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Nuclear degradation of nonsense mutated β-globin mRNA: a post-transcriptional mechanism to protect heterozygotes from severe clinical manifestations of β-thalassemia?

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ABSTRACT

Nonsense mutations of the β-globin gene are a common cause of β-thalassemia. It is a hallmark of these mutations not only to cause a lack of protein synthesis but also a reduction of mRNA expression. Both the pathophysiologic significance and the underlying mechanisms for this surprising phenomenon have so far remained enigmatic. We report that the reduction of the fully spliced mutant β-globin mRNA already manifests itself within the nucleus. In contrast, the levels of mutant pre-mRNA are normal. The promoter and the 5′-untranslated region (5′-UTR) of the herpes simplex virus type 1 thymidine kinase (HSV1 Tk) gene can independently circumvent this recognition/response mechanism *in cis* and restore nonsense mutated β-globin mRNA expression to normal levels. These two genetic elements can thus exert a dominant influence on the post-transcriptional control of nonsense mutated β-globin gene expression. While wildtype mRNA levels are restored by fusion of the HSV1 Tk 5′-UTR to the nonsense mutated β-globin reading frame, translation of a wildtype reading frame in such a hybrid is precluded. In contrast, the HSV1 Tk promoter appears to efficiently deliver the mRNA to the translational apparatus. The 5′-UTR and the promoter sequences therefore control the nuclear fate of nonsense mutated β-globin mRNA by separable pathways. The nuclear mRNA degradation mechanisms examined here may prevent the synthesis of C-terminally truncated β-globin chain fragments and may protect heterozygotes from clinically relevant symptoms of β-thalassemia.

INTRODUCTION

In the course of molecular analyses of thalassemia patients it was recognized early that nonsense and frameshift mutations not only affect protein expression but also result in a reduction of mRNA levels (1–3). In addition to β-globin mRNA, this phenomenon was observed in a number of other nonsense mutated genes (reviewed in ref. 4). In the case of Igκ light chain, nonsense mutations result in an accelerated intranuclear decay of the affected RNA with an accumulation of splicing intermediates but without a change in cytoplasmic stability (5). Cytoplasmic stability and transcription rates were also shown not to be involved in triosephosphate isomerase and dihydrofolate reductase mRNA nonsense mutations (6,7), but cytoplasmic degradation of nonsense mutated human β-globin mRNA was found in transgenic mice (8). However, in the human HeLa cell line (9,10) or in patients (11) cytoplasmic degradation products could not be observed. The mechanisms for the reduction of nonsense mutated β-globin mRNA expression are therefore incompletely understood and may involve cytoplasmic and nuclear elements.

We demonstrated previously that nonsense mutated mRNA expression can be restored *in cis* by sequences in the promoter and 5′-UTR of the HSV1 Tk gene and that the fate of nonsense mutated mRNA can thus be determined by sequences outside of the reading frame and, indeed, outside of the actual mRNA (10).

We have now further delineated the necessary *cis*-acting sequences, analyzed the expression of nonsense versus wildtype pre-mRNA and mRNA in different nuclear fractions, and studied in how far elements that can affect the fate of nonsense mutated mRNA *in cis* influence the ability of the wildtype mRNA to be translated in the cytoplasm. On the basis of the results obtained we discuss possible mechanisms for the nuclear recognition and decay of nonsense mutated β-globin mRNA.

MATERIALS AND METHODS

Constructs (Table 1)

All constructs were derived from a 5 kb *Bgl*II human β-globin gene fragment in a pSP65 expression vector containing a simian virus 40 enhancer, an extended polylinker (12) and a 628 bp deletion from position –1442 to –814 (10). Constructs #1 and #2 contain the wildtype sequence or a C→T nonsense mutation of the β-globin codon 39 (NS39).

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Table 1. Constructs used for transfection experiments

Construct #	Promoter	5′-UTR	Reading frame
1	β-globin	β-globin	wildtype
2	β-globin	β-globin	NS39
3	β-globin	HSV1 Tk	wildtype
4	β-globin	HSV1 Tk	NS39
5	HSV1 Tk	HSV1 Tk	wildtype
6	HSV1 Tk	β-globin	wildtype

In constructs #3 and #4 the β-globin 5′-UTR was replaced by sequences of the HSV1 Tk 5′-UTR. The β-globin promoter and open reading frame were left unchanged. Technically, this was achieved by a PCR strategy that involved the amplification of two fragments. Fragment 1 was derived from construct #1 and contained the β-globin gene promoter up to the cap site. Fragment 2 was derived from construct pTK-βIVS(+) with either the wildtype or the NS39 mutation (ref. 13, kindly provided by Dr Janet Mertz, University of Wisconsin, USA) and contained the HSV1 Tk 5′-UTR and the β-globin coding sequence. Importantly, the primers at the desired junction between the two fragments were phosphorylated whereas the primers at the 5′-end of fragment 1 and the 3′-end of fragment 2 remained unphosphorylated. After PCR amplification equimolar amounts of fragments 1 and 2 were ligated at 20°C for 10 h. The selective phosphorylation of the central primers favored the head-to-tail dimerisation of fragments 1 and 2. This ligation product was further selected by a second PCR using the outer primers only. Finally, the ligation/amplification product was digested with *Bam*HI and *Hpa*I and used to replace the respective sequences of construct #1 to generate constructs #3 and #4. Sequence analysis was carried out to establish that only the desired changes with no additional mutations had occured. In construct #5 and #6 the β-globin promoter and 5′-UTR (#5) or the β-globin promoter only (#6) were replaced by HSV1 Tk sequences. The generation of these recombinants was described previously (10).

Cell culture and transfection

HeLa cells were grown in a CO_2 incubator in Dulbecco's modified Eagle medium (DMEM) supplemented with 100 U/ml penicillin/streptomycin and 10% fetal calf serum. Cells were passaged twice a week and seeded at a density of 2.5×10^4 cells/cm^2 24 h before transfection by calcium phosphate precipitation with 2×25 μg supercoiled plasmid DNA. The cells were washed after 16 h and harvested 24 h later.

Cell fractionation (Figure 1)

The protocol was a modification of that developed previously by others (14,15). HeLa cell pellets were resuspended in cytoskeleton buffer (CSK: 100 mM NaCl, 300 mM sucrose, 10 mM Pipes [pH 6.8], 3 mM $MgCl_2$, 1 mM EGTA, 1.2 mM phenylmethylsufonyl fluoride [PMSF], 4 mM vanadyl riboside complex [VRC], lysed by the addition of 0.5% (vol/vol) Nonidet P-40 (NP-40) and incubated at 4°C for 5 min. The crude nuclei were pelleted for 5 min at 800 *g* and separated from the supernatant containing the soluble cytoplasmic fraction. The crude nuclei were washed again with CSK-buffer without detergent, pelleted as before, and the supernatant added to the cytoplasmic fraction obtained in step 1. Next, the nuclear membrane fraction was separated from the nuclear cell fraction by resuspending the pellet containing the CSK for 5 min at 4°C in reticulocyte standard buffer (RSB: 10 mM Tris–HCl, pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$, 4 mM VRC, 1.2 mM PMSF) supplemented with 0.5% NP-40 and 0.5% sodium deoxycholate and subsequent centrifugation for 5 min at 800 *g*. The pellet containing the nuclear cell fraction was subsequently washed with RSB without detergents, pelleted as before and the supernatant added to the nuclear membrane fraction. After this treatment no remnants of the cytoplasm were evident microscopically. Finally, the chromatin fraction was separated from the nuclear matrix after digestion with DNase I (100 μg/ml) at room temperature for 30 min in a buffer identical to CSK with 50 mM NaCl. DNase I treatment was terminated by addition of ammonium sulfate to a final concentration of 0.25 M. The nuclear matrix was pelleted at 1000 *g* for 5 min and the chromatin fraction removed with the supernatant. Care was taken to minimize contamination of the nuclear RNA with cytoplasmic material. Ethidium bromide staining showed high molecular weight rRNA precursors in the nuclear matrix fraction but not in any of the other nuclear or in the cytoplasmic fraction.

RNA isolation and analysis

RNA was isolated from the cytoplasmic and nuclear fractions, and purified by guanidine thiocyanate lysis and centrifugation through a CsCl cushion (16).

Primer extension analysis was performed with 30 μg of cytoplasmic or nuclear RNA using ^{32}P 5′ end-labeled exon-specific (5′-AGAACCTCTGGGTCCAAGGG-3′) or intron-specific (5′-CTTGTAACCTTGATACCAAC-3′) primers. After hybridization at 55°C overnight, the primer was extended at 42°C for 60 min with reverse transcriptase (M-MuLV, Pharmacia). The lengths of extended DNA molecules were defined by denaturing polyacrylamide-gel electrophoresis.

Northern blot analysis was performed with 1 μg of cytoplasmic RNA using a ^{32}P labelled cRNA β-globin probe extending from position –84 to +261 (10).

Western blotting

Cytoplasmic protein was extracted from control and transfected cells lysed in RSB containing 0.5% NP-40. Just prior to use, the protease inhibitors pepstatin A and leupeptin (1 μg/ml each) as well as PMSF (0.1 mM) were added to the RSB buffer. Protein concentration was determined according to Lowry (16). SDS–polyacrylamide gels (20%) loaded with equal amounts (~200 μg) of protein were blotted onto PVDF-membranes (Immobilon-P, Millipore) at 200 mA for 1 h in 0.025 M Tris–HCl (pH 10.4)/20% methanol. Membranes were stained with Ponceau S to ensure that the transfer was complete and uniform. The membranes were blocked overnight in TBS (150 mM NaCl, 20 mM Tris–HCl pH 7.5) supplemented with 0.1% Tween 20, 5% BSA and 0.5% gelatin (buffer A). The primary antibody (β^{6-9}, A3–A6 of human hemoglobin A; Immuno-rx, Augusta, GA, USA) was diluted 1:5000 in buffer A and incubated with the membrane for 1 h, followed by four washes of 10 min each in buffer B (TBS-0.1%-Tween 20, 0.1% BSA). The secondary antibody (goat-anti mouse IgG horseradish peroxidase, Bio-Rad) was diluted 1:8000 in buffer A and incubated for 1 h with the membrane, followed by four washes of 10 min each in buffer B.

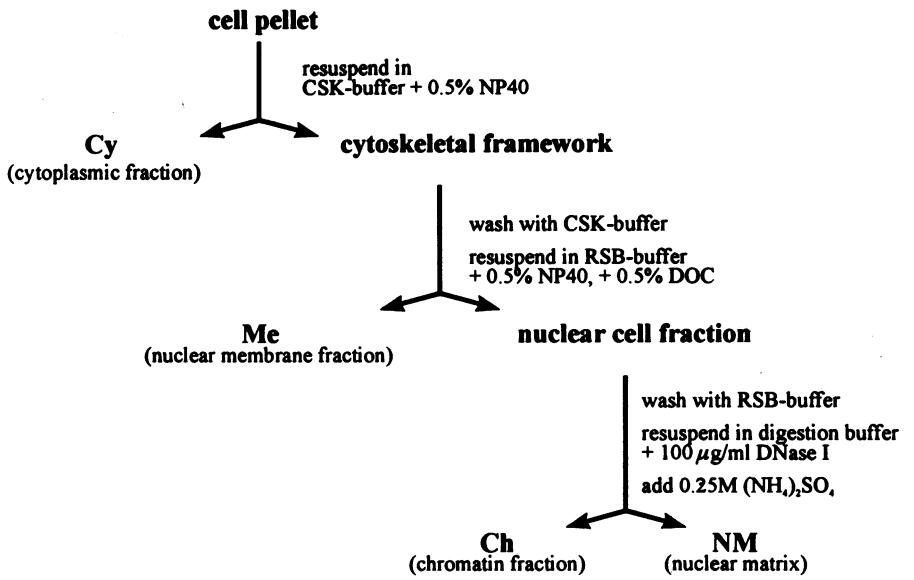

Figure 1. Fractionation protocol for the extraction of cytoplasmic and nuclear RNA from transfected HeLa cells (see Methods for details).

After the last wash, the detection reagents (chemiluminescent Western blotting reagents, ECL, Amersham) were added for 1 min and the membrane was immediately exposed for autoradiography.

RESULTS

Influence of the 5′-UTR and cellular sublocalisation on nonsense mutated mRNA decay

We found previously that sequences contained in the promoter of the HSV1 Tk gene (but not in the CMV-IE promoter) restored the expression of nonsense mutated β-globin mRNA. This rescue was quantitatively even more pronounced when both the HSV1 Tk promoter and 5′-UTR were fused to the mutated mRNA (10). We therefore studied the role of the HSV1 Tk 5′-UTR alone by comparing the expression of wildtype and NS39 mRNA expressed from the β-globin promoter either containing the β-globin (constructs #1 and #2, see Table 1) or the HSV1 Tk 5′-UTR (constructs #3 and #4, see Table 1). Messenger RNA expression was separately analyzed in the cytoplasm as well as in different nuclear fractions (see Fig. 1) of transiently transfected HeLa cells. Using a single primer complementary to exon 2 of the β-globin gene, the mRNA with the β-globin 5′-UTR can be simultaneously monitored and distinguished from that with the HSV1 Tk 5′-UTR in co-transfected cells by their 7 nt difference in length (Fig. 2: 176 nt for constructs #1 and #2 with the β-globin 5′-UTR or 183 nt for constructs #3 and #4 with the HSV1 Tk 5′-UTR). A comparison of the 176 nt wildtype (construct #1) and NS39 (construct #2) fragments, representing the mRNA containing the β-globin 5′-UTR, shows the lower abundance of the nonsense mutated mRNA in the cytoplasm (lane 4 versus lane 8). The same difference is apparent in the nuclear membrane (lane 5 versus lane 9), chromatin (lane 6 versus lane 10), and nuclear matrix fractions (lane 7 versus lane 11). In contrast, expression of the wildtype and NS39 constructs #3 and #4 containing the HSV1 Tk 5′-UTR, represented by the 183 nt fragments, does not show a reduction of NS39 expression in any of these fractions. These clearly apparent differences are internally controlled by co-transfection of the wildtype constructs #1 and #3 or the NS39 constructs #2 and #4. The ratio of β-globin/HSV1 Tk 5′-UTR mRNA expression is slightly in favor of β-globin for the wildtype and strongly in favor of HSV1 Tk for the NS39 constructs. We therefore conclude that the HSV1 Tk 5′-UTR restores the expression of nonsense mutated β-globin mRNA.

The fractionation protocol employed here was developed to monitor viral mRNA metabolism within a series of operationally defined nuclear compartments which are passed through sequentially starting from transcription in the nuclear matrix fraction (15). Minor degrees of contamination of one fraction with another cannot be excluded and are difficult to control for, but none the less it seems that the reduction of nonsense mutated mRNA expression manifests itself at an early intranuclear stage. Furthermore, the HSV1 Tk 5′-UTR is sufficient *in cis* to circumvent the mechanism that mediates the nuclear response to the recognition of translational nonsense.

Nonsense mutated pre-mRNA expression

Considering the manifestation of the response to translational nonsense in the early nuclear fractions (see Fig. 2) we next asked whether the responsible mechanism already affects unspliced pre-mRNA. We thus analyzed wildtype and NS39 RNA expression in the nuclear cell fraction (see Fig. 1) of transfected HeLa cells by extension analysis with intron 1 or exon 2 specific primers (Fig. 3). The cells were transfected with the wildtype construct #1 or the NS39 construct #2. Transfection efficiency was internally controlled by co-transfection of construct #3 containing the 7 nt longer HSV1 Tk 5′-UTR (183 and 169 nt extension products). The fragments generated by the exon 2 specific primer (lanes 4 and 5) confirm the finding of reduced nuclear NS39 mRNA expression (176 nt extension products, see also Fig. 2). We did not reproducibly observe extension products containing intron 1 with this primer. For an analysis of the

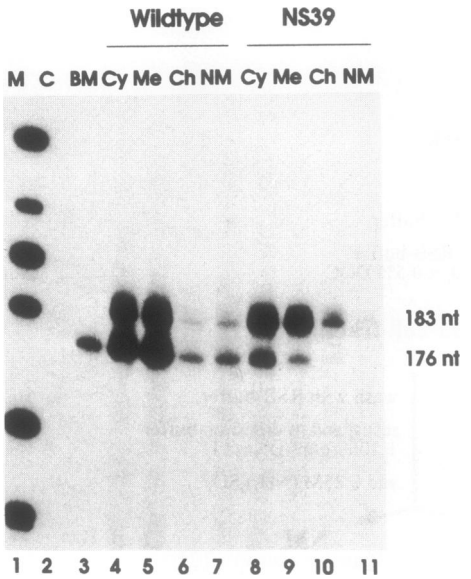

Figure 2. Cellular sublocalization of nonsense mutated β-globin mRNA decay and the influence of the HSV1 Tk 5′-UTR. Primer extension analysis was performed with RNA extracted from HeLa subcellular fractions (see Fig. 1). In lanes 4–7 the cells were co-transfected with the wildtype constructs #1 and #3, containing the β-globin or the HSV1 Tk 5′-UTR, respectively (see Table 1). In lanes 8–11 the cells were co-transfected with the NS39 constructs #2 and #4 also containing either the β-globin or the HSV1 Tk 5′-UTR. Expression of the mRNA with the β-globin or the HSV1 Tk 5′-UTR is reflected by the 176 nt or the 183 nt extension products. Lanes: M, pBR322 *Hae*III size marker; C, control RNA from untransfected HeLa cells; BM, human bone marrow RNA; Cy, cytoplasmic fraction; Me, nuclear membrane fraction; Ch, chromatin fraction; NM, nuclear matrix fraction.

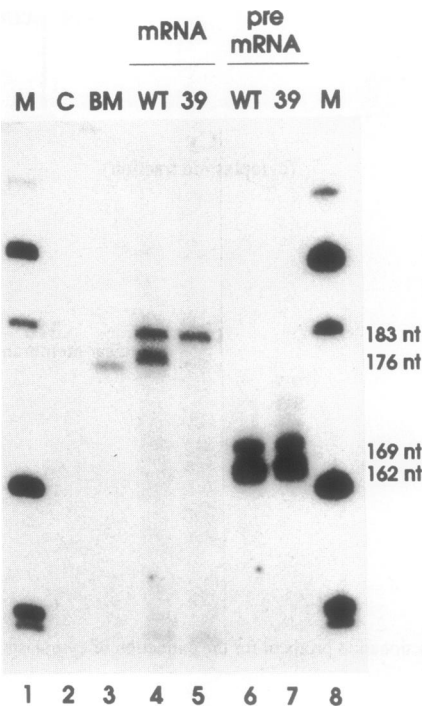

Figure 3. β-globin mRNA and pre-mRNA expression in the nuclear cell fraction (see Fig. 1). Primer extension analysis was performed with RNA extracted from HeLa cells co-transfected with the wildtype (WT) or nonsense mutated (39) constructs #1 or #2 containing the β-globin 5′-UTR and construct #3 with the wildtype reading frame fused to the HSV1 Tk 5′-UTR serving as an internal control for transfection efficiency (see Table 1). In lanes 3–5, an exon 2 specific (mRNA) and in lanes 6–8 an intron 1 specific (pre-mRNA) primer was used. The 176 and 162 nt extension products reflect expression of the RNA with the β-globin 5′-UTR whereas the 183 and 169 nt fragments are specific for the control RNA with the HSV1 Tk 5′-UTR. Lanes: M, pBR322 *Hae*III size marker; C, control RNA from untransfected HeLa cells; BM, human bone marrow RNA; WT, HeLa cells co-transfected with the wildtype contruct #1 and the control #3; 39, HeLa cells co-transfected with the NS39 construct #2 and the control #3.

pre-mRNA, we therefore used a primer that hybridizes specifically with intron 1. These experiments reveal an identical abundance of NS39 and wildtype RNA (162 nt extension products) still containing intron 1 (compare lanes 6 and 7). Abnormal splice products were not observed with either of the primers. These results directly demonstrate that the nuclear response to translational nonsense does not reduce the pre-mRNA levels but manifests itself post-transcriptionally before the fully spliced transcript is transported into the cytoplasm (see also Fig. 2).

Effect of the HSV1 Tk promoter and 5′-UTR on the translatability of β-globin mRNA

On the basis of the profound effects that the HSV1 Tk promoter (10) and 5′-UTR (Figs 2 and 3) exert on the nuclear response to translational nonsense, we next examined how far these genetic elements may influence the translation of β-globin mRNA. The constructs with the wildtype β-globin reading frame and β-globin or HSV1 Tk promoters and 5′-UTRs (constructs #1, #3, #5 and #6, see Table 1) were transiently transfected into HeLa cells and analyzed for expression of β-globin protein and mRNA. The Northern blot shown in the bottom panel of Figure 4 shows that the transfection efficiency and mRNA expression were similar for all four constructs. The Western blot analysis shown in the top panel of Figure 4 demonstrates that construct #1 containing the β-globin promoter and 5′-UTR directs β-globin synthesis (lanes 1–3). The same protein is detected after transfection with construct #6 containing the HSV1 promoter and the β-globin 5′-UTR (lane 6). In contrast, mRNAs containing the HSV1 Tk 5′-UTR (constructs #3 and #5) are not translated, irrespective of the presence of the HSV1 Tk or the β-globin promoter (lanes 4 and 5). Thus, HSV1 Tk promoter driven β-globin mRNAs are principally translatable, while β-globin mRNAs bearing the HSV1 Tk 5′-UTR appear to be precluded from translation.

DISCUSSION

Using fusion constructs between regions of the HSV1 Tk gene and nonsense mutated β-globin mRNA we have established that: (i) the cellular response to translational nonsense occurs already in the nucleus, (ii) the responsible mechanisms can be independently circumvented *in cis* by sequences in the promoter and in the 5′-UTR of the HSV1 Tk gene, and (iii) the effect of the HSV1 Tk 5′-UTR is associated with an inhibition of translation whereas the HSV1 Tk promoter efficiently delivers the mRNA to the translational apparatus. Does the HSV1 5′-UTR restore mRNA expression because it yields a non-translatable mRNA? Nonsense

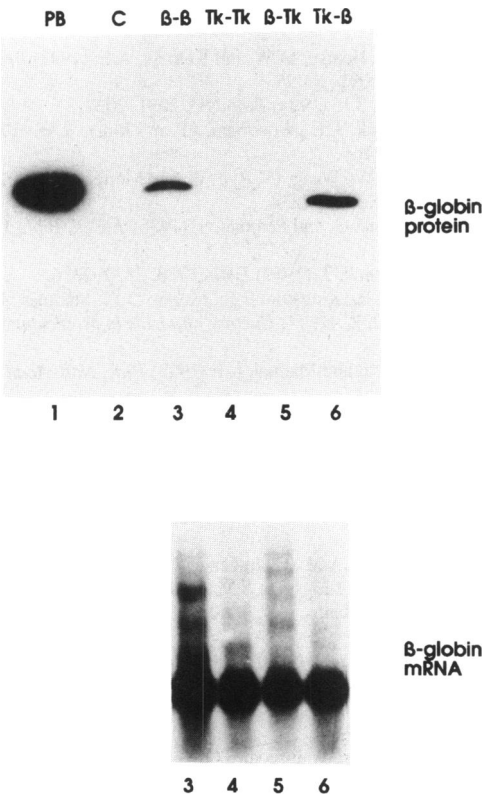

Figure 4. Translatability of different β-globin mRNAs. Western blot (top) and Northern blot (bottom) analyses were performed on protein or RNA extracted from the cytoplasm of HeLa cells transfected with contructs #1 containing the β-globin promoter and 5′-UTR (β–β), #3 containing the HSV1 Tk promoter and 5′-UTR (Tk–Tk), #5 containing the β-globin promoter and the HSV1 Tk 5′-UTR (β–Tk), or #6 containing the HSV1 Tk promoter and the β-globin 5′-UTR (Tk–β; see Table 1). β-globin protein was detected with a monoclonal antibody directed against an epitope comprising amino acids 6–9, and the mRNA with a 345 nt antisense ^{32}P labelled cRNA extending from position –84 to +261 of the β-globin gene (see Methods). Lanes: PB, protein extracted from human peripheral blood; C, protein extracted from untransfected HeLa cells.

mutated triosephosphate isomerase or avian sarcoma viral src mRNAs, which are also degraded within the nucleus, can be rescued by co-transfection of a suppressor tRNA gene or by a double mutation affecting the AUG initiation codon, indicating a pivotal role of mRNA translatability for degradation (17,18). It seems likely, therefore, that mRNAs need to be translatable to be proofread for translational sense and to be degraded within the nucleus when a nonsense mutation is present. Considering possible reasons for the lack of translatability of HSV1 Tk/β-globin mRNAs, the HSV1 Tk 5′-UTR displays a significantly higher C+G content than the β-globin 5′-UTR (34/57 [60%] versus 22/50 [44%]) and consequently contains a higher degree of secondary structure (ΔG –21.4 kcal/mol versus –10.1 kcal/mol). Furthermore, the nucleotide context surrounding the β-globin initiation codon (ACCAUGG) perfectly matches the consensus (RCCAUGG; ref. 19,20) whereas the HSV1 Tk sequence contains mismatches at positions –1 and –3 (UCTAUGG). However, the HSV1 Tk mRNA is physiologically expressed, and the HSV1 Tk 5′-UTR thus unlikely to cause non-translatability *per se*. Alternatively, the HSV1 5′-UTR may target the β-globin mRNA into a cellular subcompartment that does not support the translation of this hybrid mRNA.

In the previously reported examples (17,18) and the HSV1 Tk 5′-UTR/β-globin fusion constructs (this report), restoration of normal levels of nonsense mutated mRNAs involved inhibition of translation. It is therefore particularly intriguing that restoration by the HSV1 Tk promoter yields an apparently translatable mRNA, suggesting a different mechanism from that utilized by the 5′-UTR. In *Xenopus* oocytes the HSV1-Tk-promoter has been reported to activate nucleo–cytoplasmic mRNA transport *in cis* and *in trans* (21). Since we demonstrated previously that the HSV1-Tk promoter does not influence the kinetics of cytoplasmic wildtype or nonsense mutated β-globin mRNA accumulation (10), the HSV1 Tk promoter may direct mRNAs along an alternative nuclear export pathway that is independent of the proofreading for translational sense. Hypothetically, the current data could be explained by the presence of a major and a minor pathway of mRNA export from the nucleus. The major pathway, that is preferentially utilized by the β-globin promoter, subjects the transcripts to proofreading for the correct translational sense of the reading frame. This proofreading mechanism requires the mRNA to be translatable. If a nonsense mutation is present, this pathway may be blocked and the affected mRNA degraded within the nucleus. Considering the reduction of nonsense mutated β-globin mRNA abundance to ~20% of wildtype levels, this pathway may account for ~80% of normal mRNA expression. It is still unclear how nonsense mRNA is recognized, but in the case of mutant triosephosphate isomerase mRNA the responsible mechanism has been shown to operate *in cis* (22).

The minor export pathway would bypass the proofreading mechanism and account for ~20% of physiologic β-globin gene expression. This pathway could be used preferentially by the HSV1 Tk promoter, resulting in the restoration of nonsense mutated β-globin mRNA expression. A promoter dependent choice of different post-transcriptional expression pathways has also been suggested for the CMV-IE promoter with regard to the translation efficiency of HIV-1 U3 mRNAs (23) and the intron requirement of immunoglobulin gene expression (24).

It is worth to consider the possible (patho-) physiologic significance of the nuclear recognition and degradation of nonsense mutated mRNAs although our data do not address this issue directly. Nuclear depletion of nonsense mutated Igκ light chain RNA has been suggested to be relevant for the efficiency of allelic exclusion after VDJ rearrangement in B lymphocytes thus implying an important physiologic role of a nuclear quality control mechansim to down regulate mRNA species coding for non-functional truncated polypeptides (5). In homozygous β-thalassemia an excess of α-globin chains that precipitate in the erythroid precursor cells cause ineffective erythropoiesis. In most heterozygotes, intracellular proteolysis of excess α-globin chains allows effective erythropoiesis to occur and results in the only mild hematologic abnormalities of thalassemia minor (25). The proteolytic system operates at the limit of its capacity as demonstrated by the rare cases of symptomatic heterozygotes who express an additional functional α-globin gene (26–28).

Other molecular defects resulting in heterozygous β-thalassemia intermedia with a dominant mode of inheritance are nonsense mutations in the third exon of the β-globin gene (29,30). In contrast to the common recessively inherited forms with nonsense mutations in exons 1 and 2, these exon 3 nonsense mutations are associated with normal levels of mRNA (10,11).

When those messages are translated into β-globin chain fragments these may interact with the α-globin chains to inhibit their efficient degradation and/or impose an additional and critical load for the proteolytic system of the erythroid precursor cells resulting in dyserythropoiesis and clinical symptoms. Given the organization of many proteins, including β-globin, into functional domains, the mRNA proofreading and decay mechanism may protect heterozygous carriers of recessive traits, such as β-thalassemia, from the detrimental consequences of expressing C-terminally truncated polypeptides that could act in a dominant negative fashion.

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