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Evidence that translation reinitiation abrogates nonsense-mediated mRNA decay in mammalian cells

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Nonsense codons upstream of and including position 192 of the human gene for triosephosphate isomerase (TPI) have been found to reduce the abundance of TPI mRNA to ~25% of normal. The reduction is due to the decay of newly synthesized TPI mRNA that co-purifies with nuclei. TPI mRNA that co-purifies with cytoplasm is immune to nonsense-mediated decay. Until now, a nonsense codon at position 23 has been the 5′-most nonsense codon that has been analyzed. Here, we provide evidence that a nonsense codon at position 1, 2 or 10 reduces the abundance of nucleus-associated TPI mRNA to an average of only 84% of normal because translation reinitiates at the methionine codon at position 14. First, converting codon 14 to one for valine increased the effectiveness with which an upstream nonsense codon reduces mRNA abundance. Second, when TPI gene sequences, including codon 14, were fused upstream of and in-frame to the translational reading frame of an *Escherichia coli* chloramphenicol acetyl transferase (CAT) gene that lacked an initiation codon, a nonsense codon at TPI position 1 or 2 allowed for the production of TPI–CAT that was an estimated 14 amino acids smaller than TPI–CAT produced by a nonsense-free gene, whereas a nonsense codon at TPI position 23 precluded the production of TPI–CAT. These and related findings lend credence to the concept that the nonsense-mediated reduction in the half-life of nucleus-associated TPI mRNA involves cytoplasmic ribosomes.

Keywords: mRNA decay/nonsense codons/translation reinitiation

Introduction

For all organisms that have been examined, mRNAs that prematurely terminate translation are generally abnormally low in abundance (reviewed in Peltz *et al.*, 1994; Maquat, 1995, 1996). Nonsense codons within the gene for human triosephosphate isomerase (TPI) have been shown to reduce TPI mRNA abundance by reducing the half-life of TPI mRNA that co-purifies with nuclei (Cheng and Maquat, 1993; Belgrader *et al.*, 1994). The finding that many mammalian nonsense mRNAs are reduced in abundance in nuclear and cytoplasmic fractions to the same extent suggests that nonsense-mediated decay takes place either prior to or concomitant with nuclear mRNA export (reviewed in Maquat, 1995, 1996). At least for nonsense TPI transcripts, the possibility of decay concomitant with nuclear mRNA export has been put forth as a logical mechanism considering that (i) cytoplasmic ribosomes constitute an integral component of the only known means by which nonsense codons are recognized and (ii) nuclear mRNA may be simultaneously translocated across the pore and translated by cytoplasmic ribosomes (reviewed in Maquat, 1995). The best evidence for the concomitant export and translation of nuclear mRNA by cytoplasmic ribosomes derives from demonstrations using electron microscope tomography that the Balbiani ring granule, a large ribonucleoprotein particle of the dipteran *Chironomus tentans*, is invariably exported from the nucleus 5′-end-first and becomes associated with cytoplasmic ribosomes before the 3′-end transits the nuclear pore (Mehlin *et al.*, 1992). While translation does not appear to be required for mRNA export (Belgrader *et al.*, 1993), translation during export may offer the cell an expedient opportunity to get rid of incompletely translated, dysfunctional mRNAs, some of which have been shown to be deleterious to cell viability (Kazazian *et al.*, 1992; Thein, 1992; Pulak and Anderson, 1993; Hall and Thein, 1994).

Evidence that cytoplasmic ribosomes may not be involved in nonsense-mediated decay derives from reports that nonsense codons can be associated with an increased level of mRNA that lacks the nonsense codon-containing exon or an increased level of mRNA that lacks part of an exon or contains part or all of an intron (reviewed in Maquat, 1995, 1996). These reports have led to the proposal that nonsense codons can mediate exon skipping, cryptic splicing or intron retention by influencing splice site choice (Naeger *et al.*, 1992; Dietz *et al.*, 1993; Dietz and Kendzior, 1994; Lozano *et al.*, 1994; Aoufouchi *et al.*, 1996), implying that nonsense codon recognition can take place in the nucleus either prior to or concomitant with splicing (Urlaub *et al.*, 1989). A recent study proposes that nonsense codons can be recognized prior to splicing in a ribosome-independent mechanism. This proposal is based on the finding that nonsense mutations inhibit the splicing of immunoglobulin pre-mRNA in intact B cells as well as B-cell nuclear extracts in the presence of either puromycin or cycloheximide (Aoufouchi *et al.*, 1996). At least for TPI transcripts, however, nonsense codon recognition appears to take place after splicing, since nonsense codons that span two exons, i.e. are interrupted by an intron prior to splicing, reduce the abundance of TPI mRNA to the same extent as nonsense codons that are confined to a single exon (Zhang and Maquat, 1996).

Given that mammalian cells are not readily amenable to genetic analysis, clues to the mechanisms by which nonsense codons alter nuclear RNA metabolism have derived from the biochemical suppression of decay. To date, suppression of the nonsense-mediated decay of nucleus-associated TPI mRNA has been achieved by

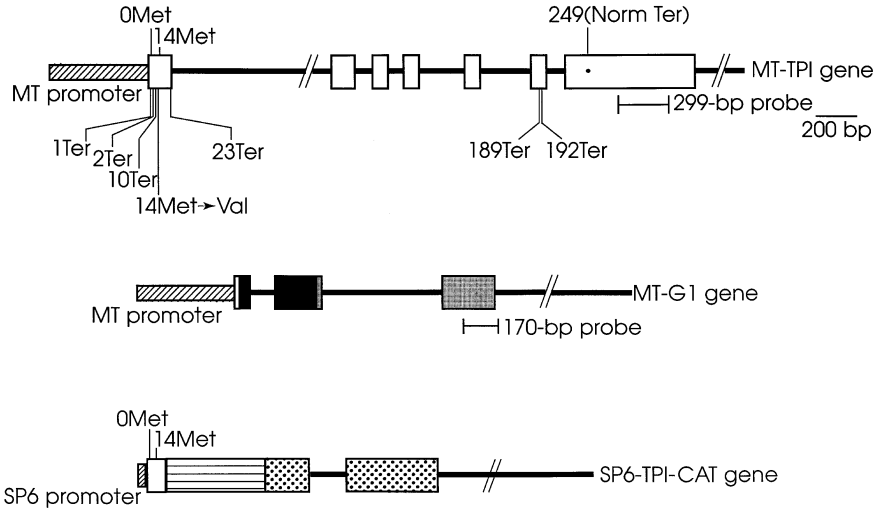

Fig. 1. Structures of the MT-TPI, MT-Gl and SP6-TPI–CAT genes. Diagonally lined boxes represent the 750 bp *Bam*HI–*Bam*HI fragment that harbors the mouse MT-1 promoter (Cheng and Maquat, 1993) or the 17 bp bacteriophage SP6 promoter of pGEM7Zf(+). Larger boxes and interspersed lines represent, respectively, exons and introns. Open boxes represent exons of the TPI gene. Black and gray boxes represent exons of a hybrid human–mouse β-globin gene (Cheng and Maquat, 1993), where the human exons are black and the mouse exons are gray. Horizontally lined and dotted boxes represent exons of a hybrid CAT–SV40 gene (Promega), where the CAT exon is horizontally lined and the SV40 exons are dotted. The horizontal line to the right of and contiguous with the final exon of each gene represents 3′ flanking DNA. Vertical lines above the MT-TPI and SP6-TPI–CAT structures specify the normal translation initiation codon (ATG) at position 0 (0Met), a second ATG codon at position 14 (14Met) and the normal translation termination codon (TGA) at position 249 (Norm Ter). Vertical lines below the MT-TPI structure specify nonsense codons at position 1 (1Ter;TAG), 2 (2Ter;TAG), 10 (10Ter;TGA), 23 (23Ter;TAG), 189 (189Ter;TGA) and 192 (192Ter;TAG), as well as the Met(ATG)→Val(GTT) missense mutation at position 14. The restriction fragments that were used as hybridization probes to detect product RNAs of the MT-TPI and MT-Gl alleles are diagramed below the structures.

expressing a suppressor tRNA that acts *in trans* to recognize a nonsense codon as a coding codon or by inserting into the 5′-untranslated region of TPI transcripts a hairpin structure that acts *in cis* to inhibit translation initiation (Belgrader *et al.*, 1993). Additionally, nonsense-associated alterations in the levels of alternatively spliced immunoglobulin (Ig)μ and α-L-iduronidase transcripts as well as the levels of T-cell receptor β and β-globin mRNAs have been suppressed by protein synthesis inhibitors that interact with either 40S or 60S ribosomal subunits (Qian *et al.*, 1993; Menon and Neufeld, 1994; Carter *et al.*, 1995). Suppression of the nonsense-mediated reduction in the abundance of T-cell receptor β mRNA can also occur by inactivating cap-dependent translation using poliovirus infection (Carter *et al.*, 1995). Finally, placing a nonsense codon out-of-frame relative to the initiation codon can suppress the nonsense-associated alterations in the levels of alternatively spliced transcripts for the R2 protein of the minute virus of mouse or fibrillin (Naeger *et al.*, 1992; Dietz and Kendzior, 1994). Each of these results implicates ribosomes in the process by which nonsense codons reduce the levels of nucleus-associated mRNA.

In seeking further comparisons of nonsense codon recognition during cytoplasmic translation and nonsense-mediated decay, we have examined how translation reinitiation downstream of a nonsense codon affects the level of nucleus-associated mRNA. Studies of reinitiation were particularly important given the proposal for *Saccharomyces cerevisiae* that reinitiation downstream of a nonsense codon within the destabilizing element may be a requirement for nonsense-mediated mRNA decay (Peltz *et al.*, 1993, 1994; Zhang *et al.*, 1995). We have found that reinitiation suppresses the nonsense-mediated reduction in the abundance of human TPI mRNA, which is consistent with recently published studies of nonsense mRNAs in yeast (Ruiz-Echevarria and Peltz, 1996). Our findings support a role for cytoplasmic ribosomes in the nonsense-mediated decay of mRNAs in mammalian cells.

Results

Nonsense codons within the first half of exon 1 do not appreciably reduce the abundance of TPI mRNA

Nonsense codons at positions 1, 2 and 10 of the human TPI gene were generated by site-directed mutagenesis, and the consequence of each nonsense codon with regard to the abundance of nuclear and cytoplasmic TPI mRNA was assessed using Northern blot hybridization. mRNA was generated from pMT-TPI plasmids in which TPI gene transcription was driven by the mouse metallothionein (MT)-1 promoter (Figure 1; Belgrader *et al.*, 1994; Cheng *et al.*, 1994). Each pMT-TPI derivative was transiently introduced into mouse L cells together with pMT-Gl, which contains a β-globin gene similarly driven by the mouse MT-1 promoter (Figure 1; Belgrader *et al.*, 1994; Cheng *et al.*, 1994). MT-Gl mRNA served to control for variations in the efficiencies of cell transfection and RNA recovery. The level of each MT-TPI mRNA was normalized to the level of MT-Gl mRNA and expressed relative to the level of normal MT-TPI mRNA, which was defined as 100%.

As has been shown previously, 23Ter within exon 1 and 189Ter within exon 6 reduced the abundance of nuclear and cytoplasmic mRNA to an average of ~25% of normal (Figure 2; Belgrader *et al.*, 1994; Cheng *et al.*, 1994). In contrast, nonsense codons at positions 1, 2 and 10 within exon 1 reduced the abundance of nuclear and

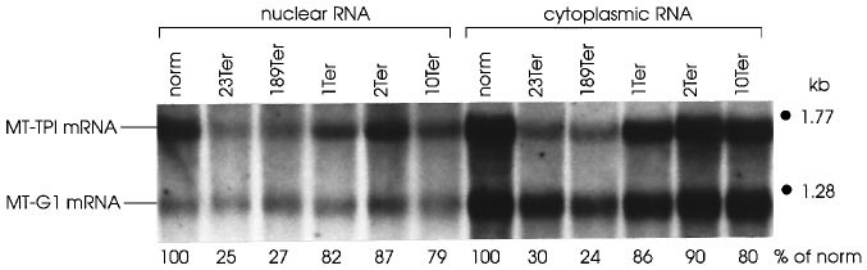

Fig. 2. 1Ter, 2Ter and 10Ter within exon 1 do not evoke the full extent of the nonsense-mediated reduction in mRNA abundance. L cells were co-transfected with a pMT-TPI construct as specified in each lane and the control pMT-Gl construct. Nuclear and cytoplasmic RNA from either transfected or untransfected L cells was purified using guanidine isothiocyanate and cesium chloride gradient centrifugation (Cheng and Maquat, 1993). RNA (25 μg) was analyzed by blot hybridization (Cheng and Maquat, 1993). The level of mRNA from each MT-TPI allele was normalized to the level of MT-Gl mRNA in order to control for variations in the efficiencies of cell transfection and RNA recovery. Normalized values were then calculated as the percentage of MT-TPI norm mRNA (% of norm), which was defined as 100, and are representative of two independently performed experiments. None of the values for the percentage of norm differed between the two experiments by >5%.

cytoplasmic mRNA to an average of only 84% of normal (Figure 2). Therefore, 1Ter, 2Ter and 10Ter are distinct from all other nonsense codons that have been generated upstream of and including position 192 (i.e. ⩾52 nucleotides upstream of intron 6), which reduce mRNA abundance to an average of 25% of normal (Cheng *et al.*, 1990, 1994).

14Met is important in abrogating the reduction in TPI mRNA abundance that is mediated by nonsense codons within the first half of exon 1

There are several possible explanations for the failure of 1Ter, 2Ter and 10Ter to elicit the full nonsense-mediated reduction of mRNA abundance. The possibility that each nonsense codon decreases the efficiency of translation initiation at 0Met does not seem realistic since only 1Ter alters nucleotides that are known to influence initiation (Figure 3). Furthermore, while the G→U transversion at position +4 would be expected to decrease the initiation frequency (Kozak, 1987a), the C→A transversion at position +5 would be expected to increase the initiation frequency (Boeck and Kolakofsky, 1994; Grünert and Jackson, 1994), conceivably making the cumulative effect of the two mutations small. Possibly, the close proximity of each nonsense codon to 0Met could preclude the loading of an RNA destabilizing factor(s) onto elongating ribosomes, assuming that cytoplasmic ribosomes are involved. Alternatively and readily amenable to analysis, translation could reinitiate at 14Met, which is the next in-frame AUG codon (Figures 1 and 3). Studies of reinitiation within the *GCN4* mRNA of *S.cerevisiae*, which constitute the most thorough analyses of reinitiation in eukaryotes, indicate that reinitiation requires (i) an internal AUG codon that need not reside within the consensus sequence for initiation sites (reviewed in Hinnebusch, 1992, 1993), (ii) rebinding of eIF-2-GTP-Met-$tRNA_i^{Met}$ to 40S ribosomes that scan the region between the termination and reinitiation sites (Grant *et al.*, 1994; Dever *et al.*, 1995; Garcia-Barrio *et al.*, 1995) and (iii) sequences between the termination and reinitiation sites that do not result in 40S ribosome release (Grant and Hinnebusch, 1994). Only a few studies have identified determinants of reinitiation in mammalian cells (e.g. see Liu *et al.*, 1984; Peabody and Berg, 1986; Peabody *et al.*, 1986; Kozak, 1987b; Tan *et al.*, 1994; Luukkonen *et al.*, 1995). These studies indicate that reinitiation, including reinitiation at AUG

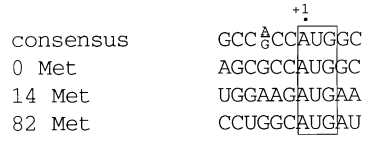

Fig. 3. Features of the putative translation reinitiation site at 14Met. Comparison of the consensus translation initiation site (Kozak, 1986; Boeck and Kolakofsky, 1994; Grünert and Jackson, 1994) and the sequence contexts of 0Met, 14Met and 82Met, the three in-frame AUG codons of TPI mRNA that reside, respectively, at codons 0, 14 and 82. Nucleotide +1 is defined as the A nucleotide of each AUG codon.

codons that reside within a sequence context suboptimal for initiation, is favored by a short upstream open translational reading frame and a sufficiently long intercistronic distance (Kozak, 1987b; Luukkonen *et al.*, 1995). In theory, the efficiency of translation termination at the nonsense codon (Mottagui-Tabar *et al.*, 1994; McCaughan *et al.*, 1995; Poole *et al.*, 1995) and a secondary structure that could stall a scanning ribosome over the reinitiation site (Kozak, 1990; see below) could also influence the efficiency of reinitiation.

If 14Met were a site of reinitiation, then its conversion to a codon for a different amino acid would be expected to inhibit reinitiation. Without reinitiation, 1Ter, 2Ter and 10Ter would be expected to reduce more completely the abundance of MT-TPI mRNA. To test for the possibility of reinitiation, 14Met(AUG) was mutagenized to 14Val-(GUU) (Figure 1) in the context of the normal, 1Ter, 2Ter and 10Ter MT-TPI alleles. 14Met→Val had no effect on the level of MT-TPI mRNA in the absence of a nonsense codon, as expected, but did reduce the level of each nonsense codon-containing mRNA from 85 to 45% of normal (Figure 4). These findings are consistent with 14Met functioning as a site of translation reinitiation when preceded by a nonsense codon. Why 14Met→Val does not fully restore nonsense-mediated decay is debatable (see Discussion). However, the possibility that translation reinitiates at 82Met (Figure 3), the only other in-frame AUG codon, is contraindicated by the finding that 23Ter reduces mRNA abundance to the same extent as a nonsense codon downstream of Met82. Notably, 14Met→Val was inconsequential to the effect on mRNA abundance of either 189Ter or 192Ter (Figure 5), each of which resides within exon 6 (Figure 1). This result indicates that 14Met is not likely to be a site of translation initiation since the

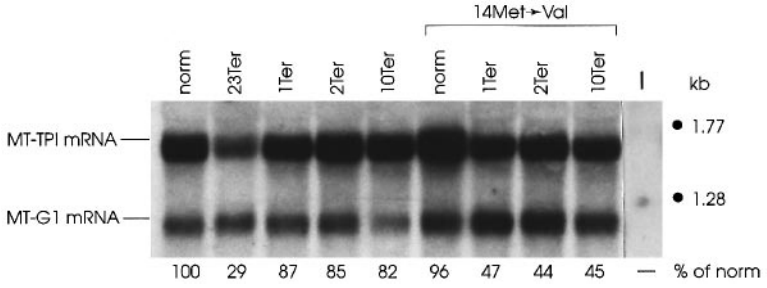

Fig. 4. 14Met(AUG)→Val(GUU) augments the reduction in mRNA abundance brought about by an upstream nonsense codon. L cell transfections and RNA purification and analysis were as described in the legend to Figure 2. None of the values for the percentage of norm differed between two independently performed experiments by >4%.

conversion of an initiation codon to a valine codon would be expected to reduce the efficiency of initiation and, in so doing, reduce the efficiencies of nonsense codon recognition and the decay of nonsense mRNA. 14Met is also not likely to be a site of translation initiation given the scanning model, which predicts that translation will initiate primarily at the 5′-most methionine codon when this codon lies in the most favorable context (Kozak, 1986, 1989). In agreement with this prediction, the results of protein sequencing indicate that TPI protein synthesis begins exclusively at 0Met (Lu *et al.*,1984; see below).

Evidence that 14Met is a site of translation reinitiation but not translation initiation

Additional evidence that 14Met is a reinitiation site derives from the analysis of constructs in which 14Met and 14Met→Val were placed out-of-frame relative to 0Met. The shift in frame was generated by a single nucleotide insertion within codon 11. All out-of-frame constructs also harbor a two nucleotide insertion within codon 17 that reverts the frame to normal. Therefore, translation would terminate at the normal termination codon if translation were to initiate at 0Met, and translation would terminate prematurely within what are normally codons 28 and 29 if translation were to reinitiate at codon 14. The frameshift insertions *per se* (i.e. in the context of 14Met) reduced the mRNA level to 60% of normal (Figure 6). While this result could reflect the use of 14Met as an initiation codon some of the time, this interpretation is contraindicated by the finding that 14Met→Val out-of-frame also reduced the level of mRNA to 60% of normal (Figure 6; see below). If 14Met were an initiation site, then 14Met→Val would be expected to reduce the initiation efficiency at this site and, therefore, increase mRNA abundance by reducing the extent of premature termination within codons 28–29. The finding that 1Ter in the context of 14Met out-of-frame generated the full nonsense-mediated reduction in mRNA abundance is consistent with 14Met being a site of reinitiation since reinitiation out-of-frame results in the premature termination of translation within codons 28–29. This finding also provides additional evidence that 82Met is not an effective reinitiation site.

In order to demonstrate that 14Met is a site of translation reinitiation in the presence of an upstream nonsense codon, MT-TPI gene sequences extending from the MT promoter through TPI codon 37 were inserted immediately upstream of codon 1 of pCAT-Basic Vector (Promega) in order to generate pMT-TPI–CAT. pCAT-Basic Vector harbors a

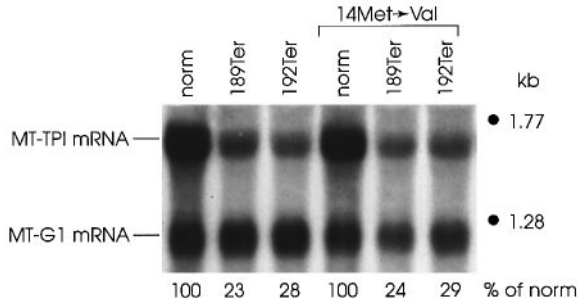

Fig. 5. 14Met→Val is inconsequential to the effect of a downstream nonsense codon. L cell transfections and RNA purification and analysis were as described in the legend to Figure 2. None of the values for the percentage of norm differed between two independently performed experiments by >4%.

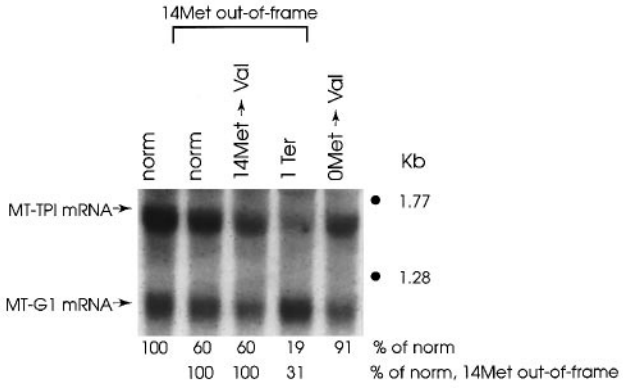

Fig. 6. 14Met out-of-frame allows for 1Ter to evoke the full nonsense-mediated reduction in mRNA abundance. L cell transfections and RNA purification and analysis were as described in the legend to Figure 2. None of the values for the percentage of norm differed between two independently performed experiments by >5%.

hybrid gene that consists of the *Escherichia coli* chloramphenicol acetyl transferase (CAT) gene followed by an intron and polyadenylation sequence from SV40 (Figure 1). pSP6-TPI–CAT constructs were next generated by inserting TPI–CAT sequences downstream of the SP6 promoter in pGEM7Zf(+). The TPI–CAT sequences extended from the TPI 5′-untranslated region into vector sequences located 3′ to SV40 DNA.

In contrast to the nonsense-free construct (norm), the construct harboring 23Ter did not produce detectable TPI–CAT protein, indicating that protein production was dependent on translation readthrough from TPI sequences

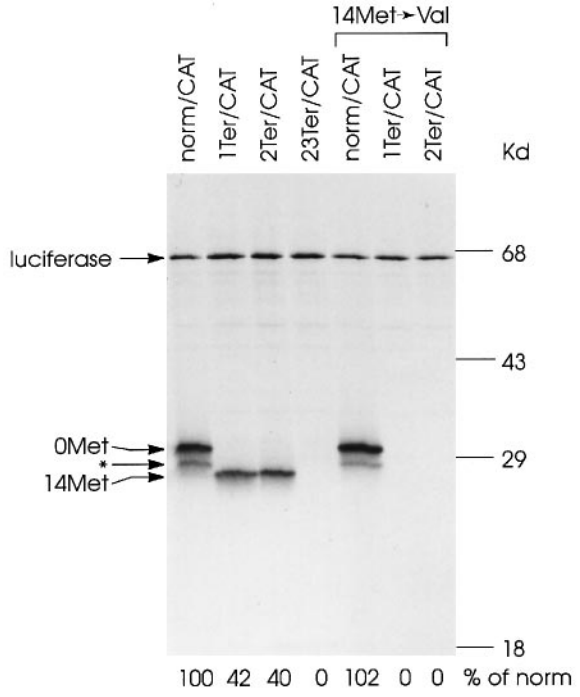

Fig. 7. 1Ter and 2Ter result in TPI–CAT protein due to reinitiation at 14Met. Test pSP6-TPI–CAT plasmids (1 μg) and reference luciferase control DNA (0.25 μg; Promega) were transcribed using SP6 RNA polymerase, and product RNAs were translated in the presence of [^{35}S]methionine in 25 μl of the TNT Coupled Reticulocyte Lysate System (Promega). A fraction (6 μl) was precipitated by reducing the pH to 5.0 using acetic acid and electrophoresed in a 12% acrylamide gel. The level of TPI–CAT from each plasmid was normalized to the level of luciferase, and normalized values were then calculated as a percentage of normal (i.e. nonsense-free) TPI–CAT (% of norm), which was defined as 100. 0Met refers to TPI–CAT that is the result of translation initiation at the normal initiation codon of TPI, and 14Met refers to TPI–CAT that is the result of translation initiation at 0Met and, subsequently, reinitiation at 14Met. The asterisk denotes a minor protein, the structure of which is unknown.

into CAT sequences (Figure 7). Constructs harboring 1Ter and 2Ter produced TPI–CAT protein that was calculated to be 15 amino acids smaller than that produced by the nonsense-free construct, consistent with translation initiation normally at 0Met and reinitiation downstream of either 1Ter or 2Ter at 14Met (Figure 7). In support of reinitiation at 14Met, 14Met→Val eliminated protein production from the 1Ter and 2Ter constructs but had no effect on protein production from the nonsense-free construct (Figure 7). Assuming comparable stabilities for TPI–CAT proteins having amino-termini at 0Met and 14Met, the finding that constructs harboring 1Ter or 2Ter produced TPI–CAT at 42% of the nonsense-free level indicates that the efficiency of reinitiation at 14Met is 42% the efficiency of initiation at 0Met.

Discussion

Our results indicate that nonsense codons at positions 1, 2 and 10 of the human TPI gene are less effective in reducing the abundance of nucleus-associated TPI mRNA than nonsense codons spanning positions 23–192 (Figures 2 and 5, Cheng *et al.*, 1994). The decrease in effectiveness appears to be attributable to translation reinitiation at 14Met since: (i) conversion of 14Met(AUG) to 14Val-(GUU) results in an increase in the efficiency with which upstream nonsense codons reduce mRNA abundance (Figure 4) but has no effect on the efficiency with which downstream nonsense codons reduce mRNA abundance (Figure 5); (ii) 14Met out-of-frame increases the efficiency with which 1Ter reduces mRNA abundance, presumably because translation terminates prematurely within codons 28–29 (Figure 6); (iii) TPI–CAT protein from a TPI–CAT gene harboring either 1Ter or 2Ter is ~14 amino acids smaller than TPI–CAT protein from a TPI–CAT gene that is nonsense-free (Figure 7); and (iv) 14Met→Val precludes the synthesis of TPI–CAT protein from a construct harboring either 1Ter or 2Ter (Figure 7). In further support of reinitiation at 14Met, 14Met is the in-frame AUG codon that resides closest to position 10 (Figure 3); the only other in-frame AUG codon, 82Met, appears to be ineffective in reinitiation since nonsense codons located between 14Met and 82Met are completely effective in reducing mRNA abundance.

Reinitiation at 14Met is surprisingly efficient and may be facilitated by one or more hairpin structures located downstream of 14Met. A stem predicted to exist by the STAR RNA folding program (C.W.A.Pleij, personal communication) begins at codon 25 and has a ΔG of –22 kcal/mol, which is above the –19 kcal/mol that improves initiation at preceding non-AUG codons (Kozak, 1990, 1991). Our studies of *in vitro*-generated mutations support the idea that this stem facilitates reinitiation at 14Met. When the stem is destroyed, the level of mRNA harboring either 1Ter or 10Ter is reduced from 85% of normal (i.e. nonsense-free) to ~55% of normal (data not shown), which is close to the 45% of normal that characterizes 1Ter or 10Ter *in cis* to 14Met→Val. Furthermore, when compensating mutations were introduced within the half of the stem that was not mutagenized so that base pairing but not the primary sequence of the stem was restored, the level of mRNA harboring either 1Ter or 10Ter was increased to 103% of normal (data not shown). Similar data were obtained when a second stem, albeit one not predicted to exist by the STAR program, was mutagenized (data not shown). This stem begins at codon 30 and has a ΔG of –19 kcal/mol. Even though formation of the first stem is predicted to preclude formation of the second stem (C.W.A.Pleij, personal communication), results suggest that some secondary structure does exist and is critical for efficient reinitiation at 14Met.

Given that a mammalian ribosome spans an estimated 30–35 nucleotides (Steitz, 1980; Kozak, 1983), it is likely that mRNAs harboring 1Ter and 2Ter are loaded with a single ribosome at the time of nonsense codon recognition, while 10Ter may be loaded with either one or two ribosomes. The finding that none of these nonsense codons generates a normal level of mRNA is consistent with the finding that constructs harboring 1Ter and 2Ter produce only 42% of the level of TPI–CAT protein produced by a nonsense-free construct. This indicates that translation reinitiation at 14Met is 58% less efficient than translation initiation at 0Met. Those mRNAs that fail to reinitiate would be expected to be degraded at the usual nonsense-mediated rate. The finding that 1Ter, 2Ter and 10Ter do not elicit the full nonsense effect in the presence of 14Met→Val, which precludes reinitiation (Figure 7), suggests that these nonsense codons are too close to 0Met

to elicit the full effect. Possibly, the translating ribosomes have not had sufficient opportunity to acquire one or more factors that are required at the time of nonsense codon recognition for nonsense-mediated decay. Alternatively, the nonsense codons may be too far from a downstream element that triggers nonsense-mediated decay when recognized by some component of the translational apparatus after nonsense codon recognition (Cheng *et al.*, 1994; Maquat, 1995). In either case, the rate of nonsense-mediated decay or the number of molecules subject to nonsense-mediated decay could be reduced. Notably, since 1Ter reduces mRNA abundance as effectively as 2Ter and 10Ter, peptide bond formation is not required for nonsense-mediated decay.

Studies on translation reinitiation were pursued for two reasons. First, nonsense codon recognition within certain mammalian mRNAs has been reported to take place within nuclei (Naeger *et al.*, 1992; Dietz *et al.*, 1993; Lozano *et al.*, 1994; Dietz and Kendizor, 1994), in one case independently of translation (Aoufouchi *et al.*, 1996). Second, it has been proposed for *S.cerevisiae* that nonsense-mediated decay involves a ribosome pause or reinitiation event downstream of the nonsense codon (Peltz *et al.*, 1993, 1994). This proposal stems from the findings using *PGK1* transcripts that: (i) of the three AUG codons residing within the 106 nucleotide sequence of the coding region that functions as a destabilizing element, the first two are required for the decay of nonsense transcripts; (ii) inserting a stem–loop structure that inhibits both translation initiation and translation reinitiation between a nonsense codon and the destabilizing element results in stabilization of an otherwise unstable nonsense-containing transcript; and (iii) 3-aminotriazole, which is an inhibitor of amino acid biosynthesis that reduces the capacity of cells to reinitiate translation at downstream AUG codons, also results in stabilization of an otherwise unstable nonsense-containing transcript (Peltz *et al.*, 1993, 1994). Subsequent studies of other *S.cerevisiae* mRNAs revealed that a destabilizing element characterizes not only the coding region of *PGK1* mRNA but also the coding regions of mRNAs for *ADE3* and *HIS4* (Zhang *et al.*, 1995). In fact, a computer search has identified element-like sequences within a total of 57 *S.cerevisiae* mRNAs (Zhang *et al.*, 1995).

Most recently, the element has been found to be complementary to 18S rRNA (Hagan *et al.*, 1995). Furthermore, RNA secondary structures inserted downstream of the element have been found to enhance nonsense-mediated mRNA decay (Zhang *et al.*, 1995). While these findings could be interpreted as additional support for function of the destabilizing element as a ribosome stall or reinitiation site, the Peltz laboratory recently has demonstrated that the decay pathway is inactivated by the insertion of a *GCN4* translational reading frame downstream of a nonsense codon within *PGK* mRNA that normally destabilizes *PGK* mRNA (Ruiz-Echevarria and Peltz, 1996). The Peltz laboratory now views the element as a site that interacts with a fraction of prematurely terminating ribosomes, ribosomal subunits or ribosome-associated factors that continue to scan the mRNA after nonsense codon recognition (Ruiz-Echevarria and Peltz, 1992; Zhang *et al.*, 1995). This interpretation is consistent with our view of nonsense-mediated decay in mammalian cells. However, the *cis*-acting determinants of decay appear to differ between mammalian and yeast mRNAs. First, mammalian mRNAs do not have a yeast-like destabilizing element on the basis of sequence analysis. Second, at least for TPI mRNA, the boundary between nonsense codons that do and do not reduce mRNA abundance can be altered as a function of the position of the final intron within pre-mRNA (Cheng *et al.*, 1994; Maquat, 1995). Since fully spliced TPI mRNA is the substrate for nonsense-mediated decay (Belgrader *et al.*, 1994), any destabilizing element would appear to involve a collaboration between intron and exon sequences (Cheng *et al.*, 1994; Maquat, 1995).

Much remains to be determined concerning the mechanism of nonsense-mediated mRNA decay. Future experiments will aim to elucidate the necessary *cis*-acting sequences and, ultimately, the interactions of these sequences with components of the translational machine.

Materials and methods

Plasmid constructions

The TPI gene was mutagenized (Kunkel *et al.*, 1987) within a 420 bp *Bam*HI–*Sma*I fragment that includes sequences from exon 1 and intron 1 and that was propagated as a single strand in pGEM7Zf(+) DNA. Nonsense mutations within codons 1, 2 and 10 were introduced using the antisense mutagenic primers 5′-CCTGGAGGG**CTA**CATGGCCGAG-3′, 5′-GAACTTCCTGGA**CTA**CGCCATGGCC-3′ and 5′-CTTCCAGTT**CA**CCCAACG-3′ (in which the bold face nucleotides deviate from the corresponding TPI gene sequence and underlined nucleotides correspond to the altered codon). Additionally, codon 14 was changed from encoding methionine to encoding valine using the antisense mutagenic primer 5′-TTCCGCCCGTT**AAC**CTTCCAGTTTC-3′, either in a nonsense-free context or in the context of a nonsense codon at position 1, 2 or 10. 0Met→Val was generated using the antisense mutagenic primer 5′-GGAGGGCGC**AAC**GGCGCTGGAG-3′. 14Met out-of-frame was generated using the antisense mutagenic primer 5′-CTGCTTCC**GG**-GCCCGTTCATCTTCCAGT**C**T-3′, which inserted a G nucleotide within codon 11 and a CC dinucleotide within codon 17. 14Met→Val out-of-frame was generated from 14Met out-of-frame using 5′-CCGGGCCCG-TT**AAC**CTTCCAGTCTTC-3′.

After DNA sequencing to confirm the mutations had been introduced, pMT-TPI DNAs (Cheng and Maquat, 1993) harboring each of the mutations and pSP6-TPI–CAT DNAs harboring some of the mutations were constructed by fragment substitution. The fragment substituted in constructing the pMT-TPI derivatives was *Bam*HI–*Sma*I. The fragment substituted in constructing the pSP6-TPI–CAT derivatives was *Kpn*I–*Eag*I (see below). pSP6-TPI–CAT was constructed in several steps so that the CAT open translational reading frame began with codon 0Met of TPI and included the first 37 amino acids of TPI. First, the 270 bp *Pst*I–*Eco*RI fragment from pCAT-Basic Vector (Promega), which includes polylinker sequences and the 5′-end of the CAT gene, was substituted for the 2.26 kbp *Pst*I–*Eco*RI fragment, which extends from TPI intron 4 into 3′ flanking DNA. Next, the 1.28 kbp *Sac*II–*Pst*I fragment that spans TPI intron 1 to intron 4 was deleted, and the 1.11 kbp *Bam*HI–*Eco*RI fragment that resides immediately downstream of the MT promoter and terminates at the *Eco*RI site of the CAT gene was inserted into the *Bam*HI–*Eco*RI sites of pGEM7Zf(+). Subsequently, 750 bp that include TPI codon 38 to the *Sac*II site of intron 1 plus 57 bp of the pCAT-Basic Vector polylinker and codon 0Met of CAT were deleted using the antisense mutagenic oligonucleotide 5′-GTATATCCAGTGAATTTTTT-TCTC/GGTGTCGGCCGGCACC-3′ (where the slash specifies the site of the deletion). Finally, the mutagenized 364 bp *Bam*HI–*Eco*RI fragment was inserted into the 2.61 kbp *Bam*HI–*Eco*RI vector fragment of pMT-TPI, and the 750 bp *Bam*HI–*Bam*HI MT promoter fragment plus the 1.39 kbp *Eco*RI–*Eco*RI fragment that extends from the CAT reading frame into 3′ flanking DNA were inserted at the *Bam*HI and *Eco*RI sites, respectively, to generate pMT-TPI^{Norm}–CAT. pMT-TPI^{1Ter}–CAT, pMT-TPI^{2Ter}–CAT, pMT-TPI^{23Ter}–CAT, pMT-TPI$^{Norm,\ 14Met\rightarrow Val}$–CAT, pMT-TPI$^{1Ter,\ 14Met\rightarrow Val}$–CAT and pMT-TPI$^{2Ter,\ 14Met\rightarrow Val}$–CAT were constructed by substituting the 784 bp *Kpn*I–*Eag*I fragment, which extends from within the MT promoter to TPI exon 1, with the corresponding nonsense fragment. Subsequently, pSP6-TPI–CAT derivatives were con-

structed by inserting the 1.74 kbp *Bam*HI–*Bam*HI fragment, extending from the 5′-untranslated region of the TPI gene into SV40 DNA, into the *Bam*HI site of pGEM7Zf(+), downstream of the SP6 promoter.

Cell transfections and RNA purification

Mouse Ltk$^-$ cells were grown in minimal essential medium α containing 10% fetal bovine calf serum and 5% bovine calf serum. Cells (3×10^7/15 cm diameter dish) were transiently transfected with a test pMT-TPI plasmid (10 μg) and the reference pMT-G1 plasmid (10 μg) by using DEAE-dextran (Cheng and Maquat, 1993). After 48 h, cells were harvested. Total L cell RNA was isolated using guanidine isothiocyanate and cesium chloride gradient centrifugation (Cheng and Maquat, 1993). Nuclear and cytoplasmic RNA was isolated using Method 1 of Belgrader *et al.* (1994).

RNA blot hybridization

Total, nuclear or cytoplasmic cell RNA (25 μg) was electrophoresed in a 1.5% agarose gel, transferred to a nylon membrane and hybridized to a 299 bp *Nde*I–*Nco*I fragment that derives from the 3′-untranslated region of human TPI cDNA and a 170 bp *Bal*I–*Dra*I fragment from the mouse β^{major}-globin gene that consists of 158 bp of exon 3 plus 3′ flanking sequences (Cheng and Maquat, 1993). Prior to hybridization, each fragment was ^{32}P-labeled by random priming (Fineberg and Vogelstein, 1983). Hybridized radioactivity was quantitated using a PhosphorImager (Molecular Dynamics) and visualized using autoradiography.

Coupled transcription–translation of pSP6-TPI–CAT constructs in vitro

Test pSP6-TPI–CAT plasmid (1 μg) and reference luciferase control DNA (0.25 μg; Promega) were transcribed from the SP6 promoter, and product RNA was translated using 25 μl of the TNT Reticulocyte Lysate System (Promega) that couples transcription by SP6 RNA polymerase and translation of the resulting RNA. A portion (6 μl) of the reaction mixture was brought to pH 5 using 1 μl of 1 M acetic acid, incubated on ice for 30 min and then centrifuged at 12 000 *g* and 4°C for 15 min in order to remove hemoglobin (Shaun *et al.*, 1984), which co-migrates with TPI–CAT protein. Pellets were dissolved in 50 μl of 1× SDS sample buffer (Promega), and 10 μl were denatured at 80°C and electrophoresed in a 12% acrylamide gel. ^{35}S-Labeled TPI–CAT and luciferase proteins were quantitated using a PhosphorImager and visualized using autoradiography.

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