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β -Thalassemia Due to Intronic LINE-1 Insertion in the β -Globin Gene (*HBB*): Molecular Mechanisms Underlying Reduced Transcript Levels of the β -Globin_{L1} Allele

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Abstract

We describe the molecular etiology of β^+ -thalassemia that is caused by the insertion of the full-length transposable element LINE-1 (L1) into the intron-2 of the β -globin gene (*HBB*). The transcript level of the affected β -globin gene was severely reduced. The remaining transcripts consisted of full-length, correctly processed β -globin mRNA and a minute amount of three aberrantly spliced transcripts with a decreased half-life due to activation of the nonsense-mediated decay pathway. The lower steady-state amount of mRNA produced by the β -globin_{L1} allele also resulted from a reduced rate of transcription and decreased production of full-length β -globin primary transcripts. The promoter and enhancer sequences of the β -globin_{L1} allele were hypermethylated; however, treatment with a demethylating agent did not restore the impaired transcription. A histone deacetylase inhibitor partially reactivated the β -globin_{L1} transcription despite permanent β -globin_{L1} promoter CpG methylation. This result indicates that the decreased rate of transcription from the β -globin_{L1} allele is associated with an altered chromatin structure. Therefore, the molecular defect caused by intronic L1 insertion in the β -globin gene represents a novel etiology of β -thalassemia.

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Keywords

β -thalassemia; β -globin; *HBB*; LINE-1; epigenetic repression

We have previously reported that the insertion of a full-length LINE-1 (L1) element (GenBank accession: AF149422.1) in the antisense orientation into the 3'-end of intron-2 of the β -globin gene (*HBB*; MIM #141900; GenBank accession: NM 000518.4) causes β^+ -thalassemia in a mother and daughter of Ukrainian descent [Divoky et al., 1996]. The β -thalassemia trait in the proband and her mother was diagnosed in the laboratory of the late Dr. Huisman (Augusta, GA) [Indrak et al., 1992]. We have shown that this L1 element retained the capacity for high-frequency retrotransposition in cultured cells [Kimberland et al., 1999]. Here, we analyzed the molecular mechanisms that are responsible for the severe reduction in β -globin gene expression in the presence of the L1 insertion and cause the β^+ -thalassemia phenotype.

The β^+ -thalassemia nature of this mutation was investigated in the reticulocytes from the patient and in the interspecific hybrids created from the fusion of transformed lymphocytes of the proband with mouse erythroleukemia cells (MEL) containing either the affected or normal chromosome 11 [Papayannopoulou et al., 1986]. The cell line containing the mutant chromosome 11 from the proband was designated as "MEL *HBB*^{L1+}," and the control cell line with a normal chromosome 11 was designated as "MEL *HBB*^{wt}." Blood samples from the proband and her mother were obtained with the approval of the institutional review board committees of Palacky University in Olomouc and the Institute of Hematology and Blood Transfusion in Prague, Czech Republic. Informed consent was obtained as per the Declaration of Helsinki. The details of the experimental procedures and the sequences of the primers used for this study are found in the Supp. Methods and Supp. Tables.

The proband (25-year-old Caucasian female) had microcytic anemia (Hb 11.3 g/dL, red blood cells $5.5 \times 10^{12}/L$, mean corpuscular volume 63 fL, and mean corpuscular hemoglobin 20 pg) with a normal reticulocyte count. Her HbA₂ was increased to 5.3% and the HbF was just above 1%. No inclusion bodies of precipitated globin chains were detected in her erythroid cells after brilliant cresyl blue staining. Hematological indices of the proband's mother were comparable; neither patient had neurological or other systemic abnormalities. Southern blot and sequencing analyses revealed heterozygosity for the full-length retrotransposon (L1, i.e., L1 β -thal) inserted in the antisense orientation into the 3'-end of intron-2 (β -IVS-II) of the β -globin gene (hereafter referred to as β -globin_{L1}; Supp. Fig. S1) deposited into the HBBVar database (<http://www.lovd.nl/HBB>). The insertion occurred at 98 bp 5' - to the 3'-end of β -IVS-II and was flanked by a 9–13-bp target site duplication [Divoky et al., 1996; Kimberland et al., 1999].

The β -globin transcripts from affected and unaffected β -globin alleles of the proband were distinguishable by a silent codon 2 polymorphism [Antonarakis et al., 1985; Orkin et al., 1982]. This polymorphism allowed the quantification of the transcript amount and the determination of the β^+ -thalassemia phenotype (Fig. 1A). The proband was a codon 2 CAT/CAC heterozygote, whereas the mother of the proband, who also carried the L1 insertion, was a codon CAC homozygote. Thus, the L1-containing allele was derived from

framework 1 [Antonarakis et al., 1985] with a codon 2 CAC sequence that creates a recognition site for the *Bs*HKAI enzyme.

To assess whether the β^+ -thalassemia is due to abnormal splicing of the β -globin message, reticulocyte and MEL *HBB*^{L1+} mRNAs were analyzed by RT-PCR using several primers. Sequencing of the RT-PCR products revealed both correctly processed transcripts as well as three rare messages with unusual sequences produced by the β -globin_{L1} allele (Fig. 1B). All of the aberrantly spliced β -globin transcripts contained a premature translation termination codon, which makes them susceptible to nonsense-mediated mRNA decay (NMD). To determine whether the NMD pathway degrades the β -globin_{L1} mRNA and thus is responsible for the reduction in the steady-state mRNA level produced by the mutant allele, we cultivated the induced MEL hybrids cells overnight with the NMD inhibitor emetine [Yang et al., 2007]. Quantitative real-time PCR (qPCR) showed a 2.4-fold increase ($P < 0.05$ vs. control) in the accumulation of transcripts from the β -globin_{L1} allele in the presence of emetine (Fig. 1C), whereas no increase in the amount of wild-type β -globin_{L1} transcripts was observed under the same conditions. To further confirm that the low stability of the β -globin_{L1} transcripts was due to NMD, transcription in the MEL *HBB*^{L1+} and MEL *HBB*^{wt} hybrids was blocked with actinomycin D in the presence and absence of emetine. Indeed, the decreased stability of the β -globin_{L1} mRNA was mainly due to NMD. The half-life of the β -globin_{L1} mRNA was restored almost to normal when emetine was used (Supp. Fig. S2).

In addition to a decrease in RNA stability, a decreased rate of transcription could be responsible for the reduced amount of β -globin_{L1} mRNA. This possibility was examined using a nuclear run-on assay [Yang et al., 2007]. The increase in the β -globin nuclear fraction primary transcript level during 40 min of *in vitro* transcription in the presence or absence of ribonucleotide triphosphates (rNTPs, see Supp. Methods for experimental details) was measured. The data showed that the β -globin_{L1} allele has a transcription rate that is reduced 4.7-fold versus the wild type (Fig. 1D).

Previous studies have demonstrated that L1 insertions disrupt the formation of the full-length transcript of the target gene by producing prematurely polyadenylated mRNA. These truncated transcripts form because of the use of the L1 polyadenylation signal(s). Additionally, the release of RNAPol-II from the L1 sequence causes inefficient elongation [Han et al., 2004]. We measured the relative amount of the β -globin_{L1} primary transcript using qPCR with primers flanking the insertion (Fig. 1E). The nuclear fraction RNA was collected from MEL *HBB*^{wt} and MEL *HBB*^{L1+} hybrids, and the relative amount of wild-type and β -globin_{L1} primary transcripts was measured at exon-2 and exon-3 and expressed as the exon-3/exon-2 ratio normalized to the control as described [Pfaffl, 2001] (see Supp. Methods). The ratio was 0.805, which suggests that 19.5% of the MEL *HBB*^{L1+} primary transcript is not extended beyond the L1 insertion. The truncated transcripts could result from either premature polyadenylation or incomplete elongation.

Retrotransposons are considered to be epigenetic mediators of gene expression in mammalian cells [Whitelaw and Martin, 2001]. We examined whether the decreased rate of β -globin_{L1} allele transcription is associated with the β -globin_{L1} promoter and 3'-enhancer methylation that could be reversed by a demethylating agent. The locations of the CpG

dinucleotides (CpGs) within the β -globin_{L1} promoter and enhancer and also within the L1 promoter are depicted in Figure 2A. The results from bisulfite sequencing (Fig. 2B) show that the β -globin_{L1} promoter sequence was hypermethylated (65% Me-CpG vs. 35% Me-CpG in the control MEL *HBB*^{wt} cell line). The 3'-enhancer sequence was fully methylated in comparison with the control sequences. Treatment of the induced hybrid MEL cells with decitabine (5-aza-2'-deoxycytidine, DAC, 2 μ M) for 24 hr did not change the methylation status of the β -globin_{L1} promoter, nor did it lead to an increase in expression of β -globin_{L1} mRNA (Fig. 2B and C). Different treatment conditions (treatment time 48 hr or 5 μ M DAC) gave similar results. Expression of the γ -globin gene, which was used as a treatment control, was increased after DAC exposure as expected [Mabaera et al., 2008] (Fig. 2C). The partial demethylation of the β -globin_{L1} erythroid-specific 3'-enhancer sequence [Boehringer et al., 1987] (Fig. 2B) suggested that insensitivity to DAC under these assay conditions was a feature of the β -globin_{L1} and L1 promoters. Even the more stringent DAC-treatment conditions [Qin et al., 2009] did not reactivate β -globin_{L1} expression (data not shown).

The histone deacetylation of the surrounding chromatin was implicated in the inactivation of L1 host genes [Garcia-Perez et al., 2010]. To test whether transcriptional silencing of the β -globin_{L1} allele is related to repressive chromatin modifications such as deacetylation, we treated induced MEL *HBB*^{L1+} cells with trichostatin A (TSA), a histone-deacetylase (HDAC) inhibitor (Fig. 2D). The TSA-induced reactivation of developmentally silenced γ -globin expression in the somatic cell hybrids was previously documented [Swank et al., 2003], and therefore, it was used as an internal treatment control (Fig. 2E). Although exposure of the induced MEL *HBB*^{L1+} cells to TSA (alone or in combination with DAC) did not change the densely methylated profile of the β -globin_{L1} promoter sequence (Fig. 2D), this treatment partially restored β -globin_{L1} expression (Fig. 2E). This result suggests that histone deacetylation is (in part) responsible for the β -globin_{L1} silencing that could be relieved by TSA.

L1 β -thal displays characteristics of an active retrotransposon [Kimberland et al., 1999]. Therefore, it is possible that transcripts originating from the L1 β -thal promoter could interfere with the β -globin gene that is transcribed in the opposite orientation to this L1 [Whitelaw and Martin, 2001]. The ϵ -globin gene silencing by a transcribed Alu element provides precedence for in cis transcriptional interference by neighboring transcripts from the antisense strand in globin gene regulation [Wu et al., 1990]. Thus, we assayed for the presence of L1 β -thal-specific transcripts in patient mRNA that was isolated from harvested BFU-E colonies and in mRNA from the interspecific hybrids (see Supp. Methods and Supp. Tables for details). No L1 β -thal-specific transcripts could be detected in the BFU-Es from the patients. Several L1 transcripts were detected in the induced MEL *HBB*^{L1+} cells; however, their sequence analyses revealed other than L1 β -thal origin (data not shown). Correspondingly, bisulfite sequencing revealed a highly methylated pattern along the L1 β -thal promoter region (Fig. 2B and D), suggesting that the absence of endogenous L1 transcripts is a consequence of the L1 β -thal promoter hypermethylation. We also investigated whether the L1 β -thal insertion splits the *HBB* gene's transcript, that is, the "gene-breaking" model [Wheelan et al., 2005]. We identified neither upstream *HBB*-specific transcripts that terminate in the major antisense polyadenylation signal [Han et al., 2004] nor evidence for

L1 antisense promoter-derived *HBB*-specific transcripts (see Supp. Methods and Supp. Tables for details).

In conclusion, we report a combination of molecular events leading to β^+ -thalassemia caused by an intronic L1 insertion in the *β -globin* gene. In addition to the known effects of the intronic L1 sequence on mRNA processing [reviewed by Hancks and Kazazian, 2012], our data reveal that transcriptional repression of the host gene is due to regional spreading of methylation that affects both the 5'-promoter and the 3'-enhancer. Our attempts to reactivate the silenced *β -globin_{L1}* expression revealed that the HDAC inhibitor TSA, but not the demethylating agent DAC, leads to a partial restoration of host gene transcription. This result indicates that the decreased rate of transcription from *β -globin_{L1}* is associated with altered chromatin that is likely caused by the spreading of repressive marks induced by L1 to the neighboring *β -globin_{L1}* gene promoter [Garcia-Perez et al., 2010; Montoya-Durango et al., 2009]. These results have therapeutic implications for diseases caused by the deleterious effects of retrotransposons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Analyses of transcription from the β -globin_{L1} allele. **A:** Total reticulocyte RNA of the proband (lane 2, codon 2 CAT/CAC polymorphism heterozygous) and the wild-type control (lane 1, codon 2 CAC homozygous) was subjected to RT-PCR, which amplified exons 1 and 2 of all existing mature β -globin transcripts. Aliquots of the labeled PCR products were digested with the *Bst*HKAI enzyme, which cuts the CAC allele. The ratio of digested to undigested cDNA was 10%–15% to 90%–85% instead of the expected heterozygous ratio of 50% to 50%. Concurrent experiments performed with genomic DNA served as a control for digestion and quantification of PCR products. **B:** The structure of the β -globin_{L1} gene is depicted at the top. Gray rectangles represent the β -globin exons, black rectangles indicate the β -globin introns, and the β -IVS-II is divided by L1 insertion (white rectangle not drawn to scale). The correctly spliced β -globin message (1) and three

abnormally spliced β -globin_{L1} transcripts (2–4) were detected by RT-PCR using three forward (β -1F, β -2F, and β -3F) and four reverse (β -1R, L1-2R, L1-3R, L1-4R) primers (primers are listed in Supp. Table S1, locations are delineated as arrows at the top). The solid lines indicate transcript sequences, the dotted lines are sequences eliminated by splicing, and the dashed lines are portions of transcripts that were not sequenced. One splicing variant resulted from activation of a cryptic acceptor site within β -IVS-II (tttcttcag/g), and the second variant resulted from activation of a cryptic acceptor site within the L1 sequence (tatttctag/g). The third aberrantly processed β -globin transcript displayed retention of the globin intron including the inserted L1 sequence. The arrows (↓) denote stop codons in the aberrant β -globin reading frame. **C:** MEL HBB^{L1+} and MEL HBB^{wt} hybrids were treated with an NMD inhibitor emetine (0.5 μ M). The qPCR quantification showed no increase in wild-type β -globin mRNA in the MEL HBB^{wt} cell line versus a 2.4-fold increase (*, $P < 0.05$ vs. control) in β -globin_{L1} mRNA in MEL HBB^{L1+} cells. Data are the mean $\pm$ SD of three independent experiments. **D:** A PCR-based nuclear run-on assay revealed a 4.7-fold decrease in the β -globin_{L1} allele transcription rate compared with the wild-type β -globin allele. To include all transcript variants originating at the β -globin_{L1} promoter, exon-1 primers were used for quantification. The rate of transcription is displayed as the fold increase of primary β -globin/ β -globin_{L1} transcript over its basal level (not containing rNTPs) after 40 min of in vitro transcription. Data are the mean $\pm$ SD of two independent experiments. **E:** Schematic representation of the primary transcripts of the β -globin and β -globin_{L1} alleles (full-length [a, solid line] and truncated [b, partially dotted line]). The thickness of the lines represents the overall relative amount of the produced primary transcript. According to the PCR-based nuclear run-on assay, the wild-type allele creates 4.7-fold more primary transcript than the mutant β -globin_{L1} allele. Additionally, the nuclear RNA was evaluated by β -globin exon-2 and exon-3 quantification. The results indicated that a portion of the primary β -globin_{L1} transcripts (19.5%) do not contain the sequences downstream of the L1 insertion. The details of the experimental procedures are described in the Supp. Methods.

Figure 2.

Analysis of epigenetic silencing of the β -globin_{L1} allele. **A:** Diagram of the human β -globin gene promoter and enhancer regions showing the locations of binding sites for transcription factors, poly(A) signal, and CpG dinucleotides [adapted from Mabaera et al., 2007]. The position of the CpGs within the L1 promoter is also shown. **B:** Genomic DNA from MEL hybrid cells was isolated and bisulfite treated to determine the methylation status of the six CpGs between -415 and +110 bp (bases downstream and upstream of the transcription start site) on the promoter region, 13 CpGs on the promoter of the inserted L1 and three CpGs between +442 and +592 bp on the enhancer region (bases upstream of the poly(A) signal) of the mutated and normal β -globin alleles (plotted horizontally). Treatment of DMSO-induced MEL HBB^{L1+} cells with a demethylating agent (DAC, 2 μ M) did not change the methylation profile of the β -globin_{L1} gene and the L1 promoters but did decrease the methylation of the enhancer. • represents methylated sequence, ○ represents unmethylated sequence. **C:** Consequently, DAC treatment did not increase the expression of β -globin_{L1} mRNA, whereas the expression of the γ -globin gene (*HBB1*; MIM #142200, GenBank: NM_000559.2) was increased. Data are the mean $\pm$ SD of three independent experiments (*, $P < 0.05$ vs. untreated cells). For the effect of DAC and TSA treatment expressed as the γ - to β -globin

mRNA ratio [Swank et al., 2003], see Supp. Figure S3. **D:** Treatment of induced hybrid MEL *HBB*^{L1+} cells with the HDAC inhibitor TSA (100 nM) alone and in combination with the demethylating agent (DAC, 2 μ M) did not change the hypermethylation profile of the β -*globin*_{L1} promoter. However, the combined treatment partially reversed the methylation of the β -*globin*_{L1} enhancer. • represents methylated sequence, ○ represents unmethylated sequence. **E:** Despite the permanent methylation pattern of the β -*globin*_{L1} promoter, TSA partially reactivated expression from the mutant β -*globin*_{L1} gene (see also Supp. Fig. S3) indicating that silencing is mediated by histone deacetylation. The data are the mean $\pm$ SD of three independent experiments (*, $P < 0.05$ vs. untreated cells). The details of the experimental procedures are described in the Supp. Methods.