Nonsense Mutations in Close Proximity to the Initiation Codon Fail to Trigger Full Nonsense-mediated mRNA Decay*

Received for publication, May 5, 2004, and in revised form, May 25, 2004
Published, JBC Papers in Press, May 25, 2004, DOI 10.1074/jbc.M405024200

Ángela Inácio‡§, Ana Luisa Silva‡§, Joana Pinto‡§, Xinjun Ji‡, Ana Morgado‡§, Fátima Almeida‡§, Paula Faustino‡, João Lavinha‡, Stephen A. Liebhaber¶, and Luísa Romão‡¶

From the Centro de Genética Humana, Instituto Nacional de Saúde Dr. Ricardo Jorge, Av. Padre Cruz, 1649-016 Lisboa, Portugal and the Departments of Genetics and Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Nonsense-mediated mRNA decay (NMD) is a surveillance mechanism that degrades mRNAs containing premature translation termination codons. In mammalian cells, a termination codon is ordinarily recognized as "premature" if it is located greater than 50–54 nucleotides 5' to the final exon-exon junction. We have described a set of naturally occurring human β-globin gene mutations that apparently contradict this rule. The corresponding β-thalassemia genes contain nonsense mutations within exon 1, and yet their encoded mRNAs accumulate to levels approaching wild-type β-globin (βWT) mRNA. In the present report we demonstrate that the stabilities of these mRNAs with nonsense mutations in exon 1 are intermediate between βWT mRNA and β-globin mRNA carrying a prototype NMD-sensitive mutation in exon 2 (codon 39 nonsense; β39). Functional analyses of these mRNAs with 5'-proximal nonsense mutations demonstrate that their relative resistance to NMD does not reflect abnormal RNA splicing or translation re-initiation and is independent of promoter identity and erythroid specificity. Instead, the proximity of the nonsense codon to the translation initiation AUG constitutes a major determinant of NMD. Positioning a termination mutation at the 5' terminus of the coding region blunts mRNA destabilization, and this effect is dominant to the "50–54 nt boundary rule." These observations impact on current models of NMD.

Nonsense-mediated mRNA decay (NMD) is an mRNA surveillance mechanism that rapidly degrades mRNAs carrying premature translation termination codons (1). Nonsense-containing mRNAs targeted by NMD can be generated by naturally occurring frameshift and nonsense mutations, splicing errors, leaky 40 S scanning, and utilization of minor AUG initiation sites (2, 3). A major function of the NMD pathway is to block the synthesis of truncated proteins that could have dominant negative effects on cell function (2, 4).

Recent studies have shown that the NMD pathway in mammalian cells is linked to splicing-dependent deposition of a protein complex 20–24 nucleotides (nt) 5' of each exon-exon junction (exon-junction complex; EJC). The EJC contains the general splicing activator RNP51, the RNA export factor Aly/REF, the shuttling protein Y14, the nuclear matrix-localized serine-arginine-containing protein SRm160, the oncprotein DEK, and the Y14 binding protein magoh. The interaction of magoh with Y14 may have a role in cytoplasmic localization of mRNAs and in anchoring the NMD-specific factors Upf3 and Upf2 to the mRNA (5–18). Previous published data have shown that Upf3 and Upf2 join the EJC in different subcellular compartments: Upf3 (Upf3a and Upf3b) is loaded onto mRNAs in the nucleus during splicing via interactions with components of the EJC. In contrast, Upf2 joins the complex soon after cytoplasmic export is initiated (14, 19, 20). According to the present models, translating ribosomes displace EJCs from the open reading frame (ORF) during the "pioneer" round of cytoplasmic translation (21). If, however, the mRNA contains a premature termination codon located more than 50–54 nt 5' of at least one EJC, complex components 3' to the termination mutation will remain on the mRNA. The retention of one or more EJCs on the mRNA triggers the NMD response by an as yet undefined mechanism. According to current models, recognition of the nonsense codon as "premature" involves a direct interaction of the phosphorylated Upf1 RNA helicase with EJC-bound Upf2 (20, 22, 23). Interactions between Upf1 and other Upf factors may also be involved in this process (14, 19, 20, 23–26). The interactions of the translation termination factors eRF1 and eRF3 with Upf1 and of eRF3 with Upf2 and Upf3 appear to provide a mechanistic link between NMD and translation termination at the premature stop codon (reviewed in Refs. 1 and 27).

Studies supporting the "50–54 nt boundary rule" suggest that an exon-exon junction serves as the major cis-acting NMD-regulatory element (28–30). Although EJC-regulated NMD is supported by multiple lines of evidence, exceptions to the 50–54 nt boundary rule have been reported. These exceptions suggest that additional determinants may be involved in determining the net stability of nonsense-containing mRNAs. For example, nonsense codons positioned close to the initiation AUG of the TPI, immunoglobulin μ heavy chain, and BRCA1 mRNAs, fail to specify NMD (31–33). For the TPI and immunoglobulin μ heavy chain mRNAs, NMD appears to be circumvented by re-initiation of translation 3' to the nonsense codon. This mechanism of NMD avoidance has also been speculated for nonsense codons located early in the BRCA1 transcript (33).
Proximity of Nonsense Codon to AUG Inhibits NMD

**Table 1**

DNA oligonucleotides used in the current study

| Primer* | Sequence (5' → 3') | Genomic position |
|---------|-------------------|-----------------|
| L5-β-S | TAAGCCAGTGGCCAAGAG | β 5' flanking |
| DelA-AS | CAACTGCGCGGCGACGTCCACATCGTTG | β Exon 1 |
| DelA-AS | CACCTGCGCGGCGACGTCCACATCGTTG | β Exon 1 |
| R5-β-AS | TCCCCATTTCAATGTTACCC | β Intron 2 |
| Seqhet-AS | GCCATGCGCGGCGACGTCCACATCGTTG | Compound β/α |
| Seqhet-AS | TACACGCTCGCGGCGACGTCCACATCGTTG | Compound β/α |
| 3' exon1-AS | GCCATGCGCGGCGACGTCCACATCGTTG | β Exon 1/Intron 1 |
| 3' exon2-AS | TCAGATGCGCGGCGACGTCCACATCGTTG | β Exon 2 |
| ATG-S | GACAGATTGTTCAATTA | β 5' UTR/exon 1 |
| 62-AS | TGGACATGCTTACATCGTACAGG | β Exon 2 |
| 62-S | CTTAAAGGGGAAATGATTGCACTGGAGA | β Exon 2 |
| 15non39-AS | CTTGACATGCTTACATCGTACAGG | β Exon 2 |
| 15non39-S | CTTGACATGCTTACATCGTACAGG | β Exon 1 |
| 55-AS | GGTGGTCTTACATCGTACAGG | β Exon 2 |
| 55-S | GGTGGTCTTACATCGTACAGG | β Exon 2 |
| 73-74-AS | GCCATGCGCGGCGACGTCCACATCGTTG | β Exon 2 |
| 73-74-S | GCCATGCGCGGCGACGTCCACATCGTTG | β Exon 1 |
| 63-64/S | GGTGGTCTTACATCGTACAGG | β Exon 2 |
| 63-64/AS | GGTGGTCTTACATCGTACAGG | β Exon 2 |
| LinkClaI-S | CCATGCTTACATCGTACAGG | β UTR |
| LinkClaI-AS | CCATGCTTACATCGTACAGG | β UTR |
| WT15:39-AS | GTTCACCTTGCCCCAGGGCAGTAACGGC | β 3' flanking |
| WT15:39-S | GTTCACCTTGCCCCAGGGCAGTAACGGC | β 3' flanking |
| 15non39-AS | CTGCCCTGTGAGGCAAG | β 3' flanking |
| 15non39-S | CTGCCCTGTGAGGCAAG | β 3' flanking |
| pTetXhoI1/15-S | CCCCTCGAGTTTACCACT | pTet-Splice |
| pTetClaI51/370-AS | CCATGCTTACATCGTACAGG | pTet-Splice |
| WT15:39-S | GGCTTACCTGCCTCGGCCCCCTGGGTCGGGCAAGGTGACCC | β Exon 1 |
| WT15:39-AS | GGCTTACCTGCCTCGGCCCCCTGGGTCGGGCAAGGTGACCC | β Exon 1 |
| 15non39-AS | GGCTTACCTGCCTCGGCCCCCTGGGTCGGGCAAGGTGACCC | β Exon 1 |
| 15non39-S | GGCTTACCTGCCTCGGCCCCCTGGGTCGGGCAAGGTGACCC | β Exon 1 |
| HindIII Amp-S | TTTAACTTATATCCAGGTGATCTCAAC | Amp* |
| HindIII Amp-AS | TTTAACTTATATCCAGGTGATCTCAAC | Amp* |
| GA-S | AAGGGGGCACTGCTTCTCGGCGGCAAG | Amp* spacer |
| GA-AS | AAGGGGGCACTGCTTCTCGGCGGCAAG | Amp* spacer |
| Primer extension assay | CTGCCTGACATGCTTACATCGTACAGG | Mouse α exon 1 |
| Hplexon3-AS | CACTCTTCTGATAGGCCCGCTGC | β Exon 3 |
| Hplexon3-UTR | GAAAGGGCACTTACATCGTACAGG | β 3' UTR |
| Mu-AS | GCCATGCGCGGCGACGTCCACATCGTTG | Mouse α exon 1 |
| RT-PCR assay | GCCATGCGCGGCGACGTCCACATCGTTG | Mouse α exon 1 |
| Hβ3-RNA-AS | GGAATGGAAAAATATGTTTATTTATA | β 3' UTR |
| Hβ3-RNA-AS | AATTTGATGCTTCACACAC | β 5' UTR |
| Hβ3exon2-AS | TGATGCGCTCGGCTACATCGT | β Exon 2 |
| Hβ3exon2-AS | TGATGCGCTCGGCTACATCGT | β Exon 2 |

* Primer designations reflect the corresponding utilization; S means sense and AS means antisense.

- The underlined sequences correspond to the introduced mutation.
- The underlined sequences correspond to the restriction site indicated in the primer designation.
- The underlined sequences correspond to the HindIII-Apal restriction sites.

Such re-initiation would allow the ribosome to disrupt EJC's located 3' to the nonsense mutation (31, 32). Nonsense codons in the T-cell receptor-β transcript also fail to elicit canonical NMD; nonsense codons located more 5' in the mRNA trigger robust NMD, whereas nonsense codons closer to the terminal exon-exon junction induce a much weaker NMD response (34). Finally, premature termination codons located in different exons of the fibrinogen Aα-chain gene or at exon 2 of the human ALG3 gene can avoid NMD (35, 36). The basis for NMD avoidance by the fibrinogen Aα-chain and ALG3 mRNAs remains undefined. Thus, present evidence indicates that determinants of NMD, at least in some systems, are more complex than would be predicted on the basis of the single 50–54 nt boundary rule.

We have previously reported that human β-globin mRNAs containing naturally occurring nonsense mutations in the 5'-part of exon 1 accumulate to levels similar to those of normal β-globin transcripts (37). The aim of the present study is to investigate the mechanism by which these mutant β-globin transcripts circumvent the full impact of NMD. Our results reveal that this unusual NMD behavior reflects the proximity of the nonsense mutations to the AUG initiation codon. Remarkably, the impact of this 5' localization is dominant to the 50–54 nt boundary rule.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—The plasmids containing the normal (pWT), β15 [CD 15 (TGG—>TGA)], or β39 [CD 39 (C—>T)] human β-globin genes were previously described (37). All variants were created within the 428-bp NcoI-BamHI fragment of the β-globin gene template by overlap-extension PCR (37). PCR reactions were performed by using Invitrogen primers (Barcelona, Spain; see Table 1). To create variants carrying DelA (deletion of 69 bp between codons 3 and 27, exclusively), PCR reaction 1 was performed by using L5-β-S and DelA-AS primers, and DNA template from the previously cloned pWT gene. PCR reaction 2 was performed by using DelA-S and R5-β-AS primers and the β39 genes as template. To clone variant βWTseqhet and βWT39seqhet constructs, which contain a different sequence between codons 3 and 27, from the second exon of the human α2-globin gene (from codon 34 through codon 56), PCR reaction 1 was performed by using Seqhet-S and Seqhet-AS primers, and the h2-globin gene as the DNA template (pTet-Wt detailed in Ref. 38). PCR reaction 2 was performed by using 3' exon1-1S and 3' exon2-AS primers and the βWT or β39 gene DNAs. To obtain β62 and β62ΔDeLA variants carrying the nonsense mutation (GCT—>TAG) at codon position 62, PCR reaction 1 was performed with ATG-S and 62-AS primers and the previously cloned βWT or DeLA gene DNAs. PCR reaction 2 was performed with 62-S and 3' exon2-AS primers and the βWT gene as DNA template. To clone the double nonsense mutated gene β15non39, carrying both nonsense mutations at codons 15 and 39, PCR reaction 1 was performed by using L5-β-S and 15non39-AS primers and the β39 gene DNA. PCR reaction 2 was performed by using 15non39-S and R5-β-AS primers and the same DNA.
Proximity of Nonsense Codon to AUG Inhibits NMD

To obtain the constructs β7-D55–63/64–73/74, β15-D55–63/64–73/74, and β39-DelA-D55–63/64–73/74, the three different mutations were introduced sequentially and the βWT-D55, β15-D55, β39-D55, and β39DelA-D55 genes (βWT, β15, β39, or β39DelA genes carrying a missense mutation that converts Met to Ile (AUG to AUA) at codon 55) were created first. Thus, PCR reaction 1 was performed by using the L5′-β-S and 55-AS primers, and the βWT, β15, β39, or β39DelA gene DNA. PCR reaction 2 was performed by using the 55-S and the RS′-β-AS primers and the βWT DNA template. To create the βWT-D55–73/74, β15-D55–73/74, β39-D55–73/74, and β39DelA-D55–73/74 constructs (corresponding to the βWT-D55, β15-D55, β39-D55, or β39DelA-D55 human β-globin genes carrying an additional missense mutation that converts the out-of-frame Met to Thr (AUG to AUG) at codon 73) thoroughly, Electric shock was performed using the L5′-β-S and 73/74-AS primers, and the βWT-D55, β15-D55, β39-D55, or β39DelA-D55 genes as DNA template. PCR reaction 2 was performed by using the 73/74-S and the RS′-β-AS primers and the βWT DNA template. In all cases, PCR reaction 3 was performed using a mix of PCR reactions 1 and 2 and primers sense from PCR reaction 1 and antisense from PCR reaction 2. The obtained PCR products were digested with NcoI-BamHI and replaced into the plasmid containing the cloned normal human β-globin gene previously digested with the same enzymes. The mutation at position 63/64 (ATG to ATT) was introduced into the genes βWT-D55–73/74, β15-D55–73/74, β39-D55–73/74, and β39DelA-D55–73/74 by using the QuikChange™ site-directed mutagenesis kit using the GA-S and the pGEM-3 plasmid (Promega) as DNA template. The cloned NcoI-XhoI1/15-S and pTetClaI351/370-AS primers (Table I). The human fragment by the 374-bp DNA fragment that was amplified using pTet-βWT plasmid containing the WT-CD55, human GA-AS oligonucleotides (Table I). The pTet plasmid containing the βWT vector was linearized and transcribed in the presence of [32P]CTP. Amplification primers contained restriction sites that facilitated insertion into the vector polylinker. To generate RNA probes, each transcription vector was linearized and transcribed in the presence of [32P]CTP. For RNA isolation—Total RNA from transfected cells was prepared using the RNAeasy total kit (Qiagen) following the manufacturer’s instructions. Before analysis, RNA samples were treated with RNase-free DNase I (Ambion) and purified by phenol-chloroform extraction.  

**Primer Extension Analysis**—Primer extension assays were performed using 5′-end-labeled primers: Hβexon3-5′ or Hβ3′ UTR-AS and Me-AS (Table I). Both primers were mixed, hybridized with 10 μg of RNA from cells transfected with the pTet2β-plasmid (for transcription efficiency and the appropriate pTet2-β-plasmid, using Lipofect Plus reagent (Invitrogen), following the manufacturer’s instructions, using 1 × 10⁵ cells per transfection and 1 μg of each plasmid. Cells were harvested after 1 day transfection pulse. For steady-state mRNA quantification, cells were harvested after a 12-h transcription pulse. For mRNA stability analysis, cells were split into four 60-mm diameter dishes. Cells were pulsed with β-globin mRNA for 12 h by growth in Tet media. Following this period, transcription from the plasmid was then blocked by the addition of Tet to the media. Cells from each culture dish were harvested in different time points for further analysis.

The adherent HeLa cells, stably expressing the tet transactivator (HeLa/TTA; described in Ref. 39), were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Transient cotransfections were performed in polyethylene (Teflon) tubes, and digested with NcoI-BamHI and replaced into the plasmid containing the 63/64-S and 63/64-AS mutagenic primers and the DNA template. To obtain the constructs, the transcription initiation site to 200 bp 3′ to the polyadenylation site was amplified by PCR using HindIIIAmp-S and ApaIAmp r-AS primers (Table I), using DNase I (Ambion). Digestions were terminated by addition of 18 μl of an SDS-buffered saline and resuspended in cold serum-free minimal essential medium supplemented with 10% fetal bovine serum and 100 ng/ml tetacycline.

Cells were washed three times in cold Dulbecco’s phosphate-buffered saline and resuspended in cold serum-free minimal essential medium at a concentration of 3 × 10⁵ cells/ml. Two micrograms of pTet2-β-plasmid or each pTet2-β variant were added to the cell suspension. Transfection was performed using the calcium phosphate precipitation protocol at 30 μg/l and digested with microfrazors, and low resistance in an Invitrogen Cell-Porator system. For steady-state mRNA quantification, cells were harvested after a 12-h transcription pulse. For mRNA stability analysis, cells were split into four 60-mm diameter dishes. Cells were pulsed with β-globin mRNA for 12 h by growth in Tet media. Following this period, transcription from the plasmid was then blocked by the addition of Tet to the media. Cells from each culture dish were harvested in different time points for further analysis.

The adherent HeLa cells, stably expressing the tet transactivator (HeLa/TTA; described in Ref. 39), were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Transient cotransfections were performed in polyethylene tubes, and digested with NcoI-BamHI and replaced into the plasmid containing the 63/64-S and 63/64-AS mutagenic primers and the DNA template. To obtain the constructs, the transcription initiation site to 200 bp 3′ to the polyadenylation site was amplified by PCR using HindIIIAmp-S and ApaIAmp r-AS primers (Table I), using DNase I (Ambion). Digestions were terminated by addition of 18 μl of an SDS-buffered saline and resuspended in cold serum-free minimal essential medium supplemented with 10% fetal bovine serum and 100 ng/ml tetacycline. Cells were washed three times in cold Dulbecco’s phosphate-buffered saline and resuspended in cold serum-free minimal essential medium supplemented with 10% fetal bovine serum and 100 ng/ml tetacycline.

Cells were washed three times in cold Dulbecco’s phosphate-buffered saline and resuspended in cold serum-free minimal essential medium supplemented with 10% fetal bovine serum and 100 ng/ml tetacycline.

Cells were washed three times in cold Dulbecco’s phosphate-buffered saline and resuspended in cold serum-free minimal essential medium supplemented with 10% fetal bovine serum and 100 ng/ml tetacycline.
script II (Invitrogen) at 42°C for 2 min. Five-μl aliquots of cDNA product were PCR-amplified in a 50-μl reaction volume, using 0.3 μl (1.5 units) of AmpliTaq DNA polymerase (Applied Biosystems) for 30 cycles at 95°C for 1 min, 54°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 10 min. Reaction mixture contents were: 37.3 μl of water, 5 μl of 10× buffer (15 mM MgCl₂) (Applied Biosystems), 0.4 μl of dNTP mix (25 mM each of dATP, dGTP, dCTP, and dTTP), 15 pmol of each primer (Hβ3′ RNA-AS plus Hβ3′ RNA-AS, Hβ3′ RNA-S plus Hβ3′ exon2-AS, or Hβ3′ exon2-AS plus Hβ3′ RNA-AS; see Table 1), and 0.3 μl of enzyme. 10-μl aliquots from each RT-PCR sample were analyzed by electrophoresis on 1% agarose gels.

Sucrose Gradient Polyosome Fractionation—Polyosome gradients were carried out as described previously (38). In brief, transiently transfected MEL cells were centrifuged at 1,000 × g for 5 min at 4°C. The cell pellet was washed with ice-cold phosphate-buffered saline, lysed in TMK100 buffer (10 mM Tris-HCl (at pH 7.4), 5 mM MgCl₂, 100 mM KCl, 2 mM DTT, 1% Triton X-100, and RNase inhibitor 100 units/ml; Promega), the nuclei were cleared at 10,000 × g for 10 min at 4°C, and the supernatants (S10) were removed and layered onto the prepared 10–50% linear sucrose gradients and centrifuged at 40,000 rpm for 85 min at 4°C. The gradients were collected in 15 fractions from the top to the bottom by displacing them upwards with 60% sucrose, and the gradient profile was monitored via UV absorbance at 254 nm with an ISCO UA-5 detector (ISCO, Lincoln, NE). RNA was extracted from sucrose fractions and ethanol-precipitated. mRNA content was determined by RPA, using probes generated by in vitro transcription of plasmids containing genomic DNA inserts for hβ-globin (38) and mGAPDH (Ambion). The final protected fragments were resolved on a 6% acrylamide–8 M urea gel. Radioactivity in bands of interest was quantified by phosphorimaging analysis (Storm 840, Amersham Biosciences).

RESULTS

β15 and β17 mRNAs Are More Stable Than the NMD-targeted β39 mRNA—We have previously reported that a set of thalassemic hβ-globin mRNAs bearing nonsense mutations in exon 1 (β5, β15, and β17, respectively) accumulate to steady-state levels in vivo that are significantly higher than the β-globin mRNA with a prototype NMD-sensitive nonsense mutation (β39) (37, 41). This observation suggested that these nonsense-containing β-globin mRNAs avoid the full impact of NMD. To firmly establish that this is the case, the half-lives of two of the β-globin mRNAs with nonsense mutations in exon 1, β15 and β17, were determined and compared with βWT and β39 mRNAs. Each β-globin gene (normal or mutant) was cloned behind a tet-controlled promoter and transfected into a MEL cell line that stably expresses the tet-transcriptional transactivator (MEL/tTA cells) (39). The cells were transcriptionally pulsed for 12 h with each β-globin mRNA, and the corresponding mRNA decay rate was subsequently established. An example of this study is shown in Fig. 1A, and a compilation of four independent experiments is shown in Fig. 1B. The half-life (t1/2) of normal β-globin mRNA (βWT) in this system is 10 h, and that of the NMD-sensitive β39 mRNA is 3.5 h. These half-lives are in agreement with a recent study by Couttet and Grange (42). The half-lives of the β15 and β17 mRNAs were similar to each other and were intermediate to the βWT and β39 mRNAs; t1/2 of β15 mRNA is ~6.3 h, and t1/2 of β17 mRNA is ~5.8 h (Fig. 1B). These half-lives are consistent with the steady-state levels of β15 and
β17 mRNAs in erythroid cells (37) and suggest that hβ-globin mRNAs with nonsense-mutations located in close proximity to the initiation AUG can escape the full impact of NMD.

Close Proximity of Nonsense Mutations to the Initiation AUG Minimizes NMD of hβ-Globin mRNA—The above data confirm that the β15 and β17 mRNAs are relatively resistant to NMD. We hypothesized that resistance might reflect the positioning of the nonsense codon relative to the initiation AUG. To test this hypothesis, a deletion was introduced into the β39 mRNA that removed 69 bp between codons 3 and 27 (β39DelA). This deletion relocates the β39 nonsense mutation to a position 16 codons from the AUG while leaving the mutation within exon 2 and preserving the distance between the β39 nonsense codon and the terminal (exons 2–3) splice junction (195 nt) (Fig. 2A). MEL cells were stably co-transfected with equimolar amounts of β39DelA plus β39 or βWTDelA plus βWT, and the levels of the corresponding mRNAs were determined after the MEL cells were stimulated to terminally differentiate (37) (Fig. 2B). The results from three independent experiments revealed that the DelA deletion increased the level of β39 mRNA by 7.3-fold. This increase was significantly greater than the 1.8-fold increase in the level of βWT mRNA. Thus, shortening the distance between the β39 mutation and the AUG resulted in a significant and preferential mRNA stabilization.

The stabilizing effect of DelA on the β39 mRNA was confirmed by a second approach. MEL cells were stably transfected with each individual construct, the cells were induced to erythroid differentiation, and the steady-state level of each mRNA was assessed relative to an internal control (mouse (m) β-globin mRNA) (Fig. 2C). Results from four independent experiments indicate that β39DelA transcripts accumulate at about 84% of βWTDelA transcript levels, whereas β39 transcripts show levels of accumulation at ~9% of βWT (Fig. 2C). The studies support the conclusion that NMD is significantly abrogated when β-globin nonsense mutations are in close proximity to the initiation AUG.

The half-life of β39DelA mRNA Is Similar to That of β15 or β17 mRNAs—Based on steady-state mRNA levels (Fig. 2), we hypothesized that DelA stabilizes the NMD-sensitive β39 mRNA. To directly test this prediction, β39 and β39DelA genes were placed under control of the tetracycline-regulated promoter. MEL/TA cells transfected with the β39 and β39DelA genes were transcriptionally pulsed for 12 h and the decay rates of the β-globin mRNAs were monitored over time (Fig. 3A). Results from three independent experiments revealed a τ½ of 5.2 h for β39DelA mRNA (Fig. 3B). This half-life is equivalent to that determined for the β15 or β17 mRNAs and significantly longer than that determined for the β39 mRNA. These data confirm that DelA stabilizes the β39 mRNA.

The Relative Stability of β-Globin mRNAs with 5′-Proximal Nonsense Mutations Is Independent of Promoter and Tissue Specificity—Work from others suggests that the decay rate of nonsense-containing β-globin mRNAs can be impacted by an erythroid environment (43). For this reason, we next compared the NMD profiles established in MEL cells (Figs. 2 and 3) with those in HeLa cells. HeLa cells were transiently co-transfected with equimolar amounts of ha- and β39DelA-globin genes under control of the CMV promoter. Twenty-four hours post-transfection, RNA was quantified by RPA using probes specific for the human α- and β-globin mRNAs. The mRNA levels of βWT, β15, β39, and βWTDelA genes were assessed in parallel (Fig. 4). Results from three independent experiments revealed accelerated decay of β39 mRNA in non-erythroid cells; levels of β39 mRNA were 37% of βWT mRNA. In contrast, mRNA with a 5′ proximal nonsense mutation (β15) accumulated to the same level as βWT. Furthermore, deletion of sequences between codons 3 and 27 (DelA) of β39 mRNA increased its accumulation in HeLa cells from 37% to 86% of βWT. Thus β15 mRNA evades NMD in the non-erythroid (HeLa) as well as erythroid (MEL) cells, whereas β39 is NMD-sensitive in both settings and is stabilized by DelA. Because the stabilizing impact of DelA on β39 mRNA is equivalent in MEL and HeLa cells, using either the homologous β-globin promoter or the heterologous CMV promoter in erythroid and non-erythroid cells, the rela-
values and standard deviations (S.D.s) are shown.

values were then calculated as the percentage of
in the cell transfection efficiency and RNA recovery. Normalized

FIG. 3. Decay rate of β39DelA mRNA in TTA/MEL cells. The tet-regulated promoter-driven β39DelA construct was transiently transfected into MEL/TTA cells, and the decay of the pulsed mRNA was followed over time (hours, bottom) by RPA analysis. A, representative RPA analysis; B, compilation of data from three studies (means and standard deviations). The half-life (t½) of the β39DelA mRNA was determined by regression analysis.

FIG. 4. The impact of NMD on the various β-globin mRNAs is cell-type and promoter independent. HeLa cells were transiently co-transfected with a tet-regulated β-globin (specified above each respective lane) and normal ha-globin gene. After 24 h of transcription the RNA from transfected and untransfected (t-) HeLa cells was isolated and analyzed by RPA using specific probes for β- and ha-globin mRNAs (see “Experimental Procedures”). Increasing amounts of RNA from HeLa cells transfected with normal hβ- and ha-globin genes (indicated above the lanes by a triangle) were also analyzed to demonstrate that the experimental RPA was carried out in probe excess and was in the linear range of detection. The level of mRNA from each β-globin allele was normalized to the level of ha-globin to control for variations in the cell transfection efficiency and RNA recovery. Normalized values were then calculated as the percentage of βWT-globin mRNA; the values below each lane represent three independent experiments. Mean values and standard deviations (S.D.s) are shown.

tive impact of NMD on the various mutant mRNAs appears to be independent of promoter as well as tissue specificity. Taken together, these data substantiate the stability of the 5’ nonsense mutations and the stabilizing effect of deleting codons 3–27 from β39 mRNA.

Stabilities of β39DelA and 5’-Proximal Nonsense mRNAs Do Not Reflect Activation of Abnormal Splicing Pathways—Mechanisms that might contribute to stabilization of the β-globin mRNAs with 5’-proximal nonsense mutations were explored. We initially tested the possibility that these mutations activated cryptic splicing pathway(s) with consequent alteration in mRNA sequence and circumvention of the premature termination codon. This possibility was of particular interest in light of the numerous cryptic splice-donor sites in exon 1 that can be activated in vivo (44–47). Representative hβ-globin genes were expressed in transfected MEL cells, and the encoded mRNAs were analyzed by RT-PCR, using a set of primers that encompass the full-length β-globin mRNA (Fig. 5A) (see “Experimental Procedures”). A single full-length product of 625 bp was amplified from all non-deletional transcripts and a 556-bp fragment was amplified from cDNAs carrying DelA. In all cases, the cDNAs that were amplified correspond to the expected sizes of normally spliced β-globin mRNA. These results were confirmed by amplifying 5’ and 3’ halves of the hβ-globin cDNA from mRNA transiently expressed in HeLa cells (Fig. 5B; see “Experimental Procedures”). As expected, the 5’ half of the cDNA generated a product of 351 bp for βWT, β39, and β15 transcripts and a fragment of 282 bp corresponding to the

βWTDelA and β39DelA mRNA samples. Analysis of the 3’ cDNA fragment revealed the amplification of a 359-bp product in all samples (Fig. 5B). These results demonstrate normal splicing patterns for the transcripts containing the various nonsense and deletion mutations.

β39DelA and 5’-Proximal Nonsense mRNAs Fail to Undergo Appreciable Levels of Translation Re-initiation—NMD can be abrogated if translation re-initiates downstream of the nonsense codon (31). With this in mind, we investigated whether translational re-initiation was occurring on the β-globin mRNAs with 5’-proximal nonsense mutations. Polysome distributions were determined for βWT, β15, β39, βWTDelA, and β39DelA mRNAs expressed in transiently transfected MEL cells (Fig. 6, A and B). Data revealed a normal polysome profile for the βWT mRNA (ORF 146 codons) with a mean polysome size of four ribosomes. The mean polysome size for the βWTDelA mRNA was three ribosomes, a size consistent with its slightly shortened ORF (123 codons). In contrast, β15 and β39 mRNAs were both limited to a mean polysome size of 1–5 ribosomes and β39DelA mRNA peaked at a mean polysome size of one ribosome (Fig. 6C). These results support the conclusion that β15, β39, and β39DelA mRNAs terminate translation at their respective nonsense codons without appreciable levels of downstream re-initiation.

The question of translational re-initiation was further addressed by a second approach. If residual re-initiation, even at low levels, did occur and was able to circumvent NMD, this effect should be inhibited by inactivating putative initiating AUG codons located 3’ to the nonsense codon. The most proximal hβ-globin AUGs in a favored Kozak consensus (48) are at positions 55 (in-frame) and 73/74 (out of frame). A third AUG codon at 63/64 is in a less optimal sequence context (49). All three putative re-initiation AUGs were mutated in cis within the βWT, β15, β39, and β39DelA genes; Met-55 was converted to isoleucine (AUG → AUA), in cis to AUG → ACG substitutions at positions 63/64 and 73/74 (out of frame). A third AUG codon at 63/64 is in a less optimal sequence context (49). A third AUG codon at 63/64 is in a less optimal sequence context (49).

The Proximity of Nonsense Codons to the Translation Initiation AUG Is a Major Determinant of NMD—The stabilization of the β39 mRNA by DelA could be explained by two models. The first model reflects the repositioning of the β39 mutation closer to the initiation codon. The second model reflects deletion of a cis
Proximity of Nonsense Codon to AUG Inhibits NMD

A. MEL cells

B. HeLa cells

Fig. 5. The structures of β39DelA, β15, and β39 mRNAs indicate that the corresponding transcripts are normally spliced. A, structural analysis of full-length β-globin mRNAs stably expressed in MEL cells. The identity of each gene is indicated above the respective lane. RNA from untransfected (t) cells was used as the negative control for RT-PCR reactions. Plasmid DNA carrying the human βWT gene constitutes the positive PCR control. The molecular weight marker (M) is the 100-bp DNA ladder marker (Invitrogen). RT-PCR products were resolved on an ethidium bromide-stained agarose gel. Primers (Table I) for RT and PCR reactions are represented in the diagram below the gel. A single full-length product of 625 bp was amplified from all non-dele-
tional transcripts, and a 556-bp fragment was amplified from cDNAs carrying deletion between codons 3 and 27. The 1605-bp fragment was amplified from plasmid DNA (pDNA) containing the human βWT-globin gene. B, structural analysis of mRNA transiently expressed in HeLa cells. The top gel shows the analysis of amplification between exon 1 and exon 2; a single 351-bp product was amplified from all non-dele-
tional transcripts, and a 282-bp fragment was amplified from cDNAs carrying DelA. The 482-bp fragment was amplified from plasmid DNA containing the human βWT-globin gene. The lower gel shows the analysis of amplification between exon 2 and exon 3; a single 359-bp product is observed for each construct. The 1209-bp fragment was amplified from plasmid DNA containing the human βWT-globin gene. Plasmid DNA (pDNA) and water comprise the positive and negative RT-PCR controls. M represents the 100-bp DNA ladder marker. Primers localization (Table I) for RT and PCR reactions are represented on the bottom of each ethidium bromide-stained agarose gel.
The hβ-globin mRNA and its nonsense derivatives are localized to polysome peaks consistent with the sizes of their predicted ORFs. A, representative sucrose gradient profile. The absorbance profile (A₂₅₄) of the gradient is shown. The top of the gradient is to the left; the positions of absorbance peaks corresponding to pre-ribosomal RNPs, 40S, 60S, and 80S, and polysomes (2-, 3-, 4-, 5-, 6-, and 7-somes) are indicated. The 15 fractions collected for subsequent analysis are identified below the gradient profile. B, representative RPA to detect the globin RNAs across the polysome gradient. MEL cells were transiently transfected with the hβ-globin genes. Each gradient fraction was assessed for human hβ-globin and mouse GAPDH mRNAs by RPA with the corresponding ³²P-labeled riboprobes; hβ-globin- and mGAPDH-protected RNAs fragments are shown. C, polysome distribution of human βWT-globin, β15, β39, βWTDelA, or β39DelA mRNAs. The contents of hβ-globin mRNAs in each fraction (see B) were quantified by phosphorimaging analysis (data concerning βWTDelA and β39DelA mRNAs not shown in B). The amount of each mRNA species in each fraction is depicted as a percentage (ordinate) of the total for the corresponding species across the gradient. D, the stability of the β15 or β39DelA mRNAs is unaffected by elimination of potential re-initiating AUGs. The three potential reinitiating AUGs at codons 55, 63/64, and 73/74, were converted to coding triplets in each of the index mRNAs. Shown is a representative RPA of RNA isolated from HeLa cells untransfected (t-) or transiently co-transfected with βWT, β15, β39, β39DelA, βWT-CD55–63/64–73/74, β15-CD55–63/64–73/74, β39-CD55–63/64–73/74, or β39DelA-CD55–63/64–73/74 human globin genes along with an equal quantity of the hα-globin gene. The positions of the hβ- and hα-globin protected fragments are indicated to the right of the autoradiograph. Levels of hβ-globin mRNA were quantified relative to the hα-globin mRNA, and these values are shown beneath each respective lane (average and standard deviations (S.D.s)) normalized to the expression level of the βWT gene. All assays were carried out in riboprobe excess. For each case, three independent experiments were performed.
Proximity of Nonsense Codon to AUG Inhibits NMD

AUG. HeLa/tTA cells were co-transfected with each of these hybrid constructs and the ha-globin gene. Cells were grown for 24 h in the absence of tetracycline, and RNA levels were subsequently quantified (Fig. 7B). These studies reveal that WT (15:39) mRNA accumulates at $-112\%$ of normal ($\beta^{WT}$) and $\beta15(15:39)$ mRNA accumulates at $-37\%$ of $\beta^{WT}$. These results further support the role of AUG proximity in NMD by demonstrating that increasing the distance between the $\beta 15$ mutation and the AUG results in mRNA destabilization.

Finally, we analyzed if the impact of AUG proximity on NMD is dominant over the general 50–54 nt boundary rule. Here, we introduced the $\beta 15$ mutation in cis to the $\beta 39$ (construct $\beta 15\text{non} \beta 39$). This gene was stably transfected in MEL cells, and the level of accumulation was determined after induction of differentiation. We find that the $\beta 15\text{non} \beta 39$ mRNA accumulates to approximately the same level as the $\beta 15$ mRNA (respectively, 63 and $60\%$ of $\beta^{WT}$; Fig. 7A). Concordant results were also obtained in HeLa cells, where $\beta 15\text{non} \beta 39$ mRNA is at about $97\%$ of $\beta^{WT}$ (Fig. 7B). These data unequivocally demonstrate that the effect of the proximity of the nonsense mutation to the AUG is a dominant determinant in the inhibition of the NMD pathway.

DISCUSSION

Termination codons are recognized by the NMD apparatus as premature if they are located more than 50–54 nt 5’ to the 3’-most exon-exon junction (28, 29). We have previously reported that human $\beta$-globin mRNAs bearing nonsense mutations in the 5′-region of exon 1 accumulate to levels similar to those of wild type $\beta$-globin mRNA (37). This finding is in apparent contradiction to the 50–54 nt boundary rule, because these mutations are substantially 5’ to the terminal exon 2–3 junction. In the present report we demonstrate that these mRNAs with AUG-proximal nonsense mutations are in fact significantly more stable than prototype NMD-sensitive $\beta$-globin mRNAs and that the proximity of nonsense mutation to the AUG initiation codon comprises the basis of their relative NMD resistance. Thus, AUG proximity can override the 50–54 nt boundary rule in establishing the overall efficiency of NMD for a particular mutant mRNA.

The AUG proximity effect studied in the current report is based on the $\beta$-globin mRNA model. The study stems from observations originally made in patients with specific $\beta$-thalassemic mutations. The cell culture studies currently reported appear to accurately recapitulate our prior in vivo observations. The effect of AUG proximity on NMD mechanism in $\beta$-globin mRNA is independent of promoter sequence and is not restricted to erythroid cells. Thus, it is likely that these findings are not restricted to the globin genes or to the erythroid cell environment and may in fact constitute general determinants of the NMD pathway.
To our knowledge, the AUG proximity effect that we report represents a newly recognized parameter of the NMD response. However, a number of apparent exceptions to the 50–54 nt boundary rule have been previously reported by others. The mechanisms involved in these individual cases of NMD resistance include translation re-initiation downstream of the nonsense codon (TPi and immunoglobulin μ heavy chain mRNAs (31, 32)) and the presence of a sequence cis-acting element that confers immunity to the 50–54 nt boundary rule (T-cell receptor-β mRNA (34)). In additional cases the rule is apparently circumvented, although the underlying mechanism responsible for inhibition of the NMD response remains undefined (BRCA1, fibrinogen Ao-chain, and ALG3 mRNAs (33, 35, 36)). We propose, based on the likelihood that the current findings are not limited to the β-globin mRNA, that the NMD resistance in some of these model systems reflects the AUG proximity effect.

The observed lack of cell-type specificity for NMD phenotypes in the β-globin mRNAs is consistent with the understanding that NMD constitutes an essential surveillance process operating in all tissues. Nonetheless, a comparison of results shown in Figs. 2C and 4 suggests that NMD of human β-globin mRNA may be somewhat more efficient in terminally differentiated erythroid cells when compared with HeLa cells. This may be contributed by the observation of Stevens et al. (43) that the degradation of nonsense-mutated β-globin mRNAs in erythroid cells is accomplished by a tissue-specific endonuclease with preference for UG dinucleotides (43). Thus, although specialized decay pathways may exist in the erythroid compartment, the general conservation of the NMD phenotypes that we observe in MEL and HeLa cells suggests that the AUG proximity effect reflects a general attribute of the NMD pathway.

The relevance of the AUG proximity of a nonsense codon to NMD is consistent with prior studies on 5′ ORF structure in NMD in other systems.2 For example, thrombopoiettin mRNA is normally insensitive to NMD, but can be rendered NMD-sensitive by extending the length of its upstream ORF. These data led to the suggestion that mammalian mRNAs with naturally occurring short upstream ORFs may have a general resistance to NMD.2 These data, in conjunction with our present study, would support a model in which termination of translation in close proximity to the initiation codon results in a resistance to NMD. This notion, combined with the knowledge that the NMD pathway is translation-dependent, may lead to a further proposal that the observed NMD insensitivity of mRNAs with a short 5′ ORF, whether natural or resulting from a nonsense mutation, can mechanistically reflect ineffective and/or brief translation. In this regard, it is interesting to note that the smallest naturally occurring euukaryotic ORF that produces significant levels of protein product comprises 24 codons (51). Based on our results and those published by Zhang et al. (28), this boundary for human β-globin mRNAs appears to map between codons 17 and 21/22 (817 is the 3′-most mutation that fails to trigger full NMD, and the β21/22 mutation is the 5′-most mutation able to fully commit mRNA to the NMD pathway (28, 37)). Thus, the reported minimal size of an ORF for effective translation roughly correlates with the boundary between NMD-resistant and NMD-sensitive mutations.

The apparent concordance between the minimal length of an ORF for effective protein synthesis and the minimal length for effective NMD may be relevant to disease pathophysiology. In the case of thalassemia, the production of truncated proteins via nonsense mutations can have a dominant-negative effect on red cell structure and function (52, 53). C-terminal truncated proteins, if produced at significant levels, can block proteolytic pathways in differentiating erythroblast and/or can interfere with critical aspects of the assembly and function of hemoglobin tetramers (53). The NMD pathway is generally considered to clear cellular mRNAs that can encode such “toxic” protein fragments. However, mutant β-globin mRNAs with AUG-proximal nonsense mutations are relatively stable. This apparent defect in NMD surveillance would theoretically place the cell in jeopardy. Nevertheless, we note that the thalassemic phenotype of heterozygotes and homozygotes carrying AUG-proximal nonsense mutations do not appear to be any more severe than in patients carrying more distal nonsense mutations that are effectively targeted by NMD. The lack of a detectable dominant-negative effect in these patients may reflect the inefficiency with which mutant mRNAs with short 5′ ORFs are translated. Thus, the inability of the NMD pathway to effectively clear mRNAs with 5′-proximal nonsense mutations may be mitigated, at least in part, by the inability of these mRNAs with very short ORFs to be effectively translated.

The relative NMD resistance of mRNAs with AUG-proximal nonsense mutations can be related to critical steps in translational biochemistry. The short ORF may result in a temporal overlap between translational initiation and termination. The ribosome may arrive at the premature termination (nonsense) codon before it has discharged its initiation factors and/or stabilized interactions with elongation/termination factors. This effect would result in interference with elongation/termination reactions that may be central to the NMD pathway. Present evidence indicates that the translation termination process is intimately linked with the NMD pathway. Analyses in other systems have shown that, during the process of translation of short upstream ORFs, interactions involving ribosomal or ribosome-associated factors, including but not limited to releasing factors, can inhibit translation elongation as well as termination (50, 54–56). In the case of NMD, UpF1 must interact with eRF1 and eRF3 to terminate translation and, in the proper setting, to interact with additional factors to trigger NMD (reviewed in Refs. 1 and 27). Therefore, it is possible that the dynamics of the mRNP remodeling necessary for NMD cannot occur or is defective in this compressed environment of the AUG-proximal nonsense-mutated mRNAs. The present study further supports the notion that NMD is a multifaceted process that reflects the overall complexity of euukaryotic translational biochemistry.

REFERENCES

1. Wagner, E., and Lykke-Andersen, J. (2002) J. Cell Sci. 115, 3033–3038
2. Maquat, L. E. (1996) RNA (N. Y.) 1, 453–465
3. Renak, M. (2002) Membrane. 13, 401–410
4. Maquat, L. E. (2004) Nat. Rev. Mol. Cell. Biol. 5, 89–99
5. Blencowe, B. J., Issner, R., Nickerson, J. A., and Sharp, P. A. (1998) Genes Dev. 12, 996–1009
6. Mayaeda, A., Badolato, J., Kubayashi, R., Zhang, M. Q., Gardiner, E. M., and Krainer, A. R. (1999) EMBO J. 18, 4560–4570
7. Kataoka, N., Yong, J., Kim, V. N., Velazquez, F., Perkinsin, R. A., Wang, F., and Dreyfuss, G. (2000) Mol. Cell. 6, 673–682
8. Le Hir, H., Izaurralde, E., Maquat, L. E., and Moore, M. J. (2000) EMBO J. 19, 6660–6669
9. Le Hir, H., Moore, M. J., and Maquat, L. E. (2000) Genes Dev. 14, 1098–1108
10. Zhou, Z., Luo, M. J., Strasser, K., Katahira, J., Hurt, E., and Reed, R. (2000) Nature 407, 401–405
11. Kim, V. N., Kataoka, N., and Dreyfuss, G. (2001) Science 293, 1832–1836
12. Kim, V. N., Yong, J., Kataoka, N., Abel, L., Diem, M. D., and Dreyfuss, G. (2001) EMBO J. 20, 2062–2068
13. Lykke-Andersen, J., Shu, M.-D., and Stenaz, J. A. (2001) Science 293, 1836–1839
14. Le Hir, H., Gatfield, D., Izaurralde, E., and Moore, M. J. (2001) EMBO J. 20, 4967–4977
15. Le Hir, H., Gatfield, D., Braun, I. C., Forler, D., and Izaurralde, E. (2001) EMBO Rep. 2, 1119–1124
16. Kataoka, N., Diem, M. D., Kim, V. N., Yong, J., and Dreyfuss, G. (2001) EMBO J. 20, 6424–6433
17. Gehring, N. H., Neuvilly, G., Schell, T., Hentze, M. W., and Kuloniz, A. E. (2003) Mol. Cell 11, 939–949

C. Stockklausner, personal communication.
Proximity of Nonsense Codon to AUG Inhibits NMD

32.180

18. Kataoka, N., and Dreyfuss, G. (2004) J. Biol. Chem. 20, 7099–7013
19. Lykke-Andersen, J., Shi, M.-D., and Steitz, J. A. (2000) Cell 103, 1121–1131
20. Serin, G., Gersappe, A., Black, J. D., Aronoff, R., and Maquat, L. E. (2001) Mol. Cell. Biol. 21, 209–225
21. Ishigaki, Y., Li, X., Serin, G., and Maquat, L. E. (2001) J. Biol. Chem. 276, 5422–5427
22. Ishigaki, Y., Li, X., Serin, G., and Maquat, L. E. (2001) Mol. Cell. Biol. 21, 209–225
23. Ohnishi, T., Yamashita, A., Kashima, A., Schell, T., Andres K. R., Grimson, A., Hachaya, T., Hentze, M. W., Anderson, P., and Ohno, S. (2003) Mol. Cell 12, 1187–1200
24. He, F., Brown, A. H., and Jacobson, A. (1997) Mol. Cell. Biol. 17, 1590–1594
25. Mendell, J. T., Medghalchi, S. M., Lake, R. G., Noensie, E. N., and Dietz, H. C. (2000) Mol. Cell. Biol. 20, 8944–8957
26. Schell, T., Kocher, T., Wilm, M., Seraphin, B., Kulozik, A., and Hentze, M. W. (2003) Biochem. J. 373, 775–783
27. Czapainski, K., Ruiz-Echevarria, M. J., Paushkin, S. V., Han, X., Weng, Y., Perlick, H. A., Dietz, H. C., Ter-Avanessyan, M. D., and Peltz, S. W. (1998) Genes Dev. 12, 1665–1677
28. Zhang, J., Sun, X., Qian, Y., and Maquat, L. E. (1998) RNA (N. Y.) 4, 801–815
29. Thermann, R., Neu-Yilik, G., Deters, A., Frede, U., Wehr, K., Hagleimer, C., Hentze, M. W., and Kulozik, A. E. (1998) EMBO J. 17, 3484–3494
30. Zhang, J., Sun, X., Qian, Y., and Maquat, L. E. (1998) Mol. Cell. Biol. 18, 5272–5283
31. Zhang, J., and Maquat, L. E. (1997) EMBO J. 16, 826–833
32. Buzina, A., and Rudman, M. J. (1999) Mol. Cell. Biol. 19, 515–524
33. Perrin-Vidot, L., Sinilnikova, O. M., Stoppa-Lyonnet, D., Lenoir, G. M., and Mazoyer, S. (2002) Hum. Mol. Genet. 11, 2805–2814
34. Wang, J., Gadkote, J. P., Olivas, O. R., and Wilkinson, M. F. (2002) EMBO Rep. 3, 274–279
35. Asselta, R., Duga, S., Spera, S., Santagostino, E., Peyvandi, F., Pischedda, G., Targhetta, R., Malcovati, M., Mannucci, P. M., and Turchini, M. L. (2001) Blood 98, 3683–3692
36. Denecke, J., Kranz, C., Kemming, D., Koch, H.-G., and Marquardt, T. (2004) Hum. Mutat. 23, 477–486
37. Romão, L., Inácio, A., Santos, S., Ávila, M., Faustino, P., Paschoal, P., and Lavinha, J. (2000) Blood 96, 2895–2901
38. J. X., Kong, J., and Liebhaber, S. A. (2003) Mol. Cell. Biol. 23, 899–907
39. Kong, J., Ji, X., and Liebhaber, S. A. (2003) Mol. Cell. Biol. 23, 1125–1134
40. Liebhaber, S. A., Wang, Z., Cash, F. E., Monks, B., and Russell, J. E. (1996) Mol. Cell. Biol. 16, 2637–2646
41. Baserga, S., and Benz, E. J., Jr. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2056–2060
42. Coutt, P., and Grange, T. (2004) Nucleic Acids Res. 32, 488–494
43. Stevens, A., Wang, Y., Bremer, K., Zhang, J., Hoepfner, R., Antoniou, M., Schenben, D. B., and Maquat, L. E. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 12741–12746
44. Orkin, S. H., Kazazian, H. H., Jr., Antonarakis, S. E., Oster, H., Goff, S., and Sexton, J. P. (1982) Nature 300, 768–769
45. Goldsmith, M. E., Humphries, R. K., Ley, T., Cline, A., Kantor, J. A., and Nierhuis, A. W. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 2318–2322
46. Orkin, S. H., Antonarakis, S. E., and Loukopolus, D. (1984) Blood 64, 311–313
47. Yang, K. G., Kutlar, F., George, E., Wilson, J. B., Kutlar, A., Stoming, T. A., Gonzalez-Redondo, J. M., and Huisman, T. H. J. (1989) Br. J. Haematol. 73, 73–80
48. Kazak, M. (1997) EMBO J. 16, 2482–2492
49. Hinenzu, A. G. (1995) Mol. Cell. Biol. 10, 215–223
50. Janzen, D. M., Frolova, L., and Geballe, A. P. (2002) Mol. Cell. Biol. 22, 5862–5870
51. Yu, X., and Warner, J. R. (2001) J. Biol. Chem. 276, 33821–33825
52. Thein, S. L., Hesketh, C., Taylor, P., Temperley, J. J., Hutchinson, R. M., Old, J., Wood, W. G., Clegg, J. B., and Weatherall D. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3923–3928
53. Hall, G. H., and Thein, S. (1994) Blood 83, 2001–2007
54. Lovett, P. S., and Rogers, E. J. (1996) Microbiol. Rev. 60, 864–986
55. Morris, D. R., and Geballe, P. (2000) Mol. Cell. Biol. 20, 8365–8364
56. Tenson, T., and Ehrenberg, M. (2002) Cell 108, 591–594
Nonsense Mutations in Close Proximity to the Initiation Codon Fail to Trigger Full Nonsense-mediated mRNA Decay

Ângela Inácio, Ana Luísa Silva, Joana Pinto, Xinjun Ji, Ana Morgado, Fátima Almeida, Paula Faustino, João Lavinha, Stephen A. Liebhaber and Luísa Romão

J. Biol. Chem. 2004, 279:32170-32180.
doi: 10.1074/jbc.M405024200 originally published online May 25, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M405024200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 56 references, 36 of which can be accessed free at http://www.jbc.org/content/279/31/32170.full.html#ref-list-1