

## Nonsense-Mediated Decay of Human *HEXA* mRNA

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**Nonsense-mediated mRNA decay (NMD), the loss of mRNAs carrying premature stop codons, is a process by which cells recognize and degrade nonsense mRNAs to prevent possibly toxic effects of truncated peptides. Most mammalian nonsense mRNAs are degraded while associated with the nucleus, but a few are degraded in the cytoplasm; at either site, there is a requirement for translation and for an intron downstream of the early stop codon. We have examined the NMD of a mutant *HEXA* message in lymphoblasts derived from a Tay-Sachs disease patient homozygous for the common frameshift mutation 1278ins4. The mutant mRNA was nearly undetectable in these cells and increased to approximately 40% of normal in the presence of the translation inhibitor cycloheximide. The stabilized transcript was found in the cytoplasm in association with polysomes. Within 5 h of cycloheximide removal, the polysome-associated nonsense message was completely degraded, while the normal message was stable. The increased lability of the polysome-associated mutant *HEXA* mRNA shows that NMD of this endogenous mRNA occurred in the cytoplasm. Transfection of Chinese hamster ovary cells showed that expression of an intronless *HEXA* minigene harboring the frameshift mutation or a closely located nonsense codon resulted in half the normal mRNA level. Inclusion of multiple downstream introns decreased the abundance further, to about 20% of normal. Thus, in contrast to other systems, introns are not absolutely required for NMD of *HEXA* mRNA, although they enhance the low-*HEXA*-mRNA phenotype.**

Investigators studying genetic disorders have often found that some mutations are associated with very low levels of mRNA. Barring gross DNA alterations, these are usually frameshift or nonsense mutations (15, 21–23, 25, 28, 34, 35, 43, 46, 48, 61, 62). Among them are mutations of the *HEXA* gene, leading to Tay-Sachs disease (TSD), an autosomally inherited neurodegenerative disorder (26). While numerous mutations have been identified as the cause of TSD, the most common is a 4-bp insertion in exon 11 (1278ins4) of the 14-exon *HEXA* gene, shortening the reading frame by 100 codons. Patient fibroblasts homozygous for this frameshift mutation (or in compound heterozygosity with a splice site mutation) have undetectable levels of the nonsense-containing mRNA (48), even though transcription is normal (52). It is not the frameshift itself but the premature termination codon 9 nucleotides (nt) downstream that causes the low-mRNA phenotype (12).

The degradation of mRNAs containing premature stop codons, termed nonsense-mediated mRNA decay (NMD), is a process of much broader biological significance than removal of mRNA in genetic disease (4, 10, 33, 40–42, 44, 54, 56, 57, 66; L. E. Maquat, Editorial, *Am. J. Hum. Genet.* **59**:279–286, 1996). Organisms as diverse as yeast (54), worms (51, 56), plants (55), and mammals (41; Maquat, editorial), demonstrate this phenomenon. It is presumed that NMD prevents the synthesis of truncated peptides that could have dominant negative effects on the organism. The process serves as a general surveillance mechanism to abolish aberrant transcripts resulting not only from rare mutations but also from mistakes in RNA processing (56).

Numerous studies in the last decade have addressed the mechanism of NMD. In *Saccharomyces cerevisiae*, NMD occurs in the cytoplasm while the transcript is associated with polysomes (73). It has been suggested that when an early stop codon is reached, translation stalls and the message is scanned by specific factors. If a certain downstream element is encountered (53, 72), and if specific *trans* factors are present (1, 2, 20, 36, 53, 60), the message undergoes a type of conformational change that renders it sensitive to a 5' decapping enzyme independent of the normal poly(A) tail shortening, followed by a 5'-to-3' exonuclease-mediated degradation (27, 45). Thus, the data show that in yeast, NMD occurs in the cytoplasm (1, 2, 7, 18, 19, 30, 45, 53, 54, 57–60, 73).

However, very few mammalian nonsense messages studied to date show a cytoplasmic mode of decay. These include transcripts for  $\beta$ -globin from bone marrow cells of transgenic mice (38, 39), Rous sarcoma virus *gag* (4, 5), and selenium-dependent glutathione peroxidase I (44; P. M. Moriarty, C. C. Reddy, and L. E. Maquat, Letter, *RNA* **3**:1369–1373, 1997). Instead, most mammalian nonsense mRNAs display a nuclear or nucleus-associated mechanism of decay. These include transcripts for human triosephosphate isomerase (8, 9, 16, 17, 21, 69, 70), human  $\beta$ -globin in nonerythroid cells (6, 71), hamster dihydrofolate reductase (66), hamster adenine phosphoribosyltransferase (33), mouse major urinary protein (10), and mouse T-cell receptor  $\beta$  (13, 14). With these transcripts, the level of the nonsense mRNA that fractionates with the nucleus is reduced to the same degree as the level in the cytoplasm; furthermore, the nonsense mRNA that escapes into the cytoplasm has normal stability (6, 9, 10, 17, 21, 33, 62, 66). This implies that NMD occurs while the message is still associated with the nucleus. But translation remains essential, as shown by the stabilization of nonsense mRNAs in the presence of trans-

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lation-inhibiting drugs (13, 14), tRNA suppressors (8), and 5' stem-loop structures that prevent mRNA translation (8, 65).

Additional suggestions of nuclear involvement in NMD come from observations that exons harboring an early stop codon are often skipped (3, 22, 23), implying recognition of the reading frame in the nucleus. However, this interpretation is not universally accepted, and the suggestion of a reverse transcription-PCR artifact has been offered as an explanation for nonsense-mediated exon skipping (67, 68).

The strongest evidence that nuclear processes are important for NMD in mammalian cells comes from the failure of intronless minigenes (cDNAs) to reproduce the decay phenotype (50, 66, 70; X. Sun, and L. E. Maquat, Letter, *RNA* 6:1-8, 2000) and from the requirement for downstream introns (10, 14, 16, 65, 70, 71). To reconcile the nuclear involvement and the requirement for cytoplasmic translation, it has been proposed that exon-exon junctions are tagged upon completion of splicing, producing an mRNP capable of signaling decay when an early stop codon is present at a proper distance from the tag (31). Some transcripts would be translated, recognized as nonsense, and degraded in the cytoplasm, and others would undergo this process while still associated with the nucleus. In this paper, we address the site of decay and the requirement for introns for the NMD of the *HEXA* transcript.

#### MATERIALS AND METHODS

**Cell culture.** Normal (GM 03299D) and TSD (GM 11852) lymphoblasts (Coriell Institute) were cultured in suspension under 5% CO<sub>2</sub> in T75 flasks with RPMI 1640 medium (Life Technologies) containing 10% fetal bovine serum (Irvine Scientific), penicillin and streptomycin, nonessential amino acids, and sodium pyruvate. Cells were passaged 1 to 2 days earlier, so that they would be at a density of about  $2 \times 10^6$  cells/ml on the day of the experiment. To inhibit translation,  $2 \times 10^7$  to  $3 \times 10^7$  lymphoblasts were pelleted, washed once with phosphate-buffered saline, and resuspended in medium containing 28  $\mu$ g of cycloheximide/ml. To subsequently remove the cycloheximide, cells were pelleted, washed twice with phosphate-buffered saline, and resuspended in medium without the inhibitor. The dose of cycloheximide selected, 28  $\mu$ g/ml, inhibited translation by 93% after a 4-h incubation, as monitored by [<sup>35</sup>S]methionine-cysteine incorporation. After removal of cycloheximide, total translation increased to 63% of the initial level within 1 h and remained at that level for an additional 5 h. To inhibit transcription, 5  $\mu$ g of actinomycin D/ml (29, 33, 66) was added to the cycloheximide-free medium. Chinese hamster ovary cells were cultured under 5% CO<sub>2</sub> in 10-cm plates with alpha minimal essential medium (Life Technologies) and 5% fetal bovine serum.

**RNA extraction.** Total cellular RNA was extracted with RNA STAT 60 (Tel-Test) as per the supplier's protocol. Nuclear and cytoplasmic RNAs were isolated by methods described by Jakubowski and Roberts (32). Briefly, cells were lysed with 500  $\mu$ l of lysis buffer (10 mM Tris-HCl [pH 7.5], 1.5 mM MgCl<sub>2</sub>, 0.3 M sucrose, 0.5% NP-40, 0.25% sodium deoxycholate, and 0.5 U of RNase inhibitor [Boehringer Mannheim]/ $\mu$ l) and loaded onto 500  $\mu$ l of a cushion buffer (10 mM Tris-HCl [pH 7.5], 1.5 mM MgCl<sub>2</sub>, and 0.4 M sucrose). The samples were centrifuged for 10 min at 800  $\times$  g. The cytoplasmic supernatant was treated with proteinase K, extracted with phenol-chloroform, and precipitated with ethanol. The nuclear pellet was resuspended in 150  $\mu$ l of high-salt DNase buffer (10 mM Tris-HCl [pH 7.4], 500 mM NaCl, 5 mM MgCl<sub>2</sub>, and 0.1 mM CaCl<sub>2</sub>) and incubated for 30 min at 37°C with 100 U of DNase I (Life Technologies). The samples were then treated with proteinase K, extracted with phenol-chloroform, and precipitated with ethanol.

**Northern blot analysis.** Fifteen-microgram aliquots of total, nuclear, and cytoplasmic RNA were used for Northern blot analysis. The RNA was subjected to electrophoresis on a 1% agarose-formaldehyde gel and blotted onto a nylon membrane. The blots were stained with methylene blue to visualize the 18S and 28S rRNA and then probed with a <sup>32</sup>P-labeled full-length *HEXA* cDNA, a 360-bp 5' *HEXA* cDNA, a 600-bp  $\beta$ -actin cDNA, or an 800-bp neomycin resistance probe. A riboprobe was synthesized from the plasmid pSPU4b, kindly provided by Douglas Black (University of California, Los Angeles), for the 94-nt U4

snRNA probe (11). Radioactivity was visualized and quantitated with a PhosphorImager (Molecular Dynamics).

**Polysome profile analysis.** Polysomes were isolated following methods described by Duncan and Burgoyne (24). In brief,  $2 \times 10^7$  to  $3 \times 10^7$  lymphoblasts were lysed with 900  $\mu$ l of lysis buffer (50 mM Tris-HCl [pH 7.4], 100 mM KCl, 5 mM MgCl<sub>2</sub>, 1% Triton X-100, 1% sodium deoxycholate, 1 mg of heparin/ml, and 20  $\mu$ g of cycloheximide/ml) and loaded onto a 15-to-50% linear sucrose gradient. The sucrose solutions were made with gradient buffer (50 mM Tris [pH 7.4], 100 mM KCl, and 5 mM MgCl<sub>2</sub>) and poured into 14- by 89-cm Beckman UltraClear centrifuge tubes using a gradient mixer attached to a peristaltic pump. To disrupt polysomes, 30 mM EDTA was added to the lysis buffer. Samples were centrifuged at 4°C in an SW41 rotor at 40,000 rpm for 2.5 h. Polysome profiles were created with an ISCO model 185 gradient fractionator with a tube piercer, attached to a UA-6 absorbance monitor and fraction collector. Fourteen fractions were collected, and sodium dodecyl sulfate was added to each to a concentration of 0.5%. RNA was isolated by extraction with phenol-chloroform and then chloroform alone and precipitated with 3 M ammonium acetate and ethanol. The entire RNA sample from each fraction was used for Northern analysis.

**Construction of minigenes.** Standard molecular biology techniques were used in the construction of the normal and mutant *HEXA* minigenes and cDNAs. Restriction enzymes were purchased from Pharmacia, and the PCR enzymes TaqPlus Precision PCR system and *Pfu* DNA polymerase were purchased from Stratagene. All constructs were cloned into the pcDNA3.1(-) vector (Invitrogen) and were thus driven by the cytomegalovirus (CMV) promoter and carried a neomycin resistance (*neo*<sup>r</sup>) gene. All the mutant constructs except the W392X intronless minigene carried the 1278ins4 mutation in exon 11. The W392X mutation creates a stop codon in exon 11, about 114 nt upstream of the stop codon resulting from 1278ins4.

(i) **Construct 1.** Two *HEXA* minigenes (12) with four introns upstream and three downstream of the mutation were a kind gift from Richard L. Proia (National Institutes of Health). These minigenes, normal (TK $\alpha$ ) and mutant (Mut TK $\alpha$ ), were digested with *Sa*I, and the 8.8-kb fragment was cloned into the *Xho*I linearized pcDNA3.1(-) in order to transfer them from a herpes simplex thymidine kinase promoter to a CMV promoter.

(ii) **Construct 2.** The two minigenes were digested with *Kpn*I and partially digested with *Sa*I to purify a 6.5-kb fragment, which was ligated to a 1.45-kb *Sa*I/*Kpn*I fragment PCR amplified from the normal *HEXA* cDNA. This intermediate plasmid was linearized with *Kpn*I and ligated to a 200-bp *Kpn*I fragment from the original normal and mutant minigenes, digested with *Sa*I, and finally cloned into *Xho*I-linearized pcDNA3.1(-).

(iii) **Construct 3.** Two bands of about 3 and 7.9 kb were gel purified after digestion of the normal and mutant construct 2 with *Hpa*I and *Hind*III. The 3-kb fragment was further cut with *Psp*1406I to yield a 550-bp fragment, which was then ligated to the 7.9-kb *Hpa*I/*Hind*III fragment and a 420-bp *Hind*III/*Psp*1406I fragment PCR amplified from the normal *HEXA* cDNA.

(iv) **Construct 4.** Normal 2-kb *HEXA* cDNA was gel purified after digestion with *Xba*I and *Hind*III and then cloned into pcDNA3.1(-). The intermediate construct was cut with *Kpn*I to release a 72-bp fragment, which was then replaced with a 273-bp genomic *Kpn*I fragment with or without the 4-bp mutation.

(v) **Construct 5.** A 5.4-kb *Pvu*II/*Eco*RI fragment of construct 1 was cloned into pGEMEX-1 (Promega) and then digested with *Pvu*II and *Bam*HI to release a 4.6-kb vector. This vector was ligated to a 820-bp *Pvu*II/*Bam*HI fragment obtained either from the normal or mutant *HEXA* cDNA. This intermediate construct was digested with *Eco*RI yielding a 1.4-kb band that was ligated to an 8-kb *Eco*RI fragment of construct 1.

(vi) **Construct 6.** The mutant cDNAs (harboring the 1278ins4 or the W392X mutation) were both created with the PCR-based QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. Briefly, the normal *HEXA* cDNA in the pGEM-3Zi(+) (Promega) vector was PCR amplified by *Pfu* DNA polymerase (Stratagene), known for its high fidelity, with primers harboring the desired mutation. The nicked DNA was used to transform XL1-Blue ultracompetent cells after the parental DNA had been digested with *Dpn*I. The new mutant cDNAs were sequenced to confirm the presence of the mutation. They were then digested with *Xba*I and *Pst*I to release a 2-kb fragment, which was cloned into pBluescript II KS(+) (Stratagene). This intermediate construct was cut with *Xba*I and *Eco*RI to yield a 2.1-kb band that was finally cloned into pcDNA3.1(-).

**Transfections.** CHO cells were transiently transfected with the various constructs using the Lipofectamine Plus system (Life Technologies) according to the manufacturer's directions. Transfection was carried out in 10-cm plates with 4  $\mu$ g of DNA in serum-free medium for 3 h. Serum was added to 5%, and about 40 h later, RNA was extracted for analysis as described above.

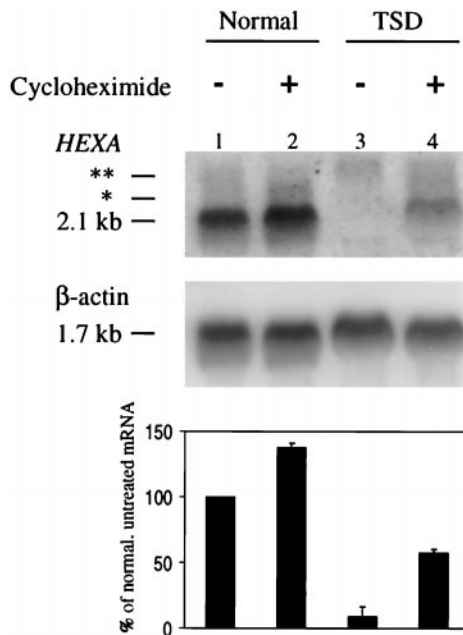


FIG. 1. Stabilization of the nonsense TSD mRNA with cycloheximide. (Top) Northern blot analysis of total RNA isolated from lymphoblasts, either normal (lanes 1 and 2) or TSD (lanes 3 and 4), with and without a 4-h cycloheximide treatment. (Bottom) PhosphorImager quantitation of the average amount of *HEXA* mRNA in the above lanes and in two other experiments, normalized to  $\beta$ -actin. Error bars indicate standard deviations. The mRNA measured is the major species of 2.1 kb. The two minor species (2.4 [\*] and 2.6 [\*\*] kb, respectively) are described in the text.

## RESULTS

### Translation is required for decay of nonsense *HEXA* mRNA.

The effect of inhibiting translation on nonsense mRNA abundance was examined by treating normal and TSD lymphoblasts with cycloheximide. Total RNA was analyzed by Northern blotting, which in these and subsequent experiments showed three distinguishable *HEXA* species—a major species of 2.1 kb and two minor species of 2.6 and 2.4 kb (Fig. 1 and 2). The 2.1-kb mRNA migrated in the region of the 18S rRNA, which occasionally disrupted the hybridization signal. The minor 2.6-kb transcript hybridized with several nonoverlapping *HEXA* probes and disappeared within 2 h of actinomycin D treatment (data not shown); it is therefore thought to be a pre-mRNA. It was present in normal and mutant cells, appeared to be insensitive to NMD, and was not studied further. The other minor species, of 2.4 kb, hybridized to an alternative 3' untranslated region probe (data not shown), showing that it was the previously described *HEXA* mRNA with a longer 3' untranslated region (47). Though the 2.4-kb message was subject to NMD, only the abundant full-length 2.1-kb mRNA was used for all analyses.

As seen in Fig. 1, the *HEXA* mRNA was nearly undetectable in TSD lymphoblasts, less than 10% of nonsense-free mRNA, prior to treatment with cycloheximide. After 4 h of treatment, the level of TSD mRNA had increased to 56% of untreated normal mRNA or 40% of cycloheximide-treated normal mRNA. Cycloheximide withdrawal, which allowed translation

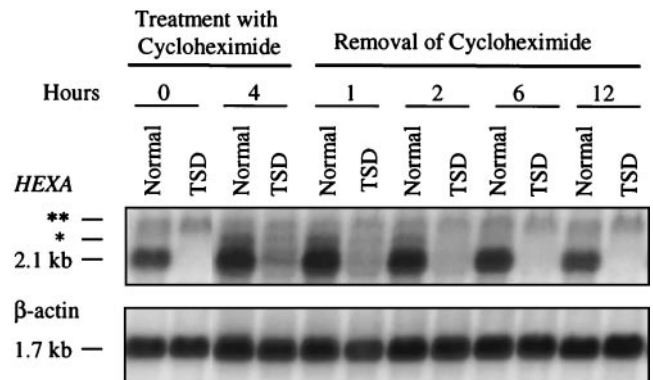


FIG. 2. Decay of the cycloheximide-stabilized pool of *HEXA* mRNA. Northern blot analysis of total RNA isolated from lymphoblasts before treatment (0 h), after a 4-h treatment with cycloheximide, and 1, 2, 6, and 12 h following cycloheximide removal. Blots were rehybridized with a  $\beta$ -actin cDNA probe for normalization. Asterisks indicate minor mRNA species as in Fig. 1.

to resume within an hour, caused the level of the accumulated mutant mRNA to decrease progressively until it reached the low steady-state level in 6 h (Fig. 2). Quantitation of the data in Fig. 2 revealed that half the mutant message had disappeared in about 3 h (data not shown). On the other hand, the half-life of the normal mRNA was estimated using actinomycin D to be more than 30 h (data not shown), far longer than the time the lymphoblasts could be maintained with the transcriptional inhibitor.

**Cycloheximide-stabilized nonsense *HEXA* mRNA is found only in the cytoplasmic fraction.** Cellular fractionation and Northern blot analysis were used to determine the cellular distribution of the normal and nonsense *HEXA* mRNA. As shown in Fig. 3, in the absence of cycloheximide, the *HEXA* mRNA was lacking in both the nuclear and cytoplasmic fractions of TSD cells, while it was present almost exclusively in the

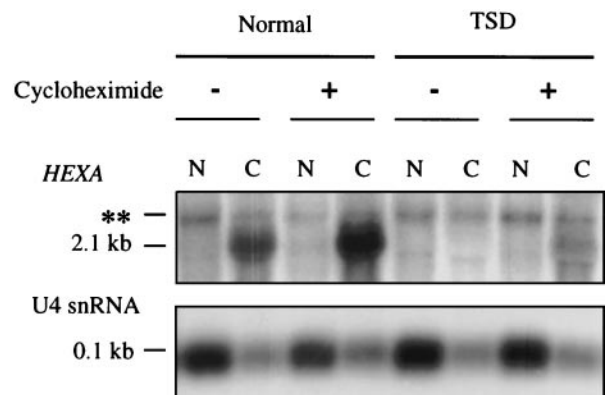


FIG. 3. Northern blot analysis of nuclear and cytoplasmic *HEXA* mRNA distribution. Lymphoblasts, before or after a 4-h treatment with cycloheximide, were lysed with buffer containing 0.5% NP-40 and loaded onto a 0.4 M sucrose pad to pellet nuclei. RNA was extracted from the supernatant (cytoplasmic [C]) and the pelleted (nuclear [N]) fractions. Approximately 15  $\mu$ g of nuclear and cytoplasmic RNA, or about a tenth of the cytoplasmic and about half of the nuclear yield, was used for Northern analysis. Blots were reprobed for the nuclear fraction-enriched U4 snRNA. \*\*, 2.6-kb NMD-insensitive transcript.

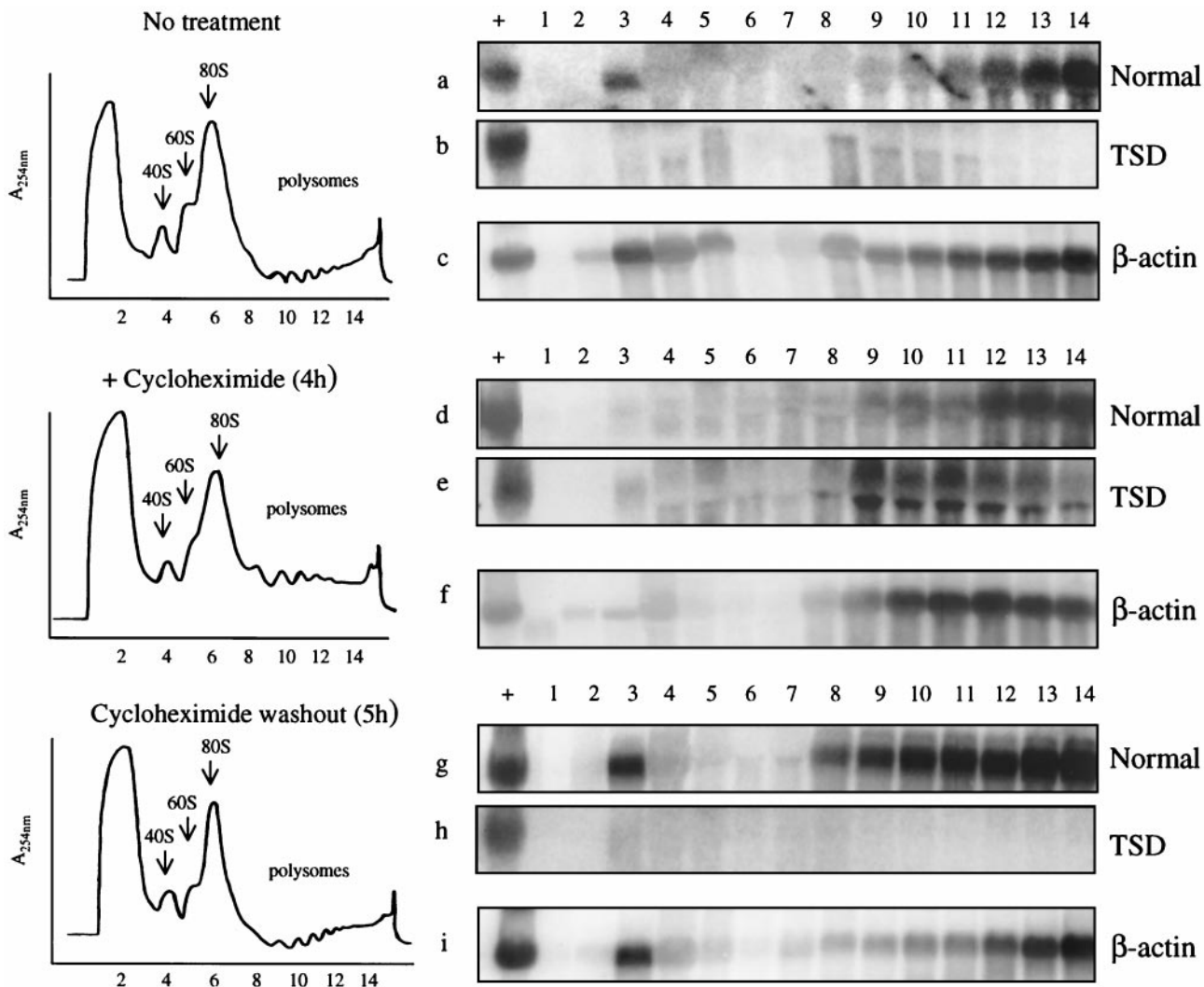


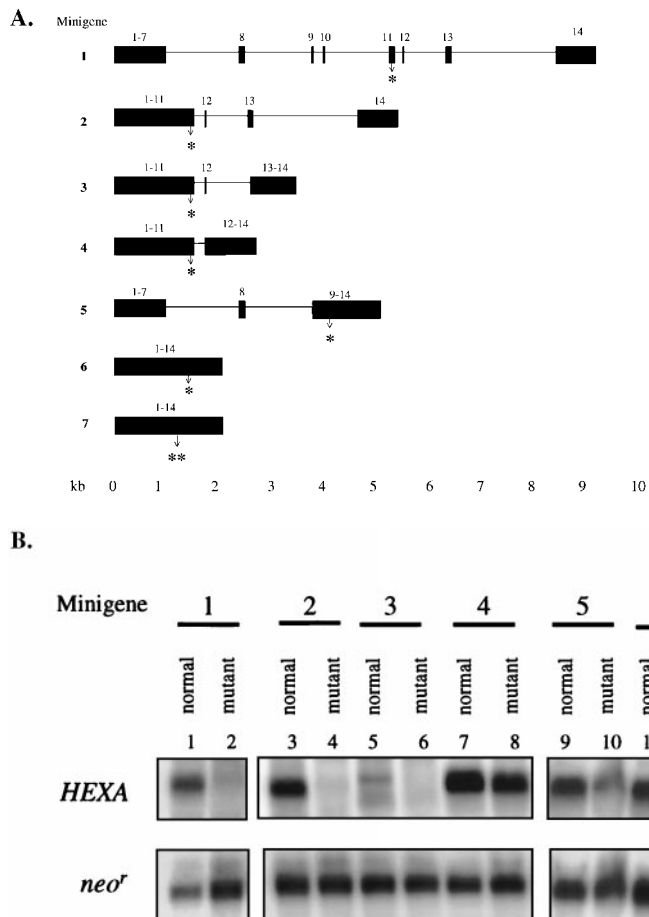
FIG. 4. Polysome profiles and Northern blot analysis of density gradient fractions of normal and TSD lymphoblasts. Cells were untreated, treated with cycloheximide for 4 h, or treated and then released from cycloheximide for 5 h. Samples were then lysed and centrifuged through a 15-to-50% linear sucrose gradient. A UV absorbance monitor continuously monitored the gradient before separation into 14 fractions. The locations of the 40S and 60S ribosomal subunits, the 80S monosomes, and the polysomes are indicated. RNA was extracted from each fraction for Northern blot analysis. Fifteen micrograms of total RNA from normal GM 03299D cells was loaded on all gels as a positive control for hybridization (lanes +). The entire RNA yield from the gradient fractions was used for the blots. Blots were initially probed with the full-length 2-kb *HEXA* cDNA probe (a, b, d, e, g, and h) and then reprobed with a  $\beta$ -actin probe (c, f, and i). Polysome profiles and  $\beta$ -actin Northern blots are shown only for preparations from TSD cells, but equivalent preparations from normal cells gave similar results.

cytoplasmic fraction of normal cells. After treatment with cycloheximide, the stabilized TSD mRNA was elevated exclusively in the cytoplasmic fraction, with no noticeable changes in the nuclear fraction. (The NMD-insensitive 2.6-kb transcript was seen in all fractions and is not included in the analysis.) Although some leakage from nucleus to cytoplasm may have occurred, the nuclear fraction appeared to be generally intact, as shown by enrichment of the snRNAs U4 (Fig. 3) and U6 (data not shown).

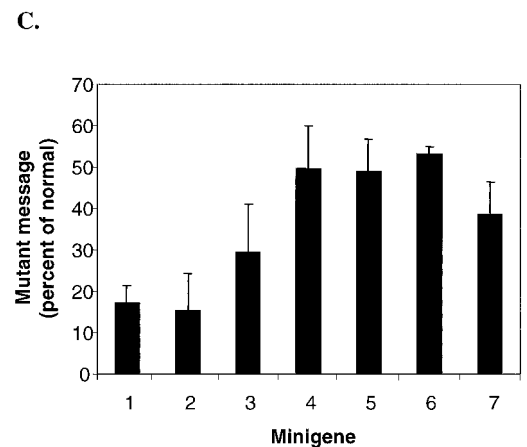
**Nonsense *HEXA* mRNA accumulates on polysomes in the presence of cycloheximide and disappears from polysomes upon removal of the inhibitor.** The cytoplasmic *HEXA* mRNA distribution was determined by Northern blot analysis of ribosome fractions from normal and TSD lymphoblasts. Fractionation of cell lysates through a sucrose gradient separated

ribosomal subunits (40S and 60S), monosomes (80S), and polysomes into 14 fractions. The absorbance profile of each sample at 254 nm showed polysomes to be abundant in fractions 9 to 14 (Fig. 4, left panels). The presence of polysomes in these fractions was verified by the addition of 30 mM EDTA, which disrupts polysomes, to a sample lysate prior to centrifugation. In the presence of EDTA, absorbance shifted from the polysome peaks (fractions 9 to 14) to the upper monosome or soluble phase (fractions 2 to 7), and most of the polysome-associated *HEXA* message shifted to the lighter fractions (data not shown).

The effect of cycloheximide treatment and removal on the nonsense mRNA associated with polysomes is shown in the Northern blots depicted in Fig. 4. Cycloheximide inhibits peptide elongation and freezes polysomes on mRNA. Prior to



**FIG. 5.** Transient transfection of CHO cells with normal and mutant *HEXA* minigenes. (A) Seven constructs containing the indicated exons (black box) and introns (black horizontal line) were made. All intron-containing mutant minigenes carried the 1278ins4 mutation (\*) (constructs 1 to 5). This placed the early stop codon 41 nt upstream of intron 11, as in the naturally occurring mutation. Of the two intronless minigenes (6 and 7), the first has the 1278ins4 mutation (\*) and the other has the W392X mutation (\*\*). The sites of these mutations are shown with an arrow. (B) Representative Northern blot analysis of RNA extracted 40 h after transient transfection of CHO cells with the minigenes in panel A. Fifteen micrograms of total RNA was used, and the blots were first probed with the 360-bp *HEXA* 5' probe and then reprobred for the neomycin resistance (*neo<sup>r</sup>*) mRNA. (C) *HEXA* mRNA levels were quantitated with a PhosphorImager, normalized to *neo<sup>r</sup>* mRNA levels, and then expressed as a percentage of the normal counterpart. The average of three experiments is shown, with standard deviations.



cycloheximide treatment, there was negligible mutant mRNA associated with polysomes (Fig. 4b), in contrast to a substantial amount of normal mRNA (Fig. 4a). After 4 h of cycloheximide treatment, TSD mRNA appeared in polysomal fractions 8 to 14 (Fig. 4e). Upon removal of the inhibitor, the TSD mRNA disappeared from all polysomal fractions (Fig. 4h) while the normal mRNA continued to associate with polysomes either in the presence of actinomycin D (Fig. 4g) or in its absence (data not shown). Northern blots of the  $\beta$ -actin mRNA from TSD cells showed approximately equal loading (Fig. 4c, f, and i), as did blots of  $\beta$ -actin mRNA from normal cells (data not shown). These experiments show that polysome-associated mutant *HEXA* mRNA is subject to degradation when translation is resumed.

**Introns are not absolutely required but enhance NMD of *HEXA* mRNA.** Intron-containing and intronless *HEXA* minigenes (Fig. 5A), driven by the CMV promoter, were transiently expressed in CHO cells. All but one of the mutant minigenes had the common 1278ins4 mutation, which shortens the reading frame by 100 codons to four-fifths of the normal frame. An additional nonsense cDNA, W392X, was tested for comparison, as it also had been shown to result in low mRNA in cells of TSD patients (61). The nonsense codon W392X is located 117 nt upstream of the nonsense codon of 1278ins4 (Fig. 5A, constructs 6 and 7). For each construct, the *HEXA* mRNA was subject to Northern blot analysis (Fig. 5B), quantitated by

PhosphorImager, and normalized to the *neo<sup>r</sup>* mRNA transcribed from the same plasmid in order to account for differences in transfection and RNA recovery (Fig. 5C).

The mutant minigenes with four introns upstream and/or only three introns downstream of the frameshift mutation, minigenes 1 and 2, expressed nonsense message at a fifth of the normal mRNA, showing that upstream introns are not essential for NMD. Further deletion of the downstream intron 13 (minigene 3) resulted in nonsense mRNA at about a third of normal mRNA level. Minigene 4, with only intron 11, or minigene 5, with the upstream introns 7 and 8 resulted in nonsense mRNA at about half the normal mRNA level. Even minigenes without any introns (cDNAs) produced nonsense mRNAs that were present at lower levels than normal; the 1278ins4 nonsense message was present on average at 53% of normal, while the W392X nonsense message was found to be at 39% of normal (minigenes 6 and 7, respectively). These data show that some NMD of the *HEXA* mRNA is seen when the nonsense codon is in the terminal or penultimate exon or in an intronless minigene. Maximal NMD is seen if there are two or more downstream introns.

## DISCUSSION

NMD of mammalian messages occurs most often while the transcript is still associated with the nucleus (6, 8–10, 13, 14, 16, 17, 21, 33, 66, 69–71), though in a few cases it occurs when the

mRNA is in the cytoplasm (4, 5, 38, 39, 44; Moriarty et al., letter). Our studies show that NMD of the *HEXA* mRNA is similar to all other mammalian mRNAs in that ongoing translation is required for decay (Fig. 1 to 4). However, it differs from nucleus-associated NMD (33, 63) in that the cytoplasmic, polysome-associated nonsense *HEXA* message remains sensitive to decay (Fig. 4); thus, the *HEXA* transcript may be added to the short list of mammalian messages that follow a cytoplasmic mode of decay. In addition, NMD of the *HEXA* message differs from that of all mammalian transcripts studied to date (49, 64) in that downstream introns are not absolutely required, although they enhance the process.

Although we have shown degradation of cytoplasmic nonsense *HEXA* mRNA, we cannot exclude the possibility that some TSD transcripts are degraded while associated with the nucleus. First, we could demonstrate stabilization of the nonsense mRNA pool to only 40% of normal. Second, any increase in nucleus-associated nonsense mRNA in the presence of cycloheximide would not have been observed, because the *HEXA* mRNA was not detected even in the nuclear fraction of normal cells, implying rapid processing and export of this transcript.

Of the few mammalian mRNAs that follow a cytoplasmic mechanism of decay (5, 38, 39), the glutathione peroxidase I transcript, the one best studied, requires an intron at least 59 nt downstream of the early stop codon (44, 64; Moriarty et al., letter), as do transcripts that decay while associated with the nucleus (10, 49, 65, 70, 71). To explain the necessity of introns and the requirement of cytoplasmic translation, a model has been proposed in which transcripts, upon completion of splicing, are marked at each exon-exon junction (31, 65, 70, 71). This mRNP would be exported out of the nucleus, translated, and degraded if an early stop codon was reached and there was an exon-exon junction marker at a proper distance downstream. If the proper mRNP structure was not created (i.e., there was no splicing to create a downstream exon-exon junction marker [14], or if the marker was too close to the early stop codon [49, 64]), then that message could escape decay.

Our data showing that intronless *HEXA* minigenes that harbor nonsense codons give rise to low mRNA add an interesting complexity to the mechanism of NMD; how can the same mechanism operate on intron-containing and intronless genes containing early stop codons with the same effect? As mentioned above, it has been proposed that certain factors bind to exon-exon junctions, and spliceosomal proteins (i.e., SRm160 and hPrp8p) have been found to associate with spliced mRNA at such junctions (37), creating a certain mRNP structure. It could be envisioned that some nonsense transcripts would rely on the process of splicing to create the proper mRNP complex to facilitate NMD, but others, resembling nonsense mRNAs in yeast, would rely partially or exclusively on sequences in *cis* to create the functionally equivalent mRNP structure. In addition, the mRNP structures derived either with or without splicing could facilitate NMD with different efficiencies. Such may be the case for the *HEXA* message, for which the decrease in abundance in the absence of introns was not as severe as when introns were present.

To our knowledge, this is the first time intronless nonsense

*HEXA* minigenes have been shown to reproduce, if incompletely, the low-mRNA phenotype. Previously, the level of nonsense *HEXA* mRNA was found to be normal in transfected COS-1 cells (50). But COS-1 cells may not be suitable for the study of NMD, at least of this transcript. They failed to show NMD when transfected with an intron-containing *HEXA* minigene, even though the same minigene was subject to NMD in mouse L cells (12).

These results, in the context of the published literature, confirm that NMD in mammalian cells is a multipathway phenomenon, with some transcripts following a nuclear or nucleus-associated pathway and others following a cytoplasmic pathway. It is also possible that the pathways are hierarchical and that nonsense codons can be recognized and degraded, with various efficiencies, at different stages following transcription.

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