Intron function in the nonsense-mediated decay of β -globin mRNA: Indications that pre-mRNA splicing in the nucleus can influence mRNA translation in the cytoplasm

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ABSTRACT

Generally, mRNAs that prematurely terminate translation are abnormally low in abundance. In the case of mammalian cells, nonsense codons most often mediate a reduction in the abundance of newly synthesized, nucleus-associated mRNA by a mechanism that is not well understood. With the aim of defining *cis*-acting sequences that are important to the reduction process, the effects of particular β -globin gene rearrangements on the metabolism of β -globin mRNAs harboring one of a series of nonsense codons have been assessed. Results indicate that nonsense codons located 54 bp or more upstream of the 3'-most intron, intron 2, reduce the abundance of nucleus-associated mRNA to 10–15% of normal without altering the level of either of the two introns within pre-mRNA. The level of cytoplasmic mRNA is also reduced to 10–15% of normal, indicating that decay does not take place once the mRNA is released from an association with nuclei into the cytoplasm. A nonsense codon within exon 2 that does not reduce mRNA abundance can be converted to the type that does by (1) inserting a sufficiently large in-frame sequence immediately upstream of intron 2 or (2) deleting and reinserting intron 2 a sufficient distance downstream of its usual position. These findings indicate that only those nonsense codons located more than 54 bp upstream of the 3'-most intron reduce β -globin mRNA abundance, which is remarkably consistent with which nonsense codons within the triose-phosphate isomerase (TPI) gene reduce TPI mRNA abundance. We propose that the 3'-most exon–exon junction of β -globin mRNA and, possibly, most mRNAs is marked by the removal of the 3'-most intron during pre-mRNA splicing and that the “mark” accompanies mRNA during transport to the cytoplasm. When cytoplasmic ribosomes terminate translation more than 54 nt upstream of the mark during or immediately after transport, the mRNA is subjected to nonsense-mediated decay. The finding that deletion of β -globin intron 2 does not appreciably alter the effect of any nonsense codon on β -globin mRNA abundance suggests that another *cis*-acting sequence functions in nonsense-mediated decay comparably to intron 2, at least in the absence of intron 2, possibly as a fail-safe mechanism. The analysis of deletions and insertions indicates that this sequence resides within the coding region and can be functionally substituted by intron 2.

Keywords: introns; mRNA destabilizing elements; nonsense codons; nucleus-associated mRNA

INTRODUCTION

All organisms appear to have established mechanisms to eliminate the production of mRNAs that prematurely terminate translation, presumably to prevent the production of potentially deleterious proteins that arise from

DNA mutation or defects in RNA metabolism (reviewed in Peltz et al., 1994; Maquat, 1995, 1996; Ruiz-Echevarria & Peltz, 1996). Reports for mammalian cells that nonsense codons can mediate a reduction in the abundance of mRNA that co-purifies with nuclei and, in some cases, can influence the level of the corresponding pre-mRNA have led to the proposal that nonsense codons can be recognized in the nucleoplasm (see, e.g., Naeger et al., 1992; Dietz et al., 1993; Dietz & Kendzior, 1994; Lozano et al., 1994; Kugler et al., 1995; Aoufouchi et al., 1996; Carter et al., 1996; Li et al., 1997). There are also examples of the nonsense-mediated decay of cytoplasmic mRNA (Shyu et al., 1989; Barker & Beemon, 1991, 1994; Lim et al., 1992; Moriarty et al., 1998).

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Conceptual difficulties in reconciling the idea of nonsense codon recognition in the nucleoplasm with the fact that protein synthesis takes place in the cytoplasm have led us to propose for mRNA encoding triosephosphate isomerase (TPI) that nonsense codon recognition and the consequential mRNA decay take place during or immediately after mRNA transit across the nuclear pore, at a point where the mRNA co-purifies with nuclei but is accessible to cytoplasmic ribosomes (reviewed in Maquat, 1995, 1996). In support of this proposal, the splicing of TPI pre-mRNA is unaffected by a nonsense codon (Cheng & Maquat, 1993), nonsense codon recognition appears to take place after splicing (Belgrader et al., 1994; Zhang & Maquat, 1996) in a mechanism that is indistinguishable from cytoplasmic translation (Belgrader et al., 1993; Zhang & Maquat, 1997), and fully spliced, nucleus-associated mRNA is the target of decay (Belgrader et al., 1994). In fact, the Balbiani ring granule of *Chironomus tentans* provides a precedent for the concomitant export and translation of an mRNA (Mehlin et al., 1992).

Nevertheless, introns appear to play a role in the nonsense-mediated decay process. First, deletion of all introns from the TPI gene eliminates decay by a mechanism that is not due to a block in mRNA export to the cytoplasm (Zhang et al., 1998). Second, nonsense codons close to and including the normal termination codon of the TPI gene do not mediate a reduction in mRNA abundance because of their proximity to the 3'-most intron (Cheng et al., 1994; Zhang et al., 1998). This second point became apparent by moving the 3'-most intron downstream of its usual position or inserting any one of several introns downstream of the 3'-most intron: each change repositioned the boundary between nonsense codons that do and do not reduce mRNA abundance so as to reside 50–55 nt upstream of the 3'-most exon–exon junction of mRNA (Cheng et al., 1994; Zhang et al., 1998). Furthermore, deletion of the 3'-most intron and some, but not all, of the upstream introns within either the TPI or T-cell receptor (TCR)- β gene repositioned the boundary (Carter et al., 1996; Zhang et al., 1998), and repositioning in the case of the TPI gene was shown, with one exception, to be dictated by the position of the new 3'-most intron.

The finding that the nonsense-mediated reduction in the abundance of both TPI and TCR- β mRNAs involves what appears to be a spliceable intron within pre-mRNA (Carter et al., 1996; Zhang et al., 1998) raised the interesting concept that components of the nuclear splicing complex may be exported to the cytoplasm in association with spliced mRNA so as to interact with components of the cytoplasmic translation complex. A precedent for this is found in studies of *C. tentans*. Ct-hrp36, a relative of human hnRNP A/B proteins, is incorporated into nascent pre-mRNA complexes, subsequently transported in association with mRNA through the nuclear pore to the cytoplasm, and found in poly-

somes (Visa et al., 1996). Therefore, Ct-hrp36 is one candidate for a factor that loads onto pre-mRNA as a component of the splicing complex and has the potential to interact with the translation complex as a component of cytoplasmic mRNP.

In order to determine the generality of the role of introns in the nonsense-mediated reduction of mRNA abundance, and to understand if the boundary between nonsense codons that do and do not reduce mRNA abundance generally resides ~50–55 nt upstream of the 3'-most exon–exon junction of mRNA, we expanded our studies to include the β -globin gene. These studies are important given the difference in boundary determinations for the TPI and TCR- β genes. In contrast to what would be predicted on the basis of findings for the TPI gene, the boundary between nonsense codons that effectively reduce TCR- β mRNA abundance and nonsense codons that only ineffectively reduce TCR- β mRNA abundance was reported to reside between 2 and 10 bp upstream of intron 2 when all introns downstream of intron 2 had been deleted (Carter et al., 1996). The position of TCR- β intron 2 was not varied in order to test the possibility that this intron positioned the boundary, as would be predicted from studies of the TPI gene (Zhang et al., 1998).

Results described here for the β -globin gene corroborate findings for the TPI gene: nonsense codons located 54 nt or more upstream of the 3'-most exon–exon junction of β -globin mRNA reduce the abundance of nucleus-associated β -globin mRNA without affecting the level of introns within β -globin pre-mRNA or eliciting an additional reduction in the abundance of cytoplasmic mRNA. Also analogous to the TPI gene, deleting the 3'-most intron of the β -globin gene still allows for the nonsense-mediated reduction, indicating that another *cis*-acting sequence functions in decay similarly to this intron, at least in the absence of this intron. Because the efficiency with which the *cis*-acting sequence functions in place of the 3'-most intron is much higher for the β -globin gene than for the TPI gene, the β -globin gene provides a unique opportunity to map the sequence. Deletion analysis revealed that the sequence is situated within the coding region. Furthermore, reinsertion of the 3'-most β -globin intron functionally compensated for the deletion of the sequence, suggesting that the exonic and intronic sequences are functionally equivalent.

RESULTS

The boundary between nonsense codons that do and do not reduce the abundance of nucleus-associated β -globin mRNA normally resides within exon 2

In order to supplement our detailed studies of the nonsense-mediated decay of human TPI mRNA, and

with a goal of understanding discrepancies between results obtained with nonsense-containing TPI mRNA and nonsense-containing TCR- β mRNA, nonsense codons were generated within a hybrid human-mouse β -globin gene. Years ago, we discovered that expression of the human β -globin gene in mouse L cells could be significantly enhanced if sequences extending from the *Bam*H I site of exon 2 into 3' flanking DNA were substituted with analogous sequences from the mouse β -globin gene (Cheng et al., 1990).

Nonsense codons were generated within the hybrid gene at position 21–22 within exon 1, positions 39, 60–61, or 101 within exon 2, or position 141 within exon 3. These nonsense codons are referred to as 21–22Ter, 39Ter, 60–61Ter, 101Ter, and 141Ter, respectively. The first three were the consequence of frameshift or nonsense mutations within β^0 -thalassemic β -globin alleles that had been shown to reduce β -globin mRNA abundance in erythroid tissues of humans and mice (see Lim et al., 1989 and references therein). The remaining two were generated in vitro for the purposes of these studies and had yet to be tested in any type

of cell. Each of the mutated alleles was transiently introduced into mouse NIH3T3 cells in the form of a pmCMV-GI plasmid so that β -globin gene transcription is driven by the mouse cytomegalovirus (mCMV) promoter (Zhang et al., 1998; Fig. 1). In order to control for variations in the efficiencies of cell transfection and RNA recovery, a pmCMV-TPI plasmid (Zhang et al., 1998) that contains the human TPI gene similarly driven by the mCMV promoter was simultaneously introduced. Cells were harvested after 48 h, and the amounts of mCMV-GI and mCMV-TPI mRNAs in nuclear and cytoplasmic fractions were quantitated using northern blot analysis. For each transfection, the amount of mCMV-GI mRNA was normalized to the amount of mCMV-TPI mRNA and presented as a percentage of the normalized amount of the corresponding nonsense-free (Norm) mRNA.

When introduced into NIH3T3 cells, the first three nonsense codons reduced the abundance of β -globin mRNA in both nuclear and cytoplasmic fractions to an average of 12% of normal, whereas the remaining two nonsense codons were of relatively little consequence

FIGURE 1. Boundary between nonsense codons that do and do not reduce the abundance of β -globin mRNA resides within β -globin exon 2, and the reduction is limited to mRNA that maintains an association with nuclei. NIH3T3 cells were transfected with a pmCMV-TPI reference construct and the pmCMV-GI construct specified above each lane, where Ter indicates a nonsense codon. RNA was purified from nuclear and cytoplasmic fractions using citric acid (Applequist et al., 1997) and cesium chloride centrifugation (Cheng & Maquat, 1993; Zhang & Maquat, 1996), and 25 μ g was analyzed by blot hybridization to DNA fragments 32 P-labeled by random priming. TPI RNA was detected 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 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., 1990). Hybridization was quantitated by PhosphorImaging. The level of mRNA from each mCMV-GI allele was normalized to the level of mCMV-TPI mRNA in order for the analysis to be quantitative. Normalized values were then calculated as a percentage of the normalized value for mCMV-GI^{Norm} mRNA (i.e., nonsense-free mRNA), which was considered to be 100. Percentages from two independently performed experiments did not differ by more than 7%.

to mRNA abundance (Fig. 1; data not shown for 21–22Ter). Therefore, a boundary between nonsense codons that do and do not appreciably reduce β -globin mRNA abundance exists within exon 2. Because the extent of the nonsense-mediated reduction in mRNA abundance was essentially the same in nuclear and cytoplasmic fractions, nonsense-mediated decay in NIH3T3 cells must be confined to β -globin transcripts that maintain an association with nuclei. Therefore, the nonsense effect on β -globin and TPI transcripts in non-erythroid cells is similar in two ways: (1) the boundary between nonsense codons that do and do not reduce mRNA abundance resides within the exon that precedes the normal termination codon, and (2) the reduction in mRNA abundance is comparable using nuclear and cytoplasmic RNA, indicating that the reduction takes place prior to the release of newly synthesized mRNA from an association with nuclei into the cytoplasm. Consistent with our findings, 39Ter was previously found to reduce the abundance of nucleus-associated β -globin mRNA produced in HeLa cells from a human β -globin gene driven by the human CMV immediate early promoter (Kugler et al., 1995).

The nonsense-mediated reduction in β -globin mRNA abundance does not affect the level of either intron within pre-mRNA

Given that a nonsense codon can be associated with an altered level of one or more introns within pre-mRNA (reviewed in Maquat, 1995 and 1996), the consequence of 39Ter and 60–61Ter to the level of each intron within β -globin pre-mRNA was assessed using the level of intron 5 within TPI pre-mRNA as an internal control (Cheng & Maquat, 1993; Zhang & Maquat, 1996). When presented as a percentage of the normalized amount of the corresponding intron within nonsense-free β -globin pre-mRNA, the level of intron 1 for either nonsense-containing RNA was 116% of normal (Fig. 2A), whereas the level of intron 2 was 100% of normal for 39Ter-containing RNA and 105% of normal for 60–61Ter-containing RNA (Fig. 2B). Therefore, the nonsense-mediated reduction in β -globin mRNA abundance is not attributable to an alteration in the level of intron-containing β -globin pre-mRNA.

Sufficiently large insertions between codon 101 and intron 2 of the β -globin gene convert 101Ter from the type of nonsense codon that has little effect on mRNA abundance to the type of nonsense codon that reduces mRNA abundance

The boundary between nonsense codons within β -globin mRNA that do and do not appreciably reduce

FIGURE 2. Nonsense-mediated reduction in β -globin mRNA abundance is not attributable to an effect on the level of either intron within β -globin pre-mRNA. The RNAs analyzed are the same as those analyzed in Figure 1. cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase, random hexamers, and nuclear RNA (0.22–6.0 μ g) from transfected NIH3T3 cells. PCR was then used to amplify and incorporate [α - 32 P]dATP into sequences that derived from either part of β -globin intron 1 plus part of exon 2 (**A**) or part of β -globin intron 2 plus part of exon 3 (**B**) of the specified mCMV-GI cDNA and, as a control, sequences that derived from part of intron 5 and part of exon 6 of mCMV-TPI cDNA. The structures and sizes (bp) of the RT-PCR products are shown to the right of each panel, where introns (lines) and exons (boxes) are numbered. The level of each mCMV-GI pre-mRNA product was normalized to the level of the mCMV-TPI product and calculated as a percentage of the normalized value of the mCMV-GI^{Norm} pre-mRNA product, which was considered to be 100. The conditions of RT-PCR were demonstrated to be quantitative using RNA standards (Stds) that consisted of serial dilutions of nuclear RNA from transfected cells. Values shown are an average of the values obtained from two independently performed experiments, which did not differ by more than 5%.

β -globin mRNA abundance maps between codon 60–61 and codon 101, i.e., between 9 and 131 nt upstream of the 3'-most exon–exon junction within β -globin mRNA (Fig. 1). Notably, the boundary within TPI mRNA maps approximately 52 nt upstream of the 3'-most exon–exon (Cheng et al., 1994). Because the boundary within TPI mRNA has been shown to be a function of the position of the 3'-most intron (Cheng et al., 1994; Zhang et al., 1998), it is possible that the position of β -globin intron 2 determines the position of the boundary within β -globin mRNA.

As a first approach to test this possibility, and with the aim of mapping the boundary within β -globin mRNA more accurately, an insertion of 45 bp or 75 bp was made between codons 102 and 103 in the context of the nonsense-free and 101Ter alleles. Each insertion consisted of a multiple of three base pairs, did not har-

bor an in-frame nonsense codon, and increased the distance between codon 101 and intron 2 from 9 bp to, respectively, 54 or 84 bp. Relative to the corresponding nonsense-free construct, 101Ter reduced mRNA abundance to 22% of normal in the context of the 45-bp insertion and 14% of normal in the context of the 75-bp insertion (Fig. 3). Therefore, insertions that increase the distance between 101Ter and intron 2 to 54 bp or more convert 101Ter from the type of nonsense codon that does not appreciably reduce mRNA abundance to the type of nonsense codon that does reduce mRNA. These results are remarkably similar to those found for the TPI gene, where a nonsense codon residing 43 bp upstream of the 3'-most intron had no effect on mRNA abundance, a nonsense codon residing 52 bp upstream of the intron had an intermediate effect on mRNA abundance (i.e., 31% of normal), and a nonsense codon residing 61 bp upstream of the intron reduced the mRNA level to what is typically the lowest nonsense-mediated level for TPI RNA (i.e., 15% of normal) (Cheng et al., 1994).

Moving β -globin intron 2 moves the boundary between nonsense codons that do and do not appreciably reduce mRNA abundance, indicating that the position of intron 2 within pre-mRNA positions the boundary within mRNA

In order to prove that the position of intron 2 rather than some other sequence within β -globin RNA normally positions the boundary between nonsense codons that do and do not appreciably reduce mRNA abundance, intron 2 was deleted and reinserted 8 or 51 bp downstream of its usual position in the context of the nonsense-free allele or alleles harboring 60–61Ter, 101Ter, or, as a control, 141Ter (Fig. 4). Therefore, intron 2 was moved to reside, respectively, 139 or 182 bp downstream of codon 60–61 and 17 or 60 bp downstream of codon 101. If the position of intron 2 is the boundary determinant, then movement to a position more than 54 bp downstream of a nonsense codon should be expected to reduce mRNA abundance.

FIGURE 3. Sufficiently large insertions between codon 101 and intron 2 of the β -globin gene convert 101Ter from the type of nonsense codon that has little effect on mRNA abundance to the type that appreciably reduces mRNA abundance, indicating that a sequence downstream of the insertions positions the boundary between nonsense codons that do and do not reduce mRNA abundance. NIH3T3-cell transfections and RNA analyses were as described in the legend to Figure 1. Total RNA was purified using Trizol (Gibco BRL). Darkened regions within exon 2 specify the insertions. Normalized values for mCMV-GI mRNAs that derived from constructs harboring 101Ter and an insertion were calculated as a percentage of the normalized value for the corresponding nonsense-free construct, which was considered to be 100. Values shown are an average of the values obtained for two independently performed experiments, which did not differ by more than 7%.

FIGURE 4. Moving β -globin intron 2 moves the boundary between nonsense codons that do and do not appreciably reduce mRNA abundance, indicating that position of intron 2 within pre-mRNA positions the boundary within mRNA. NIH3T3-cell transfections and RNA analyses were as described in the legend to Figure 3. Values shown are an average of values obtained from three independently performed experiments, which did not differ by more than 5%.

In fact, this is what the data demonstrate. The two alleles harboring 60–61Ter as well as the allele harboring 101Ter located 60 bp upstream of intron 2 generated, respectively, 8%, 10%, and 33% the amount of mRNA generated by the corresponding nonsense-free allele (Fig. 4). In contrast, the allele harboring 101Ter located only 17 bp upstream of the intron and the two alleles harboring 141Ter generated, respectively, 94%, 110%, and 104% the amount of mRNA generated by the corresponding nonsense-free allele (Fig. 4). Therefore, the position of intron 2 within β -globin pre-mRNA must normally position the boundary between nonsense codons that do and do not reduce β -globin mRNA abundance.

In the absence of intron 2, the boundary between nonsense codons that do and do not appreciably reduce β -globin mRNA abundance is maintained, indicating that another *cis*-acting sequence positions the boundary in the absence of the intron

When the 3'-most intron, intron 6, of the human TPI gene is deleted, a boundary is still evident in roughly the same position as when the intron is present, although the difference in the extent to which nonsense

codons to either side of the boundary affect mRNA abundance is decreased (Cheng et al., 1994). The simplest interpretation of this result is that a *cis*-acting sequence other than the new 3'-most intron, intron 5, positions the boundary in the absence of intron 6. In order to determine if the same is characteristic of the β -globin gene, intron 2 was deleted from alleles that were either nonsense-free or harbored 21–22Ter within exon 1, 39Ter, 60–61Ter or 101Ter within exon 2, or 141Ter within exon 3 (Fig. 5).

Alleles harboring 21–22Ter, 39Ter, or 60–61Ter and the intron 2 deletion generated, respectively, only 22%, 13%, and 11% the amount of mRNA generated by the nonsense-free allele harboring the intron 2 deletion (Fig. 5). Cell fractionation studies indicated that the intron deletion does not alter the cellular site of decay, i.e., decay remains nucleus-associated (E. Nagy & L.E. Maquat, data not shown). In contrast, alleles harboring either 101Ter or 141Ter and the intron 2 deletion generated, respectively, 76% or 88% the amount of mRNA generated by the nonsense-free allele harboring the intron 2 deletion (Fig. 5). The reason for 101Ter and 141Ter generating less than normal amounts of mRNA in the absence of the 3'-most intron is unknown. However, in the absence of the 3'-most intron of the TPI gene, subnormal levels (i.e., levels of only 72–81% of normal) are also characteristic of nonsense codons that

FIGURE 5. In the absence of β -globin intron 2, the boundary between nonsense codons that do and do not appreciably reduce mRNA abundance is maintained, indicating another *cis*-acting sequence must position the boundary in the absence of the intron. NIH3T3-cell transfections and RNA analyses were as described in the legend to Figure 3. Δ (intron 2) specifies the deletion of intron 2. Values shown are an average of values obtained from two independently performed experiments, which did not differ by more than 8%.

do not normally reduce the abundance of TPI mRNA (Cheng et al., 1994). We conclude that β -globin intron 2 can be deleted without considerable consequence to either the position of the boundary or the degree of the nonsense-mediated reduction in abundance even though the intron normally functions to position the boundary between nonsense codons that do and do not appreciably reduce mRNA abundance. Therefore, another *cis*-acting sequence must function in the absence of intron 2 to position the boundary and evoke a nonsense-mediated decay.

The *cis*-acting sequence that functions to position the boundary in the absence of intron 2 resides within the coding region rather than the 3' untranslated region of β -globin mRNA

In order to localize the *cis*-acting sequence that functions in the nonsense-mediated reduction of β -globin mRNA abundance in the absence of intron 2, three deletions were generated within pmCMV-GI alleles that lacked intron 2 and were nonsense-free or harbored either 39Ter or 60–61Ter. All reside downstream of codon 60–61Ter, the 3'-most nonsense codon demonstrated to efficiently reduce mRNA abundance in the

absence of intron 2. One deletion, referred to as Δ #1, extends from the last 14 bp of exon 2 through the first 100 bp of exon 3 (i.e., from the Klenow-filled *Bam*H I site to the Klenow-filled *Msc* I site, which corresponds to codons 99–137; Fig. 6). The others, referred to as Δ #2 and Δ #3, extend, respectively, from bp +2 through +53 or bp +54 through +105, where +1 is defined as the base pair residing immediately downstream of the translation termination codon and +106 is the base pair residing immediately upstream of the AATAAA polyadenylation sequence (Fig. 6).

Alleles harboring 39Ter or 60–61Ter and Δ #1 produced, respectively, 57% and 58% the amount of mRNA produced by the nonsense-free allele harboring Δ #1 (Fig. 6), indicating that the deletion partially abrogates the nonsense-mediated reduction in mRNA abundance. In contrast, alleles harboring either nonsense codon and Δ #2 or Δ #3 produced 17–18% the amount of mRNA produced by the nonsense-free allele harboring the corresponding deletion (Fig. 6), indicating that neither deletion affects the nonsense-mediated reduction in mRNA abundance. Δ #2 drastically reduced the level of nonsense-free mRNA, but not to a point that precluded a quantitative analysis. These data indicate that sequences that function in the nonsense-mediated reduction in β -globin mRNA abundance in the absence of intron 2 do not reside within the 3' untranslated region,

FIGURE 6. Deletion of codons 99–137 ($\Delta\#1$) partially abrogates the nonsense-mediated reduction in β -globin mRNA abundance, whereas deletion of either half of the 3' untranslated region ($\Delta\#2$ or $\Delta\#3$) is of no consequence to the reduction. NIH3T3-cell transfections and RNA analyses were as described in the legend to Figure 3. Values shown are an average of values obtained from three independently performed experiments, which did not differ by more than 7%. $\Delta\#1$ was generated by ligating the filled *Bam*H I (B) site to the filled *Msc*I (M) site, which regenerated the *Bam*H I site.

but overlap with the last 14 nt of exon 2 through the first 100 nt of exon 3.

With the aim of localizing these sequences more accurately, two additional deletions were generated in the context of pmCMV-GI alleles that lack intron 2 and are either nonsense-free or harbor 39Ter or 60–61Ter. In case sequences upstream of the last 14 bp of exon 2 are important, one deletion, referred to as $\Delta\#4$, was designed to remove the last 129 bp of exon 2 (i.e., codons 62–104; Fig. 7). The other deletion, referred to as $\Delta\#5$, removed the first 129 bp of exon 3 (i.e., codons 105–146, where codon 147 constitutes the translation termination codon; Fig. 7).

Alleles harboring either 39Ter or 60–61Ter and $\Delta\#4$ generated, respectively, 48% and 44% the level of mRNA produced by the corresponding nonsense-free allele, suggesting that the deleted region overlaps with the region important for the nonsense-mediated reduction in mRNA abundance (Fig. 7). In contrast, alleles harboring $\Delta\#5$ either with or without a nonsense codon generated too little RNA to be informative (Fig. 7). Taken together, the results obtained with exon deletions on the order of 100 bp suggest that sequences important for the nonsense-mediated reduction in β -globin mRNA abundance in the absence of intron 2 reside within exon 2 and, possibly, exon 3 because neither exon 2 deletion completely abrogates the reduction.

The *cis*-acting sequence removed by $\Delta\#1$ can be functionally replaced by intron 2

Most *Saccharomyces cerevisiae* genes lack introns, and nonsense-mediated decay in *S. cerevisiae* appears to be mediated by exonic *cis*-acting sequences (reviewed in Ruiz-Echevarria et al., 1996). The analysis of β -globin and TPI gene deletions (Figs. 5, 6, 7; Cheng et al., 1994; Zhang et al., 1998) indicates that the nonsense-mediated decay of at least some mammalian mRNAs can be mediated by either intronic or exonic sequences. Despite a lack of detectable structural similarity, the exonic sequences that mediate the decay of mammalian mRNAs may be functionally analogous to the exonic sequences that mediate the decay of *S. cerevisiae* mRNAs. With the goal of understanding how the intronic and exonic sequences within the β -globin gene functionally relate, β -globin intron 2 was generated by PCR and inserted at the site of $\Delta\#1$ in the context of pmCMV-GI alleles that were either nonsense-free or harbored 39Ter or 60–61Ter (Fig. 8). Results indicate that the inserted intron 2 restores the nonsense-mediated reduction in β -globin mRNA abundance so that the mRNA level averages 26% of normal (Fig. 8). This result is consistent with aforementioned data demonstrating that intron 2 mediates a reduction in mRNA abundance more effectively than the *cis*-acting exonic sequence.

FIGURE 7. Deletion of codons 62–104 of exon 2 (Δ #4) partially abrogates the nonsense-mediated reduction in β -globin mRNA abundance, whereas deletion of codons 105–146 of exon 3 (Δ #5) precludes the generation of a detectable level of mRNA. NIH3T3-cell transfections and RNA analyses were as described in the legend to Figure 3. Values shown are an average of values obtained from four independently performed experiments, which did not differ by more than 8%. N.D., not detectable.

DISCUSSION

The premature termination of translation of β -globin mRNA more than 50–55 nt upstream of the 3'-most exon–exon junction reduces the abundance of nucleus-associated mRNA: Models for the reduction process

We show here that a nonsense codon reduces the abundance of β -globin mRNA 5–10-fold provided it resides 54 nt or more upstream of the 3'-most exon–exon junction, as would be predicted from studies of human TPI transcripts. Our results are consistent with reports that nonsense codons within the terminal β -globin exon of β -thalassemic patients fail to reduce the abundance of β -globin mRNA and are associated with a dominant form of the anemia (Hall & Thein, 1994). In contrast to the nonsense-mediated decay of β -globin mRNA in erythroid cells, which appears to be exclusively cytoplasmic (Lim et al., 1989, 1992; Lim & Maquat, 1992), the reduction in β -globin mRNA abundance in non-erythroid cells takes place prior to the release of newly synthesized mRNA from an association with nuclei (Fig. 1; data not shown for mouse L cells; Kugler et al., 1995), but after intron removal (Fig. 2). Notably, β -globin mRNA that has been released from an asso-

ciation with nuclei into the cytoplasm is immune to nonsense-mediated decay (Fig. 1; Kugler et al., 1995). Therefore, the metabolism of nonsense-containing β -globin RNA is similar to the metabolism of nonsense-containing TPI RNA (Cheng & Maquat, 1993; Belgrader et al., 1994).

The boundary between nonsense codons that do and do not reduce β -globin mRNA abundance is normally likely to be determined by the position of intron 2 within β -globin pre-mRNA, as evidenced by analyzing the consequences of (1) insertions into the proximal end of exon 2 (Fig. 3) and (2) moving intron 2 into exon 3 (Fig. 4). Therefore, as has been demonstrated for the nonsense-mediated decay of TPI mRNA (Cheng et al., 1994; Zhang et al., 1998), the position of the 3'-most intron within pre-mRNA can influence the metabolism of intronless (i.e., spliced) product mRNA (Cheng et al., 1994; Carter et al., 1996; Zhang et al., 1998). We propose (Fig. 9) that once translation initiates (Step 1) on an mRNA being transported from the nucleus to the cytoplasm and terminates prematurely more than 50–55 nt upstream of the 3'-most exon–exon junction (Step 2), mRNA decay may be mediated by the failure of translating ribosomes to interact with one or more posttranscriptional modifications or "marks" that are deposited at the junction as a consequence of splicing the

FIGURE 8. Insertion of β -globin intron 2 into the site of $\Delta\#1$ restores the nonsense-mediated reduction in mRNA abundance. NIH3T3-cell transfections and RNA analyses were as described in the legend to Figure 3. Values shown are an average of values obtained from two independently performed experiments, which did not differ by more than 6%. A slash through the *Bam*H I (B) site indicates the site was destroyed when intron 2 was inserted.

3'-most intron (Step 3, Model 1). Alternatively, mRNA decay may be mediated by the interaction of a "scanner" with mark(s) at the 3'-most exon-exon junction (Fig. 9, Step 3, Model 2). We imagine for either model that decay is limited to the first round of translation. In the case of the second model, it may be that 50–55 nt between a termination event, which is hypothesized to trigger scanner formation, and the 3'-most exon-exon junction is the minimum distance required for the scanner to interact with the junction. The scanner may consist of the 40S ribosome together with eIF-2-GTP-Met-tRNA^{Met} that is capable of translation reinitiation downstream of at least some (i.e., relatively early) termination codons (Zhang & Maquat, 1997 and references therein). However, while this article was out for review, Ruiz-Echevarria et al. (1998) demonstrated for *S. cerevisiae* that termination at an open translational reading frame that essentially precludes reinitiation at a downstream site does not preclude nonsense-mediated decay. Alternatively or in addition to a 40S ribosome complex, the scanner may consist of the mammalian homologue to Upf1p (Perlick et al., 1996; Applequist et al., 1997; X. Sun, H.A. Perlick, H.C. Dietz, & L.E. Maquat, unpubl. data), a factor essential for nonsense-mediated decay in *S. cerevisiae* that enhances translation termination and manifests RNA binding and ATPase/helicase activities (Weng et al., 1996a,

1996b). In the case of either model, we propose that decay initiates at the 5' end of the mRNA (Step 4), as has been demonstrated for nonsense-containing β -globin mRNA in erythroid cells (Lim et al., 1992; Lim & Maquat, 1992) and nonsense-containing mRNAs in general in *S. cerevisiae* (Muhlrad & Parker, 1994; reviewed in Ruiz-Echevarria et al., 1996).

Our findings reported here for β -globin transcripts and elsewhere for TPI transcripts (Cheng et al., 1994; Zhang et al., 1998) that nonsense codons located fewer than 50–55 nt upstream of the 3'-most exon-exon junction within an mRNA do not mediate a reduction in mRNA abundance can be used to explain why the normal termination codon in the penultimate exon of the mouse major urinary protein (MUP) gene does not elicit a reduction in MUP mRNA abundance (Belgrader & Maquat, 1994). For reasons unknown, however, our determinations are different than those obtained for the TCR- β gene that stipulate a distance of fewer than 8–10 bp upstream of the 3'-most intron (Carter et al., 1996). On the basis of our data, we predict that most mammalian mRNAs would be characterized by a normal termination codon that resides no more than 50–55 nt upstream of the 3'-most exon-exon junction. Consistent with this prediction, most genes harbor the normal termination codon within the 3'-most exon. Furthermore, a survey of 1,500 genes from species as

FIGURE 9. Models for the nonsense-mediated decay of mammalian mRNA. 40S and 60S specify ribosome subunits, ter specifies a premature termination codon, the thick bar represents the 3'-most exon-exon junction, Norm Ter specifies the normal termination codon, and the filled circle represents one or more factors that assemble as a consequence of translation termination. Once translation initiates (Step 1) on an mRNA being transported from the nucleus to the cytoplasm and terminates prematurely more than 50–55 nt upstream of the 3'-most exon-exon junction (Step 2), mRNA decay may be mediated by the failure of ribosomes to interact with a mark that is deposited at the junction in the nucleus and exported with the mRNA to the cytoplasm (Step 3, Model 1). Alternatively, 50–55 nt may be the minimum distance between a termination event, which presumably triggers scanner formation, and the 3'-most exon-exon junction in order for the scanner to interact with the junction (Step 3, Model 2). In either case, decay is proposed to initiate at the 5' end of the mRNA (Step 4), as has been demonstrated for the cytoplasmic decay of nonsense-containing β -globin mRNA in erythroid cells (Lim et al., 1992; Lim & Maquat, 1992). Notably, translation elongation is not required for decay (Zhang & Maquat, 1997).

diverse as fungi, plants, insects, and mammals revealed that the normal termination codon within mRNAs from all but two (i.e., 98%) of the 101 genes found to have one or more 3' untranslated exons resides less than 50 nt upstream of the 3'-most exon-exon junction (Nagy & Maquat, 1998).

In the absence of β -globin intron 2, another *cis*-acting sequence functions comparably to intron 2 in the nonsense-mediated reduction of β -globin mRNA abundance, and this sequence is exonic

Although the position of β -globin intron 2 normally appears to determine which nonsense codons mediate a reduction in mRNA abundance, deletion of intron 2 reveals that another sequence functions comparably to the intron in the absence of the intron (Fig. 5). Similarly, deletion of the 3'-most intron of the human TPI gene uncovered the presence of a sequence that functions comparably to the 3'-most intron in the nonsense-mediated decay of TPI mRNA (Cheng et al., 1994). Data indicating that deletion of the splice sites of the 3'-most intron of the TCR- β gene results in less than

complete abrogation of the reduction in mRNA abundance brought about by a nonsense codon within the penultimate exon (Carter et al., 1996) suggest there may be an analogous situation for this gene. Conceivably, sequences that functionally substitute for the 3'-most intron in nonsense-mediated decay may exist as a fail-safe mechanism, possibly in case removal of the intron during splicing fails to result in association of the spliced product with the appropriate mark.

The efficiency with which the other sequence functionally substitutes for β -globin intron 2 when the intron is deleted offers a unique opportunity to map the sequence. Results of analyzing a series of deletions located downstream of codons 60–61, the 3'-most nonsense codon shown here to effectively reduce mRNA abundance in the absence of intron 2, revealed that the sequence does not reside within the 3' untranslated region (Fig. 6). Less clear was a delineation of where the sequence does reside, primarily because deletions on the order of 50–100 bp within the translated portion of the gene more often than not eliminated the production of detectable mRNA. Data indicate that some of the sequence functioning on behalf of intron 2 resides within the region spanning codons 99–

137, which includes the last 14 bp of exon 2 through the first 100 bp of exon 3, and within the region spanning codons 62–104, which includes the last 129 bp of exon 2. Analysis of smaller deletions and substitutions will be required to map the sequence more definitively.

Comparisons of the *cis*-acting sequences required for nonsense-mediated decay in mammals and *S. cerevisiae*

Comparing the *cis*-acting sequences that function in nonsense-mediated mRNA decay in mammals and *S. cerevisiae* (reviewed in Caponigro & Parker, 1996; Jacobson & Peltz, 1996; Ruiz-Echevarria & Peltz, 1996) reveals analogies and differences that are worth pointing out in the context of the studies reported here. Somewhat analogous to the situation in mammals, decay in *S. cerevisiae* involves a destabilizing element (Peltz et al., 1993; Zhang et al., 1995; Ruiz-Echevarria & Peltz, 1996). However, the element does not coincide with the 3'-most exon–exon junction but, rather, coincides with a particular sequence of the mRNA coding region (Zhang et al., 1995), as would be expected for an organism in which most mRNAs derive from intronless genes. This element may be comparable to the exonic sequence that functions within β -globin mRNA when intron 2 has been deleted from the β -globin gene. However, a search for sequence similarities between β -globin RNA and the roughly defined element of *S. cerevisiae* revealed nothing of significance. The yeast destabilizing element does not function if located more than 200 nt downstream of the nonsense codon (Ruiz-Echevarria et al., 1998). In the case of mammals, data suggest that exon–exon junctions in addition to the 3'-most exon–exon junction function in nonsense-mediated decay (Zhang et al., 1998). If Model 1 is valid, then a comparable mark at more than the 3'-most junction would not be problematic because the boundary would still be determined by the 3'-most junction. If Model 2 is valid, which studies of the TPI gene suggest (Zhang et al., 1998), then a mark at multiple exon–exon junctions would indicate that nonsense codons located less than 50–55 nt upstream of the 3'-most exon–exon junction fail to elicit decay because scanning begins downstream of the junction. Although a 5'-most exon can be more than 1 kbp, the coding part of a 5'-most exon is, on average, about the same size as an internal exon (Hawkins, 1988). Therefore, if every exon–exon junction functions as a destabilizing element, the maximum distance between a nonsense codon and a functional destabilizing element would generally be less than 300 nt, the size limit of an internal exon (Hawkins, 1988). In contrast, if only the 3'-most exon–exon junction functions as a destabilizing element, the maximum distance between a nonsense codon and a destabilizing element would be the distance within mRNA between codon 1 (Zhang & Maquat, 1997) and the 3'-most exon–

exon junction. This distance would vary considerably between different mRNAs.

Nonsense-mediated decay in *S. cerevisiae* is inactivated by one of two types of stabilizer elements (Peltz et al., 1993; Hagan et al., 1995; Ruiz-Echevarria et al., 1998). For one type, once the element is translated by ribosomes, nonsense codons downstream are incapable of eliciting decay. For the second type, location between a nonsense codon and the destabilizing element confers immunity to nonsense-mediated decay. No comparable stabilizers exist within TPI mRNA or β -globin mRNA, because a nonsense codon can be converted from the type that has no effect on mRNA abundance to the type that reduces mRNA abundance with either (1) a sufficiently large insertion between the nonsense codon and the 3'-most intron or (2) movement of the 3'-most intron a sufficient distance downstream of the nonsense codon (Cheng et al., 1994; Zhang et al., 1998; Figs. 3, 4).

Future studies of mammalian cells and *S. cerevisiae* that aim to define the *cis*-acting sequences, associated mRNP proteins, and *trans*-acting factors that function in nonsense-mediated decay are required in order to understand more completely similarities and differences between the decay mechanisms of higher and lower eukaryotes.

MATERIALS AND METHODS

DNA mutagenesis and plasmid constructions

All PCR products and mutagenized fragments were characterized by DNA sequencing prior to expression in mammalian cells.

Introducing frameshift and nonsense mutations into the β -globin gene

pMT-GI and pmCMV-GI constructs consist of a hybrid β -globin gene in which sequences upstream of the *Bam*H I site in exon 2 derive from the human β -globin gene and sequences downstream of the *Bam*H I site derive from the mouse β^{maj} -globin gene. pmCMV-GI derivatives harboring a 2-bp deletion within codon 8 (Δ AA on the sense strand) that generates a nonsense codon spanning codons 21–22, a nonsense mutation (CAG $\rightarrow$ TAG on the sense strand) within codon 39, or a 1-bp deletion within codon 44 (Δ C on the sense strand) that generates a nonsense codon spanning codons 60–61 were constructed by substituting the 429-bp *Nco* I–*Bam*H I fragment of pMT-GI^{Norm} that extends from exon 1 into exon 2 with the corresponding fragment from a β° -thalassemic β -globin allele of, respectively, Turkish, Italian, or Kurdish origin (Lim et al., 1989). To this end, the appropriate 429-bp fragment was ligated to two fragments of pMT-GI^{Norm}: the 1.52-kbp *Bam*H I–*Xba* I fragment that extends from β -globin exon 2 into 3'-flanking DNA, and the 3.57-kbp *Xba* I–*Nco* I fragment that includes 3'-flanking DNA, vector sequences, the MT promoter, and part of β -globin exon 1. A pMT-GI derivative har-

boring 101Ter due to a T/A → G/C transversion was generated by subcloning into pBluescriptKS[−] the 613-bp *Bam*H I–*Pst* I fragment that extends from exon 2 into intron 2. 101Ter was introduced with the antisense oligonucleotide 5′-CACCTG AAGTTCTAAGGAT-3′, in which nucleotides corresponding to the mutagenized codon are underlined, and the mutagenic nucleotide is italicized. pMT-GI^{101Ter} was subsequently constructed by ligating the mutagenized 613-bp fragment to two fragments from pMT-GI^{Norm}: the 429-bp *Nco* I–*Bam*H I fragment, and the 4.46-kbp *Pst* I–*Nco* I fragment that extends from β-globin intron 2 into 3′-flanking DNA, vector sequences, the MT promoter, and exon 1. A pMT-GI derivative harboring 141Ter due to an A/T → T/A transversion was generated by subcloning into pBluescriptKS⁺ the 906-bp *Pst* I–*Xba* I fragment that extends from intron 2 into 3′-flanking DNA. 141Ter was introduced by with the antisense oligonucleotide 5′-GGTACTTGTGAGCCTAGGCA-3′. pMT-GI^{141Ter} was subsequently constructed by ligating the mutagenized 906-bp *Pst* I–*Xba* I fragment to the 3.57-kbp *Xba* I–*Nco* I fragment and the 1.03-kbp *Nco* I–*Pst* I fragment, each of which derive from pMT-GI^{Norm}. pmCMV-GI derivatives harboring each of the frameshift or nonsense mutations were then generated by replacing the 1.9-kbp *Nco* I–*Xba* I fragment of pmCMV-GI^{Norm} with the corresponding fragment from each pMT-GI construct.

Placing insertions between β-globin codon 101 and intron 2

The 288-bp *Bam*H I–*Eco*R V fragment that extends from β-globin exon 2 into intron 2 and either does or does not harbor 101Ter was subcloned into pBluescriptKS⁺. An *Eco*R I site was then introduced into codons 102–103 with the sense oligonucleotide 5′-AGAAATCCAGGGTGAGTC-3′. Restriction fragments of 41 bp (5′-CTCTTTCTTGTTCACCCCTCCCTCCATCTGTATCTCTGCCC-3′), which is the Klenow-treated *Ecl*136 II–*Pst* I fragment from the TPI gene of pMT-TPI, and 71 bp (5′-CATGGCGCCCTCCAGGAAGTTCTTCGTTGGGGAAACTGGAAGGTTAACGGGCGGAAGCAGAGTCTGGGG-3′), which is the Klenow-treated *Nco* I–*Sac* I fragment from the TPI gene of pMT-TPI^{14Met→Val}, were inserted into the Klenow-filled *Eco*R I site so that translation terminates at the normal position in the case of the nonsense-free construct

Moving globin intron 2 to positions downstream of its normal position

The 868-bp *Bam*H I–*Xba* I fragments from pmCMV-GI^{Norm,Δ(intron2)} and pmCMV-GI^{141Ter,Δ(intron2)}, which extend from exon 2 into 3′-flanking sequences, were inserted into the *Bam*H I and *Xba* I sites of pGEM-7Zf(+). Prior to insertion, the unique *Ecl*136 II site of the plasmid was destroyed by Klenow-chewing back the ends generated by *Sac* I, an isoschizomer of *Ecl*136 II. 101Ter was introduced into the nonsense-free pGEM construct with the antisense mutagenic oligonucleotide 5′-GAAGTTCTAAGGATCC-3′. An *Ecl*136 II site was subsequently introduced into each of the three pGEM constructs into one of two places: codons 107–108 with the antisense oligonucleotide 5′-GATCATAGAGCTCAGGAG-3′ or codons 121–122 with the antisense oligonucleotide 5′-CG

GGGGTGAGCTCCTTGCCAAG-3′. PCR-produced β-globin intron 2 was generated with the sense oligonucleotide 5′-TGAGAACTTCCACGTGAGTCTGT-3′ and the antisense oligonucleotide 5′-TTGCCAGCAGCTGTGGGAAT-3′. After inserting the intron into each of the *Ecl*136 II sites, derivatives of pmCMV-GI were constructed by substituting the 868-bp *Bam*H I–*Xba* I fragment of pmCMV-GI^{Norm,Δ(intron2)} with the corresponding intron 2-containing fragment.

Deleting β-globin intron 2

Intron 2 was effectively deleted from pmCMV-GI^{Norm}, pmCMV-GI^{21–22Ter}, pmCMV-GI^{39Ter}, and pCMV-GI^{60–61Ter} by ligating the 429-bp *Nco* I–*Bam*H I fragment that harbors the respective mutation to two fragments from pmCMV-GI^{Norm}: the 120-bp *Bam*H I–*Msc* I fragment that extends from β-globin exon 2 into exon 3, and the 4.43-kbp *Msc* I–*Nco* I fragment that includes exon 3, 3′-flanking DNA, vector sequences, the mCMV promoter, and part of β-globin exon 1. Intron 2 was effectively deleted from pmCMV-GI^{141Ter} by substituting the 752-bp *Msc* I–*Xba* I fragment from pmCMV-GI^{Norm,Δ(intron2)} that extends from exon 3 into 3′-flanking DNA with the corresponding fragment from pMT-GI^{141Ter}. To construct pmCMV-GI^{101Ter,Δ(intron2)}, the 868-bp *Bam*H I–*Xba* I fragment from pmCMV-GI^{Norm,Δ(intron2)} that extends from β-globin exon 2 into 3′-flanking DNA was subcloned into pGEM-7Zf(+). Codon 101 was mutagenized with the antisense oligonucleotide 5′-GAAGTTCTAAGGATCC-3′. pmCMV-GI^{101Ter,Δ(intron2)} was subsequently constructed by ligating the mutagenized 868-bp fragment to the 3.65-kbp *Xba* I–*Bam*H I fragment that extends from 3′-flanking DNA to the mCMV promoter and the 1.0-kbp *Bam*H I–*Bam*H I fragment that extends from the mCMV promoter into β-globin exon 2, each of which derive from pmCMV-GI^{Norm}.

Deleting exon sequences from the β-globin gene and reinserting β-globin intron 2

To delete the last 14 bp of exon 2 and the first 100 bp of exon 3 (i.e., codon 99 through the first base pair of codon 138; Δ#1) within pmCMV-GI^{Norm,Δ(intron2)}, pmCMV-GI^{39Ter,Δ(intron2)}, and pmCMV-GI^{60–61Ter,Δ(intron2)}, the *Bam*H I site within exon 2 was Klenow-filled and ligated to the *Msc* I site within exon 3. Ligation regenerates the *Bam*H I site, and PCR-generated β-globin intron 2 was inserted into this site of the plasmids. In order to replace bp +2 through +53 and bp +54 through +105 of the 3′ untranslated region with an *Hpa* I site (Δ#2 and Δ#3, respectively, where +1 is the base pair residing immediately downstream of the termination codon, and +106 is the base pair residing immediately upstream of the AATAAA polyadenylation sequences), the 868-bp *Bam*H I–*Xba* I fragment from pmCMV-GI^{Norm,Δ(intron2)} was subcloned into pGEM-7Zf(+). Mutagenesis was accomplished with the antisense oligonucleotide 5′-CTGACAGATAGTTAACΔTTAGTGGTAC-3′ (Δ#2) or 5′-TGCTTTTATTAGTTAACΔGCTCTCTTGGG-3′ (Δ#3), for each of which underlined nucleotides create an *Hpa* I site and bold nucleotides specify either the translation termination codon or the polyadenylation sequence. pmCMV-GI alleles harboring each deletion and either Norm, 39Ter, or 60–61Ter were constructed using the mutagenized fragments. To delete codons 62–104 of exon 2 (i.e., the last

129 bp of exon 2; Δ #4) or codons 105–146 of exon 3 (i.e., the first 129 bp of exon 3; Δ #5), the 1.06-kbp *Acc*I–*Xba*I fragment from pmCMV-GI^{Norm} Δ (intron2), pmCMV-GI^{39Ter} Δ (intron2), and pmCMV-GI^{60–61Ter} Δ (intron2) that extends from exon 2 into 3'-flanking DNA was inserted into the *Acc*I and *Xba*I site of pBluescript-KS⁺. Mutagenesis was accomplished with the sense oligonucleotide 5'-CCTAAGGTGAAG Δ CTCCTGGGC AATA-3' for Δ #4 or 5'-GAGAACTTCAGG Δ TAAACCCCTTTC-3' for Δ #5. pmCMV-GI alleles harboring each deletion and either Norm, 39Ter, or 60–61Ter were constructed using the mutagenized *Acc*I–*Xba*I fragments.

Cell culture, cell transfection, and RNA purification

Mouse NIH3T3 cells were propagated in minimal essential medium containing 10% fetal calf serum and 5% bovine calf serum and transiently transfected using calcium phosphate (Zhang et al., 1998). Nuclear and cytoplasmic RNAs were purified using citric acid (Appelquist et al., 1997) and cesium chloride centrifugation (Cheng & Maquat, 1993), and total RNA was purified using Trizol following manufacturer specifications (Gibco BRL).

RNA blotting

Total, nuclear, or cytoplasmic RNA (25 μ g) was denatured, electrophoresed in a 1.5% agarose gel, and transferred to a nylon membrane (Zeta-bind). Blot hybridization (Cheng & Maquat, 1993) was performed with two DNA fragments that had been ³²P-labeled by random priming. mCMV-TPI RNA was detected using a 299-bp *Nco*I–*Nde*I fragment that derives from the 3' untranslated region of human TPI cDNA. mCMV-GI RNA was detected using a 170-bp *Bal*I–*Dra*I fragment that includes 158 bp of exon 3 and 3' flanking sequences from the mouse β -globin gene. Hybridization and wash conditions allowed for the detection of human but not mouse TPI RNA.

RT-PCR analysis

To quantitate the levels of each intron within mCMV-GI pre-mRNA, 10 μ g of nuclear RNA from NIH3T3 cells that had been transfected with a pmCMV-GI test plasmid and the pmCMV-TPI reference plasmid were treated with 1 U of RNase-free RQ DNase I (Promega). cDNA was then synthesized from 0.22–6.0 μ g of the RNA using Moloney murine leukemia virus reverse transcriptase (Superscript; BRL). cDNA harboring either β -globin intron 1 or β -globin intron 2 and, as a control, cDNA harboring TPI intron 5 were amplified using PCR as described previously (Belgrader et al., 1994; Zhang & Maquat, 1996). Primers for the amplification of mCMV-GI cDNA were 5'-GCCTATTGGTCTATTTTCCC-3' (intron 1, sense) and 5'-CCTGAAGTTCTCAGGATC-3' (exon 2, antisense) or 5'-TTATGCCAGGGTGACAGGG-3' (intron 2, sense) and 5'-GAAGACAGATTTTCAAATGT-3' (exon 3, antisense). Primers for the amplification of mCMV-TPI cDNA were 5'-AGACTGCAACACCCCAAC-3' (intron 5, sense) and 5'-AGC CACCGCATCAGAGACGTTGGACTTCAGCCA-3' (exon 6,

antisense). One-tenth of each PCR reaction was electrophoresed in a 5% polyacrylamide gel, and RT-PCR products were quantitated by PhosphorImaging.

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