RNA (2000), *6*:1–8. Cambridge University Press. Printed in the USA.

LETTER TO THE EDITOR

mRNA surveillance in mammalian cells: The relationship between introns and translation termination

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Keywords: 3′-end formation; introns; mRNA biogenesis; nonsense-mediated mRNA decay

INTRODUCTION

In mammals, as in all organisms examined, mRNAs that prematurely terminate translation are abnormally reduced in abundance by a mechanism called nonsense-mediated mRNA decay (NMD) or mRNA surveillance (for reviews, see Maquat, 1995, 1996; Ruiz-Echevarria & Peltz, 1996; Li & Wilkinson, 1998; Culbertson, 1999; Hentze & Kulozik, 1999; Hilleren & Parker, 1999). This mechanism is thought to have evolved to eliminate nonsense-containing RNAs that arise as a consequence of (1) mutations in germ-line or somatic DNA or (2) routine abnormalities in gene expression due to abnormalities in, for example, transcription initiation, splicing, and somatic rearrangements of the type that characterize the immunoglobulin and T-cell receptor genes. The elimination of nonsense-containing mRNAs protects cells from the potentially deleterious effects of the encoded truncated proteins, which can manifest new or dominant-negative functions (Kazazian et al., 1992; Pulak & Anderson, 1993; Hall & Thein, 1994; Cali & Anderson, 1998). In addition to eliminating abnormal transcripts, NMD also regulates the expression of certain mRNAs that are not abnormal. Examples in mammalian cells are provided by certain selenoprotein mRNAs that terminate translation at a UGA codon for the inefficiently incorporated amino acid selenocysteine (Sec; Moriarty et al., 1997, 1998). Other examples will undoubtedly resemble natural substrates found in other organisms such as the alternatively spliced mRNAs of *Caenorhabditis elegans* that retain an internal exon harboring an in-frame termination codon (Morrison et al., 1997), and transcripts of *Saccharomyces cerevisiae* that contain a small open reading frame upstream of the primary open reading frame (Leeds et al., 1992; Pierrat et al., 1993).

Based on studies of disease-associated and in vitro-mutagenized genes, a rule for termination-codon position within intron-containing genes has been established for mammalian cells: only those termination codons located more than 50–55 nt upstream of the 3′-most exon–exon junction mediate mRNA decay (Cheng et al., 1994; Thermann et al., 1998; Zhang et al., 1998a, 1998b). In support of this rule, more than 98% of genes having one or more 3′-untranslated exons terminate translation less than 50 nt upstream of the 3′-most exon–exon junction (Nagy & Maquat, 1998). There is a growing consensus that exon–exon junctions function in NMD. According to current thinking, the process of pre-mRNA splicing influences product mRNP structure and, in so doing, NMD, by leaving a mark at the junctions (Chang et al., 1994; Carter et al., 1996; Thermann et al., 1998; Zhang et al., 1998a, 1998b; Hilleren & Parker, 1999). In fact, two possible constituents of the mark are mammalian Prp8p, the 220-kDa component of U5 snRNP, and SRm160, a nuclear matrix-associated coactivator of splicing: both have recently been shown by RNA crosslinking in HeLa-cell nuclear extract to bind to each of five exon–exon junctions tested in a splicing-dependent manner and to remain bound to at least some of these junctions after spliceosome dissociation (H. Le Hir, M.J. Moore & L.E. Maquat, unpubl. data). mRNP structure located downstream of a translation termination event, albeit structure not established by splicing, also appears to function in NMD in *S. cerevisiae* (Peltz et al., 1993; Hagen et al., 1995; Zhang et al., 1995; Ruiz-Echevarria & Peltz, 1996; Ruiz-Echevarria et al., 1998; Hilleren & Parker, 1999). Nevertheless, for mammalian cells, as well as yeast cells, the mechanism by which either exon–exon junctions or other types of mRNA elements located downstream of a translation termination event influence translating ribosomes so

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as to elicit NMD remains uncertain. This uncertainty was noted most recently in a thought-provoking commentary by Hilleren and Parker (1999).

Hilleren and Parker (1999) essentially reiterate two models put forth initially by us to explain how the 3′-most exon–exon junction of mammalian mRNAs could function in NMD. In one model (Model #1 of Zhang et al., 1998a, 1998b), the junction is a positive element when positioned either upstream of or less than 50–55 nt downstream of a termination codon that stabilizes mRNA once encountered by a translating ribosome. Put another way, NMD is due to the failure of translating ribosomes to interact with the marked junction either before or during translation termination and, therefore, the failure of translating ribosomes to specify a proper translation termination event. In an alternative model (Model #2 of Zhang et al., 1998a, 1998b), the 3′-most exon–exon junction is a negative element when positioned more than 50–55 nt downstream of a termination codon that destabilizes the mRNA once it is encountered by one or more components of the translational machine that assemble as a consequence of translation termination. In other words, NMD is due to an interaction between components of the translational machine and the marked junction during or after translation termination that defines the termination event as improper. Notably, both models envision nuclear processes influencing (1) mRNP structure, which could include formation of a higher-order structure with a marked junction, (2) translation termination, either that is or is not premature, and (3) mRNA half-life. Hilleren and Parker (1999) favor the first model, which evokes the philosophically appealing premise that the completion of splicing results in positive rather than negative effects on mRNA biogenesis, possibly by forming an mRNP domain that reflects how the 3′-terminal exon was defined during splicing. They draw an analogy to this possibility and the structural element within the 3′-untranslated region of selenoprotein mRNAs that stimulates the incorporation of Sec into prescribed UGA codon(s). Nevertheless, results of experiments done either prior to or as a consequence of contemplating their commentary lead us to continue to favor the second model.

EVIDENCE THAT THE 3′-MOST EXON–EXON JUNCTION IS NOT A POSITIVE EFFECTOR OF GENE EXPRESSION THAT PRECLUDES NMD

Removing introns from a gene decreases mRNA production almost exclusively by increasing RNA decay prior to 3′ end formation rather than by eliciting NMD

Hilleren and Parker (1999) posit that the 3′-most exon–exon junction is a positive element that stabilizes mRNA when positioned either upstream of or near the termination codon in part because genes bearing at least one intron are generally expressed at higher levels than their intronless counterparts: many studies have demonstrated that an increased level of mRNA is produced from a gene with an increased number of introns (see, e.g., Hamer & Leder, 1979; Buchman & Berg, 1988; Neuberger & Williams, 1988; Ryu & Mertz, 1989; Jonsson et al., 1992; Nesic et al., 1993; Zhang et al., 1998a, 1998b). The authors go on to point out that if the 3′-most exon–exon junction of mRNAs normally obviates NMD by providing a proper context for translation termination, then an intronless gene should generate mRNA that is subject to NMD. They also acknowledge that NMD may not be the sole mechanism by which an intronless gene produces an abnormally low level of mRNA given that nascent transcripts of an intronless gene for triosephosphate isomerase (TPI) undergo decay before 3′-end formation (Nesic & Maquat, 1994). However, our finding that the absence of all six TPI-gene introns reduces the level of nascent (i.e., uncleaved and unpolyadenylated) TPI transcripts and the level of cytoplasmic TPI mRNA to the same extent (Nesic & Maquat, 1994) indicates that the reduction in TPI mRNA abundance is essentially entirely attributable to decay before 3′-end formation. Therefore, introns appear to be positive effectors of TPI gene expression by increasing the efficiency of TPI pre-mRNA processing rather than by increasing TPI mRNA stability in a translation-dependent manner.

The idea that an intronless gene generates mRNA that is subject to NMD has also been negated by our studies demonstrating that expression of an intronless gene for glutathione peroxidase (GPx) 1 under conditions of selenium deficiency fails to increase the efficiency of NMD despite increasing the efficiency with which the Sec (TGA) codon is recognized as nonsense (Moriarty et al., 1997). Furthermore, converting the Sec codon to a TAA nonsense codon in the context of an intronless GPx1 allele does not change the level of GPx1 mRNA, despite the fact that the TAA codon is recognized as nonsense more efficiently than the Sec codon, regardless of the Se concentration (Moriarty et al., 1997). In contrast, expression of the full-length GPx1 gene under conditions of Se deficiency or expression of the full-length GPx1 gene harboring a TAA nonsense codon in place of the Sec codon does increase the efficiency of NMD, as would be expected considering that the sole intron is situated 105 bp (i.e., more than 50–55 bp) downstream of the Sec codon (Moriarty et al., 1997, 1998).

The prediction of Hilleren and Parker (1999) that an intronless gene produces mRNA that is subject to NMD can also be tested by comparing the expression of intron-containing and intronless genes in the presence of either wild-type (Wt) or R844C hUpf1 protein (p), the latter of which harbors an arginine-to-cysteine change within the RNA helicase domain at position 844 that

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inhibits NMD in a dominant-negative fashion (Sun et al., 1998). To undertake this test, Cos cells were transiently transfected with a test pmCMV-GPx1 plasmid, a reference phCMV-MUP plasmid (Belgrader & Maquat, 1994), and either the pCI-neo-hUPF1 Wt or the pCI-neo-hUPF1 R844C plasmid. The test plasmid contains a GPx1 gene that harbors either a cysteine (TGC) codon, which never directs translation termination, or a nonsense (TAA) codon, which always directs translation termination, in place of the Sec codon at position 46 (Fig. 1A). TGC-containing alleles either do or do not contain the sole intron of the GPx1 gene, whereas the TAA-containing allele does contain the intron (Fig. 1A). The reference plasmid allows for quantitative analysis of test plasmid expression by controlling for variations in transfection efficiencies and RNA recovery, and the pCI-neo-hUPF1 plasmids support high levels of hUpf1p production in Cos cells in part by allowing for transient episomal replication (Sun et al., 1998). Total Cos-cell RNA was analyzed by using RT-PCR, in which one primer pair amplified GPx1 RNA across both exons (Moriarty et al., 1997) and another primer pair amplified MUP RNA from exon 4 into exon 7 (Belgrader & Maquat, 1994).

Results confirmed for intron-containing GPx1 alleles that (1) expression of either pCI-neo-hUPF1 Wt or pCI-neo-hUPF1 R844C is of no consequence to the level of GPx1-UGC(46) mRNA (Fig. 1B, compare lanes labeled 100% and 103%; Sun et al., 1998) and (2) expression of pCI-neo-hUPF1 R844C elicits an approximately two- to threefold increase in the level of GPx1-UAA(46) mRNA (Fig. 1B, compare lanes labeled 40% and 87%; Sun et al., 1998), in contrast to expression of pCI-neo-hUPF1 Wt, which is of no consequence to the nonsense-mediated reduction in the level of GPx1-UAA(46) mRNA (Moriarty et al., 1998; Sun et al., 1998). Also consistent with previous results, removal of the sole intron of the GPx1 gene reduced the level of GPx1-UGC(46) mRNA approximately fivefold (Fig. 1B, compare lanes labeled 100% and 22%; Moriarty et al., 1997). Pertinent to the prediction that the absence of an intron elicits NMD, expression of either pCI-neo-hUPF1 Wt or pCI-neo-hUPF1 R844C had no effect on expression of the intronless GPx1-TGC (46) allele (Fig. 1B, compare lanes labeled 22% and 23%). Therefore, a cDNA version of the GPx1 gene does not produce mRNA that is subject to NMD.

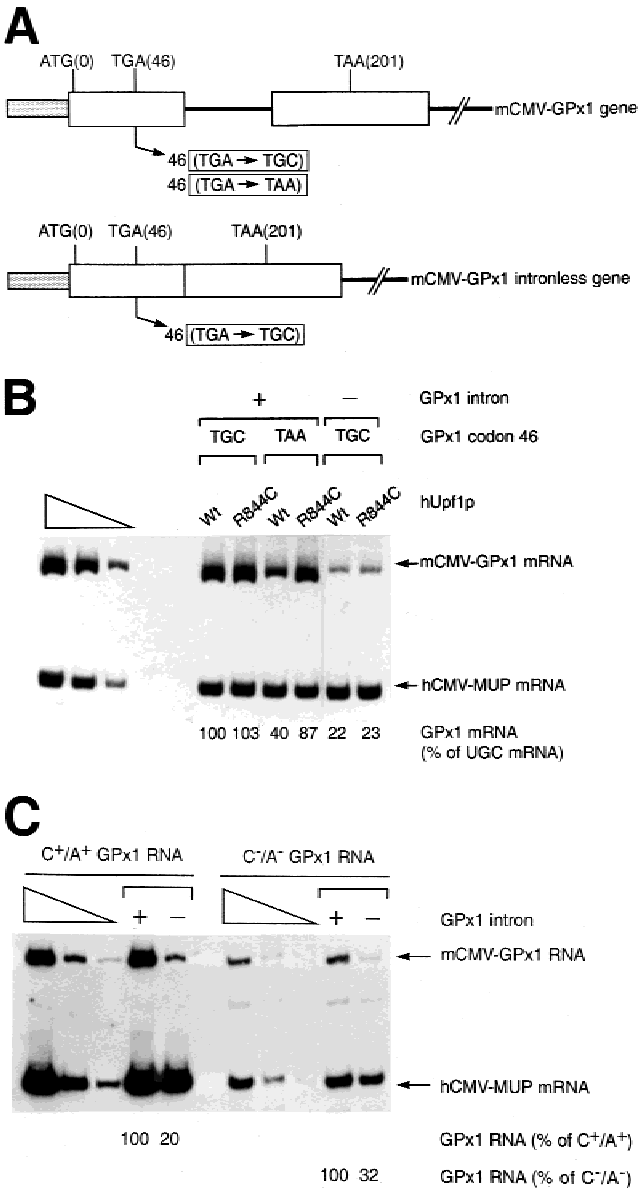

FIGURE 1. Expression of R(844)C hUPF1 cDNA in Cos cells is of no consequence to the level of glutathione peroxidase 1 (GPx1) mRNA produced from an intronless GPx1 gene, indicating that the absence of an intron does not elicit the NMD of GPx1 mRNA. Cos cells were transiently transfected by using LipofectAMINE PLUS Reagent (Life Technologies) with a total of 2 μg of plasmid DNA. Plasmids consisted of the either intron-containing or intronless versions of the pmCMV-GPx1 test plasmid [(**A**) either TGC, which harbors a TGC codon in place of the TGA Sec codon at position 46 so as to be nonsense-free, or TAA, which harbors a nonsense codon in place of the TGA Sec codon so as to always terminate translation], the phCMV-MUP reference plasmid, and pCI-neo-hUPF1 (either Wt, which harbors an unmutagenized hUpf1p reading frame, or R(844)C, which harbors the arginine-to-cysteine change at amino acid position 844). The amounts of the three plasmids were, respectively, 0.7 μg, 0.3 μg, and 1 μg. Total-cell RNA was purified using Trizol (Gibco BRL), and 2.5 μg were analyzed by RT-PCR to detect GPx1 and mouse major urinary protein (MUP) RNAs. RT-PCR and phosphorimaging were used to quantitate total-cell GPx1 RNA (**B**) and either cleaved and polyadenylated (C^+/A^+) or uncleaved and unadenylated (C^-/A^-) GPx1 RNAs (**C**). The level of each specified GPx1 RNA from either the mCMV-GPx1-TGC(46), mCMV-GPx1-TAA(46), or intronless mCMV-GPx1-TGC(46) allele was normalized to the level of MUP mRNA, and normalized values were then calculated as a percentage of the normalized value of either intron-containing or intronless GPx1-UGC(46) mRNA in the presence of Wt hUPF1 cDNA, which was considered as 100. The left-most three lanes labeled C^+/A^+ and C^-/A^- GPx1 RNA consist of serial dilutions of total Cos-cell RNA and demonstrate that the RT-PCR analysis is quantitative. For all experimental lanes, percentages differed between at least two independently performed experiment by no more than 5%.

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To determine if the abnormally low level of mRNA produced by the intronless GPx1 gene is due to RNA decay prior to 3′-end formation, as was found for the intronless TPI gene (Nesic & Maquat, 1994), RT-PCR was used to quantitate the level of either cleaved and polyadenylated (C^+/A^+) or uncleaved and unpolyadenylated (C^-/A^-) GPx1 transcripts. To quantitate C^+/A^+ GPx1 RNA, cDNA was synthesized using oligo$(dT)_{12–18}$ and amplified for 19 cycles across GPx1 exons 1 and 2. To quantitate C^-/A^- GPx1 RNA, cDNA was synthesized using a DNA primer that anneals downstream of the cleavage and polyadenylation site and amplified as was C^+/A^+ GPx1 RNA. Using measurements of MUP RNA to ensure a quantitative analysis, the level of C^+/A^+ RNA from the intronless GPx1-TGC(46) allele was 20% the level of C^+/A^+ RNA from the corresponding intron-containing allele (Fig. 1C). Similarly, the level of C^-/A^- RNA from the intronless GPx1-TGC(46) allele was 32% the level of C^-/A^- RNA from the corresponding intron-containing allele (Fig. 1C). Therefore, the primary cause for the abnormally low level of mRNA from the intronless GPx1 allele is not NMD, but decay prior to RNA 3′-end formation. In theory, the slightly more efficient production of C^-/A^- RNA relative to C^+/A^+ RNA by the intronless GPx1 allele could be due to nuclear RNA retention that, besides inefficient 3′-end formation and degradation by nonproductive splicing, has been reported for intronless versions of normally intron-containing genes (see Huang et al., 1999, and references therein).

Of course, one unavoidable caveat associated with these experiments is the possibility that the absence of an intron precludes NMD by altering the pathway of mRNA biogenesis. For example, it may be that mRNP is immune to NMD when formed by a route that does not involve pre-mRNA splicing. Currently, we have no way of assessing this possibility. However, a detectable level of GPx1 mRNA synthesized from an intronless gene is exported to the cytoplasm (Moriarty et al., 1997), where the NMD of GPx1 mRNA takes places (Moriarty et al., 1998), indicating that NMD is not precluded because of a block in export. Furthermore, the removal of introns from a gene does not necessarily preclude translation of the product mRNA, as evidenced from the number of intronless cDNA expression vectors that are commercially available (see, e.g., the pCMV-Tag vectors from Stratagene for recently available protein-producing vectors), indicating that NMD is not likely to be precluded by a block in nonsense codon recognition. And the abnormally low level of mRNA produced from an intronless gene does not a priori inhibit NMD, since the level of mRNA produced from a TPI gene lacking half of its introns (i.e., lacking introns 3–6) is only 14–16% of normal, yet when the gene contains a nonsense codon, the degree of NMD is comparable to that of the corresponding intron-containing gene (Zhang et al., 1998a).

EVIDENCE THAT THE 3′-MOST EXON–EXON JUNCTION DOES NOT NORMALLY DEFINE A PROPER TRANSLATION TERMINATION SITE

Function of exon–exon junctions in NMD is apparently not restricted to the 3′-most junction

According to Hilleren & Parker (1999), a ribosome would need to encounter the 3′-most exon–exon junction before or during translation termination for termination to be considered proper and the mRNA to be stabilized. However, a second argument against the idea that introns confer a proper translation termination context and in support of the idea that introns located more than 50–55 nt downstream of a termination codon elicit NMD by negatively affecting gene expression derives from data suggesting that exon–exon junctions other than the 3′-most can function in NMD. Hilleren and Parker (1999) propose that the 3′-most intron holds special status in NMD because it, unlike upstream introns, is defined by interactions involving RNA 3′-end formation. It follows that this special status sets the 3′-most exon–exon junction apart from upstream junctions by influencing the type of mark the junction bears after intron removal. Countering this proposal, however, is the finding that a termination codon (Ter) at position 23 within exon 1 of the TPI gene (23Ter) elicits NMD in the context of the full-length gene but not when all introns except the 3′-most, intron 6, have been deleted (Zhang et al., 1998a). Therefore, in the case of 23Ter, one or more of the deleted introns, rather than the 3′-most intron, may normally provide the mark that functions in NMD, possibly because the 559-nt distance between the 23Ter and the 3′-most exon–exon junction is too large to support NMD (Zhang et al., 1998a).

This finding has been extended by testing termination codons located less than 559-nt upstream of the 3′-most TPI exon–exon junction in the context of Δ(introns 1–5) for the ability to support NMD. To this end, NIH-3T3 cells were transiently transfected with a test pmCMV-TPI Δ(introns 1–5) plasmid that is either nonsense-free (Norm) or nonsense(Ter)-containing and a reference pmCMV-GI plasmid (Zhang et al., 1998b). Results indicate that a distance of 416 nt between a termination codon and the 3′-most exon–exon junction supports NMD essentially to the same extent as a distance of 61 nt (Fig. 2). In theory, differences in the ability of 23Ter and either 70–71Ter, 159Ter, or 189Ter to elicit NMD in the absence of introns 1–5 could be attributable to the unique ability of 23Ter to support translation reinitiation at 82Met: extrapolating from studies of other mRNAs, the length of the open reading frame and the distance between the translation termination and putative reinitiation sites would be less favorable for 70–71Ter than for 23Ter (see, e.g., Kozak,

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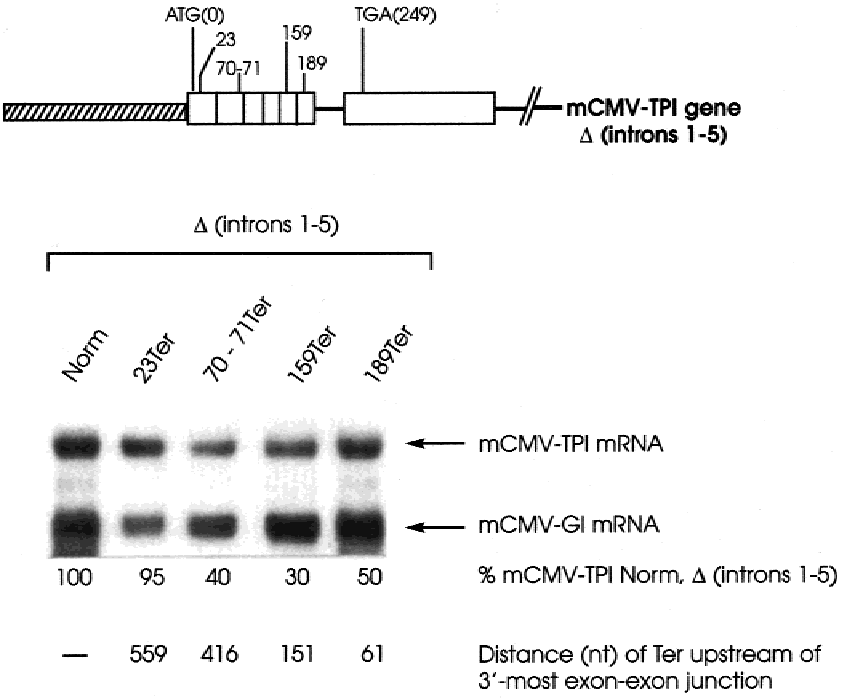

FIGURE 2. 23Ter fails to elicit NMD in the absence of TPI introns 1–5, suggesting that one or more exon–exon junctions created by the splicing of introns 1–5 normally function in 23Ter-directed NMD. NIH-3T3 cells were transfected by using calcium chloride (Zhang et al., 1998a) with 12 μg of a pmCMV-GI reference construct and 28 μg of the pmCMV-TPI test construct that lacked introns 1–5 (Δintrons 1–5) and was either nonsense-free or harbored a termination codon (Ter) at one of the specified positions. Total-cell RNA was purified, and 25 μg were analyzed by blot hybridization to DNA fragments that were ^{32}P-labeled by random priming. TPI RNA was detected by using a 299-bp *Nde*I-*Nco*I fragment that derives from the 3′ untranslated region of human TPI cDNA, and β-globin RNA was detected by using a 170-bp *Bal*I-*Dra*I fragment that includes 158 bp of exon 3 from the mouse β^{maj}-globin gene (Cheng et al., 1994). Hybridization was quantitated by phosphorimaging. The level of mRNA from each mCMV-TPI allele was normalized to the level of mCMV-GI mRNA so the analysis would be quantitative. Normalized values were then calculated as a percentage of the normalized value for mCMV-TPI $^{Norm,\Delta(introns\ 1–5)}$ mRNA, which was considered to be 100. Percentages from two independently performed experiments did not differ by more than 7%. The distance between each termination codon and the 3′-most exon–exon junction within mRNA is specified at the bottom of the figure.

1987; Luukkonen et al., 1995), and 159Ter, and 189Ter are not followed by an in-frame methionine codon. However, converting 82Met(ATG) to 82Asp(GAT) in the context of 23Ter Δ(introns 1–5) did not elicit NMD (Fig. 3). Therefore, the failure of 23Ter to elicit NMD in the absence of introns 1–5 is not because of reinitiation at 82Met. Consistent with this result, reinitiation at 82Met was not observed in vitro in the case of 1Ter or 2Ter (Zhang & Maquat, 1997). Taken together, these data support the notion that more than just the 3′-most exon–exon junction is marked by the process of splicing, and each marked junction can mediate NMD. Consistent with this notion, recently obtained data indicate that a termination codon located either 585-nt or 622-nt upstream of the 3′-most exon-exon junction in the context of Δ(introns 1–5), like 23Ter, also fails to elicit NMD (unpubl. data). The finding of a limit to the distance between a translation termination event and a downstream mRNP structure for NMD not only in mammalian cells but in *S. cerevisiae* has led to the proposal that one or more components of the translation termination complex elicit NMD by either scanning or looping out to the downstream structure (Ruiz-Echevarria et al., 1998; Zhang et al., 1998a, 1998b).

CONCLUSIONS

Studies of NMD have revealed a new role for introns with the discovery that introns can influence mRNA translation and decay possibly by influencing mRNP structure (Cheng et al., 1994; Carter et al., 1996; Thermann et al., 1998; Zhang et al., 1998a, 1998b). Although introns have been known for some time to function in the NMD of fully spliced TPI mRNA while the mRNA is newly synthesized and nucleus-associated (Cheng & Maquat, 1993; Belgrader et al., 1994), the recent finding that they also function in the NMD of fully spliced GPx1 mRNA that has already been released from an association with nuclei into the cytoplasm (X. Sun, P.M. Moriarty, and L.E. Maquat, unpubl. data) provides more direct evidence of a role for introns in translation-mediated mRNA decay. Two models have been put forth to explain how introns function in NMD. One model evokes a positive role in which an intron located either upstream or downstream of a termination codon within pre-mRNA creates a proper context for translation termination within product mRNA. Hilleren and Parker (1999) propose that a proper context for translation termination obviates NMD by allowing

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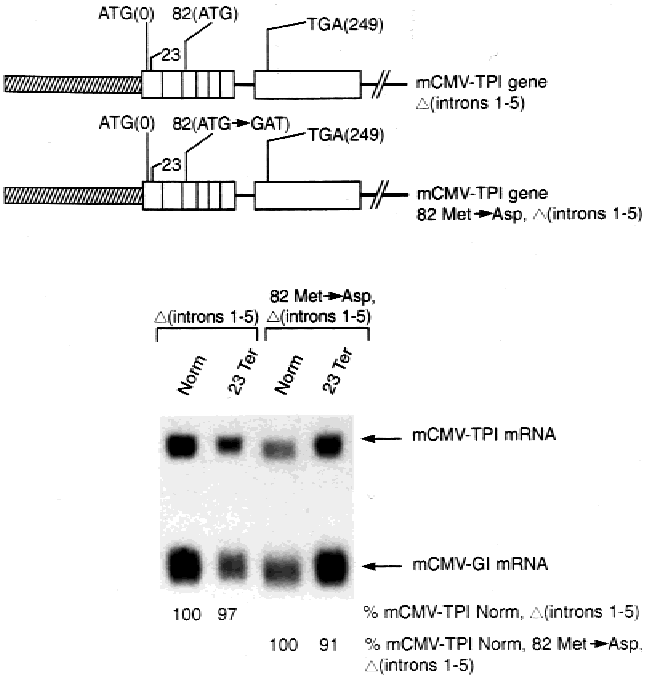

FIGURE 3. The immunity of TPI mRNA harboring 23Ter and lacking introns 1–5 is not due to translation reinitiation at 82Met. Transfections, RNA purification, and RNA blot hybridization and analysis were performed as described in the legend to Figure 2. Percentages between two independently performed experiments did not differ by more than 5%.

for the completion of termination before Upf1p mediates ATP hydrolysis. The other model evokes a negative role in which introns located more than 50–55 nt but less than 550 nt downstream of a termination codon within pre-mRNA elicit NMD. The first model predicts that the abnormally low levels of mRNA characteristic of genes from which all introns have been deleted is attributable, at least in part, to NMD elicited by the normal termination codon. However, our finding that the inefficient production of mRNA from intronless TPI (Nesic & Maquat, 1994) and GPx1 genes (Fig. 1; Moriarty et al., 1997) is either completely or almost completely attributable to RNA decay prior to 3′-end formation favors the second model. Also consistent with the second model, the abundance of GPx1 mRNA produced from an intronless GPx1 gene is not altered by (1) varying the extracellular Se concentration or converting the Sec codon to either TAA or TGC, each of which changes the efficiency of translation termination at the Sec codon (Moriarty et al., 1997) or (2) overexpressing a dominant-negative version of hUpf1p that is known to abrogate NMD (Fig. 1). Finally, evidence exists that exon–exon junctions other than the 3′-most function to elicit NMD (Zhang et al., 1998a; Figs. 2 and 3).

MATERIALS AND METHODS

Plasmid DNA constructions

pmCMV-TPI$^{70-71Ter,\ \Delta(introns\ 1-5)}$, which consists of a TPI allele harboring a nonsense codon spanning codons 70–71 and lacking introns 1–5, was generated by Klenow-filling in the *Eag*I site that spans codons 34–36 within exon 1 of pmCMV-TPI$^{Norm,\ \Delta(introns\ 1-5)}$ (Zhang et al., 1998a) to create a 4-bp insertion (5′-GGCC-3′ in the sense strand). The 735-bp *Eag*I-*Eco*RI fragment pmCMV-TPI$^{Norm,\ \Delta(introns\ 1-5)}$ that extends from exon 1 into exon 7 was cloned into the *Eag*I and *Eco*RI sites of pBS-KS(−), and a nonsense mutation was introduced into codon 159 of single-stranded DNA by using the antisense mutagenic primer 5′-CGAC*CTA*GCTCCAGTCC-3′ (where the italicized nucleotide is mutagenic and underlined nucleotides specify the nonsense codon generated). Additionally, codon 82 was converted from Met (ATG) to Asp (GAT) by using the antisense mutagenic primer 5′-GTCTTTG AT*ATC*GCCTGGGCTG-3′ (where underlined nucleotides specify the aspartate codon). pmCMV-TPI$^{159Ter,\ \Delta(introns\ 1-5)}$ was generated by using the 680-bp *Eag*I–*Msc*I fragment that extends from exon 1 into exon 7 to replace the corresponding fragment of pmCMV-TPI$^{Norm,\ \Delta(introns\ 1-5)}$. Similarly, pmCMV-TPI$^{Norm,\ \Delta(introns\ 1-5),\ 84Met-Val}$ and pmCMV-TIP$^{23Ter,\ \Delta(introns\ 1-5),\ 82Met-Val}$ were generated by using the 680-bp *Eag*I–*Msc*I fragment to replace the corresponding fragment of pmCMV-TPI$^{Norm,\ \Delta(introns\ 1-5)}$ and pmCMV-TPI$^{23Ter,\ \Delta(introns\ 1-5)}$, respectively.

Cell transfections

Monkey kidney Cos cells were propagated in minimal essential medium containing 10% fetal bovine serum (FBS) and 5% bovine calf serum (BCS). One day prior to transfection, cells (~3–4 × 10^5 per 60-mm dish) were cultured in antibiotic-free medium containing 10% FBS and, after reaching 30–40% confluency, were transfected by using LipofectAMINE PLUS Reagent (Life Technologies) following the manufacturer's directions. Briefly, 1 μg of pCI-neo-hUPF1 (Sun et al., 1998), 0.7 μg of pmCMV-GPx1 test plasmid (Moriarty et al., 1997, 1998), and 0.3 μg of phCMV-MUP reference plasmid (Belgrader & Maquat, 1994) were added to 250 μL of serum-free medium, incubated with 4 μL of PLUS Reagent for 15 min at room temperature and, subsequently, added to 250 μL of serum-free medium containing 6 μL of LipofectAMINE Reagent. After an additional incubation for 15 min at room temperature, the mixture was added to cells in 2 mL of serum-free and antibiotic-free medium, and incubated for 4 h at 37 °C. The cells were supplemented with 2.5 mL of medium containing 20% serum, making the final concentration 10%, and incubated for an additional 20 h. The medium was then replaced with 4–5 mL of antibiotic-containing medium also containing 10% FBS and 5% BCS, and cells were harvested 24 h later. Mouse NIH-3T3 cells were propagated and transfected by using calcium phosphate as described (Zhang et al., 1998a).

RNA analyses

Total-cell RNA was isolated using Trizol (Gibco BRL). Total-cell GPx1 and MUP transcripts were analyzed by RT-PCR as

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described earlier (Sun et al., 1998). C^+/A^+ GPx1 and MUP transcripts were reverse transcribed using oligo$(dT)_{12–18}$ (Gibco BRL), and cDNAs were then amplified for 19 cycles using the same primer pair used to amplify total-cell (Sun et al., 1998). C^-/A^- GPx1 transcripts were reverse transcribed using an antisense primer to sequences downstream of the cleavage and polyadenylation site (5′-CCGTTCTAG ACCAAGTTTAGACTCG-3′), which also allows for MUP cDNA synthesis, and cDNAs were then amplified as described for C^+/A^+ RNA. Notably, PCR was always dependent on the addition of RNA, RT, and the primer used to generate cDNA. TPI and β-globin (Gl) transcripts were quantitated by Northern blot hybridization as described (Zhang et al., 1998a).

ACKNOWLEDGMENTS

The authors thank Pat Hilleren and Roy Parker for very stimulating and enjoyable conversations, Xiaojie Li for expert technical assistance, Adam Plunkett for volunteer help, John Yates for helpful discussions, and members of the Maquat lab, especially Guillaume Serin and Anand Gersappe, for comments on the manuscript. This work was supported by Public Health Service Research grant DK 33933 from the National Institutes of Health.

Received August 3, 1999; returned for revision September 20, 1999; revised manuscript received October 18, 1999

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https://doi.org/10.1017/S1355838200991660 Published online by Cambridge University Press