Proc. Natl. Acad. Sci. USA  
Vol. 90, pp. 10091–10095, November 1993  
Biochemistry

Determination of mRNA fate by different RNA polymerase II promoters

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Communicated by Lennart Phillipson, August 2, 1993 (received for review June 17, 1993)

ABSTRACT Translational stop mutations of the human β-globin gene cause a reduction of cytoplasmic mRNA accumulation in thalassemia patients and in transfection models. The exact mechanism underlying this phenomenon has remained enigmatic but is known to be post-transcriptional. We have used transfected HeLa cells to study the expression of β-globin mRNAs with nonsense or frameshift mutations within the three exons of this gene. Mutations in exons 1 or 2 reduce cytoplasmic mRNA accumulation whereas a mutation in exon 3 permits essentially normal expression. We report here that the post-transcriptional fate of mutated β-globin mRNAs is differentially affected by the type of RNA polymerase II promoter driving expression. Replacement of the β-globin promoter with the herpes simplex virus type 1 thymidine kinase gene promoter but not the cytomegalovirus immediate early promoter rescues the cytoplasmic accumulation of mutated mRNA to wild-type levels. This effect is shown to be independent of the absolute quantity and the kinetics of accumulation of mutated mRNA synthesized, and primer-extension analyses confirm that both viral promoters accurately utilize identical transcription start sites. These data thus reveal an unexpected property of RNA polymerase II promoters: determination of the post-transcriptional fate of the maturing mRNA, presumably by influencing alternative choices between as yet undefined processing and/or transport pathways.

Nonsense and frameshift mutations are commonly found in the β-globin genes of thalassemia patients. Since these mutations do not affect sequences known to be important for transcription or RNA processing, one would expect to find normal cytoplasmic mRNA levels with a lack of protein due to prematurely terminated translation. Surprisingly, however, mRNA levels are reduced to about 20% of normal in the erythroid precursors of such patients and in transfected cell lines (1–11). In addition to β-globin, this reduction of nonsense mutated mRNA levels was observed in a number of other genes (ref. 12 and references therein) but in all cases the mechanism for this phenomenon remained unknown. In addition to β-globin, triosephosphate isomerase and dihydrofolate reductase gene mutations have been characterized in detail. In each of these examples, cytoplasmic stability and the transcription rate of nonsense mutated RNA have been found to be similar when compared to the wild type (10, 11, 13–15). It appeared therefore that the reduction of nonsense mutated β-globin mRNA was due to an as yet undefined nuclear or transport mechanism. We addressed this question in more detail by using a HeLa-cell transfection assay and recombinant human β-globin genes driven by its endogenous or viral promoters. The data presented in this manuscript lead to the surprising conclusion that RNA polymerase II promoters can determine the post-transcriptional fate of the maturing nonsense mutated mRNA; i.e., the metabolism of the mRNA can be influenced by sequences that are not actually part of it.

MATERIALS AND METHODS

 Constructs (Table 1). All constructs are derived from a 5-kb Bgl II wild-type human β-globin gene fragment in a pSP65 expression vector containing a simian virus 40 enhancer and an extended polylinker (16). This plasmid was modified to be suitable for convenient insertion of sequence cassettes by deletion of a 627-bp Hpa I fragment extending from positions −814 to −1442 relative to the cap site of the β-globin gene (construct 1).

 Constructs 2 and 3 were generated by replacing a 1292-bp Hpa I–BamHI fragment of construct 1 extending from positions −814 to +478 at the 3' end of exon 2 with the corresponding piece of DNA that contained either the ΔA frameshift at codon 6 in exon 1 or the codon-39 C → T nonsense mutation in exon 2. These mutated fragments were obtained by PCR amplification of genomic DNA of thalassemia patients. The +AATT frameshift at codon 121 in exon 3 of construct 4 was introduced by treatment with the Klenow fragment of DNA polymerase I and religation at the EcoRI site at that position. This 4-bp frameshift results in a premature UAA translational stop codon at position 138/139 (i.e., 27 nt 5' to the physiologic termination codon). In constructs 5 and 6, the β-globin gene promoter and its 5' untranslated sequence (UTR) extending from the Hpa I site at position −815 to an Nco I site including the translation initiation codon at position +51 was replaced by a 279-bp fragment containing the herpes simplex virus type 1 (HSV-1) thymidine kinase (Tk) promoter and 5'-UTR up to the Bgl II site at position +57. These constructs thus contained the entire open reading frame of the β-globin gene but with a HSV-1 Tk promoter and 5'-UTR.

 The Tk sequences were PCR-amplified from the construct pTK-BIVS(+) kindly provided by Janet Mertz, University of Wisconsin (17). Constructs 7 and 8 were made by joining a PCR-amplified HSV-1 Tk gene promoter fragment extending from positions −222 to −1 with a PCR-amplified β-globin gene fragment extending from positions +1 to +478 and containing either the wild-type or the NS39 mutated sequence. This manipulation introduced a CTCG tetranucleotide at positions −1 to −4 relative to the β-globin gene's cap site. These hybrid fragments were used to replace the 1292-bp Hpa I–BamHI fragment of construct 1. The resulting constructs thus contained the entire transcribed sequence of the β-globin gene including the 5'-UTR joined to the HSV-1 Tk promoter.

 Constructs 9 and 10 were generated similarly but contained a 592-bp cytomegalovirus immediate early (CMV IE) enhancer-promoter instead of the HSV-1 Tk promoter. This fragment was PCR-amplified from a plasmid containing this.

Abbreviations: HSV-1, herpes simplex virus type 1; Tk, thymidine kinase; CMV, cytomegalovirus; IE, immediate early; UTR, untranslated region; CAT, chloramphenicol acetyltransferase.

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region with primers derived from previously published sequences (18). We have also used derivatives of these constructs with deletions of the enhancer sequences contained within a Nco I fragment for transfection experiments (data not shown).

DNA sequencing of the entire promoter and transcribed region showed that all constructs were identical except for the desired translational stop mutations or for the different promoters.

Transfection. Subconfluent HeLa cells were cotransfected by calcium phosphate precipitation (19) with supercoiled plasmid DNA containing constructs 1-10 (each at 50 μg) (Table 1) and an internal control plasmid containing the Escherichia coli chloramphenicol acetyltransferase (CAT) gene expressed from a simian virus 40 enhancer (SV2-CAT). The cells were washed after 16 h and harvested 24 h later.

RNA Isolation. Total RNA was isolated by guanidinium lysis and centrifugation through a CsCl cushion (20).

Nuclear and cytoplasmic fractions were separated by standard protocols using TSM buffer with 0.2% Nonidet P-40 (21). The quality of the preparation was confirmed in ethidium bromide-stained agarose gels by the absence of precursor rRNA fragments in the cytoplasmic fraction.

RNA Analysis. Northern blot analysis was performed with 1 μg of total, cytoplasmic, or nuclear RNA by standard protocols (20) using 32P-labeled complementary RNA β-globin and CAT antisense probes. The β-globin probe was a 345-nP fragment extending from positions −84 to +261. The signals were quantified by the analysis of autoradiographs with a LKB Ultrascan XL laser densitometer. Primer-extension analysis was performed with cytoplasmic RNA preparations using Moloney murine leukemia virus reverse transcriptase and an antisense 20-mer oligodeoxynucleotide primer complementary to positions +113 to +94 in exon 1 of the β-globin gene (5'-CAGCTTCACCTTGGCCCAACA-3').

RESULTS AND DISCUSSION

HeLa cells were transfected with plasmids containing the wild-type or mutated β-globin genes with the natural promoter (constructs 1-4, Table 1) and pl-5'-CAT to control for transfection efficiency. The level of β-globin mRNAs harboring the codon-6 ΔA frameshift (FS6) or the codon-39 C → T nonsense (NS39) mutation was clearly reduced (Fig. 1, compare lane 1 with lanes 2 and 3). This reduction was specific as indicated by the constant expression of CAT mRNA (Fig. 1 Lower), confirming previous observations that the diminished accumulation of nonsense mutated mRNA is not erythroid cell-specific (9, 10). In contrast to mutations leading to translational termination signals in exons 1 (FS6) or 2 (NS39), RNA with a frameshift mutation in exon 3 (FS121) accumulates to roughly normal levels (Fig. 1, compare lanes 2 and 3 with lane 4). The reduction of mRNA accumulation by premature termination codons and its positional effect with regard to the exon-intron structure of the mutated gene have also been described in other cases and shown to be caused by a post-transcriptional mechanism (11, 14, 15, 22-24). The nature of the post-transcriptional mechanism is unknown, although the influence of the location of the mutation with regard to the position in the unspliced RNA and the most recently reported effects of nonsense mutations on exon splicing (25) suggest that the fate of these mRNAs is already determined within the nucleus.

When the β-globin gene promoter and 5'-UTR of constructs 1 and 3 (Table 1) were replaced with the respective constructs of the HSV-1 Tk promoter to generate constructs 5 and 6, we noticed a surprising effect: the accumulation of NS39 mutated β-globin RNA was rescued (Fig. 2, compare lanes 3 and 4 with 5 and 6). Thus, a defect in the open reading frame of the mRNA was compensated by an exchange of the promoter and 5'-UTR. To distinguish between an effect of the HSV-1 Tk promoter and the Tk 5'-UTR, we joined the Tk promoter precisely to the cap site of the β-globin gene. The primary transcripts from the β-globin (Table 1, constructs 1 and 3) and the HSV-1 Tk promoter (Table 1, constructs 7 and 8) should, therefore, be similar (see below). Although the experimental variation was smaller with constructs 5 and 6 (see Fig. 2), the accumulation of β-globin mRNA expressed from constructs 7 and 8 resembled that of constructs 5 and 6 after transfection into HeLa cells; i.e., mRNA with the wild-type sequence or the NS39 mutation accumulated to comparable levels (Fig. 2). The HSV-1 Tk promoter was also found to elevate expression of RNA containing the FS6

Table 1. Constructs used for transfection experiments

<table>
<thead>
<tr>
<th>Construct</th>
<th>Promoter</th>
<th>5'-UTR</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>β-Globin</td>
<td>β-Globin</td>
<td>Wild type</td>
</tr>
<tr>
<td>2</td>
<td>β-Globin</td>
<td>β-Globin</td>
<td>FS 6 ΔA</td>
</tr>
<tr>
<td>3</td>
<td>β-Globin</td>
<td>β-Globin</td>
<td>NS39 C → T</td>
</tr>
<tr>
<td>4</td>
<td>β-Globin</td>
<td>β-Globin</td>
<td>FS121 + AATT</td>
</tr>
<tr>
<td>5</td>
<td>HSV-1 Tk</td>
<td>HSV-1 Tk</td>
<td>Wild type</td>
</tr>
<tr>
<td>6</td>
<td>HSV-1 Tk</td>
<td>HSV-1 Tk</td>
<td>NS39 C → T</td>
</tr>
<tr>
<td>7</td>
<td>HSV-1 Tk</td>
<td>HSV-1 Tk</td>
<td>Wild type</td>
</tr>
<tr>
<td>8</td>
<td>HSV-1 Tk</td>
<td>HSV-1 Tk</td>
<td>NS39 C → T</td>
</tr>
<tr>
<td>9</td>
<td>CMV IE</td>
<td>β-Globin</td>
<td>Wild type</td>
</tr>
<tr>
<td>10</td>
<td>CMV IE</td>
<td>β-Globin</td>
<td>NS39 C → T</td>
</tr>
</tbody>
</table>
FIG. 2. Influence of promoter sequences on the accumulation of mutated β-globin mRNA. Northern blot analysis was performed with cytoplasmic RNA from HeLa cells transfected with constructs 1 and 3 (lanes 3 and 4), 5 and 6 (lanes 5 and 6), 7 and 8 (lanes 7 and 8), or 9 and 10 (lanes 9 and 10) and pSV/CAT as a control for transfection efficiency (lanes 3–10). When corrected for transfection efficiency and compared to the wild type, the NS39 mutated RNA from the HSV-1 Tk promoter accumulated to levels of 92–140% (n = 3, construct 5 vs. 6) or 45–157% (n = 5, construct 7 vs. 8). In contrast, the NS39 mutated RNA from the CMV promoter accumulated to low levels (19–27%, n = 4), similar to those observed for the β-globin promoter. The exposure time for the β-globin signal of lanes 1–8 was about 10 times longer than that for lanes 9 and 10, reflecting the high activity of the CMV IE promoter (see also Fig. 3). Lanes: BM, total human bone marrow RNA (lane 1); C, total RNA from untransfected HeLa cells (lane 2); WT, wild-type β-globin sequence; NS39, codon-39 C → T nonsense mutation; 3 and 4, β-globin promoter and 5′-UTR; 5 and 6, Tk promoter and 5′-UTR; 7 and 8, Tk promoter and β-globin 5′-UTR; 9 and 10, CMV promoter and β-globin 5′-UTR.

The data presented in Figs. 1 and 2 indicate that the fate of the mRNA is determined by the promoter—i.e., by sequences outside the actual transcript. The overall sizes of the transcripts were demonstrated to be identical to the resolution of Northern blot analysis, regardless of the promoter driving transcription. As shown in Fig. 3, primer-extension analyses showed that the β-globin-promoted constructs utilize exactly the same transcriptional start sites as the endogenous β-globin gene in human bone marrow (compare lane 1 with lanes 3 and 4). The CMV- and Tk-promoted mRNAs were identical to a resolution of 1 nt (compare lanes 5 and 6 with lanes 7 and 8) and differed by only 1 or 2 nt from the β-globin-promoted mRNA. No evidence of substantial upstream or downstream initiation was obtained. The β-globin and the CMV IE promoter/enhancer displayed quantitative differences between wild-type and mutated RNA (compare lane 3 with lane 4 and lane 7 with lane 8), whereas no such differences were present for the RNAs expressed by the HSV-1 Tk promoter (compare lane 5 with lane 6).

The unexpected capacity of different RNA polymerase II promoters to influence the fate of their transcripts suggests that the promoters affect a choice between alternative RNA processing/transport pathways. This points to the existence of a higher and as yet poorly defined order of gene regulation integrating different steps of gene expression. What mechanisms may mediate this characteristic of promoters? The HSV-1 Tk promoter has been shown (26) to activate nucleocytoplasmic transport of mRNA in Xenopus oocytes in cis and in trans. In contrast to these findings, the HSV-1 Tk promoter failed to rescue NS39 RNA accumulation from the β-globin promoter in trans (data not shown). The CMV IE promoter appears to overcome the requirement for an intron for immunoglobulin gene expression post-transcriptionally (27) and to influence the post-transcriptional regulatory interaction between the human immunodeficiency virus trans-activation response site sequence and the Tat protein (28). The mechanisms underlying these phenomena are also un-
were (Tk), and the 113-nt open mRNAs, efficient proofreading clear the DNA (BM, nontransfected CMV known without transcripts 39, transfected with extension analysis (Tk, extension 5, or 9 nonsense mutation. The time size markers. Lanes: WT, normal β-globin gene sequence; 39, codon-39 C → T nonsense mutation.

Fig. 3. Determination of transcription start sites. Primer-extension analysis was performed with RNA from HeLa cells transfected with constructs 1 and 3 (globin, lanes 3 and 4), 7 and 8 (Tk, lanes 5 and 6), or 9 and 10 (CMV, lanes 7 and 8) and of RNA from nontransfected control cells (Co, lane 2) and human bone marrow (BM, lane 1). To allow the visualization of the β-globin and the Tk transcripts without overexposing the CMV signal, we used 10-fold less CMV- than β-globin- or Tk-promoted RNA for this experiment. The DNA sequencing reaction products (lanes C, T, A, and G) were used as size markers. Lanes: WT, normal β-globin gene sequence; 39, codon-39 C → T nonsense mutation.

known but are different from the situation seen here as the CMV IE promoter does not rescue NS39 expression.

Our data are consistent with the existence of an intranuclear proofreading mechanism that senses the disturbance of the correct open reading frame (25). In cases of mutated mRNAs, efficient export is blocked, which leads to an increased intranuclear RNA decay. One possible mechanism for the HSV-1 Tk promoter to bypass the export block is an accelerated export of β-globin mRNA along its normal pathway and a subsequent reduction of the time for the proofreading mechanism to exert its function. To test this kinetic model, we monitored the time course of mRNA accumulation after cotransfection of the Tk- and β-globin-promoted wild-type constructs 1 and 5 (Fig. 4, lanes 3–7) or the respective NS39 constructs 3 and 6 (Fig. 4, lanes 8–12). The Tk- and the β-globin-promoted transcripts can be distinguished in a primer-extension assay as the Tk 5′-UTR of constructs 5 and 6 is 5 nt longer than the β-globin 5′-UTR of constructs 1 and 3. In contrast to the predictions of the kinetic model, the data presented in Fig. 4 show that the mRNAs expressed from the β-globin or the Tk promoter accumulated in the cytoplasm with similar kinetics (compare the lower bands of constructs 1 and 3 with the upper bands of constructs 5 and 6) regardless of the presence or the absence of the NS39 mutation. Additionally, the reduction of β-globin-promoted NS39 mRNA accumulation and the respective rescue by the Tk promoter 5′-UTR are apparent at all time points (note the lower intensities of the 113-nt bands in lanes 8–12 when compared to lanes 3–7 and the similar intensities of the 118-nt bands). These results demonstrate that RNA processing is kinetically similar for both the Tk- and the β-globin-promoter-derived mRNAs. In the light of these results and the most recently described morphologic compartmentalization of the nucleus (29–31), a topographic model appears more likely. This entails different routes of gene expression that are utilized by the HSV-1 Tk and the β-globin promoters. The experimental system described in this report should allow definition of the genetic elements and cellular factors involved. The definition of how RNA polymerase II promoters recognize or contribute to such a functional compartmentalization within the nucleus and of how the presence of premature translational terminators is already sensed within the nucleus requires further study.

We thank Professors Kleihauer, Kubanek, and Bartram for their continuous support and encouragement; Drs. Angus Lamond, Iain Mattaj, and Henk Stunnenberg for constructive comments on the manuscript; Mrs. A. Ruppert, A. Bellan-Koch, and H. Kozma for

Fig. 4. Kinetics of cytoplasmic mRNA accumulation. Primer-extension analysis was performed with HeLa-cell RNA at different time points after cotransfection with the wild-type constructs 1 and 5 (WT, lanes 3–7) or the nonsense mutated constructs 3 and 6 (NS39, lanes 8–12). The 113-nt and the 118-nt bands represent the signals generated by the β-globin mRNA with the endogeneous 5′-UTR (β-globin) or with the Tk 5′-UTR (Tk), respectively. The time points are indicated in hours at the top of the lanes. The DNA sequencing reaction products (lanes C, T, A, and G) were used as size markers. BM, human bone marrow RNA; Co, RNA from untransfected control cells.
excellent technical help; and the Deutsche Forschungsgemeinschaft (DFG) and the Federal Ministry of Research and Technology (BMFT) for financial support.