Translational readthrough potential of natural termination codons in eucaryotes – The impact of RNA sequence

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Termination of protein synthesis is not 100% efficient. A number of natural mechanisms that suppress translation termination exist. One of them is STOP codon readthrough, the process that enables the ribosome to pass through the termination codon in mRNA and continue translation to the next STOP codon in the same reading frame. The efficiency of translational readthrough depends on a variety of factors, including the identity of the termination codon, the surrounding mRNA sequence context, and the presence of stimulating compounds. Understanding the interplay between these factors provides the necessary background for the efficient application of the STOP codon suppression approach in the therapy of diseases caused by the presence of premature termination codons.

Termination of Translation

Termination of translation is one of the most complex stages in protein biosynthesis.[1] It occurs when the STOP codon in mRNA (UAA-ochre, UAG-amber or UGA-opal) enters the A-site on the small ribosomal subunit, and leads to the release of a nascent polypeptide chain from the peptidyl-tRNA positioned at the ribosomal P-site. Both in eukaryotes and bacteria, 2 classes of release factors (I and II) mediate this process, although these mechanisms are distinct.

In bacteria, there are 2 class-I release factors, RF1 or RF2; they recognize STOP codons through the direct action of a "peptide anti-codon" sequence (RF1 interacts with UAG and UAA, RF2 with UGA and UAA) and mediate the hydrolysis of the peptidyl-

tRNA ester bond.[2,3] Subsequently, a class-II release factor, RF3, which possesses GTPase activity, mediates recycling of class-I factors by ejecting them from post-termination complexes.[4-6]

Eukaryotic translation is terminated by a protein heterodimer consisting of 2 release factors, eRF1 (class-I) and eRF3 (class-II).[7] In contrast to bacteria, where 2 class-I release factors are required, in eukaryotes, eRF1 alone recognizes all 3 STOP codons,[8-10] although its interaction with UGA differs from interactions with UAA or UAG.[11] eRF1 is a tRNA-shaped protein, containing 3 domains: N-terminal, middle and C-terminal.[12] Recognition of STOP codons occurs through a complex, 3-dimensional network formed by conserved residues in the N-terminal domain of eRF1.[13-15] The highly conserved GlyGlyGln motif in the middle domain of eRF1 is responsible for the release of a peptide from the ribosome.[9] The middle domain as well as the C-terminal domain of eRF1 interact with eRF3, and the C-terminal domain of eRF3 binds GTP. Formation of a ternary eRF1•eRF3•GTP complex[16-18] leads to GTP hydrolysis.[5,19] Activation of the eRF3 GTPase activity additionally requires the interaction of the ternary complex with poly(A)-binding proteins (PABPs) present at the 3′-UTR of mRNA.[20] Hydrolysis of GTP leads to the correct positioning of eRF1 in the ribosomal peptidyl transferase center, which catalyzes the cleavage of the peptidyl–tRNA bond and releases a polypeptide chain from the ribosome.[21]

To summarize, effective termination of translation in eukaryotes requires the presence of a STOP codon in the ribosomal A-site, its interaction with 2 release factors, eRF1 and eRF3, and a close distance between the terminating ribosome and 3′UTR-bound PABPs (**Fig. 1**).

Translational readthrough of natural STOP codons

The process of protein synthesis termination, although effective, is not 100% efficient. Several natural mechanisms of termination suppression exist, including ribosomal frameshifting,[22] suppressor tRNAs (aminoacylated tRNAs with anticodons complementary to STOP codons in mRNA)[23] and STOP codon readthrough (RT).[24,25]

In this review, we will focus on the latter mechanism. STOP codon RT relies on competition between 2 distinct phenomena:
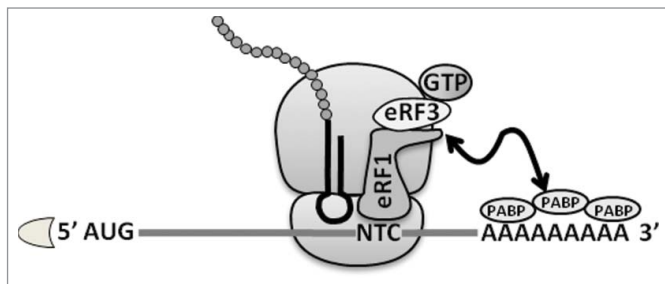
Figure 1. The major factors involved in translation termination in Eukaryota. NTC – normal termination codon; PABP – poly(A)-binding proteins; eRF1 and eRF3 – termination factors.

the recognition of a termination codon by eRF1, which triggers the proper termination of translation, and accommodation of a near-cognate tRNA (nc-tRNA) in the A-site of the ribosome, which leads to erroneous decoding of the STOP codon. Nc-tRNAs are able to pair with STOP codons at 2 of the 3 positions of a codon–anticodon sequence. This interaction may compete with the STOP codon recognition by the release factor eRF1, and thereby inhibit the process of protein synthesis termination. As a result, an amino acid is erroneously incorporated into the polypeptide chain and the ribosome continues translation to the next STOP codon in the same reading frame (**Fig. 2**).

Spontaneous suppression of the translation termination, called basal RT, has been best studied in viruses. So far, prediction of eukaryote genes undergoing RT is mostly based on phylogenetic comparisons and ribosome profiling studies,[26-28] and only few genes have been studied experimentally.[29,30] Nevertheless, it has been estimated that the level of basal RT of STOP codons in mammalian cells ranges from 0.01 to 0.1%.[31]

Efficiency of Basal RT

Type of the STOP codon

The potential efficiency of basal RT is different for each of 3 STOP codons. Experiments in mammalian cell lines, using an
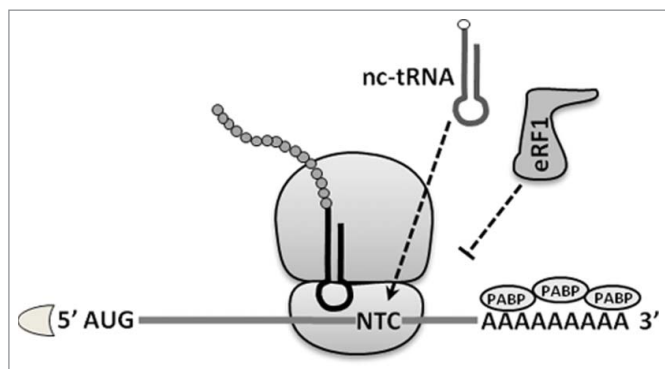

Figure 2. The principle of termination codon readthrough. Near-cognate tRNA (nc-tRNA) successfully competes with eRF1 and leads to the decoding of a STOP codon.

overexpressed dual luciferase reporter vector carrying each of the STOP codons, have demonstrated that UGA has the highest basal RT potential and thus the lowest fidelity. UAG is less "leaky," and UAA has the highest fidelity.[30,32-34]

Although the identity of a particular STOP codon is crucial for the efficiency of basal RT, this process also depends on other factors. Experimental studies in a number of organisms have shown that both downstream and upstream sequence context plays an essential role in determining the RT potential of STOP codons.

3′ context

In both bacteria and eukaryotes, the base immediately following the STOP codon (position +4, with the first nucleotide of the termination codon marked as +1) exerts the strongest influence on RT efficiency.[35-39] This has led to the hypothesis that an actual translation termination signal consists of a tetranucleotide sequence, not only the STOP codon itself.[40,41]

While different studies are consistent in demonstrating that the termination efficiency depends on the +4 nucleotide, it has been shown both in yeast,[24,42] and in higher eukaryotes that this effect depends greatly on the identity of the STOP codon. Which tetranucleotide is the most efficient in eliciting the RT in eukaryotes remains controversial (see **Table 1**). For example, the level of basal UGA-C readthrough in mammalian cells (3–4%) was shown to be 3–6 times higher than for the remaining UGA-N tetranucleotides. However, for other STOP codons, the presence of C at the +4 position did not significantly affect the efficiency of suppression: for UAG (1–2%) or for UAA (≤0 .5%).[33]

Until recently, STOP codon RT was documented in only few genes in higher eukaryotes: *syn, kelch* and *hdc* in *Drosophila melanogaster*,[26,43,44] and β-globin gene in rabbits.[45] However, the recent comparative phylogenetic analyses of *Drosophila* and other *metazoan* genomes identified over 280 genes undergoing RT.[26,46] 32% percent of these genes contained UGA codon followed by cytosine (UGA-C); these genes were nearly 10 times more likely to undergo RT than genes with other nucleotide contexts. Importantly, these results confirmed that the order of STOP codon "leakiness" in eukaryotes was UGA>UAG>UAA, and the influence of +4 nucleotide on RT was C>U>G>A.[26] The common conclusion of the studies in eukaryotes was that cytosine at position +4 promoted higher levels of basal RT (equivalent to the lower efficiency of termination); this was especially evident for the "leakiest" UGA codon. This conclusion is consistent with the earlier observation that leaky STOP codons in combination with the +4 cytosine (UGA-C and UAG-C) are rarely used termination contexts in mammals.[39] Nonetheless, the identity of a nucleotide immediately downstream of the STOP codon is still not sufficient to predict the RT efficiency for a given nonsense mutation.

The variability in the impact of the +4 nucleotide position on the efficiency of STOP codons RT can be, at least partially, explained by the influence of other surrounding sequences. Again, an important insight into the broader sequence context of a basal RT has come from studies on viruses, where 3 main types of motifs stimulating RT have been identified.[23] Type I involves

Table 1. Basal RT potential: the influence of the STOP codon identity and of the +4 nucleotide context

STOP CODON					SYSTEM USED	REFERENCE
UGA	>	UAG	>	UAA		
C>U~A>G		G>C>U=A		C>U~A>G	Yeast	Bonetti et al.[42]
C~U>G≥A		C≥U>>G≥A		C≥U>>G>A	Mammalian	McCaughan et al.[39]
C>>A>U~G		U>C>G~A		C>G>U~A	Mammalian	Manuvakhova et al.[33]
C>A~G>U		C>A~G~U		C>A~G~U	Mammalian	Floquet et al.[31]
C>A>G>U					Mammalian	Beznoskova et al.[63]

the UAG-CAAUYA motif, type II – UGA-CGG or UGA-CUA motif, and type III consists of the UAG-G motif plus a downstream stimulatory RNA pseudoknot structure.[23,47-50]

Motifs similar to those found in viruses have also been identified in eukaryotes. The CAA sequence downstream of the STOP codon was associated with the high RT level in yeast.[24,51] A more recent study of the leaky context in yeast revealed that at least 6 nucleotides (−CAAUUA) downstream of the STOP codon were involved in promoting RT.[52] Tests performed in the human kidney epithelial cell line (HEK-293T) revealed a high level of RT (7–31%) in 4 genes featuring a highly conserved -CUAG sequence downstream of the UGA codon;[30] experimental deletion of this 3′-motif almost completely abolished RT, clearly demonstrating the importance of the downstream -CUAG sequence in the UGA readthrough in mammals. The significance of the 3 sequence context was also confirmed in another study, which revealed that the C-terminally extended peroxisomal isoforms of 2 human proteins, LDHB and MDH1, result from translational RT of the respective genes containing the UAG-CU motif at the 3′end of their regular coding sequences.[53]

An interesting example of the interplay between the adjacent sequence context and STOP codon RT was reported in a study on a patient with *junctional epidermolysis bullosa*, the terminal recessive disease caused by mutations in the gene *LAMA3*.[54] The patient's condition was surprisingly good, in spite of the presence of 2 nonsense mutations in *LAMA3* (R943X/R1159X). The study revealed that expected deficiency of the full length laminin-332 protein was rescued by spontaneous RT of the R943X allele. The authors suggested that the sequence surrounding the 943X codon (AGU-UGA-CUA) was a crucial factor in the induction of premature STOP suppression.

5′context

The evolutionary conservation of the 5′-sequence context of termination codons in *Escherichia coli* and humans led to the postulate that the upstream sequence adjacent to the STOP codon also plays a role in the efficiency of translation termination.[55] Indeed, it was shown that the penultimate and/or ultimate positions could modulate the level of translational RT in bacteria and yeast.[51] Experiments on yeast demonstrated that the presence of adenine at 2 positions immediately upstream of the termination codon stimulates RT of UAG, and most probably of other STOP codons as well.[42,56] This is consistent with the observation that adenines 5′-adjacent to STOP codons are evolutionarily conserved in genes regulated by RT: among 91 plant and animal

viral RNAs subjected to RT, 65 sequences had adenine in position −1, 69 in position −2, and 50 carried adenines both in positions −1 and −2.[42,56] The importance of the upstream sequence context in the STOP codon RT is also supported by studies on mammalian cells, although the RT potential of specific 5′-adjacent sequences is still not clear. In experiments performed on mouse fibroblasts (NIH3T3) and human HEK293T cell lines, the highest RT was observed when the −1 position was occupied by adenine or generally purine; in these models, uracil at the −1 position was always associated with the lowest RT level.[30,57]

In any case, the influence of the 5′-adjacent sequence on the efficiency of the STOP codon RT is considered to be more subtle compared to the effect of the 3′-sequence context.[58]

Mechanisms explaining the impact of sequence context on the RT efficiency

In spite of intensive studies, molecular mechanisms explaining the influence of the sequence context upon the RT efficiency remain unclear.

The effect of a nucleotide following the termination codon (position +4) appears to be linked to interactions of mRNA with the translational machinery rather than to interactions of the STOP codon with nc-tRNAs.[59] Crosslinking experiments have demonstrated that the +4 nucleotide in mRNA interacts with eRF1.[60] One of the ways by which the sequence downstream of the termination codon could affect RT is the formation of secondary structures (stem–loops, pseudoknots), which can interact with the ribosome.[61,62] Such interaction of the mRNA pseudoknot with the ribosome was suggested to favor the binding of nc-tRNAs at the A-site over binding of eRF1.[25] It was even suggested that structures formed by the 3′-end of mRNA, acting as components of efficient RT cassettes in eukaryotes may in fact be the norm rather than an exception.[62]

Regarding the downstream sequence context, it has been proposed that in yeast, the sequence 5′ to the STOP codon could stimulate RT through the direct interaction with the tRNA at the ribosomal P-site. Alternatively, ultimate amino acids of the nascent polypeptide could elicit RT through interaction with the release factors and the ribosome.[51] The latter mechanism was, however, excluded in higher eukaryotes. Instead, it was suggested that adenine at position −1 or −2 could induce RT by modifying the structure of mRNA at the P-site; this would distort the structure of the ribosome, and modulate the competition between release factors and natural suppressor tRNAs.[56]

Recently, it has been proposed that eIF3, the eukaryotic translation initiation factor, could be involved in promoting programmed RT of all 3 STOP codons set in the unfavorable termination context.[63] In this mechanism, which appears to be evolutionary conserved, eIF3 interacts with the pre-termination complex, where it prevents eRF1 from recognizing the third/wobble position of the STOP codon. As a consequence, nc-tRNAs with a mismatch in the same position can decode the STOP codon, allowing the protein synthesis to be continued.

Last but not least, a study of the *S. cerevisiae* genome, searching for adjacent open reading frames separated only by a unique STOP codon (called SORFs),[64,65] identified several "leaky" STOP codons surrounded by sequences different from those believed to promote RT. This implies that our understanding of the role of 5′- and 3′-sequence context in the STOP codon suppression is still incomplete, and suggests that mechanisms other than RT might be involved in allowing ribosomes to bypass STOP codons.[65,66]

Other factors influencing the efficiency of termination codon RT

The STOP codon identity and the surrounding sequence context are not the only factors that influence the efficiency of termination codon suppression. The RT of STOP codons can be also enhanced by the increased level of mRNA that is subjected to termination suppression[67] or the depletion of termination machinery components like eRF1 and/or eRF3.[68-70] Such mechanisms involve changes in the cellular level of gene expression and are not dependent on STOP codon identity and mRNA sequence context.

It has been shown that post-translational modification of some ribosomal proteins can also influence the RT process. Hydroxylation of the proline residue at position 64 of RPS23 protein in the 40S ribosomal subunit is essential for the accurate translation process. The hydroxylation site is located in the ribosomal decoding center and can affect the termination codon recognition. Inhibition of the hydroxylases involved in RPS23 modification could stimulate the production of full-length proteins from sequences containing nonsense mutations.[71,72]

Alternatively, translational RT can be stimulated by externally provided chemical compounds, such as low molecular weight drugs. Aminoglycoside antibiotics (AAGs) are among the most important representatives of this group.[58]

STOP codon RT stimulated by AAGs

AAGs are oligosaccharides consisting of streptidine or 2-deoxystreptidine as the molecular core and a variable number of sugar rings and ammonium groups.[73] Early papers indicating the RT potency of AAGs were already published in the 1960s.[74,75] AAGs, commonly used to treat Gram-negative bacterial infections, efficiently bind to the 7 nucleotide sequence in 16S rRNA and inhibit the function of the bacterial ribosome.[11] In eukaryotes, a one-base difference (A->G at position 1408) in the 7 nucleotide motif in 18S rRNA significantly lowers the efficiency of its interaction with AAGs (e.g. a ~25–50-fold decrease in binding affinity for paromomycin).[76,77] Nonetheless, this low efficiency is sufficient to reduce discrimination between cognates and nc-tRNAs in eukaryotes, thereby stimulating translational RT.

Our current understanding does not allow unambiguous prediction of the sequence context's influence upon the susceptibility of STOP codons to AAG-mediated suppression in mammalian cells.[31-33] Nevertheless, the majority of evidence indicates that, as in basal RT, UGA is the "leakiest" termination codon and cytosine (or pyrimidine) residue at the +4 position correlates with the highest level of AAG-induced RT (see **Table 2**).

Statistical analysis of the RT potential, based on the study of cultured mouse cells (NIH3T3) transfected with 66 sequences containing a variety of termination codon contexts, suggested that the combination of UGA-C with uracil in position −1 (U-UGA-C) had the highest potential of AAG-stimulated RT. In the presence of AAGs, all codons studied with this sequence context systematically underwent RT, resulting in a full-length protein expression exceeding 0.5% of wild-type sequence expression.[31]

Biological consequences of the STOP codon RT

The process of STOP codons RT may have important biological consequences, ranging from those essential for the cell life (as in programmed RT) to detrimental ones.

Table 2. RT potential in the presence of RT-stimulating compounds; the influence of the STOP codon identity and of the +4 nucleotide context

STOP CODON				
UGA >	UAG >	UAA	RT-STIMULATING DRUG	REFERENCE
C>U>A>G	C>U~G~C	C>U>G~A	G418	Howard et al.[32]
C>U>A~G	C>U~G~C	C ~U~G~C	Gentamicin	
C>U~A>G	C ~U~G~C	C ~U~G~C	Paromomycin	
C>A~G>U	U>C~G>A	C>U>G>A	G418	Manuvakhova et al.[33]
C>A=G >U	U>C=G >A	C>G>A=U	Gentamicin	
C>A≥G>U	C>A=G =U	G=U >A=C	Paromomycin	
C>A=G =U	G>C=U >A	U>C>A=G	Neomycin	
C>A>G>U	U>C>A=G	U>A=C =G	Sisomycin	
C>U>A=G	G>C>A>U	A>U>C>G	Lividomycin	
C>A~G>U	C>A~G~U	C>A~G~U	Gentamicin	Floquet et al.[31]

Viruses have long been known to use RT to increase their coding capacity.[36,65,78,79] Some pathogenic fungi that infect plants utilize RT in order to include additional signaling sequences targeting the synthesized proteins to a specific cellular compartment, like peroxisomes.[80] In higher organisms, the biological function of programmed STOP codon RT is less clear, although recent studies have led to the identification of some functional, RT-derived extended proteins. Examples include regulated RT in a number of *Drosophila* genes,[28] peroxisomal isoforms of metabolic enzymes in diverse model organisms[53] or programmed RT in mammals.[29,30,45,53,81]

On the other hand, the RT-mediated C-terminal extension of polypeptides may cause a dominant-negative effect of mistranslated proteins, which may interfere with normal, cellular functions or lead to the gaining a new function harmful to the cell. Synthesis of useless polypeptides is also a waste of resources. There are mechanisms in the cell to prevent detrimental effects of the RT process. One of them is the existence of tandem STOPs, which are secondary in-frame STOP codons present downstream from the primary STOP codon. Tandem STOPs have been purported to limit the level of STOP codon suppression by providing a second chance for the translation termination apparatus to stop protein synthesis.[66,82] Tandem STOPs in yeast are preferentially located at the third codon after the primary STOP; the six preceding nucleotides act as a sequence context favoring translation termination at the first STOP.[83] According to studies in yeast and in ciliated species (*Paramecium tetraurelia* and *Tetrahymena thermophila*), tandem STOP codons are more frequent in highly expressed genes, where translation termination occurs more frequently.[83,84]

Decoding of STOP codons

There are several nc-tRNAs, which can recognize each of the termination codons, but their usage in the process of translational RT does not appear to be random (see **Table 3**). According to a number of studies, mainly in viral and yeast mRNAs, UGA is the most frequently decoded as tryptophan, but it can also be misread by cysteine or arginine tRNAs.[85-88] UAA is decoded as glutamine.[85,86,88] Depending on the study, UAG has been reported to be decoded as either glutamine or tryptophan,[85,86,88] or as tryptophan, tyrosine or lysine.[24] A recent study, using a novel mass spectrometry-based approach for identifying amino acids incorporated at the STOP codon in the *in vivo* reporter system in yeast, has confirmed that the UGA codon is decoded as tryptophan, cysteine or arginine, but indicated that both UAA and UAG can be decoded as glutamine, tyrosine or lysine.[15] In addition, the latter study suggested that the sequence surrounding the STOP codon had no impact on the identity or proportion of amino acids incorporated during the RT process.

Inconclusive results obtained in a variety of studies and studied organisms suggest that the identity of the amino acid inserted at the STOP codon is likely to be irrelevant. This is related to the fact that the purpose of the STOP codon suppression in the programmed RT is to access another ORF. This feature distinguishes translational RT from, for example, selenocysteine insertion at UGA codons, where character of accommodated tRNA is precisely determined.[89]

In conclusion, in spite of the observed bias in the decoding of STOP codons, it is currently impossible to predict theoretically which amino acid will be incorporated in the synthesized peptide during the process of termination codon RT.

Translational RT of premature termination codons

STOP codon RT stimulated by chemical compounds such as AAG is a promising approach to restore protein translation from pathogenic alleles containing premature termination codons (PTC). It is estimated that 20% of all genetic human diseases are caused by single base-pair substitutions, which introduce PTCs into the mRNA.[90] The idea of the RT-driven correction of PTC mutations has been tested for many inherited human diseases, especially in the context of using AAGs as RT-stimulating agents.

The first report on the PTC-RT induced by AAGs was published already in 1985. In this study, PTC-containing sequences cloned in a reporter gene overexpressed in COS-7 cells were used to demonstrate that paromomycin and G418 could restore almost 20% of the wild-type protein activity.[91] The first *in vivo*

Table 3. Near-cognate codons for the eukaryotic STOP codons. *Near-cognate codons reported to be most frequently involved in the translational RT of each of the STOP codons are shown in bold (*in vitro* studies) or underlined (*in vivo* reporter studies)

TERMINATION CODONS			EFFECT ON TRANSLATION
UAA	UAG	UGA	STOP
POSSIBLE NEAR-COGNATE CODONS*			AMINO ACID INSERTED
GAA	**GAG**		Glu E
AAA	AAG		Lys K
CAA	CAG		Gln Q
		AGA, CGA	Arg R
		GGA	Gly G
UCA	UCG	UCA	Ser S
UUA	UUG	UUA	Leu L
	UGG	**UGG**	Trp W
		UGC, UGU	Cys C
UAC, UAU	UAC, UAU		Tyr Y

proof of the therapeutic potential of AAGs was demonstrated in mdx mice, an animal model of Duchenne muscular dystrophy caused by premature PTC in the dystrophin gene.[92] Following treatment with gentamicin, up to 10–20% of the normal level expression of the full-length dystrophin was observed. Since then, the PTC-RT stimulating potential in the therapy of human genetic diseases has been tested for many aminoglycosides, their mimetics and derivatives. A variety of different models have been used in these studies, including *in vitro* transcription and translation systems,[33] cell lines,[34,93-99] and animal models.[100-104] Some of the compounds have shown promising results and have been tested in clinical trials.[105-114]

Although clinical improvement has been observed in some of the studies, the efficiency of PTC-readthrough therapies is not always satisfactory. Essentially, the same factors which determine RT of natural termination codons may also influence the efficiency of PTC suppression. The lessons from the RT of natural STOP codons provide important clues in this respect: the identity of a STOP codon and its surrounding sequence remain the most important factors. Until recently, neither of these could be changed without invasive genome modifications. While the emerging technologies based on the CRISPR/Cas9 system[115] promise a change in the therapeutic potential related to the correction of PTC at the genomic level, it remains important to know how PTCs and their surrounding sequence context influence the potential therapeutic use of translational RT. This knowledge is necessary to make an informed choice of a particular approach, best suited to a given mutation, or to apply additional measures that can influence the RT level. Even more importantly, the efficiency of various AAGs or their derivatives in promoting the suppression of natural STOP codons has to be taken into account in efforts to establish the most effective therapeutic protocol for a given PTC mutation. Finally, further studies are required to better understand and systematize the variety of sequence-unrelated factors that can modulate the suppression of translation termination.

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