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# Premature Translation Termination Mediates Triosephosphate Isomerase mRNA Degradation

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**We characterized an anemia-inducing mutation in the human gene for triosephosphate isomerase (TPI) that resulted in the production of prematurely terminated protein and mRNA with a reduced cytoplasmic half-life. The mutation converted a CGA arginine codon to a TGA nonsense codon and generated a protein of 188 amino acids, instead of the usual 248 amino acids. To determine how mRNA primary structure and translation influence mRNA stability, in vitro-mutagenized TPI alleles were introduced into cultured L cells and analyzed for their effect on TPI RNA metabolism. Results indicated that mRNA stability is decreased by all nonsense and frameshift mutations. To determine the relative contribution of the changes in mRNA structure and translation to the altered half-life, the effects of individual mutations were compared with the effects of second-site reversions that restored translation termination to normal. All mutations that resulted in premature translation termination reduced the mRNA half-life solely or mainly by altering the length of the mRNA that was translated. The only mutation that altered translation termination and that reduced the mRNA half-life mainly by affecting the mRNA structure was an insertion that shifted termination to a position downstream of the normal stop codon.**

It is well established that the stability of individual mRNAs can differ widely and, under certain metabolic conditions, may be subject to change. The molecular basis for differential mRNA degradation, however, is only poorly understood. Results of studies of both procaryotes and eucaryotes indicate that the mechanisms and rates of degradation are determined by mRNA primary and higher-order structures which may support or inhibit the activity of a *trans*-acting stabilizing or destabilizing factor (9, 27). Also, built into the mRNA structure are determinants of translational processes and, possibly, determinants of cellular compartmentalization, each of which may influence mRNA accessibility to particular *trans*-acting regulators. The determinants of translation (by, for example, associating with translationally active ribosomes) may result in the physical protection of mRNA from nucleolytic attack, may change mRNA structure to create or destroy a regulator binding site, or may influence the efficiency of mRNA transport from the nucleus to the cytoplasm (7, 8, 19, 24–26, 30, 31, 34, 53, 56). The determinants of cellular compartmentalization may localize the mRNA to a cellular region that is either rich or deficient in a regulator (60).

In this report we describe results of studies on the determinants of mRNA stability as defined by the nucleotide sequence of the translated region. Our results suggest that cells recognize and degrade mRNA molecules that prematurely terminate translation. Our interest in mRNA stability and, in particular, the association of premature translation termination with increased mRNA turnover stems from molecular studies of two inherited mRNA-deficient anemias that affect humans. One of these anemias, a $\beta^0$-thalassemia, is attributable to a frameshift mutation in the β-globin gene that reduces the β-globin mRNA half-life from 16.5 h (50) to 30 min (28, 38). The other anemia, a deficiency in the glycolytic enzyme triosephosphate isomerase (TPI; D-glyceraldehyde-3-phosphate, ketol-isomerase; EC 5.3.1.1), is shown in this report to be attributable to a nonsense mutation that reduces the TPI mRNA half-life so that defective mRNA accumulates to only 20% of the normal level.

To date, little effort has been devoted to analyzing the effects of premature translation termination on mRNA stability. The experimental approach required for such an analysis is difficult but not intractable. Difficulties exist because changes in mRNA translation that are brought about by the requisite nonsense and frameshift mutations are necessarily also changes in mRNA structure which, in themselves, could alter mRNA stability. Here we distinguish between mRNA half-life differences that are due to changes in translation and those that are due to changes in primary structure by comparing the effects of individual substitution or frameshift mutations with the effects of pairs of substitution or frameshift mutations. The pairs, when placed in *cis*, compensated for the individual changes in translation and restored termination to the normal position. The pairs thus control for the cumulative effect that the changes in structure impose on the mRNA half-life. In summary, all TPI gene mutations that resulted in abnormal translation termination reduced TPI mRNA levels by reducing mRNA stability. For most mutations it was the change in mRNA translation that was mainly responsible for the reduced mRNA level, while for one mutation it was the change in mRNA structure. Significantly, all mutations that resulted in premature translation termination reduced mRNA half-lives mainly by altering the length of the coding region rather than by altering the structure of the mRNA.

## MATERIALS AND METHODS

**Cell cultures.** Human fibroblasts, CHO, and mouse Ltk$^-$ cell lines were grown in Dulbecco modified Eagle medium containing 15% fetal calf serum (DMEM).

**Isolation and characterization of the TPI-deficient null allele.** A library of genomic DNA from TPI-deficient patient 1 was constructed in phage λ EMBL3B and screened for intron sequences that were specific to the expressed TPI gene, as described previously (17). A full-length TPI gene that lacked the single-base-pair substitution that is diagnostic

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of the previously characterized TPI$^{104\ Glu\rightarrow Asp}$ allele (17) was subcloned into phage M13 or plasmid vectors and sequenced (40, 42, 52). Concurrent with DNA sequencing, the gene plus 5'- and 3'-flanking DNA was subcloned into pBR322 DNA, introduced with the dominant selectable gene for hygromycin B phosphotransferase into cultured CHO cells, and assayed for the production of human TPI isozymes (17). Briefly, extracts prepared from pools of hygromycin-resistant transformants were electrophoresed in a starch gel. The gel was assayed for TPI activity by using D-glyceraldehyde-3-phosphate as a substrate for the enzyme in an α-glycerophosphate dehydrogenase–NADH-coupled reaction.

**Cell transformations and RNA extractions.** DEAE dextran-mediated DNA transformations of Ltk$^-$ cells were done essentially as described by Banerji et al. (5). Cells were plated at a density of $6 \times 10^6$ per 150-cm$^2$ dish in DMEM and transformed the following day with an equal number of molecules of two plasmid DNAs (a total of 20 μg) in 1.2 ml of 0.5 mg of DEAE dextran per ml per dish. The plasmid DNAs harbored a TPI allele that was normal in structure, derived from patient 1, or mutagenized in vitro. After a 30-min incubation, the DNA-DEAE dextran mixture was removed and replaced with DMEM containing 0.1 mM chloroquine diphosphate. After 4.5 h, the chloroquine medium was replaced with DMEM, and the incubation was continued for an additional 40 h. Cells were harvested by scraping at 0°C. RNA was extracted with guanidine isothiocyanate (36) and purified by cesium chloride gradient centrifugation (51). When necessary, poly(A)$^+$ RNA was prepared by chromatography on oligo(dT)-cellulose (4).

Nuclear and cytoplasmic RNA fractions were isolated by the method described by Nevins (44) with the following modifications. Transiently transformed Ltk$^-$ cells from six 150-cm$^2$ dishes were harvested, suspended in 8 ml of Iso-Hi-pH buffer, and vortexed for 5 min at 4°C. The nuclei were pelleted by centrifugation, and the cytoplasmic RNA in the resultant supernatant was purified by phenol extraction and cesium chloride gradient centrifugation (51). Nuclei were suspended in 8 ml of iso-hi-pH, pelleted by centrifugation, suspended in 5 ml of iso-hi-pH that was supplemented with a 1/10 volume of Tween-deoxycholate solution, pelleted, suspended in 7 ml of iso-hi-pH, and layered over a 5-ml sucrose pad (2.1 M sucrose, 0.001 M HEPES [*N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 6.8], 0.001 M $MgCl_2$). Nuclei were recovered by centrifugation in an SW41 rotor for 1 h at 25,000 rpm and 4°C, and washed with 5 ml of iso-hi-pH. Nuclear RNA was purified by extraction with guanidine isothiocyanate and cesium chloride gradient centrifugation. A weight ratio of 6:1 of cytoplasmic to nuclear RNA was routinely obtained by following this protocol.

**Dideoxynucleotide primer extension analysis of TPI RNA.** Two 33-deoxynucleotide primers with complementarity to human TPI mRNA were synthesized by phosphoamidite chemistry on an oligonucleotide synthesizer (Applied Biosystems). Each primer was purified by electrophoresis in a denaturing acrylamide gel and was 5' end-labeled with [γ-$^{32}$P]ATP (7,000 Ci/mmol; New England Nuclear Corp., Boston, Mass.) and T4 polynucleotide kinase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) (36). Labeled primer (80,000 cpm) and 120 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 decreased over a 16-h period to 24°C to maximize primer-TPI RNA hybridization. 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. Three deoxynucleoside triphosphates and one (dideoxynucleoside triphosphate were added to a final concentration of 0.4 mM, and the hybridized primer was extended with 20 U of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.) at 42°C for 90 min. NaOH was added to 0.2 M, and the incubation was continued for 2 h. Tris hydrochloride (pH 7.0) was added to a final concentration of 0.33 M; and samples were precipitated by adding 3 μg of carrier plasmid DNA and 1 ml of 100% ethanol, washed twice with 70% ethanol, denatured in 100% formamide, electrophoresed in 20% acrylamide–7 M urea sequencing gels, and analyzed by autoradiography.

**Hybrid gene construction and site-specific mutagenesis.** The effect of all in vitro-generated mutations on the TPI RNA half-life was analyzed by transiently expressing in L cells hybrid genes consisting of a foreign promoter inserted upstream of the various mutated TPI alleles at the *Bss*HII site at position −1. The foreign promoters consisted of either the mouse metallothionein I (MT-I) promoter (21) or the human cytomegalovirus (CMV) major immediate early (IE) promoter (54). All hybrid genes that contained the normal or naturally occurring mutant alleles were generated from pTPI (17; also referred to as pTPI$^{norm}$), pTPI$^{104\ Glu\rightarrow Asp}$ (17), or pTPI$^{189\ Arg\rightarrow Ter}$. These plasmids harbored, respectively, a normal TPI allele, the missense allele of patient 1, or the null allele of patient 1, plus 5'- and 3'-flanking DNAs in the plasmid vector pBR322. To generate the hybrid genes, each of the plasmids was fully digested with either *Sal*I or *Bam*HI (which cleave upstream of the TPI gene at the pBR322-human DNA junction), partially digested with *Bss*HII, treated with the Klenow fragment of DNA polymerase I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in the presence of deoxynucleoside triphosphates, ligated to *Bam*HI linkers (New England BioLabs, Inc., Beverly, Mass.), and digested with *Bam*HI to remove excess linkers. The fragment constituting human sequences upstream of the TPI gene through base pair −1 was replaced with either a 750-base-pair (bp) *Bam*HI fragment containing the mouse MT promoter and 9 bp of the MT gene 5'-untranslated region or a 650-bp *Sau*3A I fragment containing the human CMV IE promoter and 7 bp of the IE gene 5'-untranslated region.

Each of the in vitro-mutagenized alleles was constructed as follows.

**TPI$^{189\ Ter\rightarrow Leu}$.** The termination codon (TGA) at amino acid position 189 of the null allele from patient 1 was converted to a leucine codon (TTA) by oligonucleotide-directed mutagenesis essentially as described by Zoller and Smith (61). A 660-bp *Pst*I-*Eco*RI TPI gene fragment that harbored the null mutation and that consisted of sequences from the terminal 6 bp of intron 4 through 92 bp of exon 7 was inserted into the polylinker of M13 mp9 DNA, and single-stranded recombinant phage DNA was prepared (13). The mutagenic octadecanucleotide 5'-CATCCTAAGAG CTTCTCG-3' was synthesized and purified as described above, phosphorylated at the 5' end, and mixed with an equal molar number of the M13 universal primer (Pharmacia Fine Chemicals, Piscataway, N.J.). The primers were annealed to the single-stranded phage DNA and extended in the presence of DNA ligase at 14°C for 16 h. The extension-ligation reaction was then used to transform *Escherichia coli* JM103 (36). Phage from the resulting plaques were transferred to nitrocellulose and screened for the desired mutation by hybridization to the 5', $^{32}$P-labeled mutagenic oligonucleotide. The presence of the G→T transversion in those phage DNAs that hybridized most efficiently was confirmed

by DNA sequencing (42, 52). To construct a TPI gene that harbored the transversion, the *Eco*RI-*Pst*I fragment was subcloned into the polylinker of pUC13, and the *Hin*dIII site in the polylinker was sub-sequently converted to a *Bam*HI site. The 5′ end of the TPI$^{189\ Arg\rightarrow Ter}$ allele, including position −1, was inserted as a 2.1-kilobase-pair (kbp) *Bam*HI-*Pst*I fragment; and the 3′ end of the TPI$^{189\ Arg\rightarrow Ter}$ allele, including 3′-flanking sequences, was inserted as a 1.6-kbp *Eco*RI fragment. Either the MT-I or CMV major IE promoter was then inserted at the *Bam*HI site.

**TPI$^{23\ Glu\rightarrow Ter}$.** A 2.1-kbp *Bam*HI-*Bss*HII-*Pst*I fragment consisting of base pairs −1 through the last base pair of intron 4 of the MT-TPI$^{norm}$ construct was subcloned into the *Bam*HI and *Pst*I sites of M13 mp8 DNA and prepared as a single-stranded template for mutagenesis. The glutamate codon (GAG) at amino acid position 23 was changed to a termination codon (TAG) by using the double primer method described above. The mutagenic octadecanucleotide consisted of the sequence 5′-GATGAGCTACCCCAGACT-3′. The 565-bp *Bam*HI-*Bss*HI-*Bgl*II fragment (spanning TPI gene base pairs −1 to the middle of intron 1) from phage DNA that hybridized with the greatest intensity to the mutagenic oligonucleotide was inserted into the *Bam*HI site of M13 mp8, and the sequence was determined to confirm the presence of the desired G→T transversion. The mutated 565-bp fragment was then inserted in place of the corresponding fragment in pMT-TPI$^{norm}$.

**TPI$^{EagI\ filled}$.** A 4-bp insertion was created at the *Eag*I site within exon 1 of the normal TPI allele to shift the translational reading frame and to cause premature translation termination within codons 70 and 71. pMT-TPI$^{189\ Ter\rightarrow Leu}$ was cleaved with *Eag*I, incubated with the Klenow fragment and deoxynucleoside triphosphates (dNTPs), and recircularized by ligation.

**TPI$^{EagI\ filled\ with\ ClaI\ linker}$.** A 8-bp *Cla*I linker (5′-CATC GATG-3′) was inserted at the Klenow-filled *Eag*I site of pMT-TPI$^{189\ Ter\rightarrow Leu}$ to create a TPI allele that was characterized by a normal translation termination codon and a protein product with an extra four amino acids.

**TPI$^{FspI\ with\ a\ ClaI\ linker}$.** The 8-bp *Cla*I linker was inserted into the *Fsp*I site within exon 2 of the normal TPI allele to shift the translational reading frame and to cause premature translation termination within codons 69 and 70. pMT-TPI$^{189\ Ter\rightarrow Leu}$ was partially cleaved with *Fsp*I, ligated to *Cla*I linkers, and incubated with *Cla*I. Linear plasmid DNA was purified by electroelution from an agarose gel and circularized by ligation.

**TPI$^{EagI\ filled,\ FspI\ with\ ClaI\ linker}$.** The *Eag*I-filled mutation and the *Fsp*I with *Cla*I linker mutation were placed in *cis* to create a TPI allele that was characterized by a normal translation termination codon and an altered protein product with an additional four amino acids. The 565-bp *Bam*HI-filled *Bss*HII-*Bgl*II fragment from pMT-TPI$^{EagI\ filled}$ that consisted of TPI exon 1 to the middle of intron 1 was substituted for the equivalent fragment in pMT-TPI$^{FspI\ with\ ClaI\ linker}$.

**TPI$^{BalI\ with\ ClaI\ linker}$.** The 8-bp *Cla*I linker was inserted at the *Bal*I site within exon 7 of the normal TPI allele to create a shift in the translational reading frame and translation termination 61 nucleotides downstream of the normal stop codon; the encoded protein product had an additional 24 amino acids relative to normal TPI. pMT-TPI$^{norm}$ was partially digested with *Bal*I, ligated to a molar excess of *Cla*I linkers, and cleaved with *Cla*I. Linear plasmid DNA was purified by electroelution from an agarose gel and circularized by ligation.

**TPI$^{EcoRI\ filled}$.** A 4-bp insertion was created at the *Eco*RI site within exon 7 of the normal TPI allele to shift the translational reading frame and to cause translation termination 41 nucleotides downstream of the normal stop codon; the encoded protein was 16 amino acids longer than the normal TPI. pMT-TPI$^{norm}$ was cleaved at the two *Eco*RI sites and treated with the Klenow fragment and dNTPs, and the plasmid was reconstructed by ligating the two gel-purified fragments in the appropriate orientation.

**TPI$^{BalI\ with\ ClaI\ linker,\ EcoRI\ filled}$.** The *Bal*I with *Cla*I linker mutation and the *Eco*RI-filled mutation were placed in *cis* to create a TPI allele that was characterized by a normal translation termination codon and an altered protein product with an additional four amino acids. The *Cla*I linker was inserted at the *Bal*I site within exon 7 of pMT-TPI$^{EcoRI\ filled}$ as described above for linker insertion into the *Bal*I site of pMT-TPI$^{norm}$.

The structure of each in vitro-generated mutation was confirmed by DNA sequencing (40, 42, 52).

## RESULTS

**Isolation and characterization of a TPI null allele.** We recently described the construction and partial characterization of a genomic library from fibroblasts of patient 1, a Louisiana girl of French ancestry who was homozygous for TPI deficiency and who died at 12 years of age (17). Patient 1 exhibited the typical clinical hallmarks of this deficiency, which include chronic nonspherocytic hemolytic anemia that is present from birth, multiple hemolytic crises throughout life, progressive neuromuscular dysfunction, and an abnormally high susceptibility to infection (15, 58). Cultured fibroblasts from this patient were shown to have 19.5% of normal enzyme activity, all of which was heat labile, and approximately half of the normal TPI mRNA level (37). Complete DNA sequence analysis of one allele from this patient revealed a single-base-pair change relative to a normal allele (11, 17; I. O. Daar and L. E. Maquat, unpublished intron sequences). This change, a G · C→C · G transversion in the codon for amino acid 104, generates a thermolabile protein with an aspartate residue in place of a glutamate residue and is inconsequential to mRNA levels (17, 37).

As a first step in determining the molecular basis of the reduced TPI mRNA levels, the uncharacterized allele of this patient, together with 590 bp of 5′-flanking DNA and 100 bp of 3′-flanking DNA, was sequenced in its entirety. Three single-base-pair substitutions were present relative to the normal allele. These substitutions consisted of a G · C→A · T transition within intron 1, a A · T→G · C transition within intron 5, and a C · G→T · A transition in the codon for amino acid 189 (Fig. 1). Neither of the first two alterations created or destroyed what was recognized to be a posttranscriptional regulatory sequence. The latter alteration exemplifies a major type of sequence variation in the human population that is presumably due to deamination of the methylated cytosine residue of a CpG dinucleotide (6). It generated a premature translation termination codon (TGA) in the place of an arginine codon (CGA) and resulted in the production of a truncated protein, designated TPI$^{189\ Arg\rightarrow Ter}$, which consisted of 188 amino acids instead of the usual 248 amino acids. Significantly, it was the only one of the three substitutions to affect the TPI mRNA half-life (see below).

**Enzymatic properties of TPI$^{189\ Arg\rightarrow Ter}$.** Crystallographic and kinetic studies of TPI structure and function indicated

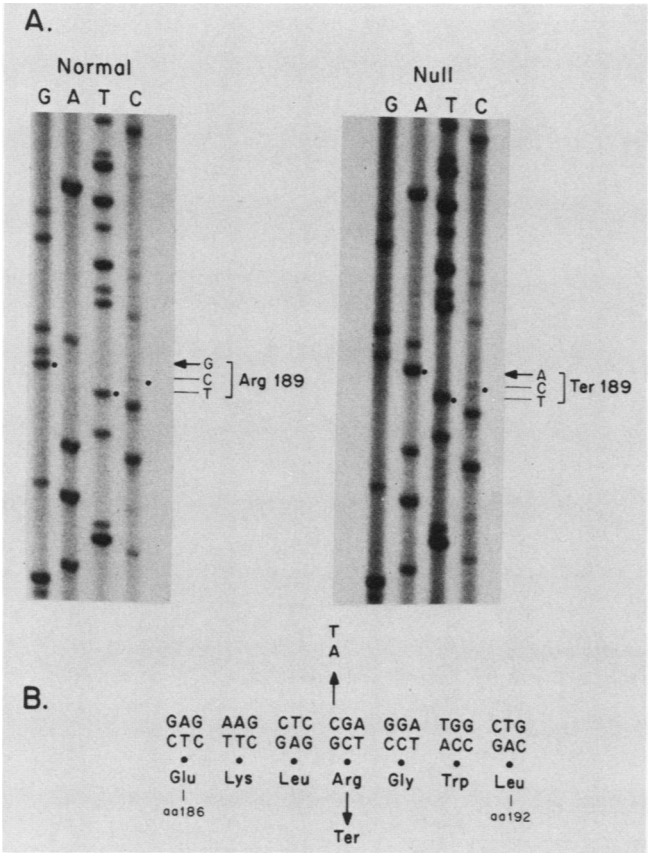


FIG. 1. Comparison of normal and null TPI allele and protein sequences. (A) Arrows indicate the single-nucleotide difference between the mRNA-complementary sequences of a normal TPI allele and the null allele isolated from TPI-deficient patient 1. (B) Base pairs of a normal TPI allele and the encoded amino acids. Arrows designate the base pair substitution within the null allele and the resultant translation stop codon at amino acid (aa) 189.

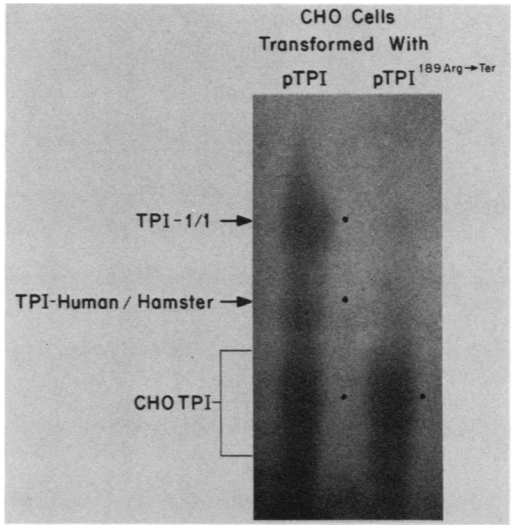


FIG. 2. TPI enzyme activities in cultured cell lines. Lysates of pools of CHO colonies that were stably transformed with pBR322-TPI constructs, either pTPI or pTPI$^{189\ Arg\rightarrow Ter}$, were analyzed for TPI enzyme activity. The human isozyme TPI-1/1 and the human-hamster TPI heterodimer that was present in CHO cells expressing human TPI are specified by arrows. The broad band of CHO TPI activity is bracketed. The human isozymes TPI-1/2 and TPI-2/2, if present, would comigrate with human-hamster TPI and CHO TPI, respectively.

that residues that are indispensable for enzyme activity exist throughout the carboxy-terminal region of the protein, with the possible exception of the ultimate few amino acids (1, 46). The importance of carboxy-terminal amino acids 189, 201, 206, 208 to 211, 228, 230 to 236, and 240, in particular, is implicated by their conservation in the TPI protein of all species that have been characterized to date, including *Bacillus stearothermophilus* (3), *E. coli* (47), *Saccharomyces cerevisiae* (2), *Aspergillus nidulans* (41), maize (39), coelacanth (29), chickens (20, 55), rabbits (16), and humans (35, 37). Therefore, TPI$^{189\ Arg\rightarrow Ter}$ is expected to be ineffective in the interconversion of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. To test this expectation, the activity of TPI$^{189\ Arg\rightarrow Ter}$ was assayed apart from any contributions of TPI$^{104\ Glu\rightarrow Asp}$, the protein product of the other allele from patient 1, following an established protocol (17). pTPI$^{189\ Arg\rightarrow Ter}$, a pBR322 recombinant that contained the null allele with 3.0 and 1.1 kbp of 5′- and 3′-flanking DNA, respectively, was introduced into cultured CHO cells with pHyg, a plasmid that encodes the dominant selectable gene for hygromycin B phosphotransferase (23). CHO cell transformants resistant to hygromycin B were pooled, lysed, and assayed for TPI. As a positive control for human enzyme activity, CHO cells were similarly transformed with pTPI (also called pTPI$^{norm}$), a pBR322 recombinant containing the normal allele plus sufficient flanking DNA to support maximal gene expression (17), and assayed for TPI. Hygromycin-resistant colonies that were transformed with pTPI$^{189\ Arg\rightarrow Ter}$ did not contain detectable human isozyme activity (in which the limit of detectability was estimated to be 1% of normal activity), whereas colonies that were transformed with pTPI did (Fig. 2). Each pBR322-TPI construct was introduced into CHO cells in a 10-fold molar excess relative to pHyg so that all colonies that survived the selection process probably contained at least one copy of the human TPI allele. Furthermore, colonies from several hundred independently transformed cells were pooled for these assays. Therefore, the absence of detectable TPI$^{189\ Arg\rightarrow Ter}$ activity was unlikely to be due to the fact that there were too few cells that expressed the TPI$^{189\ Arg\rightarrow Ter}$ allele either because the allele was absent from the cells or was present in the cells but in a DNA context that inhibited its expression. It follows that the nonsense mutation at codon 189 precluded functional enzyme production and that the TPI$^{189\ Arg\rightarrow Ter}$ allele was null.

**The level of TPI$^{189\ Arg\rightarrow Ter}$ RNA in cultured fibroblasts of patient 1 is abnormally low.** Northern blot analysis of total and poly(A)$^+$ RNA demonstrated that TPI mRNA levels are approximately half of normal levels in cultured fibroblasts of patient 1 (37). To determine more precisely the relative contribution of the TPI$^{104\ Glu\rightarrow Asp}$ (missense) and TPI$^{189\ Arg\rightarrow Ter}$ (null) alleles to the steady-state RNA population, the transcripts of each allele were distinguished and quantitated by primer extension assays. These assays differentiated the two transcripts on the basis of sequence heterogeneity. Initially, a 5′, $^{32}$P-labeled, 33-deoxynucleotide primer that was perfectly complementary to both transcripts and that had a 3′ end located six nucleotides downstream of the C · G→T · A transition within codon 189 of the null allele was hybridized in molar excess to total RNA from cultured fibroblasts of the patient. The DNA sequence of each allele indicated that primer extension with reverse transcriptase in

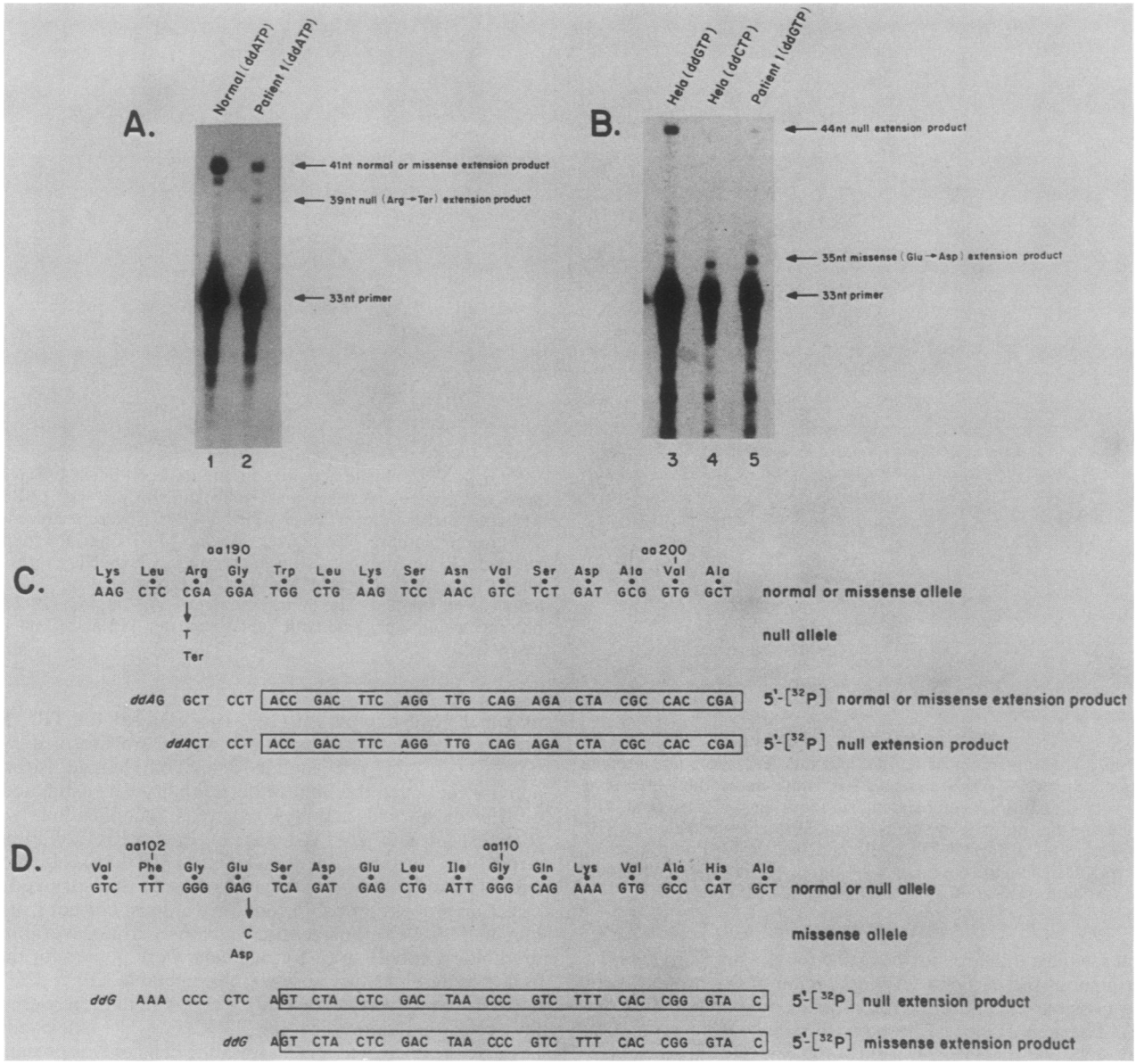


FIG. 3. Dideoxynucleotide primer extension analysis of TPI RNA in normal and TPI-deficient fibroblasts. (A) Total RNA (120 μg) from the normal fibroblast cell line L153 (lane 1) or cultured fibroblasts of patient 1 (lane 2) was hybridized to a molar excess of 5′, $^{32}$P-labeled, 33-deoxynucleotide primer (see panel C) that distinguished transcripts by sequence differences at codon 189. The primer was extended with ddATP, dGTP, dCTP, and dTTP by using reverse transcriptase. Reaction products were denatured, electrophoresed in an acrylamide-urea gel, and exposed to X-ray film. (B) Total RNA (120 μg) from a HeLa cell line (lanes 3 and 4) or cultured fibroblasts of patient 1 (lane 5) were hybridized to a molar excess of 5′, $^{32}$P-labeled, 33-deoxynucleotide primer (see panel D) that distinguished transcripts by sequence differences at codon 104. The primer was extended with ddGTP, dATP, dCTP, and dTTP (lanes 3 and 5) or ddCTP, dATP, dGTP, and dTTP (lane 4) and analyzed as described for panel A. In lanes 3 and 4, molecular weight standards for the 44- and 35-nucleotide (nt) products, respectively, are given. (C) Nucleotides of the normal and missense TPI alleles, and amino acids (aa) 186 to 201 in the corresponding TPI proteins are shown. The arrow designates the base pair substitution of the TPI-deficient null allele and the resulting termination (Ter) of translation. The expected primer extension products for normal, missense, and null TPI mRNAs are also shown. The primer regions of each extension product are boxed. (D) Nucleotides of the normal and null TPI alleles, and amino acids 101 to 116 in the corresponding TPI proteins are shown. The arrow designates the base pair substitution of the TPI-deficient missense allele and the resulting amino acid substitution. The expected primer extension products for each of the TPI mRNAs are specified where the primer regions of each extension product are boxed.

the presence of ddATP, dGTP, dCTP, and dTTP should result in the addition of six nucleotides to primers that were hybridized to TPI$^{189\ Arg\rightarrow Ter}$ transcripts and eight nucleotides to primers that were hybridized to TPI$^{104\ Glu\rightarrow Asp}$ transcripts. This was, in fact, the case, as evidenced by the separation of the differentially truncated extension products on the basis of size in an acrylamide-urea gel (Fig. 3A and C). Quantitations of each product by densitometric tracings of X-ray film exposures demonstrated that TPI$^{189\ Arg\rightarrow Ter}$ RNA levels were 20% of TPI$^{104\ Glu\rightarrow Asp}$ RNA levels in both total RNA and poly(A)$^+$ RNA (data not shown). As expected, primer extension of total RNA from normal fibroblasts gave a single product that comigrated with the TPI$^{104\ Glu\rightarrow Asp}$ product (Fig. 3A and C). To ensure that

TABLE 1. Effects of human TPI gene mutations on TPI mRNA levels and translation in L cells

| MT-TPI allele[a] | Translation termination position (codon no.)[b] | % Normal steady-state TPI mRNA in[c]: | | Determinants of reduced mRNA half-life |
|---|---|---|---|---|
| | | Total cell and cytoplasmic RNA | Nuclear RNA | |
| Normal | 249 | 100 | 100 | None |
| Null (Arg→Ter; codon 189) | 189 | 25 | 200 | Premature translation termination |
| Ter→Leu (codon 189) | 249 | 100 | 200 | None |
| Glu→Ter (codon 23) | 23 | 20 | 100 | Premature translation termination? |
| *Eag*I filled (exon 1)* | 70–71 | 20 | 200 | Primarily premature translation termination with additional contributions by altered primary structure |
| *Fsp*I with a *Cla*I linker (exon 2)* | 69–70 | 20 | 200 | Primarily premature translation termination with additional contributions by altered primary structure |
| *Eag*I filled (exon 1); *Fsp*I with a *Cla*I linker (exon 2)* | 249 | 80 | 200 | Altered primary structure |
| *Eco*RI filled (exon 7) | 263–264 | 40 | 100 | Altered primary structure |
| *Bal*I with a *Cla*I linker (exon 7) | 270–271 | 75 | 100 | Altered primary structure, translation termination downstream of the normal site, or both |
| *Bal*I with a *Cla*I linker (exon 7); *Eco*RI filled (exon 7) | 249 | 40 | ND[d] | Altered primary structure |
| *Eag*I filled with a *Cla*I linker (exon 1)* | 249 | 40 | 200 | Altered primary structure |

[a] The steady-state level of RNA produced from each TPI allele was quantitated by primer extension analysis of RNA from L cells that were transiently transformed with two MT-TPI DNA constructs. One of these constructs consisted of the mutant allele under analysis; the other construct served as a standard to control for variations in transformation efficiency, RNA recovery, or both. The normal, Ter→Leu, *Eco*RI-filled, and null allele quantitations in total RNA were confirmed by analyzing the transcripts of the corresponding CMV-TPI construct and an appropriate CMV-TPI standard. For each allele, the TPI codon or exon that was mutated is specified in parentheses. Alleles that harbored the four substitutions of the Ter→Leu allele are designated with asterisks. In the case of the Glu→Ter mutation at codon 23, contributions by the altered primary structure to the reduced mRNA half-life cannot be dismissed without analyzing the effects of a base pair substitution in *cis* that restores translation termination to the normal site.

[b] The translation termination position is numbered relative to the normal translational reading frame.

[c] All quantitations were derived from densitometric scans of autoradiographs, as exemplified in Fig 5 and 6, and are the average of at least three independent L-cell transformations. These quantitations did not deviate by more than 20% of the tabulated value.

[d] ND, Not determined.

the smaller extension product obtained with RNA from the patient was a measurement of $TPI^{189\ Arg\rightarrow Ter}$ transcript levels, additional extension reactions were performed with another primer that differentiated the transcripts of the patient on the basis of the G · C→C · G transversion within codon 104 of the missense allele. A 5′, $^{32}P$-labeled, 33-deoxynucleotide primer that had a 3′ end located two nucleotides downstream of the transversion was hybridized to the RNA from fibroblasts of the patient and then extended with reverse transcriptase in the presence of ddGTP, dATP, dCTP, and dTTP. Extension products of $TPI^{104\ Glu\rightarrow Asp}$ transcripts should contain an additional 2 nucleotides, whereas extension products of $TPI^{189\ Arg\rightarrow Ter}$ transcripts should have contained an additional 11 nucleotides. As expected from the results that were obtained with the first primer, a 44-nucleotide product was obtained at 20% of the level of a 35-nucleotide product (Fig. 3B and D).

**The abnormally low level of $TPI^{189\ Arg\rightarrow Ter}$ RNA in patient 1 is not due to reduced transcription initiation.** While the sequence of the 590 bp immediately upstream of the normal, missense, and null alleles proved to be identical and, thus, contraindicated that the alleles were differentially transcribed, the possibility that reduced transcription of the null allele was responsible for the reduced level of steady-state null RNA was definitely eliminated by assaying the expression of hybrid genes. The hybrid genes were derivatives of pTPI, $pTPI^{104\ Glu\rightarrow Asp}$, and $pTPI^{189\ Arg\rightarrow Ter}$, in which all human genomic sequences upstream of TPI gene position −1 were replaced by either the mouse MT-I promoter (Fig. 4) or the human CMV major IE promoter. The MT-I promoter consisted of approximately 750 bp and included 9 bp of the adjacent 5′-untranslated region; the CMV IE promoter consisted of approximately 650 bp and included 7 bp of the adjacent 5′-untranslated region. Each hybrid allele was transiently expressed in mouse L cells together with an equal number of molecules of another hybrid allele that served as a standard for variations in cell transformation efficiency, RNA recovery, or both. The relative levels of the resulting two hybrid transcripts were assayed by primer extension by using the deoxyoligonucleotide that distinguishes transcripts on the basis of the G · C→T · A transition at position 189. This primer was chosen because it is the only one of three that does not detect L-cell transcripts under the usual hybridization conditions (Fig. 5A, lane 3). The steady-state level of transcripts synthesized from $pMT\text{-}TPI^{189\ Arg\rightarrow Ter}$ was 25% of the steady-state level of transcripts synthesized from $pMT\text{-}TPI^{norm}$ (Fig. 5A, lane 4, and Table 1); similarly, the level of transcripts synthesized from $pCMV\text{-}TPI^{189\ Arg\rightarrow Ter}$ was 25% of the level of transcripts synthesized from $pCMV\text{-}TPI^{norm}$ (data not shown). These relative levels were found not only in total RNA but also in poly(A)$^+$ RNA (data not shown) from the transformed L cells. From these data, it can be concluded that the reduced levels of TPI RNA in fibroblasts of patient 1 are solely attributable to the null allele and are not related to promoter function. The mechanism by which the null RNA levels were reduced is not species specific because the mechanism was active in both mouse and human fibroblasts. These data also indicate that the null allele mutations comparably affect the levels of bona fide TPI mRNA, $MT\text{-}TPI^{norm}$ mRNA, and $CMV\text{-}TPI^{norm}$ mRNA.

**The C · G→T · A transition at codon 189 and, in particular, premature translation termination mediates the reduced level**

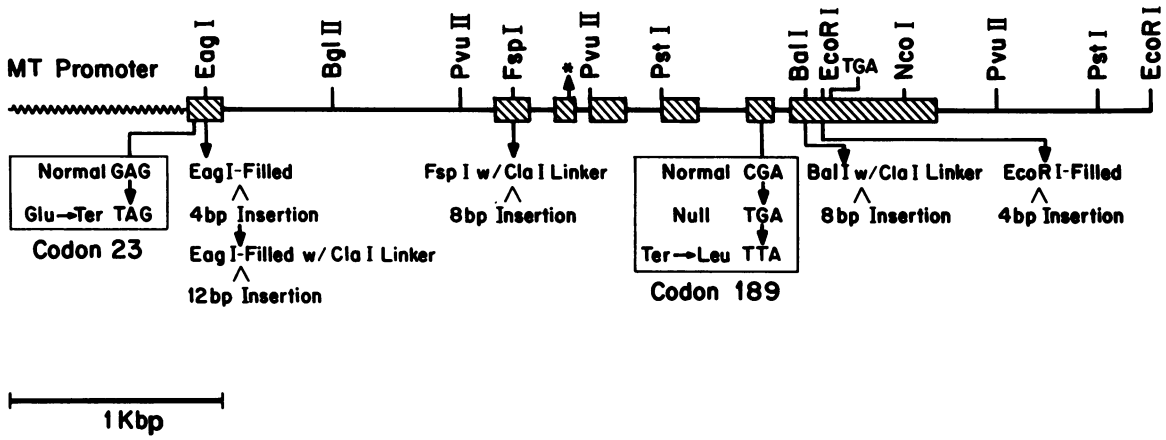


FIG. 4. Structures of the mutant MT-TPI alleles. The wavy line represents a 750-bp *Bam*HI fragment that harbored the mouse MT promoter. Hatched boxes and interspersed lines designate the human TPI gene, in which the boxes specify exons and the lines specify introns. The horizontal line to the right of and contiguous with exon 7 represents 1.1 kbp of human DNA that resides downstream of the TPI gene. The null mutation of the $TPI^{189\ Arg\rightarrow Ter}$ allele and the various in vitro-generated mutations are specified by arrows beneath the MT-TPI gene structure. Above the gene structure, the position of the missense mutation in the $TPI^{104\ Glu\rightarrow Asp}$ allele is specified by an asterisk, and the normal translation termination site in exon 7 is labeled TGA. For those mutations that added to the length of the MT-TPI gene, the number of base pairs that were inserted is also indicated. See the text for details on hybrid gene construction and in vitro mutagenesis.

**of $TPI^{189\ Arg\rightarrow Ter}$ RNA.** To determine the relative contribution of the changes in $TPI^{189\ Arg\rightarrow Ter}$ RNA primary structure and translation to the reduced level of $TPI^{189\ Arg\rightarrow Ter}$ RNA, the effect of the C · G→T · A transition on translation termination was abrogated by converting the premature translation termination codon to a coding codon. Specifically, the TGA nonsense codon was changed to a TTA leucine codon by oligonucleotide-directed mutagenesis, and the two transitions within introns 1 and 5 were maintained. Following our standard nomenclature, the leucine-containing allele was designated $TPI^{189\ Ter\rightarrow Leu}$. The steady-state level of transcripts synthesized from pMT-$TPI^{189\ Ter\rightarrow Leu}$ was fourfold the level of transcripts synthesized from pMT-$TPI^{189\ Arg\rightarrow Ter}$ (Fig. 5A, lane 8, and Table 1) and equal to the level of transcripts synthesized from pMT-$TPI^{norm}$ (Fig. 5A, lane 14, and Table 1). Therefore, the MT-$TPI^{189\ Ter\rightarrow Leu}$ RNA was present in normal amounts. This result indicates that the reduced level of null RNA is attributable solely to the C · G→T · A transition within codon 189 by a mechanism that is dependent on premature translation termination at this codon. This result also indicates that while the introduced leucine codon is recognized by a minor tRNA, any ribosome stalling that is induced by this codon is, as expected (25), insufficient to decrease the TPI mRNA level.

Structural characterizations of $TPI^{189\ Arg\rightarrow Ter}$ RNA in fibroblasts from the patient and of MT-$TPI^{189\ Arg\rightarrow Ter}$ RNA in transformed L cells by Northern blotting, primer extension, and S1 nuclease transcript mapping demonstrated that intron removal proceeds normally and that the most abundant steady-state species, albeit at an abnormally low level, is fully processed mRNA (data not shown). Therefore, the C · G→T · A transition at codon 189 destabilizes an otherwise structurally normal mRNA.

**MT-$TPI^{189\ Arg\rightarrow Ter}$ mRNA degradation is restricted to the cytoplasm.** If premature translation termination mediates $TPI^{189\ Arg\rightarrow Ter}$ mRNA degradation, then the abnormally low (25% of normal) level of this mRNA that is present in total RNA should be evidenced in cytoplasmic RNA but not nuclear RNA. L cells were transiently transformed with pMT-$TPI^{189\ Arg\rightarrow Ter}$ and pMT-$TPI^{norm}$ as usual, and RNA was purified from the nucleus and the cytoplasm. Primer extension analysis revealed that the steady-state level of MT-$TPI^{189\ Arg\rightarrow Ter}$ mRNA was 200% of normal in the nucleus and 25% of normal in the cytoplasm (Fig. 6, lanes 1 and 2). These data indicate that either the null mutation or one of the transitions within intron 1 or intron 5 slows by twofold nuclear RNA processing or transport to the cytoplasm. This slowing is inconsequential to the reduced mRNA level, however, as evidenced by two findings. First, the cytoplasmic level and the total cellular level of MT-$TPI^{189\ Arg\rightarrow Ter}$ mRNA relative to MT-$TPI^{norm}$ mRNA were similar (Fig. 6, lanes 1 and 2). Second, while the steady-state cytoplasmic level of MT-$TPI^{189\ Ter\rightarrow Leu}$ mRNA was normal, as expected (Fig. 6, lane 5), the steady-state nuclear level of this mRNA was, as for MT-$TPI^{189\ Arg\rightarrow Ter}$, 200% of normal (Fig. 6, lane 6). These data indicate that the twofold increase in nuclear RNA has no demonstrable effect on the total cellular RNA level and merely reflects a transient accumulation of one or more nuclear species that are eventually transported to the cytoplasm as fully processed mRNA molecules. These data also indicate that this transient accumulation is attributable to one of the three changes in mRNA structure rather than the change in mRNA translation. This concept is further verified by analyzing several frameshift mutations and second-site reversions of these mutations, each of which was generated in the context of the MT-$TPI^{189\ Ter\rightarrow Leu}$ allele (see below).

**An in vitro-generated nonsense codon within TPI gene exon 1 is also associated with a reduced RNA level.** To determine whether RNA destabilization by premature translation termination is a phenomenon that can be extended to nonsense codons other than the one created by the base pair substitution within codon 189 of the null allele, a mutagenic oligonucleotide was used to convert the GAG glutamate codon at position 23 to a TAG termination codon. The corresponding mutant allele was designated $TPI^{23\ Glu\rightarrow Ter}$.

The steady-state level of MT-$TPI^{23\ Glu\rightarrow Ter}$ transcripts was slightly lower than the level of MT-$TPI^{189\ Arg\rightarrow Ter}$ transcripts (Fig. 5A, lane 13) and, by comparison, 20% of the normal MT-TPI mRNA level (Table 1). Identical results were found for the corresponding cytoplasmic transcripts (data not shown). In the nucleus, MT-$TPI^{23\ Glu\rightarrow ter}$ transcripts were

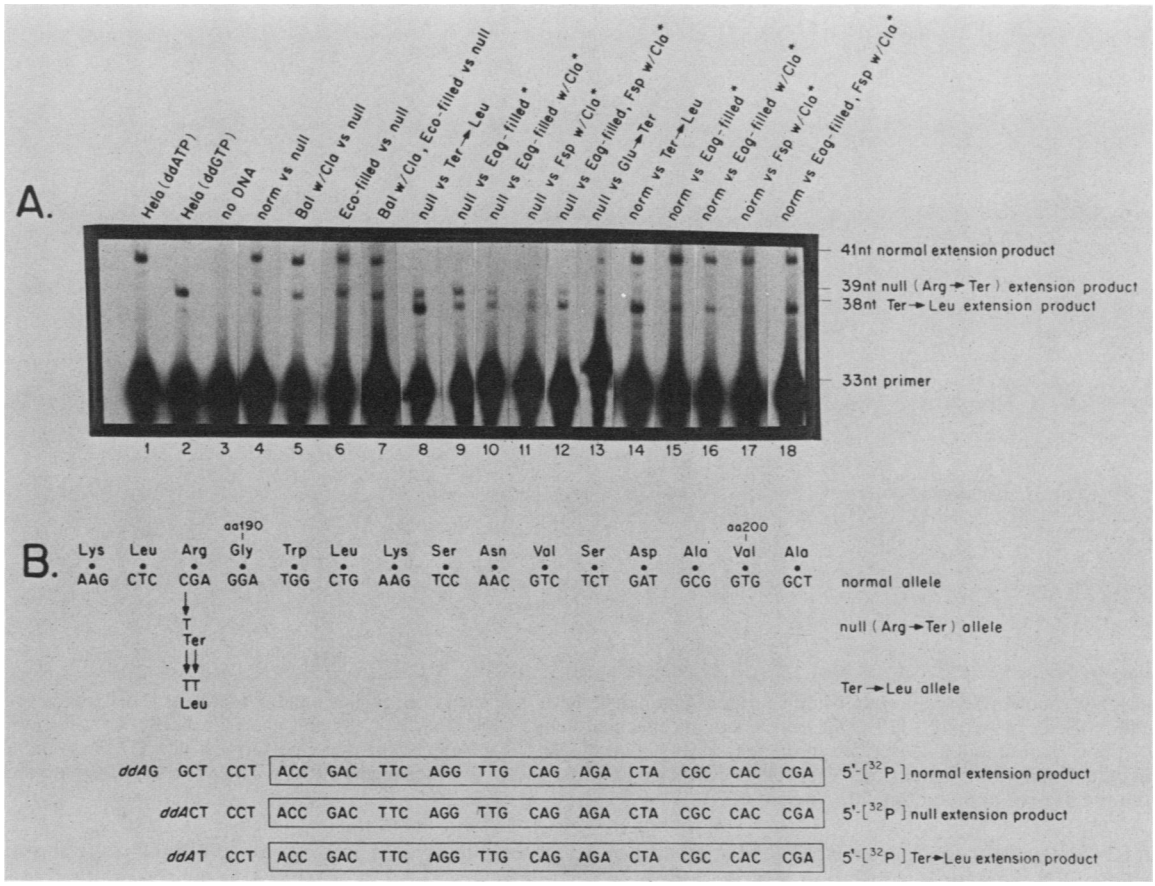


FIG. 5. Dideoxynucleotide primer extension analysis of human TPI RNA in mouse L cells. (A) Total RNA from L cells that were transiently transformed with an equal molarity of two MT-TPI DNA constructs, each of which harbored the normal or a mutant TPI allele, was hybridized to a molar excess of 5', $^{32}$P-labeled, 33-deoxynucleotide primer. The primer was extended with ddATP, dGTP, dCTP, and dTTP by using reverse transcriptase. Reaction products were denatured, separated by electrophoresis, and detected by autoradiography. Lanes 1 to 3 are the results of control experiments. Lane 1, HeLa cell RNA was extended in the presence of ddATP, dGTP, dCTP, and dTTP to generate the 41-nucleotide (nt) extension product of normal human TPI mRNA; lane 2, total HeLa cell RNA was extended in the presence of ddGTP, dATP, dCTP, and dTTP to generate a 39-nucleotide DNA that was comparable in length to the null extension product. In the remaining lanes, RNA was extended in the presence of ddATP, dGTP, dCTP, and dTTP. Lane 3, Total RNA from untransformed L cells; lanes 4 to 18, total RNA from L cells transformed with an equal molarity of two MT-TPI constructs, as specified above the autoradiograph. w/Cla denotes the *Cla*I linker. Asterisks designate those alleles that harbored the Ter→Leu mutations. None of these mutations affected total cellular or cytoplasmic MT-TPI RNA levels, but the Ter→Leu change in codon 189 resulted in a 38-nucleotide extension product that was distinguishable from the null and normal extension products. (B) Nucleotides of a normal TPI allele and the corresponding amino acids (aa) in TPI protein. The arrows designate the substitutions within the null (Arg→Ter) and Ter→Leu alleles and the resulting encoded proteins. The expected dideoxynucleotide primer extension products for the transcripts of each allele are also shown. The primer region of each extension product is boxed.

present at the normal level (data not shown). These data are consistent with the fact that premature translation termination at codon 23 destabilizes cytoplasmic TPI RNA.

**The effects of frameshift mutations on TPI RNA levels.** In theory, the association of premature translation termination and mRNA instability should be applicable to insertion and deletion mutations that create nonsense codons by shifting the translational reading frame. This concept was tested by generating frameshift mutations within the first, second, and last (seventh) exons of MT-TPI DNA. All of these mutations, with the exception of those in exon 7, were generated in the context of the MT-TPI$^{189\ Ter\rightarrow Leu}$ allele.

Initially, two frameshift mutations were created, one in exon 1 and the other in exon 2, that individually resulted in premature translation termination and that in *cis* restored termination to the normal position. The mutation in exon 1 was a 4-bp insertion generated at the *Eag*I site by cleavage with *Eag*I, treatment with the Klenow fragment and dNTPs, and ligation of the resulting blunt ends (Fig. 4). The mutation in exon 2 was an 8-bp insertion that was generated at the *Fsp*I site by cleavage with *Fsp*I, followed by ligation of the ends to an 8-bp *Cla*I linker (Fig. 4). These mutations resulted in premature translation termination within codons 70 and 71 (at the TAA sequence) and codons 69 and 70 (at the TGA sequence) of the normal reading frame, respectively. Primer extension analysis demonstrated that the steady-state RNA level was 20% of normal for the mutations that were analyzed individually and increased to 80% of normal when the mutations were assayed in *cis* (Fig. 5 and Table 1). Therefore, each mutation affects the RNA level primarily by altering RNA translation and only slightly by altering RNA structure. These data corroborate the association of premature translation termination with a reduced RNA level, as was established by experiments with mutations that directly produced a nonsense codon.

From these data it can be hypothesized that rather subtle

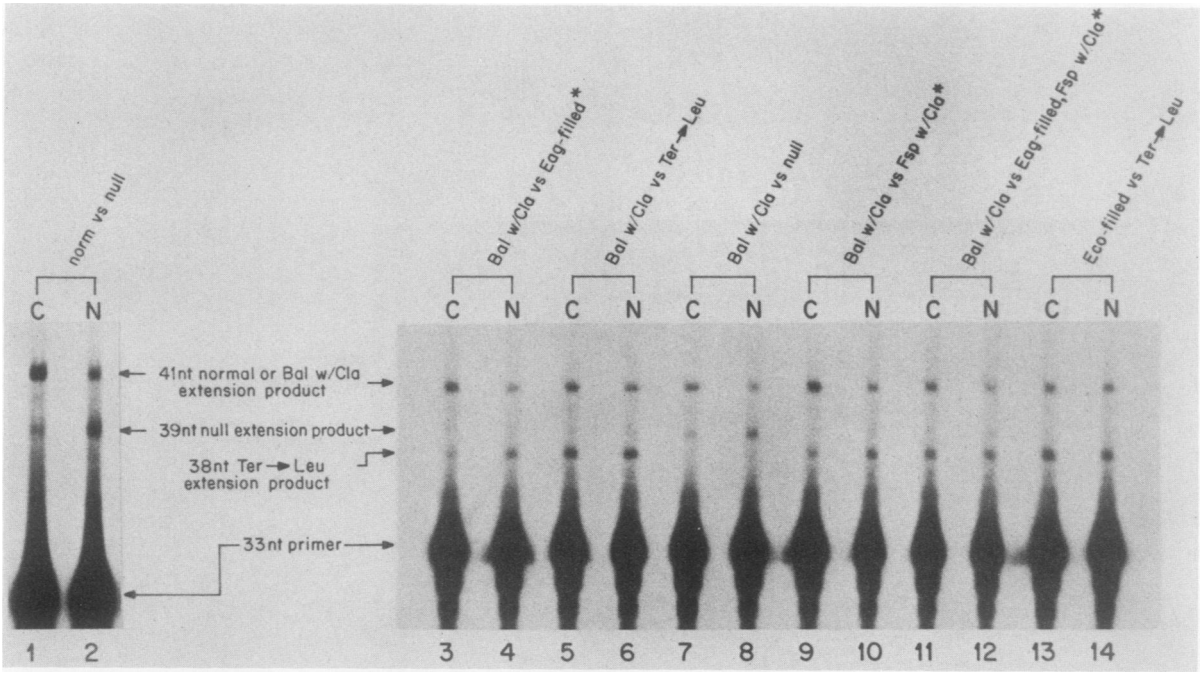


FIG. 6. Dideoxynucleotide primer extension analysis of human TPI RNA in the L-cell nucleus and cytoplasm. L cells were transformed with an equal molarity of two MT-TPI DNA constructs, as specified above each lane. Nuclear and cytoplasmic RNAs were purified, and MT-TPI RNA was quantitated as described in the legend to Fig. 5. Nuclear RNA extensions were performed with 2.7 times the number of cells used in cytoplasmic RNA extensions. C specifies cytoplasmic RNA, and N specifies nuclear RNA. The asterisks designate those alleles that harbored the Ter→Leu mutations. nt, Nucleotide.

changes in RNA structure can reduce RNA levels, albeit not with the efficacy of premature translation termination, by a mechanism that is independent of premature translation termination. To test this hypothesis directly, a 12-bp insertion at the *Eag*I site in exon 1 that did not alter the translational reading frame was analyzed. This insertion was constructed by cleavage with *Eag*I, treatment with the Klenow fragment and dNTPs, addition of a *Cla*I linker, and recircularization (Fig. 4). The steady-state level of the RNA that harbored this insertion was 40% of normal (Fig. 5A, lanes 10 and 16, and Table 1).

All nonsense and frameshift mutations described thus far resulted in premature translation termination. To investigate the effect of termination within what was normally the 3′-untranslated region, two frameshift mutations were created in exon 7 that individually extended the translated portion of the RNA and that in *cis* restored translation termination to the normal position. One mutation was a 4-bp insertion at the *Eco*RI site that was created by cleavage with *Eco*RI, treatment with the Klenow fragment and dNTPs, and ligation of the resulting blunt ends (Fig. 4). The other mutation was an 8-bp insertion of a *Cla*I linker at the *Bal*I site. Individually, these mutations reduced the MT-TPI RNA level to 40 and 75% of normal, respectively (Fig. 5A, lanes 6 and 5, respectively, and Table 1). In *cis* the two mutations reduced the MT-TPI RNA level to 40% of normal (Fig. 5A, lane 7, and Table 1). Consequently, the *Eco*RI-filled mutation exerted an effect on RNA levels solely by altering RNA structure, because its effect could not be abrogated by linker insertion at the *Bal*I site. The mechanism by which linker insertion at the *Bal*I site reduces RNA levels is unclear and may involve altered primary structure, altered translation termination, or both.

All tested MT-TPI RNAs that harbored frameshift mutations and frameshift mutations together with second-site reversions were present in total and cytoplasmic RNA relative to MT-TPI$^{norm}$ RNA at nearly identical levels (Fig. 5 and 6 and Table 1). The nuclear levels of those RNAs that harbored the three TPI$^{189\ Arg\rightarrow Ter}$ allele substitutions were consistently 200% of normal (Fig. 6 and Table 1), whereas the nuclear levels of those RNAs that lacked these substitutions were normal. These data reinforce the concepts that altered translation termination specifically affects cytoplasmic mRNA stability and that the accumulation of nuclear RNA due to the TPI$^{189\ Arg\rightarrow Ter}$ allele substitutions is transient and does not reduce the amount of total or cytoplasmic RNA.

## DISCUSSION

We have demonstrated that premature translation termination reduces TPI mRNA levels by increasing the rate of TPI mRNA turnover in the cytoplasm. Our data indicate that the reduction in mRNA levels by all nonsense and frameshift mutations that were tested was not affected by nuclear synthesis or splicing of the primary transcripts and was restricted to the cytoplasm. Consistent with these findings, none of the nonsense or frameshift mutations created or destroyed what was recognized to be an RNA splice junction or some other sequence that was involved in posttranscriptional processing. Furthermore, the drastic effects of all of the nonsense and frameshift mutations on mRNA levels were ameliorated by second-site reversions that restored the translational reading frame to normal. If these mutations reduced cytoplasmic mRNA levels by altering RNA processing or transport to the cytoplasm via a mechanism that was independent of altered translation termination, then their effects should be *cis* dominant.

A correlation between nonsense codons and reduced mRNA levels has been established for several other eucary-

otic mRNAs. For example, nonsense mutations within the yeast *URA3* gene (34) and frameshift mutations within the mouse immunoglobulin mu gene (7) have been associated with abnormally low amounts of mRNA. The study with yeast cells demonstrated that nonsense mutations do not affect the rate of mRNA synthesis and, thus, decrease mRNA levels by increasing the rate of mRNA turnover. As a rule for both the *URA3* and immunoglobulin mu genes, progressively higher mRNA levels were observed with nonsense codons that resided farther away from the initiation codon. The correlation of mRNA levels with the position of translation termination was not linear, however, since termination within the first half of the mRNA resulted in very low mRNA levels. The molecular basis for this nonlinearity is unclear, but is probably not attributable to differences in structure at the mutated nucleotide. Our data indicate that, unlike larger structural alterations, single-nucleotide nonsense or frameshift mutations affect mRNA levels primarily, if not exclusively, by the change in translation. It is possible that, as has been established for bacteria, ribosomes and the translation process function in the mechanism of mRNA decay passively by physically protecting the mRNA from nucleolytic attack (24, 27, 53). The general polarity that is evident in the *URA3* and immunoglobulin mu gene studies may, at least in part, reflect the accessibility of a larger number of nuclease-sensitive sites in mRNAs that have a nonsense codon closer to the initiation codon relative to mRNAs that have a nonsense codon farther from the initiation codon. This larger number would increase the probability and, consequently, the rate of degradation. At odds with these findings, our data demonstrate that TPI gene nonsense codons reduce TPI mRNA levels to roughly the same extent (20% of the normal level), regardless of their position relative to the initiation codon.

Other reports in which it has been indicated that nonsense and frameshift mutations in eucaryotes reduce mRNA levels have included descriptions of human thalassemic mutations that consist of a 2-bp frameshift mutation in codon 8 of the β-globin gene (45), a nonsense mutation in codon 39 of the β-globin gene (43, 45, 57), a nonsense mutation in codon 17 of the β-globin gene (14), and a single-nucleotide substitution within the α-globin gene that converts the normally used termination codon to a codon for glutamine and results in an α-globin protein that is extended by 31 amino acids (59). Of these mutations, only the last one has been reported to affect the mRNA half-life (reference 59 cited unpublished data). Interestingly, a single-nucleotide substitution within the α-globin gene that converts the initiation codon to a threonine codon is also associated with a reduced mRNA level (48). Theoretically, this substitution blocks translation initiation and, therefore, functions like a nonsense mutation. Additionally, our studies of a Kurdish Jew $\beta^0$-thalassemic mutation in codon 44 that produces a shift in the translational reading frame and a premature termination codon at position 60 have shown that the thalassemic β-globin mRNA is correctly processed yet is degraded with a half-life of 30 min in bone marrow cells (28, 38). It has not been established how the reduced α- and β-globin mRNA levels that are attributable to the above-described mutations reflect the change in mRNA translation or the change in mRNA structure. At least for the β-globin gene mutations, there is no linear correlation between the position of the nonsense codon and the steady-state mRNA level. Results of studies of the hemoglobinopathy hemoglobin (Hb) Niteroi (10) suggest that premature translation termination contributes to the mechanism of mRNA instability in the Kurdish Jew $\beta^0$-thalassemia. This hemoglobin variant is the result of a 9-bp deletion that overlaps with the thalassemic deletion. Unlike the thalassemic deletion, however, the Hb Niteroi deletion does not shift the translational reading frame. Since Hb Niteroi is present in peripheral blood at sufficiently high levels to allow structural analysis, the mRNA that encodes the β-globin chain of this protein undoubtedly has a half-life that is considerably longer than 30 min. In contrast, results of studies of β-globin RNA that harbors the nonsense codon at position 39 suggest that nuclear stability, transport to the cytoplasm, or both may be affected (26, 56).

The exact mechanism that is responsible for the decrease in TPI mRNA stability by premature translation termination remains to be determined. The finding that all nonsense codons upstream of position 189 (i.e., all nonsense codons tested) reduce the TPI mRNA half-life to approximately the same extent by virtue of premature translation termination suggests two possibilities which may be, but are not necessarily, mutually exclusive. For the first possibility, sequences that minimally reside between codon 189 (the 3′-most nonsense codon tested) and the normal stop codon at position 249 and that maximally span the entire translated region are accessible to cytoplasmic nuclease degradation when they are free of ribosomes. This possibility makes it necessary for any nontranslated TPI mRNA under normal metabolic conditions to be degraded. Data indicate that essentially all of the TPI mRNA in exponentially growing HL-60 cells is polysome-associated (A. J. Kinniburgh and S. Swartwout, personal communication). The absence of nontranslated mRNA may reflect either the possibility mentioned above or efficient translation initiation. The endonuclease that is responsible for this hypothetical degradation is unlikely to be associated with the 40S ribosome subunit, which is known to be bound to nontranslated mRNA but only as far as the initiation codon (30), unless TPI mRNA is normally folded in such a way that the initiation codon and the ribosome-free region, such as the region between codons 189 and 249 in $TPI^{189\ Arg\rightarrow Ter}$ mRNA, are in close proximity. The endonuclease may be associated with the 80S ribosome complex, as has been proposed for the 3′→5′ exonuclease that mediates histone mRNA degradation (22, 49), provided that sites exist throughout the translated region that are equally sensitive to endonucleolytic degradation, the rate-limiting degradative step, when they are free of ribosomes. Such a provision is necessary to account for the observation that translation termination at codon 23 and translation termination at codon 189 confer comparable lability to the template mRNA. Alternatively, the nuclease may not be ribosome-associated. In this case, the ribosome-free, nuclease-sensitive region necessarily resides between codons 189 and 249, to account for the finding that premature translation termination throughout the translated region results in the same reduction in mRNA half-life.

For the second possibility, 80S ribosomes that read through codons 189 to 249 confer a nuclease-resistant mRNA structure either by structurally altering the mRNA or by allowing for proper mRNA transport across the nuclear membrane. There is evidence for both bacteria and higher eucaryotes that the translation process can structurally alter the template mRNA. In bacteria, such structural alterations have been associated with changes in mRNA translatability and, perhaps, mRNA half-life. An example of a coordinate regulation of translation and mRNA decay in bacteria is the induction of *ermC* methylase gene expression by translational attenuation (18). According to the induction model, ribosomes actively function as regulatory effectors by virtue

of their concomitant interaction with mRNA and the antibiotic erythromycin. Unmethylated ribosomes that are bound by erythromycin stall while they translate the leader peptide RNA. This stalling not only alters the conformational state of the leader plus downstream RNA sequences to allow initiation of methylase synthesis but also induces *ermC* mRNA stabilization. mRNA stabilization may be mediated by the mRNA conformational change which, in turn, destroys an endonuclease target site, or by physical protection of the mRNA from degradative enzymes (8).

Coordinate changes in translation and mRNA secondary structure in higher eucaryotes are exemplified by studies in which it has been demonstrated that eucaryotic initiation factor 4F is an ATP-dependent, unwinding enzyme that is capable of catalyzing the melting of mRNA to a state of increased nuclease sensitivity (32). It is reasoned that eucaryotic initiation factor 4F functions to remove the high degree of secondary structure that is present in many if not all mRNAs and that is inhibitory to translational events subsequent to the factor-mRNA interaction such as ribosome binding and migration (30). By analogy, ribosomes that initiate and terminate TPI mRNA translation normally may preclude the formation of a structure that destabilizes the mRNA or allow the formation of a structure that confers stability to the mRNA. Alternatively, the translation of ribosomes may simply protect the mRNA from nucleolytic attack. Any of these possible events could be inhibited when translation terminates prematurely, and thus, TPI mRNA would be rapidly degraded. Furthermore, any of these events is compatible with the finding that translation termination within what is normally part of the 3′-untranslated region, as exemplified by the *Eco*RI-filled frameshift mutation, is not responsible for a reduced mRNA level. Other reports in which it has been indicated that translation termination downstream of the normal stop codon does not significantly reduce the template mRNA level include studies of two frameshift mutations within the human β-globin gene. These mutations give rise to elongated β-globin chains in Hb Tak and Hb Cranston at levels in blood that are close to normal (12, 33) and, therefore, do not appreciably lower the erythrocyte β-globin mRNA content.

It remains to be determined whether the half-life of cytoplasmic TPI mRNA is normally influenced by a nuclease-sensitive structure(s) in a ribosome-free region or an efficiently transported, stabilized structure when it is properly associated with ribosomes. Each structure may exist with a certain probability during normal TPI mRNA metabolism, and the fraction of mRNA molecules that is characterized by one or both structure(s) may determine the inherent degradation rate of TPI mRNA. Alternatively, and especially if TPI mRNA is translated with a high efficiency such that it is almost always associated with ribosomes, one or more other target sites for degradation and stabilization may specify the rate of TPI mRNA decay. Additional experiments are under way to determine the particular translational processes and *cis*-acting mRNA sequences that dictate the TPI mRNA half-life.

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