Translational errors: from yeast to new therapeutic targets

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Abstract

Errors occur randomly and at low frequency during the translation of mRNA. However, such errors may also be programmed by the sequence and structure of the mRNA. These programmed events are called 'recoding' and are found mostly in viruses, in which they are usually essential for viral replication. Translational errors at a stop codon may also be induced by drugs, raising the possibility of developing new treatment protocols for genetic diseases on the basis of nonsense mutations. Many studies have been carried out, but the molecular mechanisms governing these events remain largely unknown. Studies on the yeast *Saccharomyces cerevisiae* have contributed to characterization of the HIV-1 frameshifting site and have demonstrated that frameshifting is conserved from yeast to humans. Yeast has also proved a particularly useful model organism for deciphering the mechanisms of translation termination in eukaryotes and identifying the factors required to obtain a high level of natural suppression. These findings open up new possibilities for large-scale screening in yeast to identify new drugs for blocking HIV replication by inhibiting frameshifting or restoring production of the full-length protein from a gene inactivated by a premature termination codon. We explore these two aspects of the contribution of yeast studies to human medicine in this review.

Introduction

Ribosomes consist of two ribonucleoprotein subunits acting cooperatively during mRNA translation. The small subunit (40S in eukaryotes) contains the mRNA-binding site, the path along which the mRNA progresses, and the decoding centre, where codons are read by tRNAs. The large subunit (60S in eukaryotes) is the site of peptide bond formation and contains the polypeptide exit tunnel. tRNAs enter the ribosome at the A-site and move to the P-site after peptide bond formation; deacetylated tRNA then moves from the P-site to the E-site before leaving the ribosome (Schmeing & Ramakrishnan, 2009). The standard rules of decoding are well-known. The ribosome reads the mRNA, codon by codon, from a start site (usually AUG) to a stop signal (UAA, UAG or UGA). However, the genetic code is not quite universal, with some codons having different meanings in certain organelles and in a small number of organisms. For example, tryptophan is specified by UGA in human mitochondria (Barrell *et al.*, 1979). This reassignment affects all mRNAs present in these organelles or organisms. In addition, alternative ways of reading the genetic code have been described for specific mRNAs. These extensions of the genetic code, named 'recoding', are programmed by signals present in specific mRNAs (Baranov *et al.*, 2002; Namy *et al.*, 2004).

The translation of mRNA is usually a very accurate process, with an estimated frequency of miscoding of about 0.15% per codon in *Saccharomyces cerevisiae* and 0.34% per codon in *Escherichia coli* (Kramer & Farabaugh, 2007; Fan-Minogue & Bedwell, 2008). However, it is now well-known that the frequency of unconventional decoding (recoding) may be as high as 40% (Grentzmann *et al.*, 1998; Namy *et al.*, 2008; Pennell *et al.*, 2008). Recoding events occur in competition with standard decoding and make possible the synthesis of two or more polypeptides, at a precise ratio, from a single mRNA. Recoding may occur during translational elongation (+1 or −1 frameshifting, hopping, Stop-Go) or termination (stop codon readthrough) (Gesteland & Atkins, 1996; Namy *et al.*, 2004; Atkins *et al.*, 2007).

Recoding may extend the gene expression repertoire in several ways. It may lead to the production of a more diverse set of proteins from a single mRNA (Blinkowa & Walker,

1990; Flower & McHenry, 1990; Tsuchihashi & Kornberg, 1990; Morris & Lundblad, 1997). Typical examples can be found in *S. cerevisiae*, in which two polypeptides are generated from the mRNA corresponding to the *EST3* gene in a +1 frameshifting event (Morris & Lundblad, 1997; Asakura *et al.*, 1998). A similar mechanism is involved in the expression of *ABP140* (Asakura *et al.*, 1998). Recoding may lead to the incorporation of nonstandard amino acids, such as selenocysteine at UGA codons and pyrrolysine at UAG codons (Yuan *et al.*,; Leinfelder *et al.*, 1988; Namy *et al.*, 2004, 2007; Allmang & Krol, 2006). Recoding events mostly occur in viruses and mobile elements, playing an essential role in life cycle regulation (Baranov *et al.*, 2006; Girnary *et al.*, 2007; Mazauric *et al.*, 2008, 2009; Chung *et al.*, 2010). Several viruses make use of frameshifting to produce enzymes involved in replication. This is the case for some retroviruses, several eukaryotic positive-strand RNA viruses, double-stranded RNA viruses from yeast, some plant RNA viruses and bacteriophages (for a review, see Brierley & Dos Ramos, 2006). Ribosomal frameshifting is frequently used in the production of replicases. It is involved in the synthesis of the Gag-Pol and Gag-Pro-Pol polyproteins of retroviruses and is essential for production of the RNA-dependent RNA polymerase in a number of other viruses. Finally, recoding can regulate gene expression through an autoregulatory loop mediating rapid regulation in fluctuating environments. This is well-illustrated by the +1 frameshifting found in both bacterial prfB (RF2) and eukaryotic antizyme genes (Craigen & Caskey, 1986; Matsufuji *et al.*, 1995). The stimulatory element of the RF2 frameshifting site is the UGA stop codon, which is recognized by RF2 itself. At high RF2 levels, the competition between termination and frameshifting is shifted in favour of termination, leading to a decrease in RF2 concentration. This decrease in RF2 levels leads to a decrease in translation termination at the UGA codon, thereby increasing frameshifting efficiency. For antizyme mRNA, high polyamine levels stimulate frameshifting, which is required for the synthesis of a functional antizyme. The production of larger amounts of antizyme leads to inhibition of the first step of the polyamine biosynthesis pathway. This results in a general decrease in polyamine concentration, in turn decreasing frameshifting efficiency. Alternatively, recoding may also regulate gene expression at the mRNA level, by preventing or stimulating mRNA degradation through the nonsense-mediated mRNA decay (NMD) pathway (Plant *et al.*, 2004). It seems likely that recoding events are largely under-identified or misannotated in genomes, but they are clearly present in all life forms and significantly increase the diversity of the polypeptides produced in cells.

We focus here on two types of recoding events – the −1 frameshifting found in two viruses of major public health concern, HIV and SARS-CoV, and stop codon readthrough – both of which have major medical implications. This review aims to highlight the essential contribution of fundamental research in the yeast *S. cerevisiae* to improving our understanding of these complex events and to the possible development of novel treatments in the future.

Discussion

−1 frameshifting and viral diseases

Programmed −1 frameshifting

Programmed frameshifting signals (PRF) are among the most frequently identified recoding sites. The minimal PRF consists of a slippery sequence, at which one or two tRNAs shift the reading frame, and a stimulatory element (a secondary structure) (Brierley *et al.*, 2008). These −1 frameshifting sites may consist of a heptameric slippery sequence of the general structure X XXY YYZ or a tetrameric sequence Y YYZ (the initial reading frame is indicated), separated from the secondary structure by a spacer region. The precise length and composition of this region play an important role in the correct presentation of the stimulatory element to the ribosome for maximal frameshifting efficiency (Kollmus *et al.*, 1994; Kontos *et al.*, 2001; Bertrand *et al.*, 2002). Various secondary structures have been found to increase −1PRF efficiency (stem loop, kissing loop, pseudoknot, etc.; Fig. 1). These secondary structures induce a strong pause of the ribosome during elongation (Somogyi
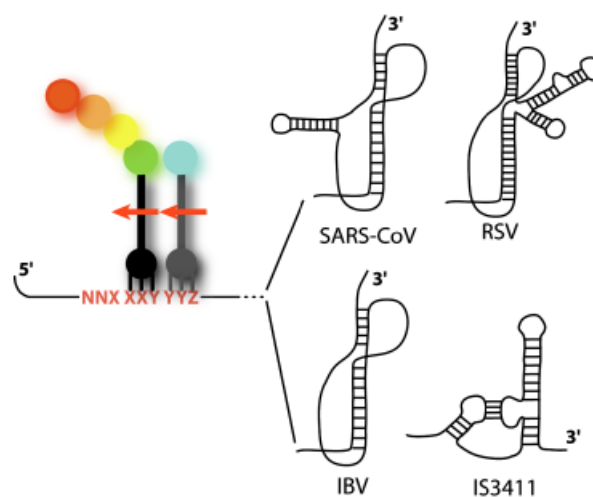

Fig. 1. Simplified representation of a −1 frameshifting site. The slippery sequence is indicated in red, with the initial reading frames indicated by spaces. The P- and A-site tRNAs are shown in black and grey, respectively. The arrows show the direction of the slippage during −1 frameshifting. Four examples of stimulatory secondary structures are shown. Three were identified in viruses [SARS-CoV (Dos Ramos *et al.*, 2004; Plant & Dinman, 2005), RSV (Jacks *et al.*, 1988; Marczinke *et al.*, 1998), IBV (Brierley *et al.*, 1989; Brierley *et al.*, 1991)], and the fourth was identified in a bacterial insertion sequence [IS3411 (Mazauric *et al.*, 2008)].

et al., 1993; Lopinski *et al.*, 2000; Kontos *et al.*, 2001), but this pause is not sufficient to induce frameshifting. The mechanisms by which these structures stimulate − 1PRF have long been a matter of debate (Napthine *et al.*, 1999; Giedroc *et al.*, 2000; Plant *et al.*, 2003; Plant & Dinman, 2005). Namy *et al.* (2006) recently provided a partial solution to this enigma, using a cryoEM approach to study the pausing ribosome at a frameshifter pseudoknot. Namy *et al.* (2006) observed the direct interaction between the pseudoknot and the ribosome and proposed a new model for the stimulation of − 1PRF by a secondary structure. These results have been confirmed by other groups, providing additional support for our original model (Ortiz *et al.*, 2006; Leger *et al.*, 2007). The pseudoknot prevents the completion of a full cycle of translocation, trapping EF2 (EF-G in prokaryotes) within the ribosome, with a distorted tRNA, in an intermediate state of translocation (Moran *et al.*, 2008; Flanagan *et al.*, 2010). Thus, − 1 frameshifting is a powerful tool for studying the mechanisms of reading frame maintenance during translational elongation.

Frameshifting and viral replication

Frameshifting sites are frequently found in viruses, including both HIV-1 and SARS-CoV, in which a slippery sequence followed by either a stem–loop or a pseudoknot is found between the Gag and Pol genes or between ORFs *1a* and *1b* (for a review, see Brierley & Dos Ramos, 2006).

The HIV-1 frameshifting was first identified, in an *in vitro* translational assay, by Jacks *et al.* (1988). It consists of a slippery sequence, U UUU UUA, followed by a stimulatory element. However, a recent study focusing on the architecture and secondary structure of the entire HIV-1 RNA genome indicated that this HIV-1 frameshifting site is part of a larger three-helix structure. The stimulatory element is called P3 and the slippery sequence pairs with an upstream region to form the second helix (P2). These two helices are stabilized by an anchoring helix, P1, which creates the larger structure (Watts *et al.*, 2009) (Fig. 2a). Frameshifting efficiency has never been tested in such a broad context, but this RNA motif may be involved in its regulation. The presence of the ribosome may induce a structural rearrangement. Indeed, the ribosome probably first melts P1 and the upper stem, which then becomes available for base-pairing with the free nucleotides located between the slippery sequence and the stem loop (in blue, Fig. 2a).

Using *S. cerevisiae* to decipher viral frameshifting mechanisms

Wilson *et al.* (1988) were the first to provide an *in vivo* demonstration of a frameshifting event, in *S. cerevisiae*. They inserted the Gag-Pol fragment containing the potential
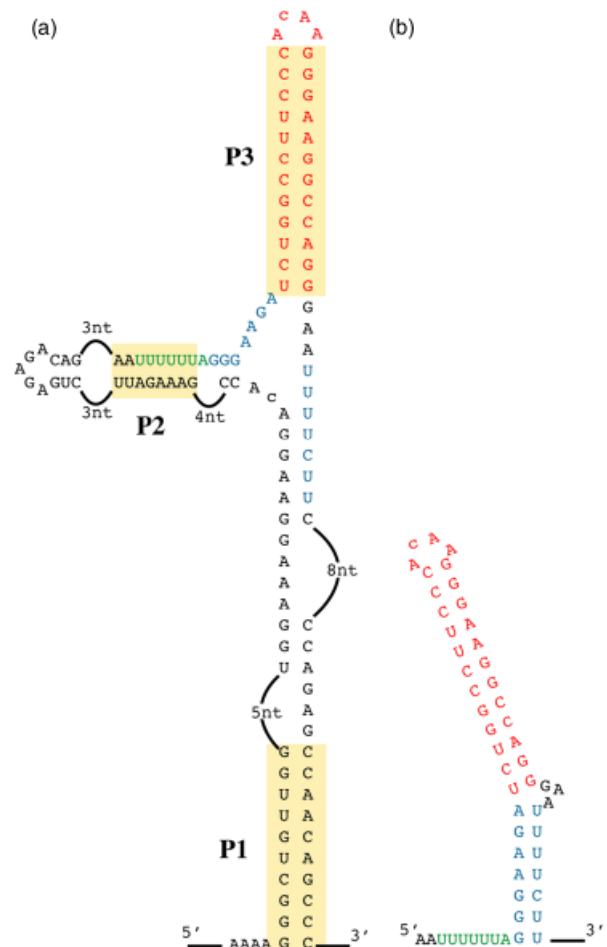

Fig. 2. Structure of the − 1 frameshifting site present in HIV-1. (a) Complete structure of the region of the HIV-1 genome comprising the frameshifting site, as determined by Watts *et al.* (2009). The three stems, P1, P2 and P3, are highlighted. The slippery sequence is indicated in green, the lower part of the predicted stem of the stimulatory structure is shown in blue and the upper stem is shown in red. (b) Representation of the structure of the stem loop proposed by Dulude and colleagues and confirmed by NMR analysis (Dulude *et al.*, 2002; Staple & Butcher, 2005). The significance of colours are as in (a).

frameshifting site of HIV-1 (without the stimulatory element) into a yeast expression plasmid, upstream from the IFN cDNA. They monitored production of the frameshifted protein by Western blotting. This work suffered from the absence of the stimulatory element, which had not yet been identified. The lack of identification of a stimulatory element in initial works had long led researchers to hypothesize that no secondary structure was present in the HIV slippery site (Jacks *et al.*, 1988; Wilson *et al.*, 1988). It is now clear that a stimulatory secondary structure is required for maximal frameshifting efficiency, although the precise nature of this structure remained unclear for a long time. NMR studies have shown that the HIV-1 stimulatory element

consists of a highly stable apical stem forming a continuous helix and capped by a noncanonical U–G base-pair and a ACAA tetraloop (Staple & Butcher, 2005). A less stable lower stem is also present, separated from the upper stem by a three-purine bulge, which introduces a bend between the two stems (Staple & Butcher, 2005) (Fig. 2b). Bidou et al. (1997) showed, with a dual reporter system in yeast, that there is a direct correlation between HIV frameshifting efficiency and the stability of the stem loop. The stem–loop analysed in these studies was the upper part of the complete stimulatory element observed by NMR. Under these conditions, the stability of this structure is clearly linked to frameshifting efficiency. A structure has recently been identified on the basis of the complete genome sequence, and it would be interesting to assess frameshifting efficiency with the complete sequence. The structure of the tetraloop is reminiscent of the tetraloop motif found in the RNaseIII recognition site from S. cerevisiae (Staple & Butcher, 2003). As the ACAA motif is poorly recognized by RNaseIII, it might be possible to engineer the S. cerevisiae RNaseIII for selective targeting of the HIV-1 tetraloop. It would then be necessary to produce this protein in HIV-1-infected cells. This therapeutic approach has yet to be developed, but would provide an interesting approach to the limitation of HIV-1 proliferation.

The similar slippage efficiencies of the HIV frameshifting site in vivo in yeast and in vitro in a mammalian system demonstrate the high level of conservation of frameshifting mechanisms. Moreover, the lack of stimulation of frameshifting in virus-infected cells demonstrated the absence of specific autoregulatory control involving host or HIV-encoded factors in HIV frameshifting (Cassan et al., 1994). The demonstration that − 1 frameshifting is conserved from yeast to humans paves the way for the use of yeast mutants to analyse retroviral frameshifting, as already reported by several groups.

SARS-CoV is a novel coronavirus carrying a − 1 frameshifting signal (Plant & Dinman, 2005). The minimal frameshifting signal in this virus is a U UUA AAC slippery sequence and a stimulatory structure folding into a pseudoknot (Dos Ramos et al., 2004) (Fig. 1). This pseudoknot has several unusual features, including the third stem in loop 2 and the presence of two unpaired adenosine residues within the structure (Baranov et al., 2005; Plant & Dinman, 2005; Brierley & Dos Ramos, 2006). Plant & Dinman (2005) demonstrated the ability of this new site to frameshift in S. cerevisiae. The frequency of frameshifting in yeast was much lower (3%) than for other coronavirus sites tested in yeast [12% for infectious bronchitis virus (IBV)]. This may indicate the existence of subtle differences in terms of frameshifting mechanisms. Indeed, the importance of the unpaired adenosine residues remains unclear, as this part of the pseudoknot is thought to lie outside the ribosome. It

would be interesting to investigate the possible binding of a trans-acting factor, although the binding of such a factor has never been detected with the well-studied IBV coronavirus pseudoknot. The yeast mak8-1 mutant is known to have a specific defect in − 1 frameshifting (Peltz et al., 1999). This mutant carries an altered form of ribosomal protein L3, in the ribosomal peptidyl-transfer centre. This strain was reported to have a slightly higher SARS-CoV frameshifting efficiency than wild-type strains (Plant & Dinman, 2005), in the first demonstration that this newly discovered frameshifting site uses the same mechanisms as those analysed previously.

Frameshifting as a target for treatment

Both HIV-1 and SARS-CoV pose major public health issues. Elucidation of the mechanisms of viral frameshifting is therefore a key first step towards the development of new antiviral strategies. It has been shown for several retroviruses, including HIV, that the modulation of frameshifting efficiency can significantly reduce viral infectivity in cultured cells (Hung et al., 1998). Indeed, viral RNA dimerization and export are strongly affected by changes in the Gag/Gag-Pol protein ratio (Kaye & Lever, 1996; Shehu-Xhilaga et al., 2001). Various compounds, including antibiotics and modified oligoribonucleotides, have been shown to affect frameshifting in vitro or ex vivo (Vickers & Ecker, 1992; Aupeix-Scheidler et al., 2000; Toulme et al., 2001). However, the extent to which these compounds are able to block viral propagation in vivo remains a matter of debate. Nevertheless, frameshifting constitutes a potential target for treatments aiming to interfere with viral multiplication. Indeed, despite the high level of variability between HIV genome sequences, the frameshifting site is strongly conserved among all subtypes of HIV group M. Moreover, frameshifting efficiencies lie within a narrow range for all of the subtypes of HIV-1 group M, suggesting that very few modifications are tolerated at this step of the virus cycle (Baril et al., 2003). Many stages in the HIV replication cycle are targeted by drugs blocking viral replication. However, past clinical experience indicates that resistant HIV-1 variants are likely to emerge, making it necessary to identify additional targets for treatment in the future. No targets for new drugs have been identified in the translational step of the viral cycle (Fig. 3), largely because the translational machinery of the cell is used to translate viral proteins. Any drug preventing the translation of viral proteins would therefore also affect cellular protein production. Targeting the frameshifting step may make it possible to circumvent this problem (Hung et al., 1998), potentially allowing the development of new treatments specifically targeting the synthesis of viral proteins. One potential issue for the development of this approach concerns the possibility that
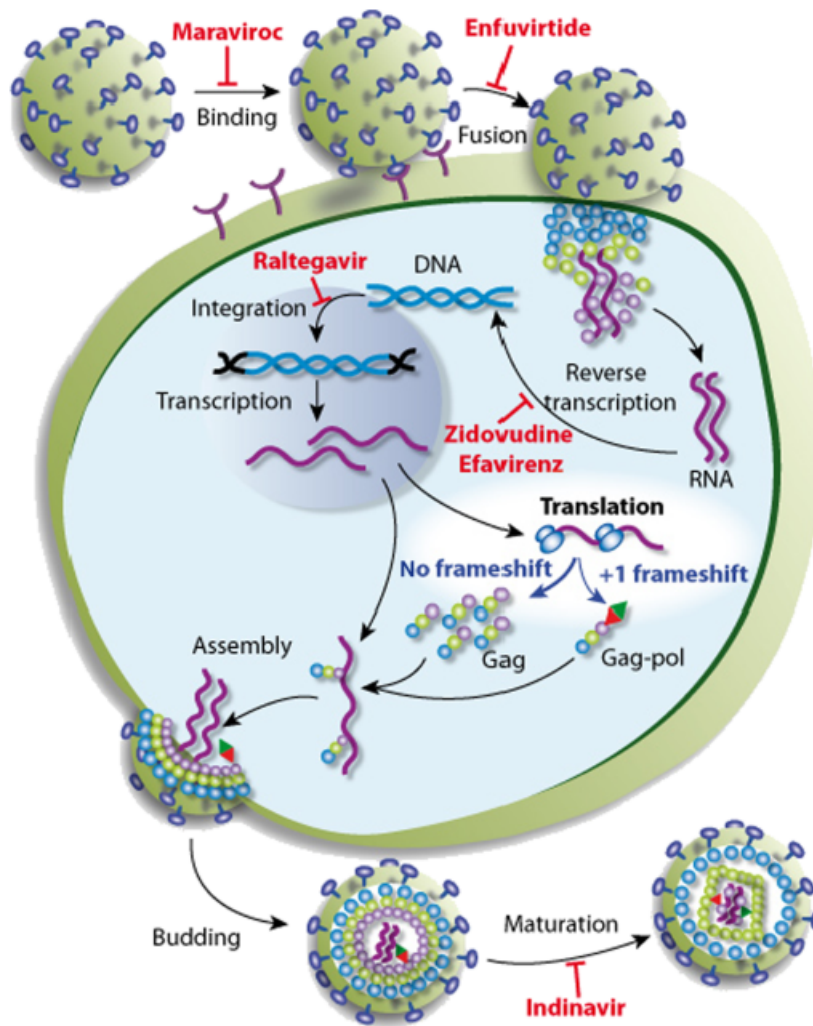
Fig. 3. Schematic diagram of the virus infection cycle. One example of the drugs in common use is indicated at each step of the virus cycle. Maraviroc is an entry inhibitor blocking the interaction between the virus and the chemokine receptor CCR5 on host cells. Enfuvirtide blocks fusion by binding to the gp41 subunit of the viral envelope glycoprotein, thereby preventing the conformational changes required for the fusion of viral and cellular membranes. Zidovudine and efavirenz belong to two different classes of reverse transcriptase inhibitors. Raltegravir is an integrase inhibitor. Indinavir inhibits maturation by blocking HIV-1 proteases. This simplified view highlights the lack of drugs targeting the translational step of the virus cycle.

cellular genes may use frameshifting during their normal expression. To date, only one mammalian gene has been shown to use -1 frameshifting (Shigemoto *et al.*, 2001; Manktelow *et al.*, 2005; Clark *et al.*, 2007). This gene is expressed exclusively during embryogenesis, but we cannot yet exclude the possibility that other, as yet unidentified, genes make use of recoding events in their expression. Careful evaluation of the risk/benefit ratio is therefore required. It is thus of prime importance to design efficient tools for identifying recoding sites in genomes. In the last decade, several bioinformatic approaches have been developed for this purpose (see references in Namy *et al.*, 2004). Most such studies to date have focused on *S. cerevisiae*, because the genome of this yeast is of high quality (low

frequency of sequencing errors), with well-identified introns clustered in a small number of genes. The extension of these approaches to more complex genomes is required, but this is currently limited by the difficulty of intron identification and the complexity of the mRNA population generated by alternative splicing. Hopefully, the development of deep sequencing approaches will partially resolve this problem, making it possible to develop an accurate mRNA database.

The conservation of -1 frameshifting mechanisms between *S. cerevisiae* and mammals makes it possible to screen for active molecules targeting this step. The yeast model occupies a key role in the search for new active drugs against the frameshifting of both HIV-1 and SARS-CoV. Indeed, two groups have already carried out genetic

screening in *S. cerevisiae* with a view to isolating mutations affecting frameshifting efficiency. They identified chromosomal mutations that they called *mof* (maintenance of frame) (Dinman & Wickner, 1994) or *ifs* (increased frameshifting) (Lee *et al.*, 1995). However, the ifs1-1 and mof4-1 mutations were subsequently found to inactivate the Upf2 and Upf1 proteins, respectively. These two proteins are involved in the degradation of mRNAs containing a premature termination codon (PTC) through the NMD pathway (Cui *et al.*, 1996) and have no effect on frameshifting efficiency *per se* (Bidou *et al.*, 2000; Harger & Dinman, 2004). Ribosomal proteins and translation factors may also affect frameshifting, but the potential of these proteins for use in treatment remains unclear. It will be essential to characterize the role of these proteins in the frameshifting mechanism for the identification of new targets for treatment.

Stop codon readthrough and 'stop codon diseases'

Translation termination in eukaryotes

In organisms using a standard genetic code, translation is terminated when one of the three stop codons, UAA, UGA or UAG, enters the ribosomal A-site (Fig. 4). Unlike sense codons, which are recognized by tRNAs, stop codons are recognized by a group of proteins called class I release factors (Frolova *et al.*, 1994). Translation termination efficiency depends on competition between stop codon recognition by release factors and decoding by near-cognate tRNAs that can pair with two of the three bases of the stop codon. In eukaryotes, two eukaryotic release factors, eRF1 and eRF3, mediate translation termination (Stansfield *et al.*, 1995). *Saccharomyces cerevisiae* has proved a powerful tool for the identification and characterization of eukaryotic release factors (Frolova *et al.*, 1994). Full and partial X-ray structures have been determined for both proteins, providing insight into their function (Song *et al.*, 2000; Cheng *et al.*, 2009). The eRF1 protein recognizes stop codons through its N-terminal domain and triggers peptidyl-tRNA hydrolysis by activating the peptidyl transferase centre of the ribosome through the highly conserved NIKS and GGQ motifs in domains 1 and 2, respectively (Frolova *et al.*, 2002). The C-terminal domain of eRF1 is involved in binding eRF3 (Eurwilaichitr *et al.*, 1999). The overall shape of human eRF1 mimics a tRNA, positioning the functional motifs GGQ and NIKS in the peptidyl centre and decoding sites of the ribosome, respectively. The molecular mechanisms underlying this process remain unclear in eukaryotes. The presence of a stop codon in the ribosomal A-site necessitates a conformational rearrangement for the activation of eRF3 GTPase activity, with GTP hydrolysis facilitating the correct positioning of the GGQ motif in the peptidyl transferase centre for the catalysis of peptidyl-tRNA cleavage; eRF3 then acts as a proofreading factor during termination (Salas-Marco & Bedwell, 2004).

Despite the competition between near-cognate tRNAs and release factors (Fig. 4), very little incorporation of these
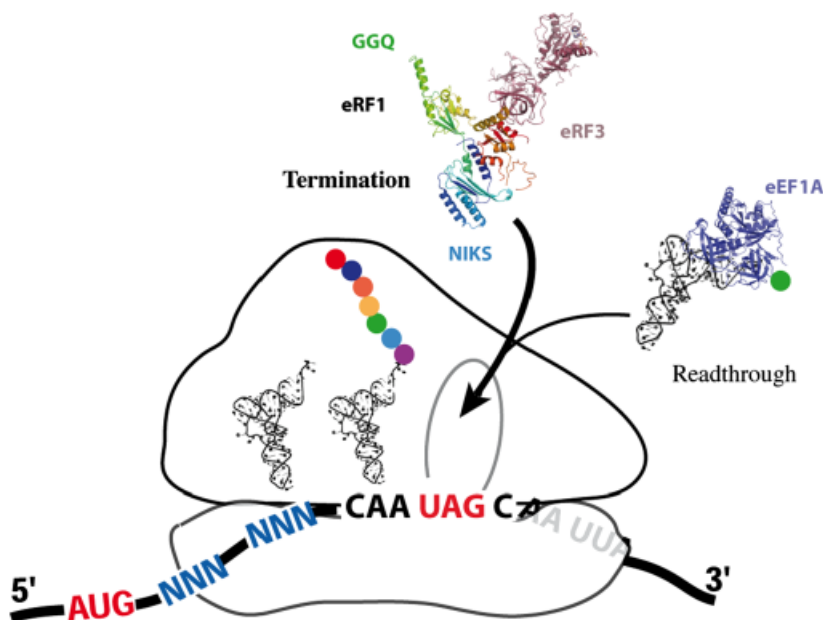

Fig. 4. Schematic diagram of the competition between termination and readthrough. Release factors are represented by their X-ray structures (PDB: 3E1Y). The stop codon (UAG) is located in the A-site, whereas the P- and E- sites are occupied by tRNAs. The incoming natural suppressor tRNA is shown bound to the elongation factor (PDB: 1B23). Aminoglycosides facilitate the acceptance of natural suppressor tRNAs by the ribosome.

tRNAs is observed under normal conditions, with an error rate of 0.3% in a standard yeast strain (Namy *et al.*, 2003). The nucleotides around the stop codon (stop codon context) are a key determinant of termination efficiency and can 'reprogramme' the stop codon to increase readthrough efficiency to levels as high as 20%. We have developed a powerful reporter system in *S. cerevisiae*, based on the insertion of a stop codon in the *ADE2* gene, for combinatorial analysis of the stop codon context (Namy *et al.*, 2001). *ADE2* encodes the P-ribosyl-amino-imidazole carboxylase (EC 4.1.1.21) responsible for degradation of the red pigment amino-imidazole ribotide. We have shown that yeast colonies change colour, from red to white, when readthrough levels at the *ADE2* gene exceed 10% (Namy *et al.*, 2001). This and other studies have demonstrated that, in *S. cerevisiae*, the six downstream nucleotides play a crucial role in stop codon suppression efficiency (Bonetti *et al.*, 1995; Namy *et al.*, 2001). Similar findings have been reported for plants (Skuzeski *et al.*, 1991) and mouse cells (Cassan & Rousset, 2001). Stop codon readthrough was first recognized in the 1970s as a mechanism by which viruses regulate the ratio between two proteins generated by translation from the same mRNA (Gesteland & Atkins, 1996). It has also been described in other organisms (Namy *et al.*, 2004), but the factors governing the selection of the near-cognate tRNA remain unknown. Nonetheless, the conservation of stop codon readthrough from yeast to mammals (Stahl *et al.*, 1995) has made it possible to use *S. cerevisiae* to characterize this mechanism in more detail, including, in particular, the role of the nucleotides surrounding the stop codon.

Translation termination: a target for treatment

Many genetic disorders and cancers involve a PTC. The use of drugs to induce PTC readthrough would pave the way to the development of many novel treatment protocols. These promising approaches could, at least theoretically, be applied to all 'stop codon' diseases. Indeed, PTCs account for 10–30% of inherited diseases, including cystic fibrosis (CF), haemophilia, retinitis pigmentosa and Duchenne muscular dystrophy (DMD) (Zingman *et al.*, 2007). Most of the mutations giving rise to these PTCs lead to a complete loss of protein function and a decrease in mRNA levels, through NMD. The first attempts to suppress PTCs in eukaryotic cells involved the use of aminoglycosides, a large family of structurally related antibiotics. In both prokaryotes and eukaryotes, aminoglycosides induce miscoding by mimicking the change in the conformation of 16S and 18S rRNA genes that would be induced by a correct codon–anticodon pair, thereby compromising the integrity of codon–anticodon proofreading during translation (Burke & Mogg, 1985; Carter *et al.*, 2000; Pape *et al.*, 2000). Palmer *et al.* (1979) were the first to demonstrate that paromomycin

could abolish some nonsense mutations in *S. cerevisiae*. A few years later, Burke & Mogg (1985) showed that paromomycin and G-418 partially restored the synthesis of a functional protein from a mutant gene with a UAG nonsense mutation in cultured mammalian cells. The feasibility of extending this approach, as a therapeutic strategy for human diseases, was first demonstrated by the work of Sweeney's group, who demonstrated that gentamicin partly restored the production of full-length dystrophin in mdx mice carrying the X-linked muscular dystrophy mutation (mdx) (Barton-Davis *et al.*, 1999). Their seminal work incited considerable interest in translation termination as a target for treatment, with many subsequent studies validating this approach for several genetic disease models (Rowe & Clancy, 2009). These strategies are based on the stimulation of PTC readthrough by various molecules, including gentamicin, PTC124, negamycin or the recently characterized tylosin, a member of the macrolide family (Welch *et al.*, 2007; Rosin-Arbesfeld *et al.*, 2009), that induces errors during the termination process (Hainrichson *et al.*, 2008). Bedwell *et al.* (1997) have also shown that both G-418 and gentamicin restore production of the cystic fibrosis transmembrane conductance regulator (CFTR) in a bronchial cell line carrying a nonsense mutation in the CFTR gene. Similarly, a Hurler syndrome fibroblast cell line heterozygous for a stop mutation displayed a significant increase in α-L-iduronidase levels when cultured in the presence of gentamicin (Keeling & Bedwell, 2002). Several clinical trials have also provided preliminary evidence that gentamicin abolishes stop mutations in DMD and CF patients, but it remains to be determined whether the resulting protein levels are sufficiently high to be of therapeutic benefit (Wilschanski *et al.*, 2000; Wagner *et al.*, 2001; Politano *et al.*, 2003).

We recently collaborated in a clinical trial on CF patients with various nonsense mutations. The patients were treated intravenously with gentamicin, every day, for 15 days. Clinical benefits were observed in a subset of these patients, with significant alterations to CFTR-mediated chloride transport in nasal and sweat gland epithelia. There was a correlation between the level of readthrough obtained and the improvement in clinical status observed. This clinical trial was the first to show that the parenteral administration of gentamicin at a dose previously demonstrated to be safe could have beneficial clinical effects (Sermet-Gaudelus *et al.*, 2007).

A new molecule (PTC124 or ataluren) discovered by PTC Therapeutics, a biotech company, is currently undergoing evaluation for use in DMD and CF, in clinical trials. However, the effect of PTC124 on readthrough efficiency remains a matter of debate, following the publication of a study demonstrating that PTC124 is a potent competitive inhibitor of the firefly luciferase, a reporter commonly used

to quantify PTC readthrough level (Auld *et al.*, 2009, 2010; Peltz *et al.*, 2009). Such biases probably occur frequently and highlight the importance of using different reporter systems during the process of drug screening, to prevent the isolation of compounds acting specifically on the reporter system.

Another major issue identified in many studies concerns differences in the sensitivity of nonsense mutations to readthrough-promoting drugs. As a result, only a subset of PTC-carrying patients would benefit from pharmacological treatments of this type (Bidou *et al.*, 2004). Interestingly, these analyses revealed that the mdx mutation directs a very low level of readthrough, despite the strong stimulation of dystrophin accumulation observed in the initial study by Barton-Davis and colleagues. This may reflect the inhibition of the NMD pathway by the antibiotic, through stabilization of the mRNA molecule being read by the ribosome (Allamand *et al.*, 2008).

The ability of a drug to suppress a PTC depends on several factors, including the identity of the stop codon and the context surrounding it. The first nucleotide after the stop codon (+4) has been reported to be associated with high levels of readthrough, in the presence or absence of aminoglycosides (Manuvakhova *et al.*, 2000). Moreover, we have shown that, in cultured mammalian cells, a C at this position could also be associated with moderate readthrough levels (Bidou *et al.*, 2004). The impact of the +4 nucleotide largely depends on the surrounding nucleotides. Unfortunately, the factors determining readthrough efficiency remain unknown, making it impossible to predict the efficiency of drugs for promoting readthrough for a given mutation.

The use of *S. cerevisiae* to decipher readthrough mechanisms

Bedwell's laboratory was among the first to analyse PTC suppression in its natural gene context. They examined the suppression of a PTC mutation in the yeast gene encoding the Ste6 protein (Ste6p), which is highly conserved among members of the ATP-binding cassette transporter family (Fearon *et al.*, 1994). The human CFTR, which is defective in individuals with CF, also belongs to this protein family. The Ste6 mutations examined were chosen because a PTC at the corresponding residue of the CFTR gene has been reported to cause less severe pulmonary involvement than some missense mutations. This suggests that low-level suppression of this stop codon may occur and, indeed, the results indicated that this PTC could be suppressed at frequencies as high as 10% in yeast. The authors showed that a limited sequence context surrounding this site contained sufficient information to abolish translation termination (Fearon *et al.*, 1994). They also highlighted the

hierarchy between the three stop codons in *S. cerevisiae*, showing that the amber UAG codon was suppressed more efficiently than the opal (UGA) or ochre (UAA) codons. A similar hierarchy is generally found in mammalian cells in culture. Experiments from Bedwell's group have also demonstrated that the mechanism responsible for suppression is tRNA mispairing, rather than the ribosome hopping previously shown to occur in some cases in *E. coli* (Weiss *et al.*, 1987). This group also analysed the amino-acid sequence of the readthrough protein and demonstrated that readthrough of the UAG codon was mediated by the incorporation of at least three near-cognate tRNAs (tyrosine, lysine, or tryptophan). Unfortunately, the incorporation of an amino acid in place of the PTC has been demonstrated in very few studies. Consequently, the potential effect of readthrough drugs and stop codon context on the incorporation of near-cognate tRNAs is unknown. Further characterization is required, as the stability and/or activity of the resulting full-length protein may be critically dependent on the amino acid incorporated (Allamand *et al.*, 2008). Further studies could be carried out in *S. cerevisiae*, for which convenient reporter systems already exist, or in mammalian cells, for which a dedicated reporter system has yet to be constructed. A key issue in these analyses is the very low level of stop codon readthrough. The presence of the [*PSI*$^+$] prion in yeast may make it possible to overcome this problem to some extent. [*PSI*$^+$] is the prion form of eRF3, which is encoded by the *SUP35* gene in *S. cerevisiae*. (Ter-Avanesyan *et al.*, 1993; Wickner, 1994). The change in the conformation of eRF3 to generate its prion-aggregated form results in an impairment of termination activity and higher levels of stop codon readthrough, facilitating the analysis considerably. Moreover, many mutant strains of yeast in which one of the release factors is affected are available and could prove very useful for studies of stop codon readthrough in a genetic context of weak termination.

Saccharomyces cerevisiae as a host for the development of a large-scale screening strategy

Aminoglycosides are toxic, with renal, cochlear and vestibular side effects, and this is a major concern for their clinical use (Kahlmeter & Dahlager, 1984). However, these side effects are not directly associated with the ability of aminoglycosides to suppress PTCs. It should therefore be possible to identify compounds inducing highly efficient codon readthrough, but with lower levels of toxicity. Very few compounds other than aminoglycosides have been shown to increase readthrough. These compounds include negamycin and PTC124 (Arakawa *et al.*, 2003; Welch *et al.*, 2007). These two molecules have been reported to have fewer side effects, but it remains unclear whether they induce readthrough significantly more efficiently than

gentamicin. Tylosin has also been shown to induce stop codon readthrough efficiently for a PTC in the APC gene (Rosin-Arbesfeld *et al.*, 2009). This drug is potentially interesting because it stimulates readthrough strongly and has only limited toxicity in animals. It belongs to the macrolide family, the members of which bind to the polypeptide exit channel of the large subunit of the ribosome (Hansen *et al.*, 2002). The disaccharide moiety of these molecules extends toward the catalytic centre of the ribosome. Macrolide binding induces the rearrangement of base A2062 (*E. coli* numbering) to generate an extended conformation allowing the formation of a covalent bond with the macrolides. This rearrangement is very different from that induced by aminoglycosides. The two classes of drugs could, therefore, potentially be used together to generate high levels of readthrough, giving beneficial therapeutic effects while making it possible to use each compound at a lower, less toxic concentration.

Considerable efforts are currently being made to develop new readthrough-inducing molecules that are more specific to the termination reaction and less toxic (Kaufman, 1999). These approaches would benefit considerably from the development of new screening procedures. *Saccharomyces cerevisiae* is a host of choice for such screening. The *ADE2* system can be used to screen chemical libraries. Under basal readthrough conditions, the Ade2p protein is produced in very small amounts and the colonies are red. In pilot experiments, we have shown that treatment with aminoglycosides results in white colonies. This system can be used for the high-throughput screening of compounds or for testing chemically modified drugs, such as aminoglycoside derivatives. Indeed, improvements in our understanding of the binding of aminoglycosides to eukaryotic RNAs would make it possible to design more specific compounds. In addition, *S. cerevisiae* is also an ideal model organism for powerful genetic screens, making it possible to identify the biological entities targeted by the isolated compounds (reverse screening assays) (Bach *et al.*, 2003; Tribouillard *et al.*, 2007).

Concluding remarks

The conservation of translational fidelity mechanisms between the yeast *S. cerevisiae* and mammals has made it possible to develop the use of yeast as a model organism. Many studies have made use of this model to decipher the roles of various components of the translation apparatus or to determine the effects of drugs modifying translation accuracy. *Saccharomyces cerevisiae* has been instrumental in the identification and characterization of eukaryotic release factors. It has also been used for the selection and characterization of a large number of mutations affecting translational fidelity at various stages, including reading frame

maintenance and termination accuracy. These mutants are currently used in studies on the mechanism of translational fidelity. One of the current challenges in this field is elucidation of the mechanisms underlying the contribution of the nucleotides surrounding the termination codon to readthrough efficiency (the so-called 'context effect'). Readthrough levels may differ by a factor of 100 between nonsense mutations, but the molecular mechanism mediating the effect of stop codon context remains unknown. Similarly, we know nothing about the signals involved in the differences in response to aminoglycoside treatment. We believe that in these cases, the yeast *S. cerevisiae* will prove to be a potent tool for deciphering the basis (actors, interactions) of the mechanisms at work.

The development of procedures for screening chemical libraries in *S. cerevisiae* to identify drugs acting at different stages of gene expression is promising. As far as frameshifting is concerned, such approaches may open up new possibilities for the development of innovative treatment protocols for combating retroviruses, such as HIV-1. Rational drug design would also be an interesting alternative, but would require accurate knowledge of the structures of the factors involved in frameshifting. Ribosomal proteins are a target of choice for the modulation of frameshifting efficiency, but little is known about their precise role in frame maintenance. It also seems likely that several of these proteins may be involved in the association of ribosomal subunits, limiting the use of drugs targeting these proteins.

The termination step is biochemically well-characterized in eukaryotes, but too little is known about the structure of the termination complex within the ribosome for the rational design of molecules interfering with this step. *Saccharomyces cerevisiae* will undoubtedly continue to be used as a powerful model for deciphering the complex structural and functional interactions between the different players (rRNA and proteins, tRNAs, release factors, etc.) dictating readthrough efficiency. It may also contribute to the identification of new molecules interfering with either frame maintenance or termination efficiency.

Acknowledgements

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