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Translation to Near the Distal End of the Penultimate Exon Is Required for Normal Levels of Spliced Triosephosphate Isomerase mRNA

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The translation of human triosephosphate isomerase (TPI) mRNA normally terminates at codon 249 within exon 7, the final exon. Frameshift and nonsense mutations within the TPI gene that cause translation to terminate prematurely at or upstream of codon 189, within exon 6, result in a decreased level of TPI mRNA (I. O. Daar and L. E. Maquat, Mol. Cell. Biol. 8:802–813, 1988). For all mutations in this group, the decrease is to the same extent, i.e., to approximately 20% of the normal level. We show here that a second group of nonsense mutations that cause translation to terminate prematurely at or downstream of codon 208, in exon 6, did not affect TPI mRNA abundance. Deletion analysis demonstrated that the abundance of translationally active TPI mRNA is a function of both the distance and the polarity of the nonsense codon relative to the final intron in TPI pre-mRNA. Our results indicate that if translating ribosomes are unable to progress to at least a certain position within the penultimate exon relative to the final intron, then the level of the corresponding mRNA will be abnormally low. Studies inhibiting RNA synthesis with dactinomycin demonstrated that a block in translation does not affect the half-life of mature TPI mRNA. The simplest interpretation of our data is that the translation of TPI mRNA in the cytoplasm facilitates the splicing of TPI pre-mRNA or the transport of TPI mRNA across the nuclear envelope or both.

In animal cells, mRNA abundance is determined by complex nuclear and cytoplasmic processes that include transcription initiation, intron removal, transport from the nucleus to the cytoplasm, and degradation in the cytoplasm. Since mRNA functions primarily to direct the synthesis of protein, it is conceivable that the process of translation could also regulate mRNA abundance. Evidence that this is, in fact, the case exists. As an example, the premature termination of mRNA translation that results from either a nonsense or a frameshift mutation often results in a reduced mRNA level. This has been demonstrated for murine immunoglobulin μ mRNA (3), human β-globin mRNA (1, 2, 16, 18, 20, 23, 25, 29, 31), adenovirus E1A mRNA (13), hamster dihydrofolate reductase (DHFR) mRNA (32), and triosephosphate isomerase (TPI) mRNA (10). For TPI mRNA, in which translation normally terminates at position 249 within exon 7 (the final exon), nonsense codons at position 23 in exon 1, position 69 to 70 in exon 2, position 70 to 71 in exon 2, or position 189 in exon 6 reduce the TPI mRNA level to the same extent, i.e., to 20% of the normal level. One possible explanation for this finding is that sequences between codon 189 (the 3′-most nonsense codon tested) and the normal stop codon at position 249 need to be translated in order to prevent a decrease in the mRNA level. To test this possibility, nonsense codons that reside between positions 189 and 249 were generated and analyzed. Also, deletions were introduced to remove part of exon 6, part of exon 7, or the entire final intron. We conclude from these experiments that nonsense codons in the final exon and, in certain cases, the penultimate exon do not affect the level of TPI mRNA. Therefore, only nonsense codons that reside upstream of the final intron reduce TPI mRNA abundance. We also conclude that this reduction is dependent upon a ribosome-free region between the nonsense codon and the final intron. Our data indicate that nonsense codons affect TPI mRNA abundance as a function of their distance upstream of the final intron in TPI pre-mRNA. The effect appears to be limited to newly synthesized RNA, since studies using dactinomycin indicate that the rate of TPI mRNA decay is not altered.

MATERIALS AND METHODS

DNA-mediated cell transfections and RNA extractions. Murine L tk$^-$ cells were transfected by using DEAE-dextran as described previously (10), except that modified Eagle medium containing 10% fetal calf serum and 5% Nu-serum were substituted for Dulbecco modified Eagle medium containing 15% fetal calf serum. The transfecting DNAs consisted of a test plasmid and a reference plasmid. In some experiments, both plasmids were derivatives of pMT-TPI in which the murine MT-I promoter, including 9 base pairs (bp) of the adjacent 5′ untranslated region, was inserted 1 bp upstream of the transcription initiation site of the complete TPI gene (10). In these experiments, L cells were transfected with an equal molar amount of each plasmid (a total of 20 μg/150-cm^2 dish). In other experiments, the test plasmid was derived from pMT-TPI, and the reference plasmid, pMT-hybrid Gl, expressed an MT hybrid β-globin mRNA. pMT-hybrid G1 was constructed by replacing the *Nco*I-*Eco*RI fragment of pMT-TPI^{norm}, extending from the TPI gene translation initiation codon to 1.1 kbp 3′ to the gene, with the *Nco*I-*Hpa*I fragment of a hybrid human-murine β-globin gene (22), extending from the human β-globin gene translation initiation codon to 779 bp 3′ to the murine β-globin gene. L cells were transfected with a 1.4-fold molar excess of pMT-hybrid Gl relative to pMT-TPI (a total of 20 μg/150-cm^2 dish). Prior to transfection, the concentration of all plasmid DNAs was initially estimated spectrophotometrically and then adjusted by the degree of ethidium bromide staining after linearization

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and agarose gel electrophoresis. Total, nuclear, and cytoplasmic RNAs were isolated as published elsewhere (10). To measure the stability of TPI mRNA, dactinomycin (5 μg/ml; 32) was added to L cells 36 h after transfection, and cells were harvested after additional growth periods of 0, 3, 6, 8, 10, 12, and 14 h.

DNA mutagenesis. The 660-bp *Pst*I-*Eco*RI fragment that includes sequences in exons 5 through 7 of the TPI gene was isolated from either pMT-TPI^{norm} or pMT-TPI$^{189\ Arg\rightarrow Ter}$ and subcloned into M13mp9. Substitution or deletion mutations were introduced by the method of Kunkel et al. (19). Nonsense codons at positions 189, 208, 223, and 237 were introduced in the normal subclone by using as a single primer the oligonucleotides 5′ CCATCCTCAGAGCTTCTCG 3′, 5′ GCCTCCTTAAATGATACG 3′, 5′ TCAGGCTAGCTGGC CAGC 3′, and 5′ ATTCGGGCTAGGGAAG 3′, respectively. A 54-bp deletion of codons 190 through 207 was generated in the normal subclone by using the oligonucleotide 5′ CCACTCACCTCCATA(Δ54)TCGGAGCTTCTCG TG 3′ and in the 189 Arg→Ter subclone by using the oligonucleotide 5′ CCACTCACCTCCATA(Δ54)TCAGAG CTTCTCGTG 3′. All mutagenized fragments were sequenced in their entirety before being used to reconstruct the pMT-TPI plasmids (10). A 54-bp deletion of codons 221 through 239 was constructed by ligating the *Bal*I and *Eco*RI sites in exon 7 after treatment with Klenow fragment and dNTPs. In so doing, the *Eco*RI site was regenerated.

To generate pMT-TPI plasmids with intron 6 deleted, the 105-bp *Hgi*AI-*Eco*RI fragment that includes sequences in exons 6 through 7 was isolated from TPI cDNA in pHTPI 5A (22) and used to substitute for the analogous intron 6-containing fragment of pMT-TPI^{norm}, pMT-TPI$^{23\ Glu\rightarrow Ter}$, and pMT-TPI$^{189\ Arg\rightarrow Ter}$.

RNA blotting. Total RNA was denatured with glyoxal, electrophoresed in an agarose gel (24), and transferred to a nylon membrane (Zeta-bind). The transferred RNA was first visualized by short UV irradiation to assess the integrity of 28S and 18S rRNAs and was subsequently hybridized (7) simultaneously to two DNAs that had been ^{32}P labeled by random priming (11). One of these DNAs was a 300-bp *Nde*I-*Nco*I fragment that was derived from the 3′ untranslated region of human TPI cDNA. By hybridizing at 48°C and washing at 68°C, this fragment did not cross-react with murine TPI mRNA. The other DNA was a 170-bp *Bal*I-*Dra*I fragment from the murine β^{major}-globin gene that consisted of 158 bp of exon III plus 3′-flanking sequences.

Dideoxynucleotide primer extension analysis of TPI RNA. A 33-deoxynucleotide primer with complementarity to human TPI mRNA at a position in exon 6 that resides downstream of codon 189 was used in the extension analysis as described previously (10). Briefly, 80,000 cpm of 5′ ^{32}P-labeled primer and either 120 μg or, in the experiment using dactinomycin, 90 μg of total cellular RNA were suspended in 40 μl of 40% deionized formamide–12 mM Tris hydrochloride (pH 7.0)–0.56 M NaCl and denatured at 70°C for 10 min. The temperature was then gradually dropped to 24°C. The hybrids were ethanol precipitated and suspended in 50 μl of 50 mM Tris hydrochloride (pH 8.1)–2 mM dithiothreitol–5 mM $MgCl_2$–40 mM KCl. dGTP, dCTP, dTTP, and ddATP were each added to final concentrations of 0.4 mM, and the hybridized primer was extended with avian myeloblastosis virus reverse transcriptase at 42°C for 90 min. NaOH was added to 0.2 M, and the incubation was continued for 2 h. Samples were neutralized, precipitated, denatured, electrophoresed in 20% acrylamide–7 M urea sequencing gels, and analyzed by autoradiography.

RESULTS

Nonsense codons downstream of and including position 208 do not affect the level of MT-TPI RNA. In previous experiments (10), the effect of in vitro-generated TPI gene mutations on TPI RNA metabolism was analyzed by replacing the promoter of each mutant allele with the relatively strong promoter of the murine MT-I gene (see Fig. 1). Each hybrid (test) allele was then transiently expressed in L cells together with an equal number of molecules of another hybrid (reference) MT-TPI allele. The reference allele served as a standard for variations in cell transfection efficiency or RNA recovery or both and differed from the test allele within codon 189. The relative levels of the resulting test and reference hybrid transcripts were analyzed by a dideoxynucleotide primer extension assay that distinguishes transcripts on the basis of the sequence divergence within codon 189. This assay was chosen because it does not detect L-cell transcripts and it provides a very sensitive quantitation of the various test and reference RNAs. By this assay, it was demonstrated that the premature termination of translation upstream of and including codon 189 (the distalmost codon tested) reduced the steady-state level of MT-TPI mRNA to approximately 20% of the level of MT-TPI mRNA that terminated translation normally at codon 249. Aberrant transcription or splicing were ruled out as possible causes for this reduction since (i) the reduction was independent of the promoter (human TPI, murine MT-I, or human cytomegalovirus major immediate early) that was used to drive transcription of the various TPI alleles, (ii) none of the mutations created or destroyed sequences that are essential for transcription (5) or splicing, (iii) the mutant MT-TPI mRNAs were the proper size (see below), and (iv) the reduction was ameliorated by second-site frameshift or substitution mutations that restored the proper translational reading frame and permitted translation termination at the normal site. From these results, it was concluded that nonsense codons upstream of and including position 189 reduce TPI mRNA abundance to the same extent.

Since the effect of all nonsense codons in this class was quantitatively the same and since a common feature of all mRNAs that harbored these nonsense codons was a ribosome-free region downstream of position 189, we wanted to test the effect of nonsense codons that resided between position 189 and the normal translation termination site at position 249. Accordingly, nonsense mutations within the MT-TPI gene were generated by oligonucleotide-directed site-specific mutagenesis at codons 208, 223, and 237 (Fig. 1). Codon 208, like codon 189, resides within the penultimate exon (exon 6), whereas codons 223 and 237, like the normal site of translation termination, reside within the last exon (exon 7). The nonsense codons were referred to, respectively, as 208 Tyr→Ter, 223 Glu→Ter, and 237 Lys→Ter; the corresponding hybrid alleles that harbored these codons were called MT-TPI$^{208\ Tyr\rightarrow Ter}$, MT-TPI$^{223\ Glu\rightarrow Ter}$, and MT-TPI$^{237\ Lys\rightarrow Ter}$.

As demonstrated previously by using the primer extension assay, the steady-state level of transcripts synthesized from MT-TPI^{null} (a hybrid allele that was constructed with the TPI allele of a TPI-deficient patient that harbors a nonsense codon at position 189 [189 Arg→Ter]) was 24% of the steady-state level of transcripts synthesized from MT-TPI^{norm} (a hybrid allele that was constructed with the TPI allele of a normal individual) (10; Fig. 2A, lanes 1 and 5; Table 1). By contrast, the steady-state level of transcripts synthesized from MT-TPI$^{208\ Tyr\rightarrow Ter}$, MT-TPI$^{223\ Glu\rightarrow Ter}$, and MT-

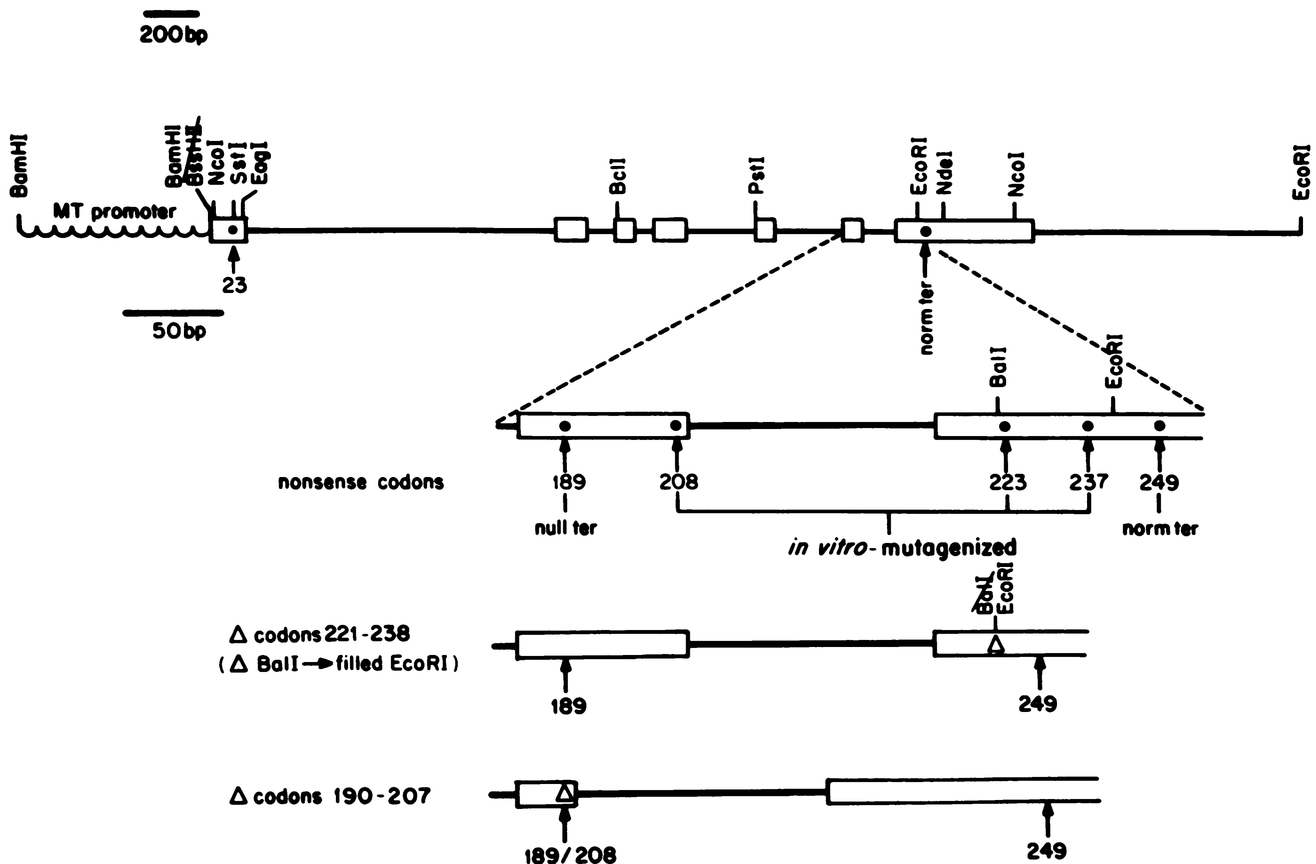

FIG. 1. Structures of the MT promoter-TPI alleles. The wavy line represents a 750-bp *Bam*HI fragment that harbors the murine MT promoter. Boxes and interspersed lines designate, respectively, the exons and introns of the human TPI gene. The horizontal line to the right of and contiguous with exon 7 represents 1.1 kbp of human DNA that resides downstream of the TPI gene. null ter and norm ter refer, respectively, to the premature termination codon (TGA) of the MT-TPI$^{189\ Arg \rightarrow Ter}$ allele and the normal termination codon (TGA) of the MT-TPI^{norm} allele, and 23 specifies the premature termination codon (TAG) of the MT-TPI$^{23\ Glu \rightarrow Ter}$ allele. The nonsense codons at positions 208 (TAA), 223 (TAG), and 237 (TAG) were generated in vitro by oligonucleotide-directed mutagenesis. Δ specifies deleted regions of the TPI gene. In deleted codons 221 to 238, the first base pair of codon 221 was ligated to the second base pair of codon 239 to delete precisely codons 221 through 238 and, in so doing, regenerate the *Eco*RI site. The deletion of codons 190 to 207 was generated with a synthetic oligonucleotide that had sequences to either side of the deleted region. A diagonal line through a restriction enzyme site indicates the loss of that site in the cloning procedure.

TPI$^{237\ Lys \rightarrow Ter}$ were normal, i.e., approximately fivefold that of MT-TPI^{null} (Fig. 2A, lanes 2, 3, and 4; Table 1). This finding was corroborated for MT-TPI$^{208\ Tyr \rightarrow Ter}$ and MT-TPI$^{223\ Lys \rightarrow Ter}$ by demonstrating that the level of transcripts from each of these alleles was comparable to that of MT-TPI$^{189\ Ter \rightarrow Leu}$ (Fig. 2A, lanes 7 and 8; Table 1), an allele that produces normal levels of hybrid mRNA (10; Fig. 2A, lane 6; Table 1). In MT-TPI$^{189\ Ter \rightarrow Leu}$, the TGA null codon was changed to a TTA leucine codon (Fig. 2B), thereby maintaining the same nucleotide that created the nonsense codon but restoring translation termination to the usual site. These data indicate that nonsense codons at and downstream of position 208 do not affect the level of MT-TPI RNA.

In the process of translation, readthrough of codons 189 to 208 prevents a decrease in the level of MT-TPI mRNA. The steady-state of each of these hybrid transcripts was also assessed by blot hybridization to ensure that the primer extension assay was a reliable measure of full-length mRNA and that none of the hybrid alleles produced aberrantly structured RNAs. To this end, each hybrid allele was introduced into L cells together with an MT hybrid β-globin gene in the form of pMT-hybrid Gl. In these experiments, the MT-globin gene served as the reference allele to control for variations in transfection efficiency or RNA recovery or both. MT-TPI RNA was detected with a 300-bp uniformly labeled *Nde*I-*Nco*I fragment from the 3′ untranslated region of human TPI cDNA, and MT-globin RNA was detected with a uniformly labeled 170-bp *Bal*I-*Dra*I fragment that included 26 bp of the coding region plus the entire 3′ untranslated region. Hybridization conditions were selected so that the TPI cDNA fragment did not detect L-cell RNA (Fig. 3, lane 1).

When variations in transfection efficiency or RNA recovery or both were calculated, it was clear that the levels of the various MT-TPI RNAs as measured by blot hybridization were as measured previously by the primer extension assay (Fig. 3; data not shown for MT-TPI$^{189\ Ter \rightarrow Leu}$). Furthermore, only RNAs corresponding to full-length MT-TPI mRNA and full-length MT-globin mRNA were detected (Fig. 3, lanes 2 to 6; data not shown for a broader RNA size range), indicating that none of the MT-TPI alleles produced abnormally sized transcripts. Therefore, the primer extension and blotting data indicate that the premature termination of translation downstream of and including codon 208 does not affect MT-TPI RNA metabolism. From this finding plus the finding that the premature termination of translation

FIG. 2. Dideoxynucleotide primer extension analysis of MT-TPI RNA in murine L cells. (A) L cells were transfected with an equal molarity of two MT-TPI DNA constructs as specified in each lane. Total cellular RNA was purified, and MT-TPI RNA was quantitated by primer extension (10). nt, Nucleotide. (B) Nucleotides of a normal TPI allele and the corresponding amino acids (aa) in TPI protein. Arrows designate the substitutions within the MT-TPI^{null} and MT-TPI$^{189\ Ter\rightarrow Leu}$ alleles and the resulting encoded proteins. (In addition to the designated substitution, the MT-TPI^{null} and MT-TPI$^{189\ Ter\rightarrow Leu}$ alleles also harbor a single base pair substitution in both introns 1 and 5.) The expected dideoxynucleotide primer extension products for the transcripts of each allele are also shown. The primer region of each extension product is boxed.

upstream of and including codon 189 reduces the concentration of MT-TPI mRNA, we conclude that the failure to translate codons 189 to 208 decreases the yield of translationally active MT-TPI mRNA.

Nonsense codons upstream of and including position 189 do not reduce the amount of MT-TPI mRNA merely because they reside too far from the normal stop codon. A possible explanation for the deleterious effect of nonsense codons upstream of and including position 189 is that they, unlike nonsense codons downstream of and including position 208,

TABLE 1. Effects of mutations on the level of MT-TPI mRNA[a]

MT-TPI allele	% of MT-TPI^{norm} mRNA by:	
	Primer extension	Blotting
norm	100	100
null	24	
23 Glu→Ter	21	14
189 Arg→Ter	22	20
189 Ter→Leu	100	
208 Tyr→Ter	98	97
223 Glu→Ter	113	99
237 Lys→Ter	106	80
norm Δ(221-238)		98
null Δ(221-238)		25
intron 1, intron 5	100	
norm Δ(190-207)		86
189 $^{Arg→Ter}$ Δ(190-207)		90
23 $^{Glu→Ter}$ Δ(190-207)		19
norm Δ(intron 6)		58
189 $^{Arg→Ter}$ Δ(intron 6)		51
23 $^{Glu→Ter}$ Δ(intron 6)		12

[a] The level of RNA produced by each MT-TPI allele was quantitated by primer extension and/or Northern (RNA) blot analysis of total RNA from L cells that were transiently cotransfected with each allele plus a reference allele that served to control for variations in transfection efficiency and/or RNA recovery. All quantitations were derived from densitometric scanning of autoradiographs, as exemplified in Fig. 2 through 5, and are averages of at least two independent L-cell transfections. These quantitations did not deviate by more than 20% of the tabulated value.

reside sufficiently far from the normal stop codon at position 249 to trigger a decrease in the amount of mRNA when contacted by a translating ribosome. To test this possibility, codons 221 through 238 (54 bp) were deleted within MT-TPI^{norm} and MT-TPI^{null} (Fig. 1) so that the distance between codons 189 and 249 was reduced from 177 to 123 bp, very close to the 120-bp distance between codons 208 and 249. The corresponding alleles, designated MT-TPI^{norm} Δ(221-238) and MT-TPI^{null} Δ(221-238), were expressed in L cells together with the reference MT-hybrid β-globin gene. Blot hybridization of the resulting transcripts revealed that the level of MT-TPI^{norm} (Δ221-238) mRNA was comparable to the level of MT-TPI^{norm} mRNA (Fig. 4, lanes 2 and 4; Table 1). And the level of MT-TPI^{null} Δ(221-238) mRNA was comparable to the level of MT-TPI^{null} mRNA (Fig. 4, lanes 3 and 5; Table 1).

Therefore, reducing the distance between codons 189 and 249 to approximate the distance between codons 208 and 249 does not ameliorate the deleterious effect of 189 Arg→Ter on the level of MT-TPI mRNA. These results suggest that the specific translation of sequences residing between codons 189 and 208 is required in order for the translational process to maintain a normal level of MT-TPI mRNA.

Deletion of codons 190 through 207 ameliorates the mRNA reduction that is mediated by nonsense codon 189 but not nonsense codon 23. As reported previously, the null allele contains not only the nonsense codon at position 189 but also a G · C→A · T transition within intron 1 and an A · T→G · C transition within intron 5 (10). One or both of these intron transitions were shown to result in a transient twofold increase in the level of nuclear MT-TPI RNA that had no demonstrable effect on the level of total cellular or cytoplasmic MT-TPI RNA (10). In order to use the level of transcripts that harbored only the nonsense codon at position 189 as a reference, this codon was separated from the two intron substitutions and vice versa. MT-TPI$^{189\ Arg→Ter}$ refers to the hybrid allele that harbors solely the nonsense codon at position 189, and MT-TPI$^{intron\ 1,\ intron\ 5}$ refers to the hybrid allele that harbors only the two intron changes. Primer extension analysis revealed that the steady-state level of MT-TPI$^{189\ Arg→Ter}$ RNA was 22% of the normal level (Fig. 5, lanes 2 and 5), whereas the steady-state level of MT-TPI$^{intron\ 1,\ intron\ 5}$ was normal (Fig. 5, lanes 3 and 6; Table 1). Therefore, as expected, neither of the intron changes contributes to the reduced abundance of MT-TPI^{null} RNA, and MT-TPI$^{189\ Arg→Ter}$ can be used as a reference allele.

In order to begin to understand the mechanistic role of codons 189 through 208 in preventing a decrease in the mRNA level when translated, codons 190 through 207 (54 bp) were deleted within MT-TPI^{norm} and MT-TPI$^{189\ Arg→Ter}$ (Fig. 1). This deletion brought codon 189 closer to the splice donor site of the final intron so that it was the 3rd rather than the 22nd codon upstream of the splice donor site. This deletion also removed the ribosome-free region that is characteristic only of those mRNAs with nonsense codons that reduce mRNA abundance. Notably, it did not alter the distance between codon 189 and the translation initiation codon. The corresponding alleles, designated MT-TPI^{norm} Δ(190-207) and MT-TPI$^{189\ Arg→Ter}$ Δ(190-207), were expressed in L cells together with the MT-hybrid β-globin gene. Blot hybridization of the resulting transcripts revealed that the levels of MT-TPI^{norm} Δ(190-207) mRNA and MT-TPI$^{189\ Arg→Ter}$ Δ(190-207) mRNA were identical (Fig. 6A, lanes 1 and 2; Table 1). In parallel L-cell transfections, it was determined that the level of each of these deletion-bearing mRNAs was approximately 88% of the level of MT-TPI^{norm} mRNA (Fig. 6A, lanes 1, 4, and 5; Table 1) and, accordingly, was approximately fivefold higher than the level of MT-TPI$^{189\ Arg→Ter}$ mRNA (Fig. 6A, lane 5; Table 1). Therefore, the deletion ameliorates the deleterious effect of the nonsense codon at position 189. Notably, this is not due to the deletion resulting in exon 6 skipping, i.e., the splicing out of the 34 nucleotides that remain in exon 6, including the nonsense codon at position 189 (J. Cheng and L. E. Maquat, data not shown). Also, since deletion of the codons at positions 190 to 207 and 221 to 238 created 3′ untranslated regions of precisely the same size yet differed in their effects on nonsense codon 189, the nonsense codon-mediated reduction in mRNA abundance is not a function of the distance between the last translating ribosome and normal site of translation termination. Furthermore, since the distance between codon 189 and the translation initiation codon in MT-TPI$^{189\ Arg→Ter}$ mRNA and MT-TPI$^{189\ Arg→Ter}$ Δ(190-207) mRNA is identical, the reduction is not a function of the distance between the first and last translating ribosomes. Therefore, the reduction either is a function of the distance between the last translating ribosome and the last intron in MT-TPI pre-mRNA or requires the presence of a sequence that resides between codons 189 and 208 in MT-TPI mRNA.

In order to differentiate between these two possibilities, codons 190 through 207 were also deleted within MT-TPI$^{23\ Glu→Ter}$, a gene that harbors a nonsense codon at position 23 in exon 1 (Fig. 1) and produces a level of MT-TPI mRNA that is 21% of the level of MT-TPI^{norm} mRNA (10). Coexpression of MT-TPI$^{23\ Glu→Ter}$ Δ(190-207) with the MT-hybrid β-globin gene in L cells demonstrated that the deletion did not ameliorate the deleterious effect of nonsense codon 23 (Fig. 6A, lane 3). In similar experiments, the deletion also did not ameliorate the deleterious effect of either of two frameshift mutations that cause translation to terminate prematurely at different positions in exon 2 (10; J. Cheng and L. E. Maquat, unpublished data). We conclude from these results that deletion of the codon at positions 190

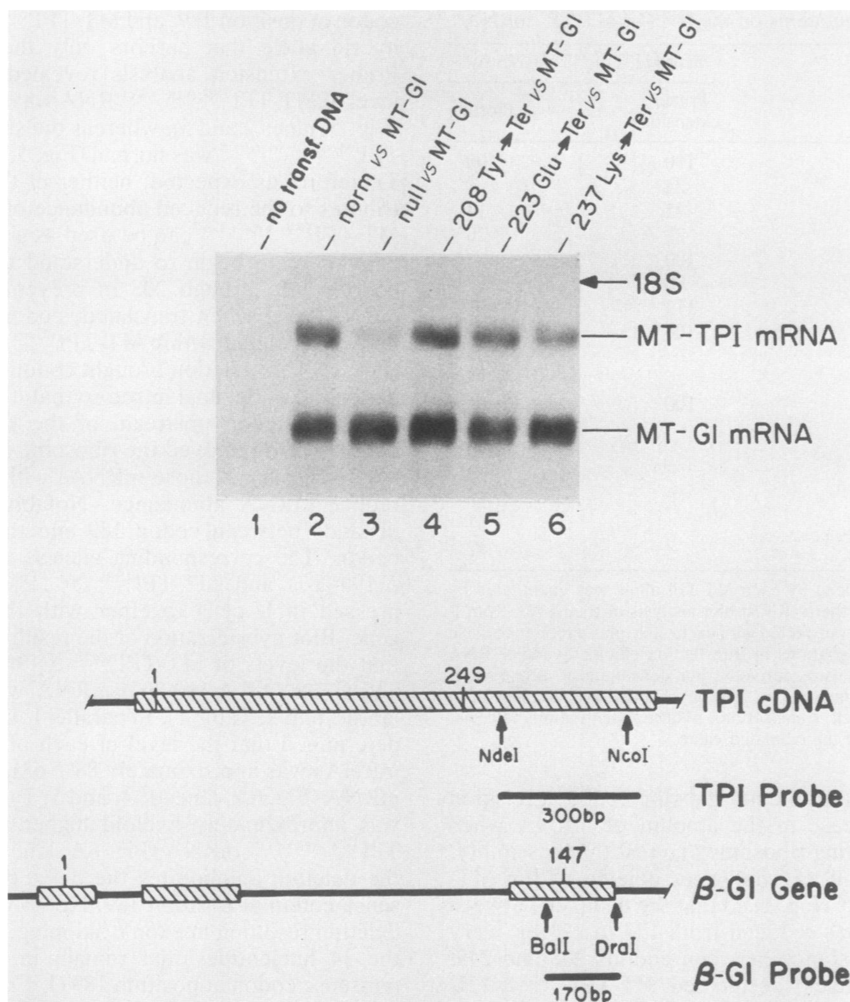

FIG. 3. RNA blot analysis of MT-TPI RNA in murine L cells. L cells were transfected with a pMT-TPI DNA and pMT-hybrid Gl DNA as specified in each lane. Total cellular RNA (25 μg) was denatured, electrophoresed in agarose, and transferred to a nylon membrane. Membrane-bound RNA was cohybridized to the ^{32}P-labeled 300-bp *Nde*I-*Nco*I fragment of TPI cDNA and the ^{32}P-labeled 170-bp *Bal*I-*Dra*I fragment of the murine β^{major}-globin gene. Under the chosen hybridization and wash conditions, the TPI cDNA fragment did not cross-react with murine TPI mRNA. 18S marks the position of 18S rRNA. Numbers 1 refer to the translation initiation codons, and numbers 249 and 147 refer to the translation termination codons with the human TPI and murine β^{major}-globin genes, respectively.

to 207 compensates for the mRNA reduction that is due to nonsense codon 189 by reducing the distance between this codon (and thus the last translating ribosome) and the final intron. By contrast, deletion of the codons at positions 190 to 207 cannot compensate for the mRNA reduction that is due to nonsense codons in exons 1 and 2 (and, presumably, exons 3 through 5) because of the presence of the one or more introns that reside downstream of the nonsense codon and upstream of the deletion.

Deletion of the final intron also ameliorates the mRNA reduction that is mediated by nonsense codon 189 but not nonsense codon 23. If the above conclusions are correct, then the deleterious effect of nonsense codon 189 but not nonsense codon 23 should be ameliorated by removal of the final intron (intron 6). Accordingly, intron 6 (126 bp) was deleted within MT-TPI^{norm}, MT-TPI$^{189\ Arg\rightarrow Ter}$, and MT-TPI$^{23\ Glu\rightarrow Ter}$. The corresponding alleles, designated MT-TPI^{norm} Δ(intron 6), MT-TPI$^{189\ Arg\rightarrow Ter}$ Δ(intron 6), and MT-TPI$^{23\ Glu\rightarrow Ter}$ Δ(intron 6), were expressed in L cells together with the MT-hybrid β-globin gene. Blot hybridization revealed that as predicted, the level of MT-TPI$^{189\ Arg\rightarrow Ter}$ Δ(intron 6) mRNA was equal to that of MT-TPI^{norm} Δ(intron 6) mRNA and approximately fivefold that of MT-TPI$^{23\ Glu\rightarrow Ter}$ Δ(intron 6) mRNA (Fig. 6B, lanes 4 to 6; Table 1). Interestingly, the level of each Δ(intron 6) construct was approximately 55% of the level of the intron-6-containing counterpart, suggesting that sequences within intron 6 contribute to efficient mRNA formation (Fig. 6B, compare lanes 1 and 4, 2 and 5, and 3 and 6; Table 1). This is in keeping with the concept that the final intron may be involved in efficient 3′ end formation and polyadenylation (9).

The stability of mature TPI mRNA is not reduced by nonsense codon 189. The data presented above suggest that nonsense codons reduce the level of TPI mRNA, depending upon their position relative to the final intron. This interpretation implies that translation and splicing are somehow linked and that a block in TPI mRNA translation affects the metabolism of newly synthesized RNA. To determine whether a block in mRNA translation affects the metabolism of steady-state RNA, the degradative rates of MT-TPI$^{189\ Arg\rightarrow Ter}$ mRNA and MT-TPI^{norm} mRNA were compared after inhibiting new RNA synthesis with dactinomy-

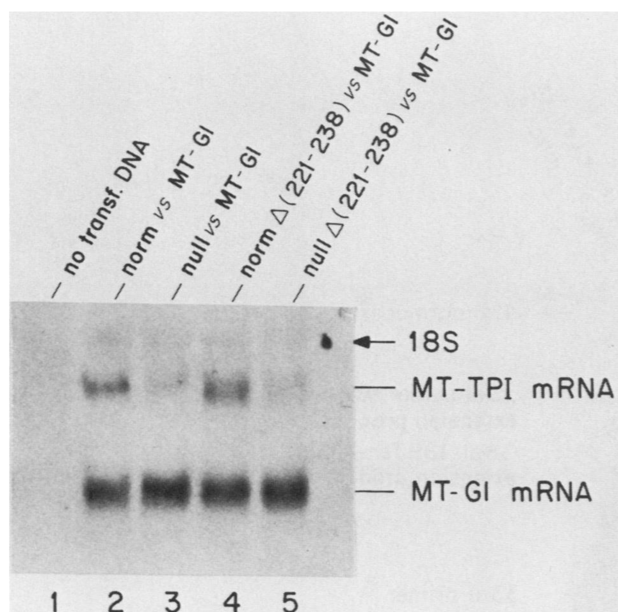

FIG. 4. RNA blot analysis of MT-TPI RNA in murine L cells. L cells were transfected with a pMT-TPI DNA and pMT-hybrid Gl DNA as specified in each lane. RNA was analyzed as described in the legend to Fig. 3.

cin. The corresponding alleles were introduced into L cells, and primer extension was used to quantitate the relative amounts of the product mRNAs at various times after the addition of dactinomycin. The ratio of the two mRNAs remained constant throughout a 14-h incubation with the inhibitor (Fig. 7). These results indicate that nonsense codon 189 does not increase the rate of TPI-mRNA degradation. Therefore, a block in TPI mRNA translation appears to affect only newly synthesized RNA. However, the interpretation of these data must be tempered by the possibility that the interruption of RNA synthesis by dactinomycin interferes with the process that destabilizes MT-TPI$^{189\ Arg \rightarrow Ter}$ mRNA and, possibly, MT-TPI^{norm} mRNA.

DISCUSSION

We have shown that the premature termination of MT-TPI mRNA translation does not always result in a reduction in the amount of MT-TPI mRNA. Therefore, depriving an mRNA segment of its usual complement of ribosomes often but not always results in a reduced mRNA level. This has also been shown for *Escherichia coli* β-lactamase mRNA (27), Chinese hamster ovary (CHO) DHFR mRNA (32), and a murine immunoglobulin heavy chain mRNA (3). Additionally, our data demonstrate that, at least for human TPI mRNA, ribosomes need not traverse the final exon but need only read into the penultimate exon in order for the normal level of template mRNA to be maintained. A similar finding has recently been described for CHO DHFR mRNA (6).

Our earlier investigations (10) demonstrated that the termination of translation at codon 23 in exon 1, codon 69 to 70 in exon 2, codon 70 to 71 in exon 2, or codon 189 in exon 6 reduces the steady-state level of MT-TPI mRNA to approximately 20% of the normal level. Here we show that the termination of translation at codon 208 in exon 6, the penultimate exon, or at codons 223 or 237 in exon 7, the final exon, does not affect the level of MT-TPI mRNA. We also show that deleting either codons 190 through 207 or intron 6 ameliorates the mRNA reduction that results from nonsense codon 189 but does not ameliorate the mRNA reduction that results from nonsense codon 23.

All of our findings support the translational translocation model that was initially proposed by Urlaub et al. (32). This model proposes that the translation of an RNA molecule facilitates the splicing and transport of that molecule. Therefore, when a ribosome stalls at a nonsense codon, the rates of splicing and transport are retarded, making the RNA more susceptible to nuclear degradation. This model is based primarily on studies of the CHO DHFR mRNA (32) which demonstrated that (i) nonsense codons in internal exons but not the last exon reduce the level of DHFR mRNA (nonsense codons in the first exon were not analyzed), (ii) decay rates are the same for wild-type and nonsense mutant DHFR mRNAs after the inhibition of RNA synthesis with dactinomycin, (iii) intronless minigene versions of the wild-type and nonsense mutant DHFR genes are expressed equally after stable transfection, and (iv) nonsense codons do not affect the rate of DHFR gene transcription initiation. This model is also consistent with the ideas of Humphries et al. (16) and Takeshita et al. (31) to explain how a nonsense codon at position 39 within exon 2 of the human β-globin gene reduces both the nuclear and the cytoplasmic levels of the product mRNA and does not affect the decay rate of mature mRNA as measured in the presence of dactinomycin.

As envisioned by Urlaub and co-workers (32), translation in the cytoplasm pulls pre-mRNA through the nuclear splicing machinery and across the nuclear envelope. Since a nonsense codon that resides 4 nucleotides upstream of the final intron at position 208 did not reduce the level of MT-TPI mRNA, we conclude that by the time a ribosome reaches position 208 on the cytoplasmic side of a translocating RNA molecule, removal in the nucleus of the final intron of this molecule is committed, if not completed, so that translocation proceeds efficiently. By contrast, since a nonsense codon that resides 61 nucleotides upstream of the final intron at position 189 does reduce the level of MT-TPI mRNA, we conclude that by the time a translating ribosome reaches position 189, splicing and transport of that molecule have not been sufficiently facilitated to proceed without further ribosome movement toward the final intron of the template RNA. Deletion of codons 190 through 207 ameliorates the mRNA reduction that is brought about by nonsense codon 189 because it brings this codon sufficiently close to, i.e., 7 nucleotides upstream of, the final intron so that splicing and transport proceed efficiently despite the block in ribosome movement. Similarly, deletion of intron 6 ameliorates the mRNA reduction that is brought about by nonsense codon 189, since it removes the only intron that is downstream of the nonsense codon. However, neither deletion ameliorates the mRNA reduction that is brought about by nonsense codon 23 because of the introns that remain between the nonsense codon and the deletion.

Translational translocation implies that mRNA will be generated efficiently from pre-mRNA only if the last intron is the last to be removed. This has yet to be proven for either human TPI or CHO DHFR pre-mRNAs. However, there does appear to be a preferred albeit nonobligatory order of intron removal in vivo for those pre-mRNAs that have been examined, with the removal of 5′ introns generally preceding the removal of 3′ introns (4, 33). Support for the removal of the last intron last also comes from the finding that efficient 3′ end formation and polyadenylation of pre-mRNA require

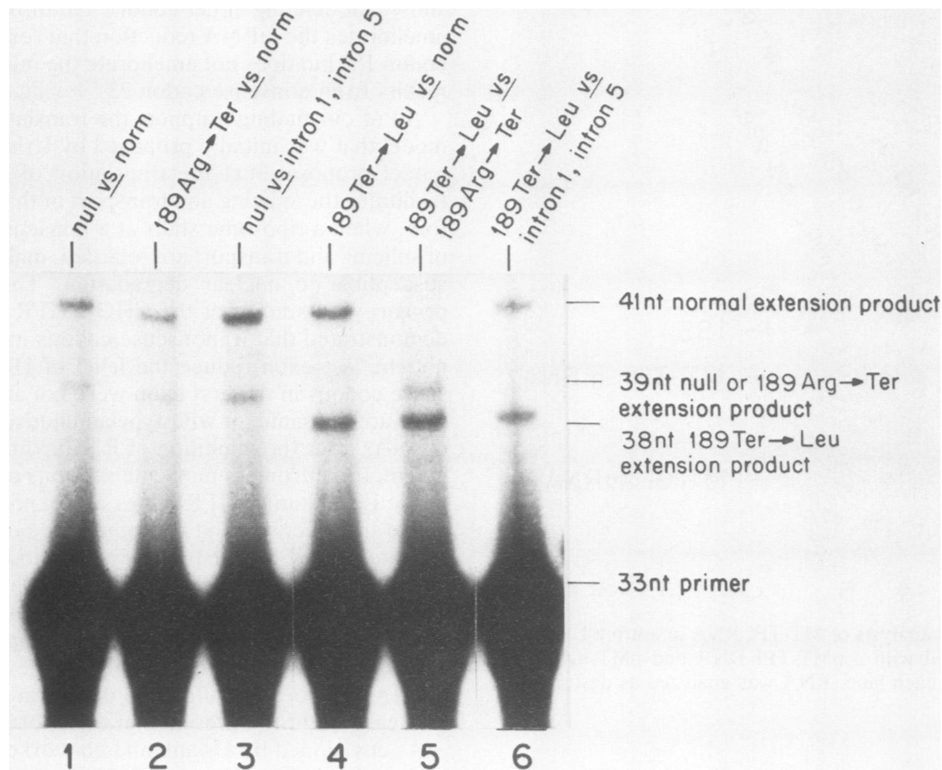

FIG. 5. Dideoxynucleotide primer extension analysis of MT-TPI RNA in murine L cells. L cells were transfected with an equal molarity of the two pMT-TPI constructs as specified above each lane. intron 1, intron 5 specifies the G · C→A · T mutation in intron 1 and the A · T→G · C mutation in intron 5. MT-TPI RNA was quantitated as described in the legend to Fig. 2. nt, Nucleotide.

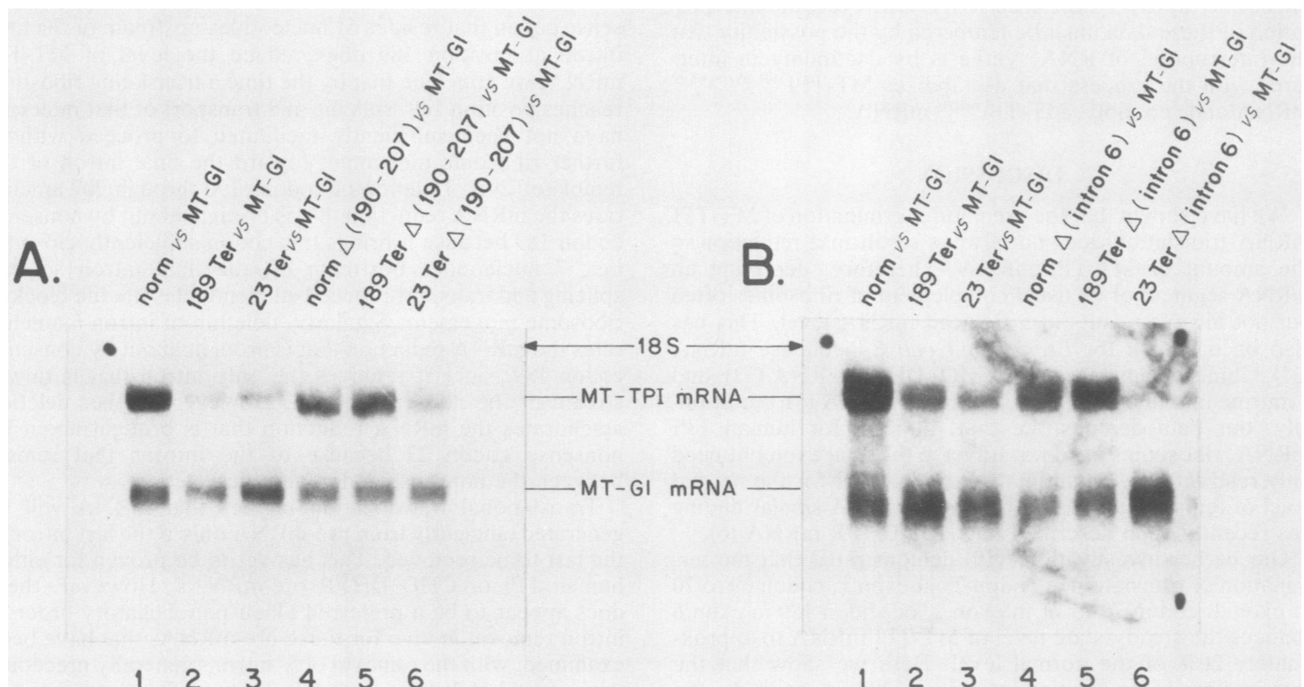

FIG. 6. RNA blot analysis of MT-TPI RNA in murine L cells. L cells were transfected with a pMT-TPI DNA and pMT-hybrid GI DNA as specified above each lane. RNA was analyzed as described in the legend to Fig. 3.

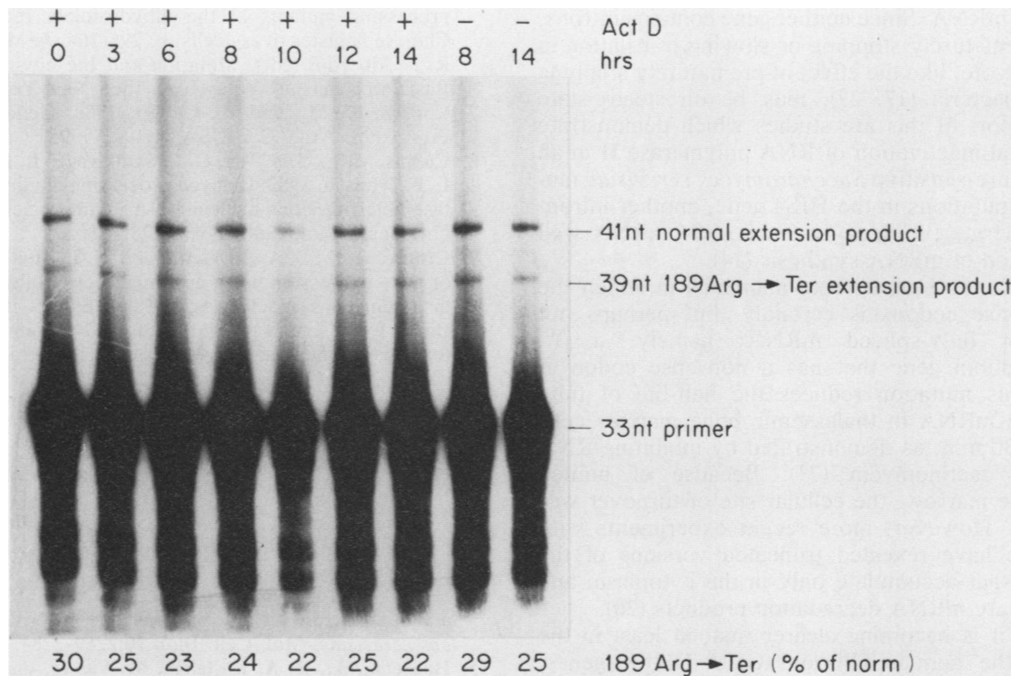

FIG. 7. Dideoxynucleotide primer extension analysis of MT-TPI$^{189\ Arg\rightarrow Ter}$ mRNA and MT-TPI^{norm} mRNA in murine L cells incubated with (+) and without (−) dactinomycin (Act D). L cells were transfected with an equal molarity of pMT-TPI$^{189\ Arg\rightarrow Ter}$ and pMT-TPI^{norm} DNAs. Thirty-six hours later, dactinomycin was added to 5 μg/ml. Total cellular RNA was then harvested at the indicated times (in hours [hrs]) and analyzed as described in the legend to Fig. 2. In control experiments, RNA from cells that were incubated without dactinomycin was analyzed. The percentage of MT-TPI$^{189\ Arg\rightarrow Ter}$ mRNA relative to MT-TPI^{norm} mRNA as determined from densitometric measurements of each lane is provided below the figure. nt, Nucleotide.

sequences within the intron preceding the site of poly(A) addition (9, 28).

Translational translocation also implies that cycloheximide or amino acid starvation, like nonsense codons, would lessen the production of mRNA. Probably the most relevant studies using cycloheximide or amino acid starvation were done by Muralidhar and Johnson (26) and Collins et al. (8) because they included studies of DHFR mRNA, albeit murine DHFR mRNA. They found that a reduction in protein synthesis by either treatment decreased the rate of the cytoplasmic accumulation of murine DHFR mRNA as well as several other mRNAs threefold. However, at least for murine DHFR mRNA, this threefold slowing of what must be processing or transport or both did not reduce the total cell content of DHFR mRNA. This finding is not what would be predicted by the translational translocation model and, most simply interpreted, implies either that not all mRNAs are subject to translational translocation or that the formation of cytoplasmic mRNA is independent of translation when total protein synthesis is blocked. However, this finding may be interpreted as support for the existence of a mechanism in the nucleus that scans at least certain pre-mRNAs for the presence of nonsense codons and that, unlike the process of translation in the cytoplasm, is not affected by either cycloheximide or the concentration of amino acids. The basic concept of nuclear scanning has been put forth previously by Urlaub et al. (32) as an alternative to translational translocation. Nuclear scanning, upon detecting a nonsense codon that resides in frame to the translation initiation codon, would result in the slowing down of pre-mRNA splicing or mRNA transport or both. It is possible that the scanning for nonsense codons may be linked to the process of splicing, especially given the recent proposal that splice site selection involves a communication across exons, possibly by exon scanning (30). It will be important to study the effect of translation inhibitors on CHO DHFR mRNA and human TPI mRNA.

The fact that nonsense codons within the final exons of the human TPI and CHO DHFR mRNAs do not reduce mRNA abundance seems more consistent with the hypothesis that either translation facilitates or nuclear scanning retards splicing and/or transport than with the alternative explanation that ribosome loading simply protects the transcript from nucleolytic attack. The fact that nonsense codons which do reduce the abundance of TPI or DHFR mRNAs do not affect the rate of mRNA decay also argues against a ribosome protection model. Our finding that the abundance of MT-TPI mRNA is affected only by nonsense codons that reside at least a certain distance upstream of the final intron may explain for most mRNAs that have a 3′ noncoding exon why translation terminates relatively close to (in 21 of 23 cases, less than 45 nucleotides upstream of) the final intron (12).

We had previously concluded that nonsense codons reduce the abundance of TPI mRNA by increasing the rate of mRNA decay in the cytoplasm (10). This conclusion was based on the result, obtained by using primer extension, that nonsense codons do not appreciably affect the level of TPI mRNA that fractionates with the nucleus (10). The significance of this result remains to be determined, since the primer extension assay does not distinguish between pre-mRNA and fully processed mRNA.

It should be noted that nonsense codons in the yeast URA3 gene (21) as well as an abundance of artificially introduced, infrequently used codons in the yeast phosphoglycerate kinase 1 gene (15) have been shown to reduce the

level of product mRNA. Since neither gene contains introns, the effect of prematurely stopping or slowing translation in this lower eucaryote, like the effect of prematurely stopping translation in bacteria (17, 27), may be on steady-state mRNA. In support of this are studies which demonstrate that after thermal inactivation of RNA polymerase II in an *rpb1-1* temperature-sensitive *Saccharomyces cerevisiae* mutant, nonsense mutations in the HIS4 gene, another intronless gene, accelerate the decay rate of *HIS4* mRNA well after the cessation of mRNA synthesis (14).

There is also one example from mammals in which the effect of nonsense codons is certainly (but perhaps not exclusively) on fully-spliced mRNA; namely, a β^0-thalassemic β-globin gene that has a nonsense codon at position 60. This mutation reduces the half-life of fully spliced β-globin mRNA in thalassemic bone marrow cells from 16.5 h to 30 min, as demonstrated by inhibiting RNA synthesis with dactinomycin (23). Because of limited amounts of bone marrow, the cellular site of turnover was not determined. However, more recent experiments with transgenic mice have revealed truncated versions of the mutant mRNA that accumulate only in the cytoplasm and that we believe are mRNA degradation products (20).

In summary, it is becoming clearer that, at least in the expression of the human TPI and CHO DHFR genes, pre-mRNA splicing in the nucleus, mRNA transport across the nuclear envelope, and mRNA translation in the cytoplasm might be functionally linked. We consider it less likely that there exists a nuclear mechanism that is capable of recognizing nonsense codons. Certainly, additional details of the link between nuclear and cytoplasmic processes will be elucidated with a better understanding of the molecular mechanisms by which nonsense codons decrease the abundance of these and other mammalian mRNAs.

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LITERATURE CITED

1. **Atweh, G. F., H. E. Brickner, X.-X. Zhu, H. H. Kazazian, Jr., and B. G. Forget.** 1988. New amber mutation in a β-thalassemic gene with nonmeasurable levels of mutant messenger RNA *in vivo*. J. Clin. Invest. **82:**557–561.
2. **Baserga, S. J., and E. J. Benz, Jr.** 1988. Nonsense mutations in the human β-globin gene affect mRNA metabolism. Proc. Natl. Acad. Sci. USA **85:**2056–2060.
3. **Baumann, B., M. J. Potash, and G. Kohler.** 1985. Consequences of frameshift mutations at the immunoglobulin heavy chain locus of the mouse. EMBO J. **4:**351–359.
4. **Beyer, A. L., and Y. N. Osheim.** 1988. Splice site selection, rate of splicing, and alternative splicing on nascent transcripts. Genes Dev. **2:**755–765.
5. **Boyer, T. G., and L. E. Maquat.** 1989. Transcriptional regulatory sequences of the housekeeping gene for human triosephosphate isomerase. J. Biol. Chem. **264:**5177–5187.
6. **Chasin, L. A., G. Urlaub, P. Mitchell, C. Ciudad, J. Barth, A. M. Carotners, R. Steigerwait, and D. Grunberger.** 1989. RNA processing mutants at the dihydrofolate reductase locus in Chinese hamster ovary cells, p. 295–304. *In* M. Mendelson and R. J. Albertini (ed.), Mutation and the environment, part A. Basic mechanisms. Wiley-Liss, Inc., New York.
7. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81:**1991–1995.
8. **Collins, M. L., J.-S. Wu, C. L. Santiago, S. L. Hendrickson, and L. F. Johnson.** 1983. Delayed processing of dihydrofolate reductase heterogeneous nuclear RNA in amino acid-starved mouse fibroblasts. Mol. Cell. Biol. **3:**1782–1802.
9. **Crouse, G. F., L. A. Stivaletta, and M. L. Smith.** 1989. Analysis of gene expression using episomal mouse dihydrofolate reductase minigenes. Nucleic Acids Res. **16:**7025–7042.
10. **Daar, I. O., and L. E. Maquat.** 1988. Premature translation termination mediates triosephosphate isomerase mRNA degradation. Mol. Cell. Biol. **8:**802–813.
11. **Fineberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
12. **Hawkins, J. D.** 1988. A survey of intron and exon lengths. Nucleic Acids Res. **16:**9893–9908.
13. **Hearing, P., and T. Shenk.** 1985. Sequence-independent autoregulation of the adenovirus type 5 E1A transcription unit. Mol. Cell. Biol. **5:**3214–3221.
14. **Herrick, D., R. Parker, and A. Jacobson.** 1990. Identification and comparison of stable and unstable mRNAs in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:**2269–2284.
15. **Hoekema, A., R. A. Kastelein, M. Vasser, and H. A. de Boer.** 1987. Codon replacement in the *PGK1* gene of *Saccharomyces cerevisiae*: experimental approach to study the role of biased codon usage in gene expression. Mol. Cell. Biol. **7:**2914–2924.
16. **Humphries, R. K., T. J. Ley, N. P. Anagnou, A. W. Baur, and A. W. Neinhuis.** 1984. β^0-39 thalassemia gene: a premature termination codon causes β-mRNA deficiency without affecting cytoplasmic β-mRNA stability. Blood **64:**23–32.
17. **Kennell, D. E.** 1986. The instability of messenger RNA in bacteria, p. 101–142. *In* W. Reznikoff and L. Gold (ed.), Maximizing gene expression. Butterworth Publishers, London.
18. **Kinniburgh, A. J., L. E. Maquat, T. Schedl, E. A. Rachmilewitz, and J. Ross.** 1982. mRNA-deficient β^0-thalassemia results from a single nucleotide deletion. Nucleic Acids Res. **10:**5421–5427.
19. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotype selection. Methods Enzymol. **154:**376–382.
20. **Lim, S. K., J. J. Mullins, C.-M. Chen, K. Gross, and L. E. Maquat.** 1988. Novel metabolism of several β^0-thalassemic β-globin mRNAs in the erythroid tissues of transgenic mice. EMBO J. **8:**2613–2619.
21. **Losson, R., and F. Lacroute.** 1979. Interference of nonsense mutations with eukaryotic messenger RNA stability. Proc. Natl. Acad. Sci. USA **76:**5134–5137.
22. **Maquat, L. E., and A. J. Kinniburgh.** 1985. A β^0-thalassemic β-globin RNA that is labile in bone marrow cells is relatively stable in HeLa cells. Nucleic Acids Res. **13:**2855–2867.
23. **Maquat, L. E., A. J. Kinniburgh, E. A. Rachmilewitz, and J. Ross.** 1981. Unstable beta-globin mRNA in mRNA deficient β^0-thalassemia. Cell **27:**543–553.
24. **McMaster, G. K., and G. G. Carmichael.** 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. USA **74:**4835–4838.
25. **Moschonas, N., E. deBoer, F. G. Grosveld, H. H. M. Dahl, S. Wright, C. C. Shewmaker, and R. A. Flavell.** 1981. Structure and expression of a cloned β^0-thalassemic globin gene. Nucleic Acids Res. **9:**4391–4400.
26. **Muralidhar, M. G., and L. F. Johnson.** 1988. Delayed processing/export of messenger RNA under conditions of reduced protein synthesis. J. Cell. Physiol. **135:**115–121.
27. **Nilsson, G., J. G. Belasco, S. N. Cohen, and A. von Gabian.** 1987. Effect of premature termination of translation of mRNA stability depends on the site of ribosome release. Proc. Natl. Acad. Sci. USA **84:**4890–4894.
28. **Niwa, M., and S. Berget.** 1990. Processing of 3′ terminal exons,

p. 201. *In* A. Hopper, J. Manley and O. Uhlenbeck (ed.), RNA processing. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

29. **Orkin, S. H., and S. Goff.** 1981. Nonsense and frameshift mutations in β^0-thalassemia detected in cloned β-globin genes. J. Biol. Chem. **256:**9782–9784.
30. **Robberson, B. L., G. J. Cote, and S. M. Berget.** 1990. Exon definition may facilitate splice site selection in RNAs with multiple exons. Mol. Cell. Biol. **10:**84–94.
31. **Takeshita, K., B. G. Forget, A. Scarpa, and E. J. Benz, Jr.** 1984. Intranuclear defect in β-globin mRNA accumulation due to a premature translation termination codon. Blood **84:**13–22.
32. **Urlaub, G., P. J. Mitchell, C. J. Ciudad, and L. A. Chasin.** 1989. Nonsense mutations in the dihydrofolate reductase gene affect RNA processing. Mol. Cell. Biol. **9:**2868–2880.
33. **Zeitlin, S., and A. Efstradiatis.** 1984. In vivo splicing products of the rabbit β-globin pre-mRNA. Cell **39:**589–602.