNA cross-linking studies have confirmed that eRF1 contacts the first nucleotide following the stop codon.⁴³

Aminoglycoside-Mediated Nonsense Suppression

The efficiency of translation termination can also be reduced through the action of a large class of structurally related antibiotics called aminoglycosides. Aminoglycosides bind to a region of the small subunit rRNA known as the decoding site⁴⁴ that normally monitors proper codon-anticodon interactions. Several nucleotides in the decoding site act to probe the conformation of the codon-anticodon helix to ensure that tRNA selection is correct. When aminoglycosides bind to the decoding site, they induce a conformational change that reduces the ability of rRNA to discriminate between cognate and near-cognate aminoacyl-tRNAs.⁴⁵⁻⁵⁰ This reduction in the accuracy of codon recognition increases the probability that translational misreading will occur, including the readthrough of stop codons.

Remarkably, the rRNA sequences that make up the prokaryotic and eukaryotic decoding sites are very similar (Fig. 1). One of the main differences lies within the major groove of the decoding site where aminoglycosides bind. A key residue for aminoglycoside binding to the prokaryotic decoding site, the A1408 nucleotide, is a G nucleotide in the corresponding position of the eukaryotic decoding site.⁵¹ Introduction of the A1408G mutation in the bacterial decoding site has been shown to reduce the affinity for aminoglycoside binding significantly.⁵² This suggests that this key nucleotide difference at least partially accounts for the specificity of aminoglycosides to inhibit the bacterial ribosome, resulting in their utility as antibiotics in humans.

However, several studies have shown that some aminoglycosides can also stimulate low levels of misreading that leads to termination suppression in eukaryotic systems.⁵³⁻⁶³ A recent yeast study revealed that aminoglycosides induced little or no misreading at sense codons,

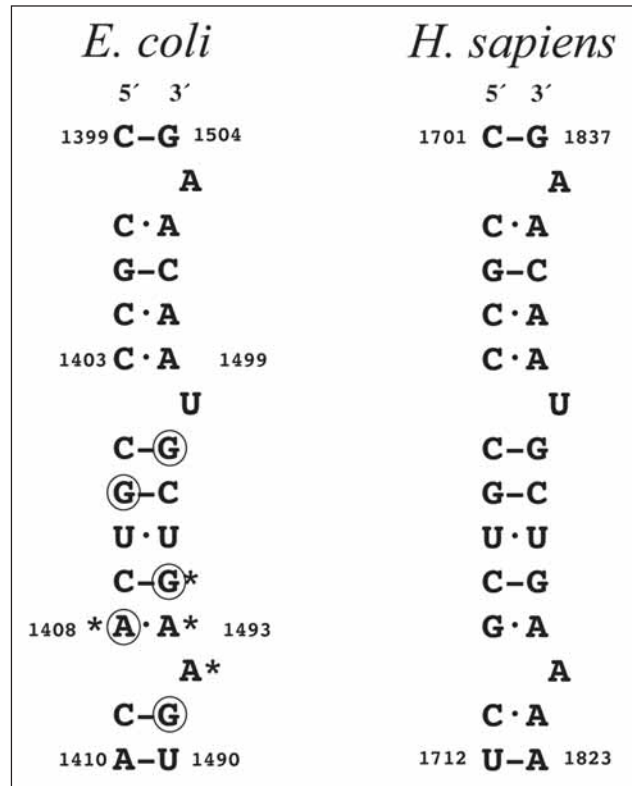


Figure 1. Comparison of the decoding sites in *E. coli* 16S rRNA and human 18S rRNA. Residues in the *E. coli* decoding site that are protected from dimethyl sulfoxide (DMS) modification by paromomycin are circled.⁴⁵ Residues that are protected from DMS modification by mRNA in a model decoding structure and also protected by an mRNA-dependent interaction of tRNA with the A site of ribosomes are marked by asterisks.¹⁵⁹

while the suppression of nonsense mutations was generally robust.⁶⁴ This result suggests that stop codons are generally much more susceptible to aminoglycoside-induced misreading than sense codons in eukaryotes. This selectivity could be due to inherent differences in the fidelity of the elongation and termination processes.

The observations that aminoglycosides suppress premature stop codons in eukaryotic systems have led to a number of investigations to determine whether aminoglycosides could provide sufficient readthrough of premature stop mutations to suppress the phenotypes associated with human diseases. In most cases this question remains to be answered. However, it has been shown in mammalian cells that aminoglycosides can induce the suppression of nonsense mutations that cause many diseases, resulting in the restoration of low levels of functional protein. Aminoglycosides have been shown to induce readthrough of nonsense mutations that cause cystic fibrosis,⁶⁵⁻⁶⁷ Duchenne muscular dystrophy,^{68,69} Hurler syndrome,^{70,71} infantile neuronal ceroid lipofuscinosis,⁷² cystinosis,⁷³ X-linked nephrogenic diabetes insipidus,⁷⁴ recessive spinal muscular atrophy,⁷⁵ and polycystic kidney disease.⁷⁶ They have also been shown to suppress nonsense mutations in the *p53*⁶⁰ and *ATM*⁷⁷ tumor suppressor genes.

Particularly promising results have been obtained using mouse models for both Duchenne muscular dystrophy (DMD) and cystic fibrosis (CF). A mouse model for Duchenne muscular

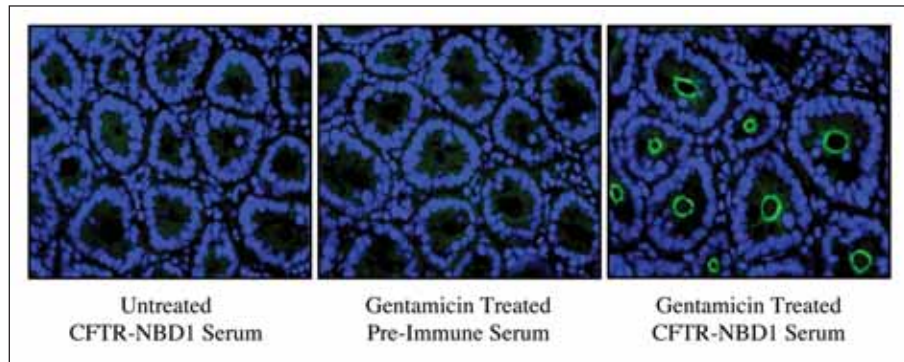


Figure 2. Immunofluorescence staining of submucosal glands in the duodenum shows the appearance of cystic fibrosis transmembrane conductance regulator (CFTR) protein following gentamicin treatment. Samples from homozygous *Cftr*^{-/-} *hCFTR*-G542X transgenic mice (harvested from untreated or gentamicin-treated animals) were stained with a nuclear stain (blue) and incubated with either preimmune serum or CFTR-NBD1 serum. After incubation with a secondary antibody conjugated to a fluorescent dye (green), samples were visualized by fluorescence microscopy. CFTR protein was observed at the apical surface of epithelial cells only in tissues incubated with CFTR-specific serum that had been harvested from gentamicin-treated animals.

dystrophy known as the *mdx* mouse carries a naturally occurring UAA nonsense mutation in the dystrophin gene. It was shown that the administration of gentamicin via subcutaneous injections resulted in the partial restoration of dystrophin protein in muscle tissue of the *mdx* mouse.⁶⁹ In addition, muscle contraction assays demonstrated that gentamicin treatment restored enough functional dystrophin protein to significantly reduce muscle contractile injury that is the hallmark of DMD. In another study, a transgenic CF mouse model that carried a UGA nonsense mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene was used to examine whether subcutaneous injections of gentamicin could restore CFTR protein expression. Treated mice showed a partial restoration of CFTR protein expression by immunofluorescence (Fig. 2) and a partial restoration of cAMP-activated chloride channel activity.⁷⁸ These findings indicate that the administration of gentamicin stimulated readthrough of the *CFTR* premature stop mutation, resulting in the production of functional CFTR protein.

Several small clinical trials have also administered aminoglycosides to CF or DMD patients who carry premature stop mutations to determine whether any restoration of protein function occurred. To date, three such trials with CF patients have been reported. In two of the trials, the administration of gentamicin via nasal droplets restored some CFTR activity in the nasal epithelia of CF patients with a *CFTR* nonsense mutation.^{79,80} In a third study, a partial restoration of CFTR function was detected in the nasal epithelium of CF patients that carried a *CFTR* nonsense mutation when gentamicin was administered intravenously.⁸¹ Decidedly more mixed results were obtained in two clinical trials in which DMD patients with nonsense mutations were administered intravenous gentamicin. In one trial with four DMD patients, no increase in dystrophin levels or physical improvement could be ascertained after aminoglycoside treatment.^{82,83} However, the results of another clinical trial were more promising, since three of the four treated patients showed a partial restoration of dystrophin protein expression.⁸³

The chemical structure of aminoglycosides determines their ability to suppress nonsense mutations (Fig. 3). Only a subset of these compounds is effective at suppressing nonsense mutations in eukaryotes, and only three aminoglycosides from this group (gentamicin, amikacin, and tobramycin) are approved for internal human use. In addition, the susceptibility of premature stop codons to aminoglycoside-mediated suppression depends on the identity of the stop codon and its surrounding mRNA sequence context.^{59,60} This codon and context dependence

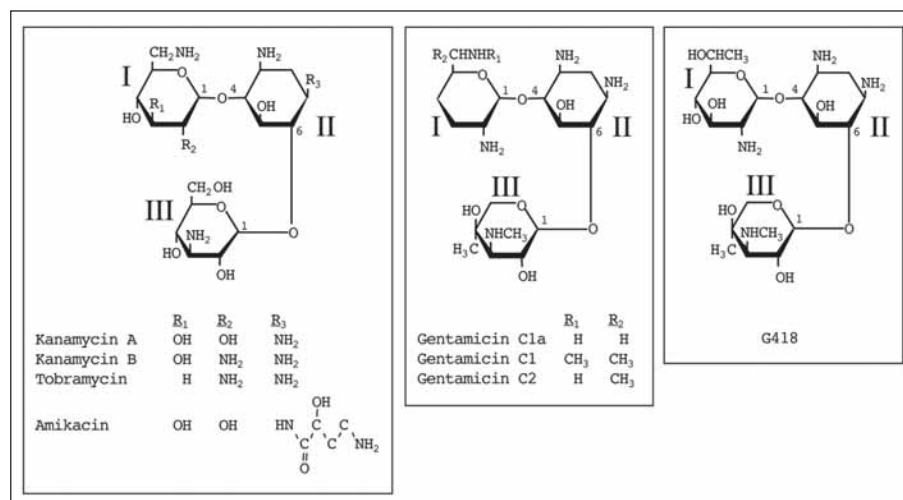


Figure 3. Structures of aminoglycosides commonly used in a clinical setting. The 2-deoxystreptamine ring is labeled ring II in each structure.

of aminoglycoside suppression is consistent with the results of a DMD clinical trial in which three DMD patients that carried UGA premature stop mutations showed a partial restoration of dystrophin expression after gentamicin treatment. However, no dystrophin could be detected in a patient with a UAA stop mutation after receiving the same gentamicin regimen.⁸³ These results suggest that aminoglycoside suppression of a UGA stop codon may result in significantly more readthrough in patients than aminoglycoside suppression of a UAA codon, as previously concluded from in vitro studies.^{59,68,84} Thus, it may be necessary to alter the efficiency or stop codon specificity of nonsense suppression if a greater level of readthrough is to be obtained for diseases caused by certain stop mutations.

A major hurdle to the long-term use of aminoglycosides as a therapy to treat diseases caused by nonsense mutations is their toxicity, which can lead to kidney damage and hearing loss. However, the majority of these side effects do not appear to be due to their ability to induce translational misreading, but rather to other consequences related to their charged nature. Aminoglycosides are taken into cells via megalin, a multi-ligand, endocytic receptor that is particularly abundant in the proximal tubules of the kidney and the hair cells of the inner ear.⁸⁵ Upon entering kidney cells, the positively charged nature of aminoglycoside molecules promotes their binding to acidic phospholipids in the lysosomal membrane,^{85,86} which alters the activity of a number of enzymes. In addition, aminoglycosides have been shown to promote the generation of free radical species that leads to tissue damage. Approaches that may reduce aminoglycoside toxicity include: altering the route and duration of their administration;^{87,88} coadministering compounds such as antioxidants to circumvent free radical damage;⁸⁹⁻⁹² and coadministering polyanions such as poly-L-aspartate^{93,94} or daptomycin^{95,96} to sequester aminoglycosides away from the lysosomal membrane.

Another potential drawback associated with the suppression of premature stop mutations using aminoglycosides is the potential suppression of native stop codons. If this occurred on a global basis, it would lead to the production of many proteins with an extended C-terminus that could result in misfolding and loss of protein function. However, a previous study found that human cells cultured in the presence of high concentrations of aminoglycosides exhibited only a small increase in the level of the Hsp70 molecular chaperone, suggesting that little protein misfolding occurred during aminoglycoside treatment.⁷⁰

The apparent lack of global readthrough at normal stop codons could be explained in several ways. Evidence of an evolutionary bias toward natural stop codons and surrounding sequence context(s) that may represent the most efficient termination signals has been observed at the end of genes in many species.⁴² No such selection for resistance to readthrough would occur at premature stop mutations. Thus, while the efficacy of aminoglycosides may be limited by the identity of the premature stop mutations and surrounding sequence context, these differences may also prevent readthrough at normal termination signals. In addition, multiple, in-frame stop codons are frequently found at the end of mRNAs.⁹⁷⁻¹⁰⁰ The presence of multiple stop codons should dramatically reduce the ability of aminoglycosides to induce readthrough of normal termination signals. Furthermore, the termination complex formed at premature stop codons appears to differ from the complex formed at native stop codons at the end of an mRNA.^{101,102} This intriguing finding suggests that the ribosome may terminate translation less efficiently at premature stop codons than native stop codons, possibly because the interactions between the termination complex at a premature stop codon and other factors bound in the 3' untranslated region of an mRNA cannot occur in a normal manner (see chapter by Amrani and Jacobson for further details).

Other Pharmacological Compounds That Suppress Nonsense Mutations

Since many of the toxic side effects caused by aminoglycosides are not directly associated with their ability to suppress stop mutations, another way to avoid these problems is to identify new, safer classes of compounds that suppress stop mutations. One such compound that has been investigated is negamycin. Negamycin is a dipeptide antibiotic that interacts with the ribosomal decoding site, much like aminoglycosides, even though it is structurally unrelated to aminoglycosides. Negamycin was shown to suppress the dystrophin premature stop mutation in the *mdx* mouse model, and was reported to be less toxic than aminoglycosides.¹⁰³ Another drug, PTC124, is a novel compound discovered by PTC Therapeutics, Inc. that has been shown to suppress nonsense mutations in cell culture and in animal models.¹⁰⁴ These results suggest that developing new pharmaceutical agents that suppress premature stop mutations without inducing the toxic side effects associated with aminoglycosides may have great potential for future therapeutic use.

Suppression of Nonsense Mutations Using Suppressor tRNAs

Another means of suppressing nonsense mutations involves expressing suppressor tRNAs. In this approach, DNA encoding a tRNA with an anticodon complementary to a stop codon is introduced into cells. This type of mutant tRNA, referred to as a suppressor tRNA, can compete with the termination factor eRF1 much more effectively than a near-cognate tRNA, resulting in a significant increase in stop codon suppression.

This approach has been shown to suppress premature stop mutations that cause β -thalassemia¹⁰⁵ and Duchenne muscular dystrophy¹⁰⁶ in mammalian cells. It has also been shown that the injection of DNA encoding a suppressor tRNA into the skeletal and heart muscles of a transgenic mouse expressing a reporter gene with a premature stop codon resulted in the suppression of the stop codon in vivo.¹⁰⁷

Besides the potential suppression of natural stop codons that was discussed in a previous section, there are other significant drawbacks to this therapeutic approach. Although tRNA genes have strong RNA polymerase III promoters and the encoded tRNA molecules are generally stable after they are transcribed and undergo maturation,¹⁰⁸ the efficient introduction and maintenance of tRNA genes into cells by gene therapy methods remains a challenge. In addition, the termination signal to be suppressed and its surrounding context also affect the efficiency of suppressor tRNA-mediated suppression.^{108,109}

Mutation Repair

Another novel approach that could be used to treat diseases caused by premature stop mutations is the repair of a mutation directly in the genome using a nucleotide exchange antisense oligonucleotide approach. Unlike nonsense suppression therapy, which requires that the mutation be in the correct open reading frame, the use of antisense oligonucleotides also has the potential to repair various point mutations, small deletions, insertions, or splicing defects. In this method, a mutant DNA sequence is replaced by the wild-type sequence. Although a number of approaches have been designed to target DNA for mutation repair, including the use of ribozymes, group II introns, and triplex-forming oligonucleotides, single-stranded DNA currently appears to generate the most robust and reproducible gene repair. This exchange is directed by a double-stranded DNA chimeric oligonucleotide that is typically 70-80 nucleotides long. This chimera is synthesized as a single-stranded molecule that is designed with sequence complementarity such that it folds into a double-hairpin structure. The double-hairpin structure prevents the molecule from nuclease digestion as well as concatenation.¹¹⁰ When these molecules are introduced into cells, the chimeric oligonucleotide hybridizes to its complementary sequence in the target gene except for the region of mismatch sequence where the mutation lies. This mismatch is recognized by the cellular mismatch-repair system, which then catalyzes the exchange of the wild-type nucleotides for the mutant nucleotides, thus repairing the mutation in the DNA.

Gene repair of mutations has been demonstrated in yeast^{111,112} and mammalian cell culture, where correction of mutations that caused sickle cell anemia¹¹³ thalassemia,¹¹⁴ alkaline phosphatase deficiency,¹¹⁵ apolipoprotein A2-linked atherosclerosis,¹¹⁶ and epidermolysis bullosa simplex¹¹⁷ were accomplished. In addition, animal models of tyrosinemia,¹¹⁸ muscular dystrophy,¹¹⁹⁻¹²² hemophilia,¹²³ and renal tubular acidosis¹²⁴ have been used to demonstrate a partial correction of mutations and a restoration of some protein expression. The amount of normal protein levels restored among the various disease phenotypes varied from 0.5% to 20% of wild-type levels.¹¹⁰ This level of functional protein could improve the phenotype of patients with these (and many other) genetic diseases.

Although this approach to correct mutations and restore wild-type protein production in mammalian cells is promising, its development for clinic application is still a work in progress. There are several problems that currently prevent the implementation of this approach.¹²⁵ First, the design of stable oligonucleotides capable of replacing the mutation efficiently can be difficult. Second, as with many gene therapy approaches, the introduction of oligonucleotides into the appropriate cell type is a major obstacle, and the efficiency of repair is cell-cycle dependent. Third, the percentage of corrected cells decreases with time. Finally, apoptosis has been observed in some cells that have undergone this form of targeted sequence alteration. Thus, further studies are required to determine the ultimate utility of this approach.

Suppression of NMD

Many strategies aimed at suppressing premature stop mutations could be compromised by the fact that mRNAs that contain premature stop mutations are often unstable, resulting in a severe reduction in their steady-state level. The reduced abundance of mRNAs that carry a stop mutation is due to the NMD pathway (see chapter by Maquat). Therefore, approaches that stabilize nonsense-containing mRNAs that are normally degraded by NMD should increase the steady-state amount of mRNA available for translation. This, in turn, could greatly enhance the level of protein produced by suppression therapy.

The NMD pathway and the NMD factors Upf1, Upf2, and Upf3 are conserved in eukaryotes ranging from yeast to humans (see chapters by Baker and Parker, Singh and Lykke-Andersen, Anderson, and Behm-Ansmant and Izauralde). Several factors involved in mammalian NMD bind to mRNAs in the nucleus during transcription and the subsequent stages of mRNA processing. In particular, some NMD components assemble with the exon-junction complex (EJC), located ~20-24 nucleotides upstream of exon-exon junctions as a consequence of pre-mRNA

splicing, and remain bound to the mRNA as it is exported to the cytoplasm¹²⁶ (see chapter by Maquat). In human cells, Upf3 binds to nuclear mRNA-protein (mRNP) complexes as a component of the EJC complex. Upf2 binds to these complexes as they leave the nucleus, while Upf1 is thought to associate with the complex in the cytoplasm.^{127,128}

According to current models, once the mRNA reaches the cytoplasm these bound nuclear factors are removed as the ribosome translates the mRNA during the initial or "pioneer round" of translation^{129,130} (see chapter by Maquat). If translation proceeds to the normal stop codon and all of the nuclear proteins are removed from the coding sequence during the pioneer round of translation, the transcript is remodeled to become steady-state mRNP and NMD can no longer occur. However, if a premature stop codon is present in the mRNA, the ribosome will not remove any nuclear proteins distal to the premature stop codon during this initial round of translation. This causes the mRNA to be identified as faulty and results in its rapid degradation by the NMD pathway. Generally, NMD occurs if an mRNA molecule carries a premature stop codon that is ≥ 50 nucleotides upstream of the 3'-most exon-exon junction.¹³¹

The destabilization of a nonsense-containing mRNA by NMD requires active translation of that mRNA. This conclusion is supported by the observations that several proteins that function in the NMD pathway associate with polysomes,¹³²⁻¹³⁵ and NMD can be inhibited by translation elongation inhibitors such as cycloheximide and puromycin. In yeast, the NMD factors have been shown to associate with the translation termination factors eRF1 and eRF3¹³⁶ (see chapters by Baker and Parker, and Amrani and Jacobson). Of the Upf factors, at least Upf1 associates with eRF1 and eRF3 in mammals (see chapters by Maquat, and Singh and Lykke-Andersen), also indicating that NMD and the process of translation termination are likely to be tightly linked.

It has also been shown that suppression of a premature stop codon can stabilize the mRNA. Overexpression of a suppressor tRNA can inhibit the degradation of nonsense-containing mRNAs in yeast and in mammalian cells,^{137,138} and suppression of premature stop codons by aminoglycosides has also been shown to stabilize nonsense-containing mRNAs in some cases. For example, a 5-fold increase in the abundance of *CFTR* mRNA containing a premature stop codon was detected in a human bronchial epithelial cell line derived from a CF patient that carried the *CFTR-W1282X* premature stop mutation after incubation with the aminoglycoside G418 for 24 hours⁶⁷ (Fig. 4). A 2-fold increase in mRNA was observed in human fibroblasts from a patient with Smith-Lemli-Opitz syndrome that carried a premature termination codon in the 7-dehydrocholesterol- δ 7-reductase (*DHCR7*) gene after G418 treatment.¹³⁹ Finally, a 2-fold increase in mRNA was observed in human fibroblasts derived from a patient with Hurler syndrome that carried nonsense mutations in the α -L-iduronidase (*IDUA*) gene after gentamicin treatment (K.M. Keeling and D.M. Bedwell, unpublished data). These results are surprising since aminoglycosides only induced readthrough of these premature stop mutations at a low frequency of 1-20%. While it is possible that premature stop mutations are more susceptible to aminoglycoside-mediated suppression during the pioneer round of translation than in subsequent rounds of translation, the mechanism by which such a modest level of readthrough can inhibit NMD is currently unknown.

Certain factors in the NMD pathway have also been identified as potential targets for pharmacological inhibition of mRNA degradation by the NMD pathway. One such target is Upf1, which is essential for the NMD process and is conserved in all eukaryotes. Upf1 is a phosphoprotein, and changes in its phosphorylation state regulate its function in the NMD pathway.^{140,141} Several modifiers of the Upf1 phosphorylation state have been identified. For example, SMG1 is a kinase that phosphorylates at least two serine residues in Upf1 and stimulates the NMD process¹⁴²⁻¹⁴⁴ (see chapter by Yamashita et al). SMG1 is a member of the phosphatidylinositol-4,5-bisphosphate (PIP2) family of protein kinases, which are frequently inhibited by caffeine and wortmannin. Accordingly, these compounds were found to abrogate the degradation of the collagen VI α 2 subunit mRNA by NMD in human fibroblasts,

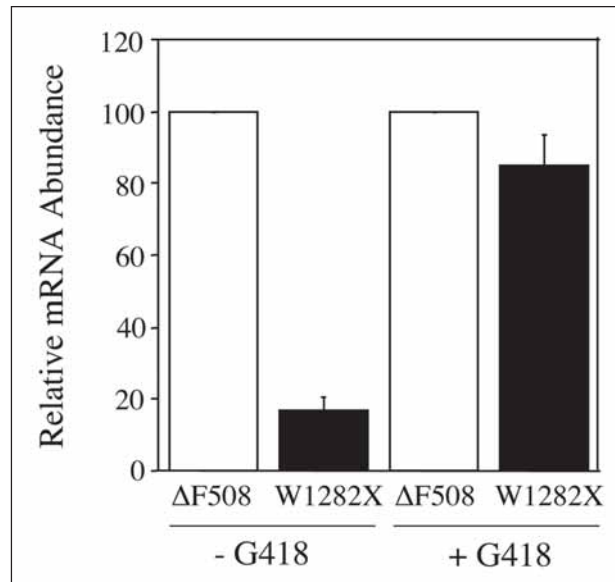


Figure 4. *CFTR*-W1282X mRNA abundance is increased in the IB3-1 bronchial epithelial cell line following G418 treatment. IB3-1 cells carry the W1282X premature stop mutation on one *CFTR* allele and the ΔF508 mutation on the other *CFTR* allele. The ΔF508 allele harbors a 3-nucleotide deletion of codon 508, so translation proceeds to the normal translation termination codon and a normal level of mRNA is produced.¹⁶⁰ In contrast, the W1282X allele contains a nonsense mutation at codon 1282 that results in NMD.^{161,162} For this experiment, IB3-1 cells were grown in the presence or absence of G418, and the abundance of mRNA from each *CFTR* allele was determined by allele-specific oligonucleotide hybridization. The level of *CFTR* mRNA from the ΔF508 allele was defined as 100%, and the level of mRNA from the W1282X allele was normalized to the ΔF508 level. Each value represents the mean ± standard deviation of four independent measurements.

presumably by inhibiting SMG1 activity.¹⁴⁵ The phosphorylation status of Upf1 is also influenced by SMG5, SMG6 and SMG7, which participate in the dephosphorylation of Upf1 and thus complete its phosphorylation-dephosphorylation cycle.¹⁴⁶⁻¹⁴⁹ These Upf1 modifiers also represent potential targets for pharmacological inhibition of NMD. Although the yeast Upf1 protein is also a phosphoprotein (K.M. Keeling and D.M. Bedwell, unpublished data), the kinase and phosphatase involved in its regulation have yet to be identified. Interestingly, a compound called diazaborine has been shown to stabilize aberrant mRNAs that are normally degraded by the NMD pathway in yeast.¹⁵⁰ While the target of this compound is not yet known, it is possible that this compound could also target a component of the Upf1 phosphorylation cycle or some other step of yeast NMD.

It is important to note that the NMD pathway has additional functions other than degrading mRNAs that carry premature stop mutations (see chapters by Abraham and Oliveira, Azzalin et al, Kaygun and Marzluff, and Kim and Maquat). Mutations in Upf1, Upf2, and Upf3 influence the abundance of many normal mRNA species in yeast (see chapter by He and Jacobson) and mammals (see chapters by Sharifi and Dietz, and Soergel et al).^{151,152} Factors in the NMD pathway may also act as checkpoints for RNA processing and nuclear export.^{153,154} Therefore, therapeutic approaches aimed at inhibition of the NMD pathway must be monitored carefully and optimized such that other vital cellular pathways are not adversely affected.

Future Development of Therapies for Nonsense-Associated Diseases

Even though the results of several clinical trials indicate that the suppression of nonsense mutations can partially restore protein function, none of these studies were designed in a manner that allowed the investigators to determine whether enough protein expression was restored to confer a therapeutic improvement in the disease phenotype. The threshold level of functional protein needed to alleviate a disease phenotype is unknown for most diseases. This is a complex issue, since the minimal level of functional protein needed to improve a particular disease phenotype will vary widely depending upon the structure and function of each protein, and the key tissue(s) in which each protein is required. For example, estimates of the amount of CFTR protein required to alleviate the CF disease phenotype range from 5%¹⁵⁵ to 30%¹⁵⁶ of wild-type CFTR levels. In contrast, as little as 1% of α -L-iduronidase can reduce the severity of the Hurler syndrome disease phenotype.^{157,158} Therefore, suppression therapy may hold more potential for treating some disorders than others.

The development of new compounds or approaches that can suppress nonsense mutations without the side effects currently associated with aminoglycosides shows great potential for the future of nonsense suppression therapy. New pharmacological targets could include regions of rRNA, ribosomal proteins, translation termination factors, and components of the NMD machinery. Future therapies for the treatment of human diseases caused by premature stop mutations may include treatments designed to correct defects occurring at multiple levels simultaneously. For example, combining the suppression of premature stop mutations with the inhibition of NMD could restore a significantly higher level of functional protein than with either approach alone, thus providing a much greater opportunity to alleviate a disease phenotype. Currently, more research on the basic mechanisms of translation termination and NMD is needed to provide a better understanding of potential targets for therapies to treat nonsense-associated diseases.

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