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A β^o-thalassemic β-globin RNA that is labile in bone marrow cells is relatively stable in HeLa cells

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ABSTRACT

We have shown previously that a β-globin RNA-deficient β^o-thalassemia is caused by a single base-pair deletion in codon 44 of the human β-globin gene[1]. The lack of β-globin RNA in erythroid cells of these affected individuals is due to extreme β-globin RNA instability ($t_{1/2}$=30 min)[2]. We have further investigated the mechanism of this extreme lability by transiently expressing the β^o-thalassemic allele in HeLa cells and assaying the stability of the β-globin RNA that is produced. Surprisingly, the β^o-thalassemic RNA is much more stable in HeLa cells than in bone marrow cells. Apparently, developing erythroid cells have a mechanism for turning over this thalassemic RNA while cervical carcinoma cells do not. We also have assayed the stability of RNA derived from in vitro-mutagenized β-globin genes. In HeLa cells, β-globin RNAs harboring deletions in and around the translation initiation codon accumulate to steady-state levels that are similar to the level of normal β-globin RNA.

INTRODUCTION

RNA turnover is an important factor in the regulation of gene expression. To date very little is known about the determinants of RNA stability. In mammalian cells, most mRNAs have a half-life of 5 to 15 hrs[3-5]. The half-life of certain mRNAs can be modulated by specific environmental or developmental stimuli. For example, prolactin, a peptide hormone that targets the mammary gland, stabilizes casein mRNA in mammary gland organ culture[6]. The addition of cyclic AMP to disaggregated Dictyostelium discoideum cells stabilizes aggregation-stage mRNAs[7]. Preferential mRNA stability is involved in the accumulation of globin and myosin mRNA during the terminal stages of erythropoesis and myogenesis, respectively[8-10].

It is reasonable to assume that, in addition to any environmental and developmental controls of mRNA stability, the component nucleotides of an mRNA influence mRNA half-life. The basis for this assumption lies in studies of the human hereditary anemias, the β-thalassemias.

Certain β^o-thalassemias are caused by mutations within an exon of the β-globin gene that result in a shortened β-globin RNA half-life[1,2,11]. All of these mutations generate a nonsense codon either directly or by

shifting the translational reading frame. Comparison of β^{o}-thalassemic RNA levels suggests that RNA instability increases with the distance of the nonsense codon from the initiation codon[1,2,11]. These findings eliminate models that correlate RNA stability exclusively with the extent of ribosome association, where an increased number of RNA-bound ribosomes increases the protection of RNA from nuclease degradation. It seems likely that the primary structure of each β^{o}-thalassemic β-globin RNA directly affects RNA stability by altering either RNA secondary structure, the association of an RNA-binding protein, or a secondary modification of the RNA. The erythroid cell may recognize any of these aberrancies and subsequently degrade the mutant RNA molecules.

To further our understanding of RNA turnover, we have begun to determine how the component nucleotides of β-globin RNA affect RNA stability. Thalassemic and *in vitro*-generated mutant β-globin alleles have been inserted into the πSVHP expression vector and introduced into HeLa cells. After transient expression, the effect of these mutations on β-globin RNA half-life has been measured.

MATERIALS AND METHODS

In vitro mutagenesis

A 7.5 kb Hind III fragment of human DNA that includes a normal β-globin gene was cloned into the Hind III site of pBR322. To create small deletions that encompass the 5' untranslated region and first few nucleotides of the translated region, the DNA was mutagenized at the unique Nco I site. This site includes the ATG translation initiation sequence. After incubation with Nco I, nucleotides were removed from either or both ends of the DNA by nuclease Bal 31 digestion (10 sec to 1 min at 29°C in 0.6 M NaCl). DNA was ligated, digested with Nco I, and introduced into *E. coli* HB101. Deletions of plasmids lacking the Nco I site were roughly sized by restriction enzyme analysis. Small deletions were precisely determined by DNA sequencing[12]. The mutated β-globin genes and flanking sequences were cloned into the Bgl II and Pst I sites of πSVHP as 4.0 kb Bgl II/Pst I fragments. The RNA-deficient β^{o}-thalassemic gene was also cloned into πSVHP as a 4.0 kb Bgl II/Pst I fragment. To introduce the thalassemic lesion in *cis* to each of these *in vitro*-generated mutations, the Ava II 216 bp fragment encoding parts of exon II and IVS-2 of the normal human β-globin gene was substituted with the corresponding Ava II 216 bp fragment from the thalassemic gene.

The EcoR I site in exon III of the normal β-globin gene was mutagenized by purifying a 4.4 kb Pst I fragment of human DNA that encodes the entire β-globin gene. The fragment was circularized with T4 DNA ligase and digested

with EcoR I. EcoR I cleaves this DNA once in exon III. DNA was incubated with nuclease Bal 31. Linear molecules were purified and circularized with T4 DNA ligase. The sample was then digested to completion with EcoR I and Pst I, and ligated to Pst I-cleaved and alkaline phosphatase-treated pBR322. Only 4.4 kb Pst I fragments having lost the EcoR I site should be cloned. DNA was introduced into E. coli HB101. Sequences were screened for the loss of the EcoR I site. Deletions were roughly sized by restriction enzyme analysis and precisely defined by DNA sequencing. These mutated β-globin genes were cloned as a 4.0 kb Bgl II/Pst I fragment into the Bgl II and Pst I sites of πSVHP.

HeLa cell growth and transformation

HeLa cells were grown in 150 mm^2 T-flasks in Eagle's Minimal Essential Medium alpha (MEMα). Cells were passaged every 5-7 days. Twenty-four hours before transformation with πSVHP derivatives, cells were split 1:5. Two hours before transformation, the medium was replaced with fresh medium. πSVHP plasmids were precipitated with $CaCl_2$ at room temperature for 30 min as described by Treisman et al.[13]. The Ca-DNA phosphate precipitate was added to the cells at a ratio of 1:10 volumes DNA:medium with 10 μg of plasmid DNA per flask. The cells were incubated at 37°C for 4-6 hrs, then shocked with 25% glycerol in MEMα (5 ml per flask) for 1 min at 37°C. When RNA half-life measurements were to be made, 1/20 volume of a 200 μg/ml actinomycin D solution (in MEMα) was added to one half of the HeLa cells. The cells were incubated for 30 min or 1 hr at 37°C and then harvested.

RNA Purification and S_1-nuclease mapping

Adherent cells were scraped into 10 ml of chilled (4°C) MEMα. The cells were collected by centrifugation at 1,500 x g for 6 min at 4°C. RNA was extracted with phenol and chloroform, and pelleted through a CsCl gradient as previously described[14]. The purified RNA was used in S_1-nuclease mapping experiments[15,16]. DNA probes were 3' end-labeled with [α-^{32}P]dATP (∿3,000 Ci/mmole) and purified in acrylamide gels[2]. Experiments were performed in probe excess[2].

RESULTS

Normally, the β-globin gene is expressed only in erythroid precursor cells. However, when the β-globin gene is cloned into the πSVHP expression vector and introduced into HeLa cells, it is transcribed[13]. πSVHP plasmids carrying either the normal or the β^o-thalassemic gene have been constructed (Fig. 1A). Both globin genes include 1700 bp of 5' flanking DNA, the entire transcribed region, and 500 bp of 3' flanking DNA. The β^o-thalassemic gene lacks a single base-pair in codon 44 which results

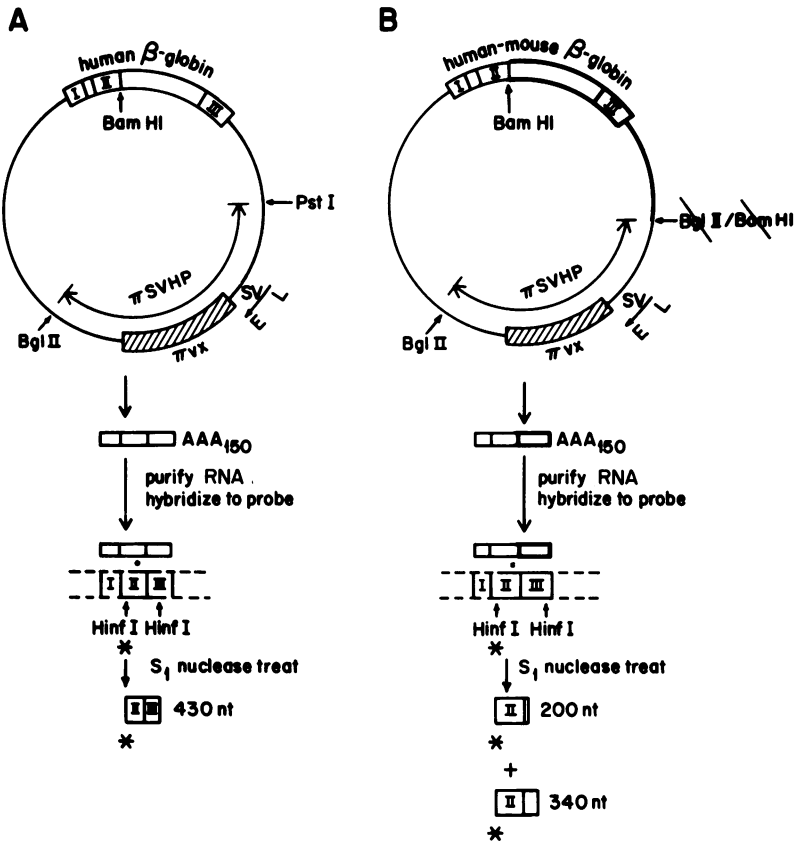

Figure 1. Structure of the β-globin gene/πSVHP recombinant plasmids and the protocol for S_1-nuclease transcript mapping. πSVHP plasmids contain either human (A) or human-mouse (B) β-globin genes. The human genes were cloned as 4.0 kb Bgl II/Pst I fragments into the Bgl II and Pst I sites of πSVHP. These genes were either normal human, β^0-thalassemic, normal human *in vitro*-mutagenized, or β^0-thalassemic *in vitro*-mutagenized β-globin sequences. The human-mouse hybrid gene consists of a 2.3 kb Bgl II/ BamH I fragment that contains the 5' end of the human β-globin gene plus 5' flanking region, and a 2.5 kb BamH I/Bgl II fragment that contains the 3' end of the mouse β-globin gene plus 3' flanking region (thick line). The human-mouse fragments were cloned into the Bgl II and BamH I sites of πSVHP. β-globin gene exons are numbered I, II, and III intervening sequences are open blocks. πSVHP consists of πVX which contains a Col E1 plasmid origin of replication, a suppressor gene (Sup F), SV 40 (SV) early (E) and late (L) DNA (that contains the tandemly repeated 72 bp sequence needed for efficient β-globin gene expression in HeLa cells), and a polylinker (that includes the Bgl II, Pst I, and BamH I sites). See ref. 13 for additional details.
RNA purified from HeLa cells co-transformed with one of the human β-globin gene/πSVHP DNAs and the human-mouse β-globin gene/πSVHP DNA was

quantitated by S_1-nuclease transcript mapping. Total cellular RNA was hybridized to a [^{32}P] end-labeled (*) Hinf I fragment derived from a cloned human β-globin cDNA. The entire length of the probe (430nt) is protected from S_1-nuclease digestion when hybridized to processed human RNA. Smaller regions of the probe (340nt and 200nt) are protected when hybridized to human-mouse hybrid globin RNA because of sequence divergence between the human and mouse genes. Therefore, human and hybrid β-globin RNA levels can be differentiated.

in a shift of the translational reading frame with the generation of a nonsense codon at amino acid position 60[1]. In bone marrow erythroblasts, RNA encoded by this gene is synthesized and correctly processed; however, fully spliced β-globin RNA is extremely unstable ($t_{1/2}$=30 min)[2].

To investigate the mechanism of β^o-thalassemic β-globin RNA instability, πSVHP plasmid DNA carrying either the normal or β^o-thalassemic β-globin gene was introduced into HeLa cells by calcium phosphate copreciptiation[13]. An equal mass of a human-mouse hybrid β-globin gene in πSVHP (Fig. 1B) was included in the coprecipitations to serve as a standard for variations in transformation frequency and/or RNA recovery. This hybrid gene contains 1700 base-pairs of 5' flanking region, exon I, intervening sequence (IVS)-1, and most of exon II of the human β-globin gene, and 18 bp of exon II, IVS-2, exon III, and 2300 base-pairs of 3' flanking region of the mouse β^{maj}-globin gene. RNA that is transcribed from the hybrid gene can be distinguished from RNA that is transcribed from the human gene using an S_1-nuclease transcript mapping technique[2,15,16]. In this technique, transformed HeLa cell RNA is hybridized with a 3' end-labeled, 430nt Hinf I fragment that is derived from a human β-globin cDNA clone (Fig. 1). This Hinf I fragment spans most of exon II and exon III and is fully protected from S_1-nuclease cleavage when hybridized to processed human β-globin RNA. In contrast, only part of the Hinf I fragment is protected from S_1-nuclease cleavage when hybridized to processed human-mouse hybrid RNA. The sequence of the hybrid RNA diverges from the human gene in exon III (which is derived from the mouse gene). Mismatches in a hybrid RNA-Hinf I 430nt DNA duplex result in S_1-nuclease sensitivity at one site that is 200nt from the 3' end-label and at a second site that is 340nt from the 3' end-label (Fig. 1). At the first site, 7 of 12 bases are different with a perfect match of at least 8 bases to either side resulting in S_1-nuclease cleavage 5-80% of the time. From the second cleavage site to the 5' end of the Hinf I fragment there is 70% mismatch between the human and mouse genes. This results in S_1-nuclease cleavage at nucleotide 340 of the probe all of the time.

To prove that the S_1-nuclease protection is specific and quantitative, several controls were performed. HeLa cells were transiently transformed with the human β-globin gene, the human-mouse β-globin gene, or

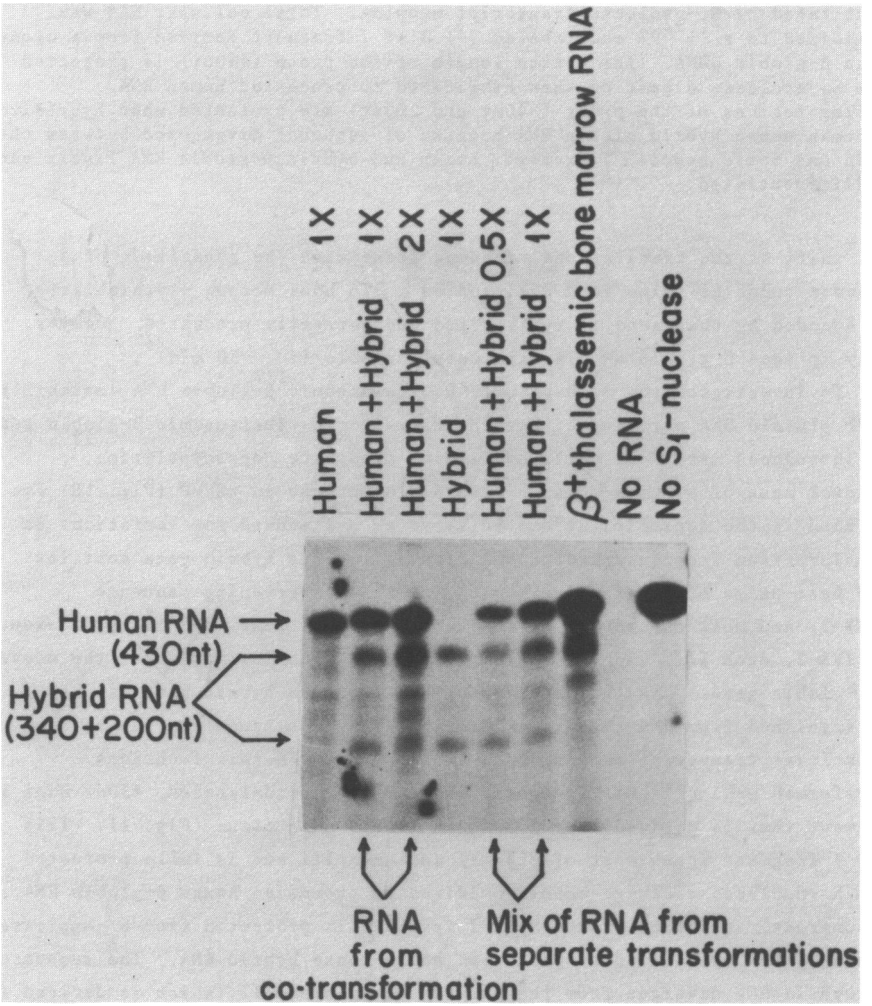

Figure 2. Demonstration that a single DNA fragment can quantitate human β-globin RNA and human-mouse β-globin RNA levels by S_1-nuclease protection. Total RNA (15 μg for 0.5X lanes, 30 μg for 1X lanes, and 60 μg for 2X lanes) from transformed HeLa cells was mixed with approximately 2×10^4 cpm of 3' end-labeled Hinf I probe (see text for description). Samples were hybridized for 18 hrs, incubated with S_1-nuclease, electrophoresed, and exposed to X-ray film.

both genes. Total cell RNA was prepared 42 hrs post-transformation. In additional experiments, equal amounts of RNA from the singly transformed cultures were mixed. RNA was analyzed by S_1-nuclease mapping using a molar excess of the 3' end-labeled 430nt Hinf I fragment. The results

demonstrate that RNA derived from double-transformed cells, as well as RNA derived from separate transformations that had been mixed prior to the S_1-nuclease mapping, protect the same amount of Hinf I fragment (Fig. 2, compare lanes labeled Human + Hybrid [1X] from co-transformed and mixed RNA samples). Expression of the two co-transformed genes is additive, and therefore the human-mouse hybrid gene can be used as an internal control for variations in gene expression and/or RNA recovery among the various transformed cell cultures. This single probe distinguishes the human RNAs (protecting a 430nt DNA fragment) from the human-mouse hybrid RNA (protecting 340nt and 200nt fragments) (Fig. 2, compare lanes labeled Human and Hybrid). The procedure is quantitative as shown by the doubling of band intensity when the quantity of RNA is doubled (Fig. 2, compare the lanes labeled 1X, 2X, and 0.5X, 1X). It should be noted that a β^+-thalassemic bone marrow RNA produces a 340nt band in addition to the 430nt band. This additional band is due to β-globin RNA hybridization to the β-globin Hinf I fragment, with subsequent S_1-nuclease cleavage in the mismatched 3' untranslated region. β-Globin gene/πSVHP plasmids similar to those described here have been shown to promote the synthesis in HeLa cells of β-globin RNA that is structurally indistinguishable from authentic β-globin mRNA[13]. We have demonstrated by S_1-nuclease transcript mapping, Northern blotting, and oligo(dT)-cellulose chromatography that both normal and β^o-thalassemic β-globin transcripts produced by this method 1) initiate at the cap site (data not shown), 2) terminate at the poly(A) addition site (see below in Fig. 5), 3) are the same length as bone marrow β-globin mRNA, and 4) are polyadenylated (Swartwout and Kinniburgh, in preparation).

Simultaneous detection of human β-globin RNA and the mouse-human hybrid β-globin RNA allowed us to quantitate RNA from cultures transformed with the RNA-deficient β^o-thalassemic gene. The half-life of the β-globin RNA was measured by comparing steady-state β-globin RNA levels to levels after a 30 or 60 min block in transcription by actinomycin D. Using a similar protocol with nucleated erythroid cells of this β^o-thalassemic patient, we were able to demonstrate a 60% reduction in the level of β-globin RNA after a 30 min block in transcription[2]. HeLa cells were transformed with the hybrid gene and either the normal gene, the β^o-thalassemic gene, the normal gene harboring an *in vitro*-generated mutation, or the β^o-thalassemic gene harboring an *in vitro*-generated mutation. These mutations include a deletion of one nucleotide directly upstream from the ATG translation initiation codon (Δ-1), a deletion of three nucleotides directly upstream from the translation initiation codon (Δ-3 to -1), a 12nt deletion that includes the ATG initiation codon (ΔATG), a

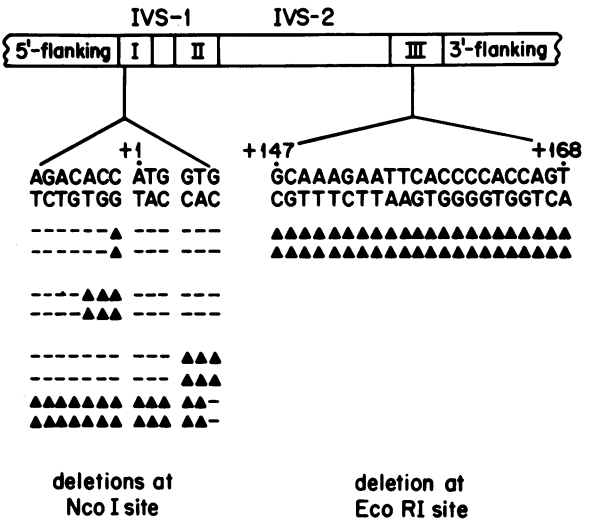

Figure 3. Summary of in vitro-generated mutations within the normal and β^othalassemic β-globin genes. Deleted nucleotides are indicated with solid triangles. +1 is defined as the first nucleotide of the ATG translation initiation codon. Roman numerals indicate exons; IVS indicates intervening sequences. All deletions were characterized by DNA sequencing.

deletion of the codon for amino acid one (Δa.a.1), and a 22nt deletion that includes the EcoR I site in the third exon yet does not extend into IVS-2 (Δ+147 to +168; this deletion was introduced into the normal but not the thalassemic β-globin gene) (Fig. 3). In additional experiments, HeLa cells were mock-transformed without DNA. Transformed cells were allowed to accumulate β-globin RNA for 42-44 hrs, and then one half of the culture was treated with actinomycin D for 1 hr to block transcription and to allow the assay of RNA turnover.

When HeLa cells are transformed with the normal human β-globin gene, the β^o-thalassemic gene, or the Δa.a.1 derivatives of these genes, β-globin RNA accumulates in the steady-state (Fig. 4). The relative proportion of RNA derived from the hybrid gene as compared to each of the human genes is similar (Fig. 4) indicating that the normal, thalassemic and Δa.a.1 gene transcripts have similar stabilities. This is in contrast to results with erythroid bone marrow genes where the steady-state level of β^o-thalassemic RNA is several orders of magnitude lower than the steady-state level of normal β-globin RNA[2]. A comparison of RNA levels in the steady-state and after treatment with actinomycin D reveal that none of the above mutations or combinations of mutations drastically affects the stability of β-globin RNA (Figs. 4 and 5, compare steady-state RNA, ss, to actinomycin D-chased cells,

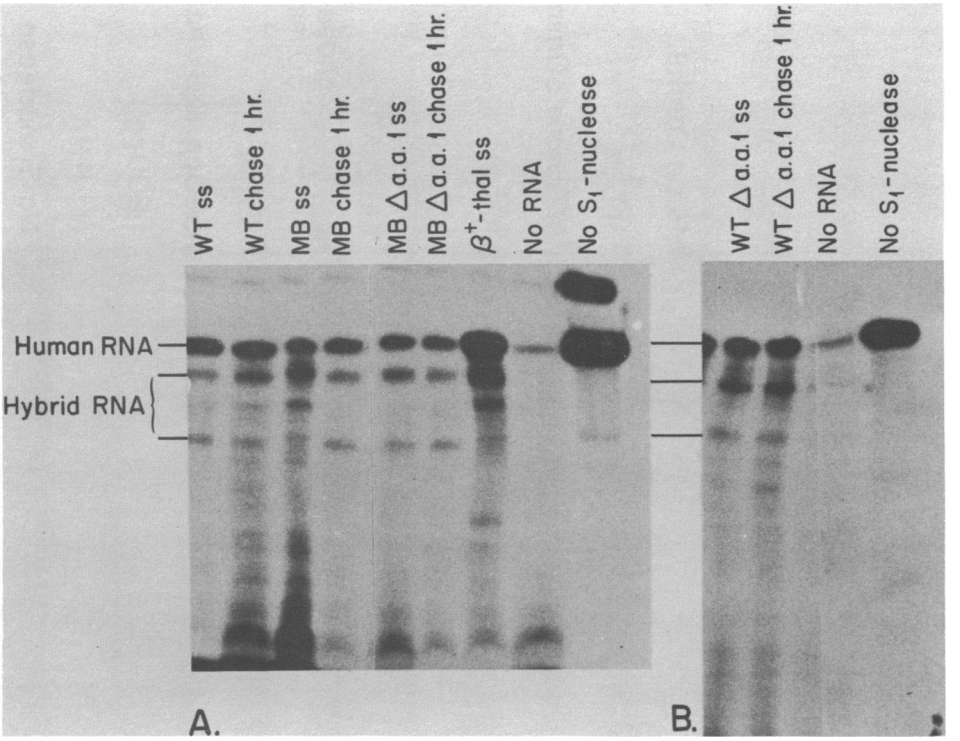

Figure 4. Steady-state levels of β^{o}-thalassemic and in vitro-mutagenized β-globin RNAs are comparable to the level of normal β-globin RNA in transformed HeLa cells. Various πSVHP constructs (designated at the top of each lane) were introduced with a human-mouse hybrid β-globin gene into HeLa cells (see Materials and Methods). RNA (30 μg) prepared from each transformed culture was used in an S_1-nuclease protection assay (see legend to Fig. 2). Total bone marrow RNA (1 μg) from a β^{+}-thalassemic individual was used as a positive control. WT, a normal human β-globin gene; MB, the β^{o}-thalassemic globin gene; MB Δa.a.1., the β^{o}-thalassemic allele with a 3 bp deletion spanning amino acid codon one; WT a.a.1, the normal human allele with a 3 bp deletion spanning amino acid codon one; ss, steady-state RNA; chase, RNA from cultures treated with actinomycin D (10 μg/ml for 1 hr). S_1-nuclease mapping experiments shown in panels A and B were performed at different times.

chase; data not shown for the EcoR I site deletion or Δ-3 to -1 deletion). In HeLa cells, RNA derived from the β^{o}-thalassemic gene (Fig. 4: MB; ss, chase), RNA derived from the β^{o}-thalassemic gene lacking the codon for amino acid one (Fig. 4: MB Δ a.a.1; ss, chase), and RNA derived from a normal gene (Fig. 4: WT; ss, chase) are at comparable steady-state levels. These RNA levels do not decrease during the chase period. In addition to the Hinf I

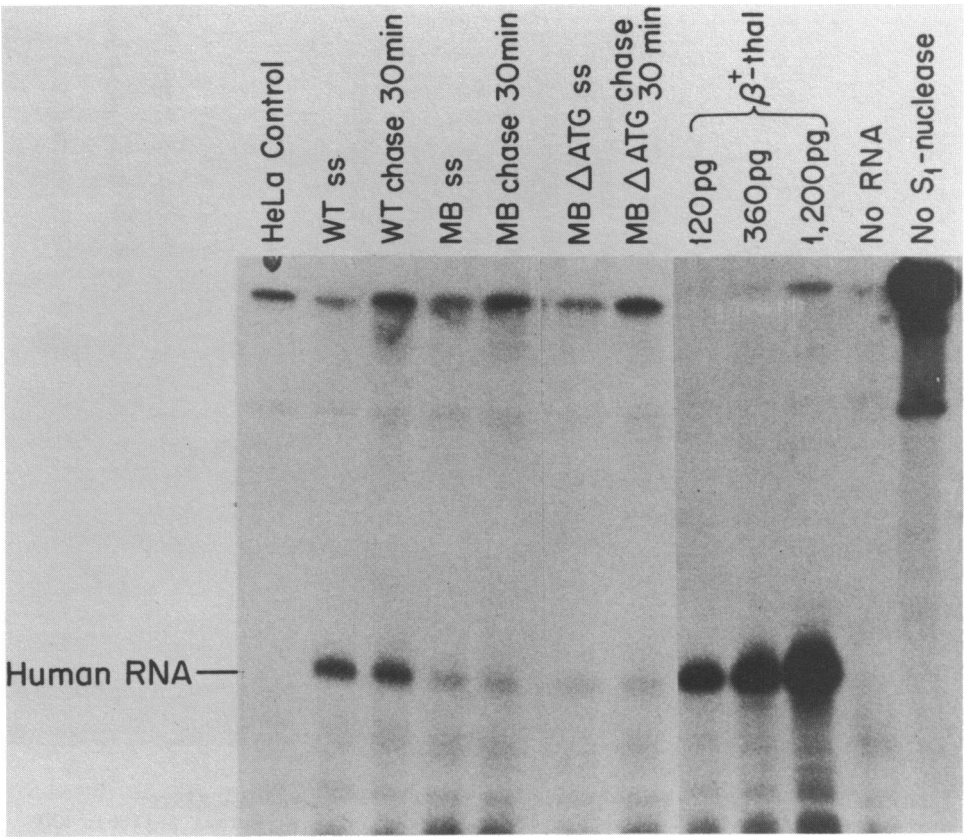

Figure 5. Further evidence for mutant β-globin RNA stability in HeLa cells. HeLa cell cultures were transformed with various human β-globin gene constructs in πSVHP (designated above lanes). RNA (30 μg) prepared from each cell culture was hybridized to a 3' end-labeled, 1.5 kb EcoR I fragment that includes 205 bp of the third β-globin exon, 550 bp of 3' flanking DNA, and 750 bp of vector (pBR322) DNA[2]. Incubation with S_1-nuclease and gel electrophoresis were as described[2]. Processed human β-globin RNA protects a 205nt of the [^{32}P] labeled EcoR I fragment from digestion by S_1-nuclease (labeled Human RNA). Sample abbreviations are as described in Figure 4.

fragment, a 3' end-labeled EcoR I fragment (that contains the 3'-most 208 of 261 bp in exon III and 500 bp of 3' flanking human β-globin DNA) was used for RNA quantitation. This fragment can be [^{32}P]-labeled at two residues of the EcoR I site (two [^{32}P]dATP residues will be incorporated using Klenow fragment) and therefore will detect relatively low levels of human β-globin RNA. It is evident (Fig. 5) that RNA derived from the β^{o}-thalassemic gene (MB; ss, chase), the initiation codon-deleted

β^{o}-thalassemic gene (MB Δ ATG; ss, chase), and a normal gene (WT ss, chase) accumulate to similar steady-state levels. These RNA levels are maintained during the chase period. Hybrid gene RNA is not detected because the region of homology between the EcoR I fragment and mouse exon III is small. However, these results are quantitative as shown by a proportional increase in band intensity with increasing amounts of β^{+}-thalassemic RNA (Fig. 5). Furthermore, the S_1-nuclease protected DNA fragment is a result of transforming plasmid gene expression as assayed by the lack of protection with mock-transformed HeLa cell RNA (Fig. 5, HeLa control). While the absolute half-lives of the normal and mutant β-globin RNAs in HeLa cells are unknown, it is clear that these half-lives are on the order of hours. This is in sharp contrast to the 30 minute half-life demonstrated for the β^{o}-thalassemic RNA in erythroid bone marrow cells. If the β^{o}-thalassemic RNA were as labile in HeLa cells as it is in erythroid bone marrow cells, then a 60% decrease in the amount of either Hinf I or EcoR I probe protected from S_1-nuclease digestion should be detected. Even if the thalassemic RNA half-life were 1-2 hrs, a 25-50% reduction in RNA during the chase would be detected. Also, there should be detectable differences in the steady-state levels of β^{o}-thalassemic and normal β-globin transcripts as compared to internal hybrid RNA controls if the thalassemic β-globin RNA were extremely labile. To ensure that the thalassemic lesion was present in the thalassemic β-globin gene/πSVHP plasmid, approximately 300 bp encompassing codon 44 were sequenced. The sequence data confirmed the single nucleotide deletion at codon 44 (data not shown).

DISCUSSION

We have transiently transformed HeLa cells with either normal, RNA-deficient β^{o}-thalassemic, or *in vitro*-generated mutant β-globin genes. Utilizing a quantitative S_1-nuclease protection assay, we have found that all β-globin genes analyzed produce RNAs that are sufficiently stable to accumulate in the steady-state (Figs. 4 and 5). We have assayed β-globin RNA stability more directly by blocking transcription with actinomycin D. Unexpectedly, neither the RNA-deficient β^{o}-thalassemic allele nor the allele harboring a deletion of the ATG translation initiation codon produce RNA that turns over during the chase period. It appears that there is some feature(s) of the β^{o}-thalassemic RNA (e.g., the actual cytidine deletion in codon 44, the frameshift-generated nonsense codon at position 60, the lack of ribosome protection between codon 60 and the termination codon) that erythroid bone marrow cells recognize but that HeLa cells do not recognize. This feature(s) results in the rapid turnover of β-globin RNA in erythroid cells but not in HeLa cells. HeLa cells, being cultured neoplastic cells,

may have undergone selection for certain characteristics, e.g., rapid growth, that may have obviated the need for a precise mechanism of eliminating RNAs that harbor nonsense codons. On the other hand, differentiating erythroid cells may have evolved an efficient mechanism for destroying nonsense globin RNAs since the synthesis of truncated globin proteins might affect red cell structure and function by disrupting normal hemoglobin packing. Another explanation for this difference in cell-type RNA turnover may be the relative level of β-globin RNA. In the developing erythroid cell, β-globin mRNA accounts for 10-50% of total mRNA. According to our calculations, β-globin RNA is only 0.25-1.0% of total RNA in transformed HeLa cells (data not shown). If HeLa cells have a mechanism for nonsense RNA turnover, this mechanism may not be activated unless the nonsense RNA is at higher levels than the levels obtained in these experiments. We are currently examining the hypothesis that there is an erythroid-specific factor(s) that recognizes the β^{o}-thalassemic lesion by testing various cell types for specific ribonucleases.

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REFERENCES

1. Kinniburgh, A. J., Maquat, L.E., Schedl, T., Rachmilewitz, E. A., and Ross, J. 1982. Nucleic Acids Res. 10: 5421-5427.
2. Maquat, L. E., Kinniburgh, A. J., Rachmilewitz, E. A., and Ross, J. 1981. Cell 27: 543-553.
3. Perry, R. and Kelley, D. E. 1973. J. Mol. Biol. 79: 681-696.
4. Puckett, L. and Darnell, J. E. 1977. J. Cell Physiol. 90: 521-534.
5. Harpold, M. M., Wilson, M. C., and Darnell, J. E., Jr. 1981. Mol. Cell. Biol. 1: 188-198.
6. Guyette, W. A., Matusik, R. J., and Rosen, J. A. 1979. Cell 17:1013-1023.
7. Mangiarotti, G, Ceccarelli, A., and Lodish, H. F. 1983. Nature 301: 616-618.
8. Volloch, V. and Housman, D. 1981. Cell 23: 509-514.
9. Affara, N. A., Robert, B., Jacquet, M., Buckingham, M. E., and Gros, F. 1980. J. Mol. Biol. 140: 441-458.
10. Buckingham, M. E., Caput, D., Cohen, A., Whalen, R. G., and Gros, F. 1974. Proc. Natl. Acad. Sci. USA 71: 1466-1470.

11. Moschonas, N., de Boer, E., Grosveld, F. G., Dahl, H. H. M., Wright, S., Shewmaker, C. K., and Flavell, R. A. 1981. Nucleic Acids Res. 9: 4391-4401.
12. Maxam, A. M. and Gilbert, W. 1980. Methods Enzymol. 65: 499-560.
13. Treisman, R., Proudfoot, N. J., Shander, M., and Maniatis, T. 1982. Cell 29: 903-911.
14. Maquat, L. E., Kinniburgh, A. J., Beach, L. R., Honig, G. R., Lazerson, J., Ershler, W. B., and Ross, J. 1980. Proc. Natl. Acad.Sci. USA 77: 4287-4291.
15. Berk, A. J. and Sharp, P. A. 1978. Proc. Natl. Acad. Sci. USA 75: 274-1278.
16. Weaver, R. F. and Weismann, C. 1979. Nucleic Acids Res. 7:1175-1193.