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Erythroid Cell-Specific mRNA Stability Elements in the α2-Globin 3′ Nontranslated Region†

INGRID M. WEISS[1,2] AND STEPHEN A. LIEBHABER[1,2,3]*

Howard Hughes Medical Institute[1] and Departments of Genetics[2] and Medicine,[3] University of Pennsylvania, Philadelphia, Pennsylvania 19104

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Very little is known about the mechanisms mediating longevities of mRNAs. As a means of identifying potential *cis*- and *trans*-acting elements which stabilize an individual mRNA, naturally occurring mutations that decreased stability of the normally long-lived globin mRNA were analyzed. Our previous studies demonstrated that a subset of mutations which allowed the translating ribosome to read through into the α2-globin 3′ nontranslated region (NTR) targeted the mutant mRNAs for accelerated turnover in erythroid cells but not in several nonerythroid cell lines (I. M. Weiss and S. A. Liebhaber, Mol. Cell. Biol. 14:8123–8132, 1994). These results suggested that translational readthrough interfered with some feature of the α2-globin 3′ NTR required for message stability in erythroid cells. To define the *cis*-acting sequences which comprise this erythroid cell-specific stability determinant, scanning mutagenesis was performed on the α2-globin 3′ NTR, and the stability of each mutant mRNA was examined during transient expression. Three cytidine-rich regions which are required for longevity of the α2-globin mRNA were identified. However, in contrast to the readthrough mutations, base substitutions in these elements destabilize the message through a translation-independent mechanism. To account for these results, we propose that the *cis*-acting elements form a complex or determinant in the normal α2-globin mRNA which protects the message from degradation in erythroid cells. Disruption of this determinant, by translational readthrough or because mutations in an element prevent or inhibit its formation, targets the message for accelerated turnover in these cells.

Eucaryotic mRNAs have a considerable range of half-lives, from as short as a few minutes to as long as several days. It has become increasingly clear that these differences in message stability have major impacts on the levels of gene expression (19). While many of the features involved in mediating the half-lives of short-lived mRNA species have been described (8, 12, 27, 37, 40, 41, 42), mechanisms responsible for the longevities of eucaryotic mRNAs are for the most part undefined. To date, very few structures have been shown to stabilize a message. Exceptions include the ^{7}mGppp cap (4, 17), poly(A) tail/poly(A)-binding protein complex (9, 24), and a GC stem structure which appears to slow the degradation pathway (14, 50). Recently, however, particular regions within several long-lived mRNAs have been implicated in message stabilization (5, 38, 43, 47). Furthermore, *trans*-acting factors have been shown to interact with one of these sequences (15), allowing for the possibility that the longevities of mRNAs are governed by specific mechanisms.

One system in which message stability plays a major role in gene expression is during erythrocyte (RBC) differentiation. mRNAs encoded by the α- and β-globin gene clusters increase from less than 1% to represent over 95% of the total message and protein in the more differentiated RBCs (6). This high-level accumulation results not only from an increase in transcription but in part from the long half-lives of globin mRNAs (24 to 60 h) and from the degradation of nonglobin mRNAs (2, 7, 29, 33, 39, 44, 45, 49). The mechanisms responsible for the longevities of globin mRNAs and the turnover of other mRNAs in RBCs are presently undefined. There is some evidence suggesting that the stabilities of globin mRNAs may be mediated by *cis*-acting elements (25, 44, 53) or structural alterations in the message (34). Additional studies demonstrate that erythroid cells have degradation pathways which preferentially act on certain sequences or mRNAs (3, 29, 30, 56).

To explore the mechanisms which regulate message stability, we developed a transient transfection system that could be used in conjunction with a reverse transcription-PCR (RT-PCR)-based assay to measure the impact of mutations on stability of the α2-globin mRNA (31, 52, 53). Initial studies focused on examining the defect in expression of a naturally occurring α2-globin mutant called Constant Spring (CS). The CS mutation is a single-base substitution which changes the translation termination signal to a glutamine codon (UAA→CAA) and allows the ribosome to translate an additional 31 codons into the 3′ nontranslated region (NTR) (13). In RBCs, there is an almost complete absence of mRNA and protein from the affected allele (13, 23, 26, 32). Our findings in murine erythroleukemia (MEL) cells demonstrated that this loss of expression resulted from destabilization of the CS mRNAs (53). The mechanism responsible for mRNA degradation was translation dependent and required that the ribosome translate between one and four codons into the 3′ NTR (53). These results indicate some feature or determinant associated with the α2-globin 3′ NTR was important in mediating mRNA stability in erythroid cells. In contrast, CS mRNAs were stable in nonerythroid cells, suggesting the determinant functions in an erythroid cell-specific manner (53).

In the present study, scanning mutagenesis was performed on the α2-globin 3′ NTR to locate and define potential *cis*-acting sequences which comprise the erythroid cell-specific stability determinant. Three cytidine-rich (C-rich) regions were found to be essential for message stability in erythroid cells but

* Corresponding author. Mailing address: Room 438, Clinical Research Building, 422 Curie Blvd., University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6145. Phone: (215) 898-7834. Fax: (215) 898-1257.

† Stephen A. Liebhaber, members of his laboratory, and all of the friends and colleagues of Ingrid M. Weiss note with sadness her unexpected death and dedicate this publication to her memory.

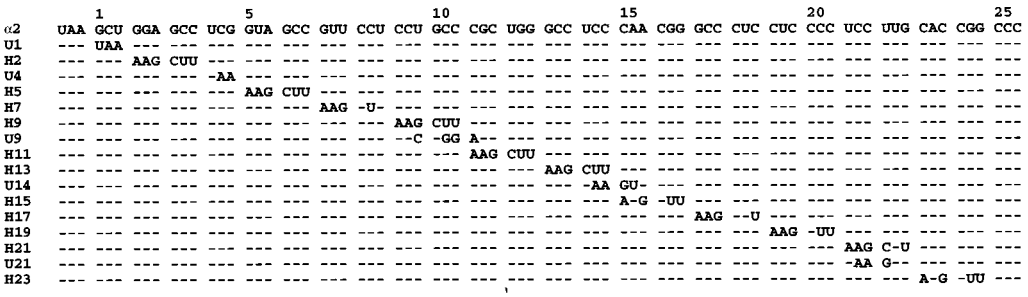

FIG. 1. Site-directed mutations in the α2-globin 3′ NTR. The first 75 bases of the normal α2-globin 3′ NTR are shown on the top line, starting from the translation termination codon at the far left. Mutations are noted below, and sequence identity is represented by a dash. Each set of four to six base substitutions creates a *Hin*dIII restriction site (5′-AAGCTT-3′) and is designated HX, where X is the codon position of the *Hin*dIII site relative to the normal translation stop codon. Several additional mutations which do not generate *Hin*dIII sites are named UX as described above.

not in nonerythroid cells. Mutations in single elements were capable of destabilizing the α2-globin mRNA, indicating that these segments may interact to form a functional determinant. In contrast to the translation termination mutants, the mechanism responsible for degradation of mRNAs containing mutations in C-rich segments does not require translation of the message. To account for these findings, we propose that the C-rich elements form a determinant required for stability of the α2-globin mRNA in erythroid cells. The disruption of this determinant, due to a translating ribosome migrating through the region or because base substitutions in an element inhibit or abolish its formation, results in accelerated degradation of the mRNA in erythroid cells. We hypothesize that the tissue-specific differences in α2-globin mRNA metabolism are because the maintenance of this determinant is important only in erythroid cells, in which it serves to protect the message from turnover. It is possible that the recognition of an intact 3′ NTR determinant is one of the mechanisms by which the erythrocyte distinguishes between globin and nonglobin mRNAs during RBC differentiation.

MATERIALS AND METHODS

Construction of the α-globin expression vectors. The pSV2Aneoα2 expression vector containing the 1,493-bp *Pst*I α2-globin gene has been described elsewhere (52). A PCR-based splice overlap extension (SOE) technique (21, 22) was used to introduce a series of base substitutions which create *Hin*dIII sites (5′-AAGCTT-3′) at defined positions within the α2-globin 3′ NTR (Fig. 1). pSV2Aneoα2 was used for template DNA, and the complementary pairs of mutagenesis primers are listed in Table 1. The 549-bp DNA fragments obtained by SOE mutagenesis were digested with *Bst*EII and *Eco*RI, and the resulting 305-bp fragments containing the various *Hin*dIII sites were exchanged for the corresponding region of the pSV2Aneoα2 expression vector. The U9, U21, and ACG.U9 mutations were generated by M13mp19 site-directed mutagenesis (59) with the following oligonucleotides (5′→3′): U9, GGCCCAGCTCCCGGGAGGAACGGC; U21, GCCGGTGCACTTAGGGGAGGAG; and ACG, GAGAGAACCCACCACGGTC. The mutagenized fragments were cloned into the expression vector as described previously (52, 53). Construction of the α2-globin gene under the transcriptional control of the c-*fos* promoter (pfos/α2) has been described elsewhere (53). The pfos/H2 and pfos/H13 expression vectors were made by exchanging the 152-bp *Bst*EII- and *Apa*I-restricted DNA fragments containing the H2 and H13 mutations for the corresponding regions of plasmid pfos/α2-globin. All of the expression vectors were sequenced to verify the presence of the mutations and the fidelity of the PCR-generated DNA inserts (46).

Cell culture, electroporation, and Northern (RNA) blot analyses. MEL cells were washed and resuspended in phosphate-buffered saline at 10^7 cells per ml. Electroporations were performed at 400 V, using a Bethesda Research Laboratories System II Porator as described previously (52, 53). Semiconfluent C127 cells were trypsinized, washed several times with minimal essential medium, resuspended in medium at 10^6 cells per ml, and electroporated at 200 V. To make stable cell lines for the RNA half-life measurements, plasmids pfos/H2 and pfos/H13 (10 mg) were linearized at the *Eco*RI site 5′ to the c-*fos* promoter. C127 cells were electroporated with the linearized DNAs and 2 days later were placed in selective medium (400 μg of G418 per ml). Medium was changed every other day, and at the end of 2 weeks, pools of transformed cells were obtained. Stable cell lines containing the *fos*/H2 or *fos*/H13 globin gene constructs were made quiescent by a 3-day incubation in minimal essential medium containing 0.5% fetal bovine serum (FBS) and were subsequently induced with 20% FBS. At various times postinduction, a 10-cm^2 plate of cells was harvested for RNA, and RT-PCR analyses were performed with 20% of the cytoplasmic RNA.

For Northern blot analyses, RNAs were transferred to GeneScreen Plus membranes, UV cross-linked, prehybridized, and hybridized as described by the manufacturer (New England Nuclear). Probes were generated by random primer labeling a 305-bp *Bst*EII-*Eco*RI α2-globin gene fragment (53), a PCR-generated fragment which spans codons 130 to 235 of the rat c-*fos* cDNA (gift of L. S. Callans and W. Lee, University of Pennsylvania, Philadelphia), and a 400-bp *Hin*dIII-*Eco*RI fragment of the ribosomal protein rpL32 (35). RNA levels were determined by quantitating the signals from the Northern blots with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). The α-globin and c-*fos* signals were normalized for loading, as determined by the rpL32 signal from the same Northern blot.

RT-PCR *Hin*dIII assays. Primer extension reaction mixtures contained 5 to 10 μg of cytoplasmic RNA, 100 pmol of the N9T9 primer (5′-TTTTTTTTTGCC GCCCAC-3′), which is complementary to the last nine bases of the α2-globin mRNA and the poly(A) tail, 1 mM deoxynucleoside triphosphates, RT-PCR

TABLE 1. Oligonucleotides used for PCR SOE mutagenesis technique[a]

Mutation	Type	Sequence
H2	Sense	GTTAAGCTAAGCTTTCGGTAGC
	Antisense	GCTACCGAAAGCTTAGCTTAAC
H5	Sense	GAGCCTCGAAGCTTGTTCCTCC
	Antisense	GGAGGAACAAGCTTCGAGGCTC
H7	Sense	CGGTAGCCAAGCTTCCTGCCCG
	Antisense	CGGGCAGGAAGCTTGGCTACCG
H9	Sense	CCGTTCCTAAGCTTCGCTGGGC
	Antisense	GCCCAGCGAAGCTTAGGAACGC
H11	Sense	CTCCTGCCAAGCTTGCCTCCCA
	Antisense	TGGGAGGCAAGCTTGGCAGGAG
H13	Sense	CCCGCTGGAAGCTTCAACGGGC
	Antisense	GCCCGTTGAAGCTTCCAGCGGG
H15	Sense	GGGCCTCCAAGCTTGCCCTCCT
	Antisense	AGGAGGGCAAGCTTGGAGGCCC
H17	Sense	CCCAACGGAAGCTTCTCCCCTC
	Antisense	GAGGGGAGAAGCTTCCGTTGGG
H19	Sense	GGGCCCTCAAGCTTTCCTTGCA
	Antisense	TGCAAGGAAAGCTTGAGGGCCC
H21	Sense	TCCTCCCCAAGCTTCACCGGCC
	Antisense	GGCCGGTGAAGCTTGGGGAGGA
H23	Sense	CCTCCTTGAAGCTTCCCTTCCT
	Antisense	AGGAAGGGAAGCTTCAAGGAGG
945	Sense	CACGTGGACGACATGCCCAA
1493	Antisense	GGGAATTCCTGCAGAGAGGTCCT

[a] 5′ DNA fragments for SOE mutagenesis were generated with the 945 and antisense primers, and 3′ fragments were made with the 1493 and sense oligonucleotides. Full-length mutagenized SOE DNAs were generated by amplifying a mixture of the 5′ and 3′ fragments of a designated mutant with the 945 and 1493 primers.

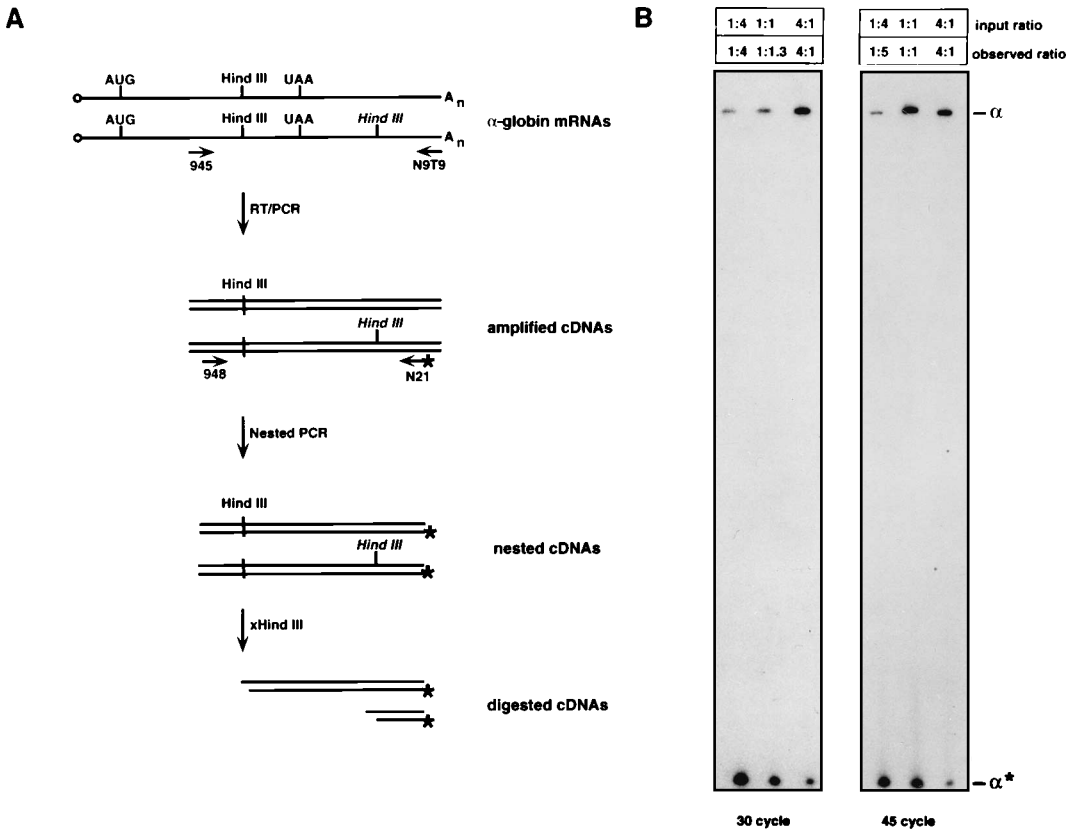

FIG. 2. RT-PCR *Hin*dIII scanning mutagenesis assay. (A) Overview of the RT-PCR assay. RNAs isolated from MEL cells coelectroporated with the mutant and α2-globin genes were reverse transcribed, and the cDNAs were amplified by using the 945 and N9T9 oligonucleotides. Because of the high degree of sequence identity between the endogenous murine and human α-globin mRNAs, the specificity of the PCR step was increased by performing five additional cycles with the nested oligonucleotides 948 and a ^{32}P-end-labeled N21 oligonucleotide as shown. Samples were then digested with *Hin*dIII, and the mutant (α*) and normal (α) α2-globin cDNAs were resolved on a 5% acrylamide–8 M urea denaturing gel. The normal α2-globin cDNAs contain a *Hin*dIII site in exon II, and each mutant contains an additional *Hin*dIII site at a unique position within the 3′ NTR. (B) Quantitation control. α2-Globin and mutant (H19) plasmid DNAs were mixed in 1:4, 1:1, and 4:1 ratios as shown. Fifty nanograms of each mix was amplified, aliquots were removed at 30 and 45 cycles, and the relative levels of normal α2 (α) and mutant (α*) DNAs were analyzed by using the PCR *Hin*dIII assay. Signals were quantitated with a PhosphorImager (Molecular Dynamics). The experimentally derived ratios accurately reflect the input plasmid DNA ratios over 45 amplification cycles. All RNA analyses were performed after 31 PCR cycles.

buffer (50 mM KCl, 20 mM Tris-HCl [pH 8.4], 1.5 mM $MgCl_2$), and 10 U of reverse transcriptase. PCR samples included 10% dimethyl sulfoxide, 8 mM β-mercaptoethanol, and the 945 oligonucleotide (Table 1). Samples were cycled once (5 min at 95°C, 1.5 min at 54°C, 3 min at 72°C) and 25 times (1 min at 95°C, 0.5 min at 54°C, 1.5 min at 72°C). Since the endogenous murine α-globin mRNAs are highly homologous to the human α2-globin mRNAs, the specificity of the PCR was increased by performing five additional PCR cycles as detailed in Fig. 2 with nested primers (5′→3′): 948, GTGGACGACATGCCCAACGC; and the ^{32}P-end-labeled N21, CCCACTCAGACTTTATTCAA. To distinguish between the mutant (α*) and normal (α) cDNAs, the PCR samples were gel purified, digested with *Hin*dIII, and resolved on a 5% acrylamide–8 M urea denaturing gel. The normal α2-globin cDNAs contain a *Hin*dIII site in exon II and serve as an internal control for a complete digestion. Murine α-globin RNAs do not have a *Hin*dIII site and, if amplified, would remain uncut. Each mutant α* cDNA has a second *Hin*dIII site at a unique position in the 3′ NTR, and the panel of mutant cDNAs generates a ladder of labeled DNA fragments (see Fig. 3). RNA levels were determined by quantitating the signals from the *Hin*dIII digests with a PhosphorImager (Molecular Dynamics). Because the mutant and normal α-globin mRNAs were reverse transcribed and amplified in the same tube with the same pair of primers, the relative levels of mRNA in the transfected cells were accurately reflected by the ratios in the PCR samples (53).

The ratios of mutant to α2-globin mRNAs obtained as raw data from the RT-PCR assays were normalized for minor variations in concentrations of each plasmid DNA in the electroporation mix. Plasmid DNA concentrations in the electroporation mixes were determined by using the PCR *Hin*dIII assay. One of the quantitation control experiments is presented (Fig. 2B).

RESULTS

***Hin*dIII scanning mutagenesis assay.** Our previous examination of α2-globin translation termination mutants suggested that an erythroid cell-specific stability determinant was associated with the 3′ NTR (53). The model most consistent with the data was that the entry of a translating ribosome into this region disrupted an mRNA-protein complex or secondary structure needed for stability. The formation of these structures would depend on specific *cis*-acting sequences within the α2-globin 3′ NTR. Given the uniqueness of an erythroid cell-specific stability determinant and the small size of the α2-globin 3′ NTR (109 bases), we decided to map these elements in detail by scanning mutagenesis.

Clusters of base substitutions which created *Hin*dIII restriction sites (5′-AAGCTT-3′) were introduced at 11 positions between the translation stop site and the polyadenylation signal (Fig. 1). Sequences surrounding the polyadenylation signal were left intact so as not to interfere with mRNA processing. The expression of each mutant gene was directly compared with that of the normal α2-globin gene during transient expression in MEL cells. We have previously shown that the destabilization of naturally occurring α2-globin mutants observed in vivo in RBCs is accurately reflected by this in vitro experimental system (31, 52, 53). Briefly, equal amounts of the mutant *Hin*dIII and normal α2-globin genes were coelectroporated into MEL cells, and ratios of the mutant to normal α2-globin mRNAs were quantitated. Because of the high homology between the human and murine α-globin mRNAs and

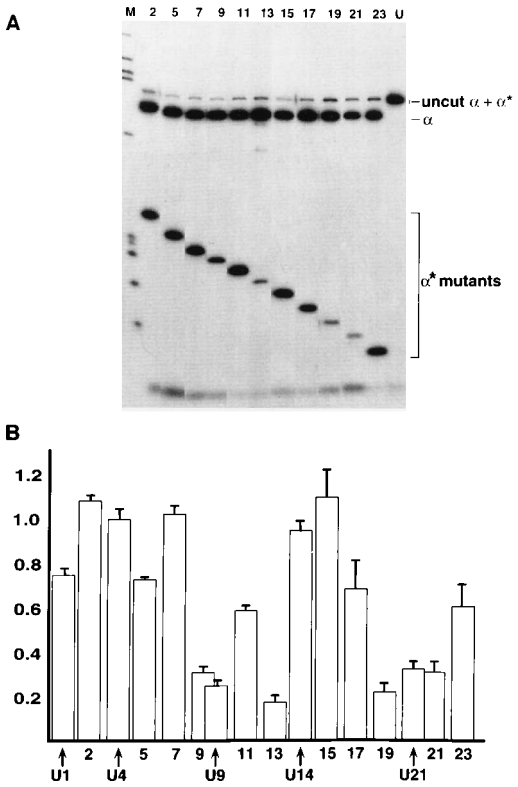

FIG. 3. Specific mutations in the 3′ NTR destabilize the α2-globin mRNA in erythroid cells. MEL cells were coelectroporated with equal quantities of the mutant (α*) and α2-globin (α) genes. Two days later, cells were harvested for RNA, and the mutant (α*)/normal (α) mRNA ratios were determined by the RT-PCR *Hin*dIII assay (Fig. 2). (A) A representative gel of RNAs isolated from cotransfected cells, analyzed by the RT-PCR *Hin*dIII assay. Alternate lanes of the gel, containing duplicate experiments, were spliced to create the composite gel. pGEM3 × *Hin*fI markers (M), uncut α plus α*, mutant (α*), and normal (α) α2-globins are noted. Mutations are labeled at the top of the gel. The effects on mRNA stability for the U1, U4, and U14 mutations have been reported elsewhere (53). (B) Quantitation of the mutant/normal α2-globin mRNA ratios. A PhosphorImager (Molecular Dynamics) was used to quantitate the signals of the *Hin*dIII-digested PCR fragments. For each mutation tested, levels of the α2-globin cDNAs were defined as 1.0, and values for mutant cDNAs were expressed relative to this value. Histogram bars represent averages of five experiments for the *Hin*dIII mutations and three datum points for the U series of mutations.

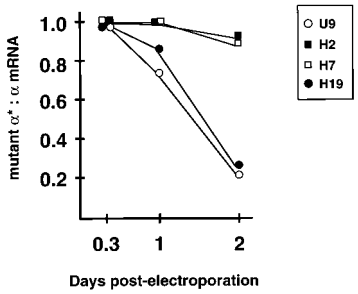

FIG. 4. Time-dependent decrease in mutant α2-globin mRNAs. MEL cells were coelectroporated with the mutant and α2-globin genes. At the indicated times after electroporation, the mutant/normal α2-globin mRNA ratios were determined by the RT-PCR *Hin*dIII assay (H2, H7, and H19) or the RT-PCR cloning assay (U9) as described previously (53). For each time point tested, values for normal (α) mRNAs were defined as 1.0, and values for mutant (α*) mRNAs were expressed relative to this value.

because message levels for the transfected genes may be low, a number of conventional techniques (Northern blot hybridization, primer extension, and RNase mapping) were impractical. Instead, a quantitative RT-PCR-based assay (31, 52, 53, 57) was used to specifically detect the human transcripts (Fig. 2). Concentrations of the *Hin*dIII mutant and normal α-globin mRNAs represented in the PCR samples were then determined by *Hin*dIII digestion and resolution of the different-size digest products by polyacrylamide gel electrophoresis. Results of this *Hin*dIII assay and a quantitation control are presented (Fig. 2).

Specific mutations destabilize the α2-globin mRNA in erythroid cells. For the initial analysis, each *Hin*dIII mutant was coelectroporated with the normal α2-globin gene into MEL cells, and ratios of mutant to normal α2-globin mRNAs were determined 2 days later, using the *Hin*dIII assay. A representative gel displaying the panel of *Hin*dIII mutants analyzed in this manner is shown (Fig. 3A), and data compiled from five separate sets of cotransfections are presented in the histogram (Fig. 3B). Data for several other 3′ NTR mutations (U1, U4, U9, U14, and U21) which were analyzed by using a different RT-PCR-based assay that we developed (53) are also included in the histogram. Of the 16 mutant α2-globin genes analyzed, 10 had either no adverse effect (H2, U4, H7, U14, and H15) or only a mild effect (U1, H5, H11, H17, and H23) on mRNA levels. However, six mutations (H9, U9, H13, H19, H21, and U21) caused a marked decrease in the levels of mutant mRNAs, with mutant/normal α2-globin mRNA ratios of between 0.18:1 and 0.38:1 (Fig. 3B).

Under the conditions used for these studies, we have previously demonstrated that mutations which destabilized the α2-globin mRNA in vivo also resulted in a reproducible, time-dependent decrease in the ratios of mutant to normal mRNAs by 2 days postelectroporation (53). Therefore, to ensure that the decreased expression observed above resulted from destabilization, the concentrations of four representative mRNAs which were found at normal (H2 and H7) or low (U9 and H19) levels were monitored following transfection. For each time point, the levels of α2-globin mRNAs were defined as 1, and the levels of mutant α2-globin mRNAs (α*) were expressed relative to this value. At early times postelectroporation (0.3 days), equal levels of mutant and normal α2-globin mRNAs were observed, suggesting the genes were transcribed at similar rates (Fig. 4) (53). While concentrations of the H2 and H7 mRNAs paralleled that of the stable α2-globin control throughout the time course, levels of the U9 and H19 mRNAs declined dramatically after 1 day, with mutant/normal mRNA ratios decreasing from about 1:1 to less than 0.3:1 (Fig. 4). This decrease is fully consistent with the values obtained previously for unstable α2-globin mutants and suggests that these mutations resulted in accelerated turnover.

Three regions are required for α2-globin mRNA stability. A couple of possibilities could account for the accelerated degradation of these mutant α2-globin mRNAs. Either the mutations introduce an instability motif or the sequences at those particular positions are important for stability. As most of the *Hin*dIII mutations had no effect on α2-globin mRNA stability (Fig. 3) and its sequence (5′-AAGCTT-3′) did not resemble any known instability motif (8), it seemed unlikely that these base substitutions were inherently unstable. In addition, two different sets of mutations introduced at positions +9 (H9 and U9) and +21 (H21 and U21) destabilized the mRNA (Fig. 1 and 3B), confirming that the primary sequences at these sites define regions essential for longevity of the α2-globin message.

Mutations that caused accelerated turnover of the α2-globin mRNA mapped to at least two noncontiguous positions within the 3′ NTR (see Fig. 5 for a detailed map). The borders of the

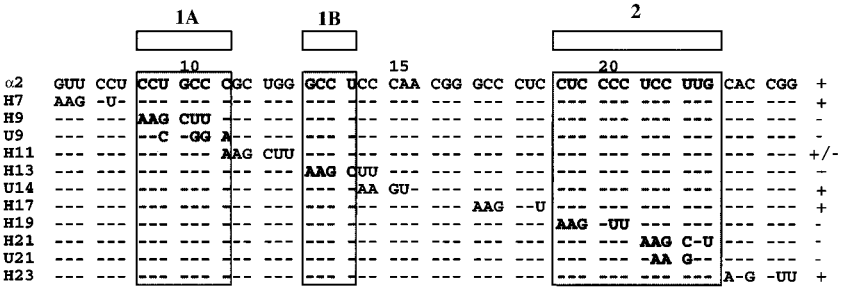

FIG. 5. C-rich stability elements 1A, 1B, and 2. Mutations which destabilize the α2-globin mRNA and delineate the stability elements 1A, 1B, and 2 are boxed. The figure is labeled as described for Fig. 1.

first region (element 1) were demarcated by the instabilities of the H9 and H13 mRNAs and by the normal levels of H7 and U14 mRNAs (Fig. 5). Depending on the interpretation of the intermediate levels of H11 mRNAs, this region could be further divided into elements 1A and 1B. Another segment (element 2) was delineated by instabilities of the H19 and H21 mRNAs and the normal to intermediate concentrations of H17 and H23 mRNAs. The most notable feature of these elements is their high cytidine content (61%) compared with the rest of the 3′ NTR, which is 38% cytidine. All six of the unstable mutants (H9, U9, H13, H19, H21, and U21) substantially decreased the cytidine content in one of these segments (Fig. 5).

C-rich regions form the erythroid cell-specific stability determinant. The scanning mutagenesis assay located several C-rich segments in the α2-globin 3′ NTR which were essential for longevity of the message in erythroid cells. To determine if these elements modulate mRNA metabolism in nonerythroid cells, eight mutant α2-globin genes were cotransfected with the normal α2-globin control into murine fibroblast (C127) cells, and message levels were examined during transient expression. Regardless of whether the mRNAs were stable (H2, H5, H7, H11, and H23) or unstable (H9, H21, and U21) in erythroid cells (Fig. 3), ratios of the mutant to wild-type α2-globin mRNAs were comparable at 2 days postelectroporation in nonerythroid cells (Fig. 6). An extended time course experiment performed with mutants H2, H7, H9, and H21 shows that the mutant α*/α2-globin mRNA ratios are similar for 3 days following transfection (data not shown). Since the α2-globin message is stable in C127 cells (53), the equal levels of these mRNAs indicate that mutations in the C-rich elements had no adverse effect on stability and were not involved in regulating mRNA turnover in nonerythroid cells.

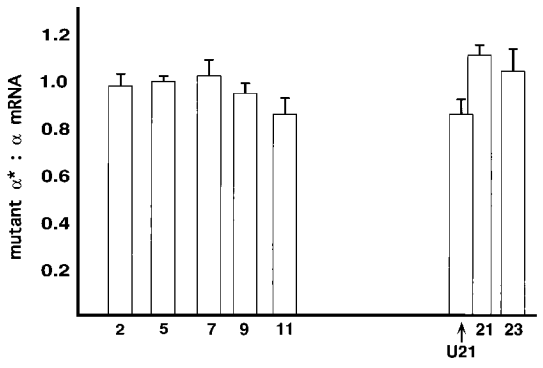

FIG. 6. C-rich elements are not involved in mediating α2-globin mRNA stability in nonerythroid cells. C127 cells were coelectroporated with equal quantities of the mutant and α2-globin genes. Two days later, cells were harvested for RNA, and the mutant/normal α2-globin mRNA ratios were determined by the RT-PCR *Hin*dIII assay (Fig. 2) or the RT-PCR cloning assay (U21 [53]). Each histogram represents the average of three separate experiments.

Confirmation that these elements comprise the erythroid cell-specific stability determinant was further obtained by determining the stabilities of two representative mRNAs in the nonerythroid, C127 cells. The H2 mRNA, which was stable in MEL cells, and the H13 mutant, which was unstable in erythroid cells, were chosen for the analyses. These genes were placed under the transcriptional control of the serum-inducible c-*fos* promoter and were used to establish stable cell lines. After prolonged exposure to low serum, the *fos* promoters were transiently activated with the addition of 20% FBS (18, 48). Stabilities of the mutant α2-globin mRNAs encoded by the chimeric genes were examined by harvesting cells at various times after serum induction and quantitating the mRNA levels by Northern blot hybridization. The general difference in the intensities of the H2 from the H13 bands on the Northern blots appears to reflect relative loading on the two gels, as the rpL32 bands show a parallel difference in intensity. In addition, the copy number of transfected plasmid integrants in these two stably transfected pools was not determined and may differ. The H2 and H13 mRNAs encoded by the chimeric genes both accumulate for several hours postinduction (Fig. 7). However, in contrast to the unstable c-*fos* mRNAs, whose message levels decrease within 2 h of induction, concentrations of the mutant α2-globin mRNAs remain high for at least 8 h after induction and are readily detectable up to 96 h later. The slow decline in the concentrations of these mRNAs within the first 8 h after induction suggests that the H2 and H13 mRNAs are relatively stable in this nonerythroid cell line (Fig. 7C and D). Accordingly, the mRNAs observed prior to transcriptional induction by high serum (T_0 lanes in Fig. 7A and B) represent mutant α2-globin mRNAs which persisted through 3 days of culture in low serum (in the absence of transcription). Under similar conditions, baseline levels of other stable c-*fos*/β-globin chimera mRNAs (20) and *fos*/α2-globin chimeric mRNAs (53) have been observed. Thus, the C-rich regions delineated by scanning mutagenesis function in an erythroid cell-specific manner to mediate stability of the α2-globin mRNA.

Translation-independent mRNA destabilization. Ongoing translation is required for the regulated turnover of a number of mRNAs (8, 11, 20, 54, 58). In particular, we have shown that the mechanism responsible for accelerated degradation of the α2-globin translation termination mutant CS in erythroid cells is translation dependent in that mRNA turnover requires entry of the translating ribosome into the 3′ NTR (53). Inhibiting translation of the CS mRNA by cycloheximide treatment or by mutating the start codon (AUG→ACG; mRNA ACG.CS) stabilized the mutant mRNAs (Fig. 8) (53). Therefore, to deter-

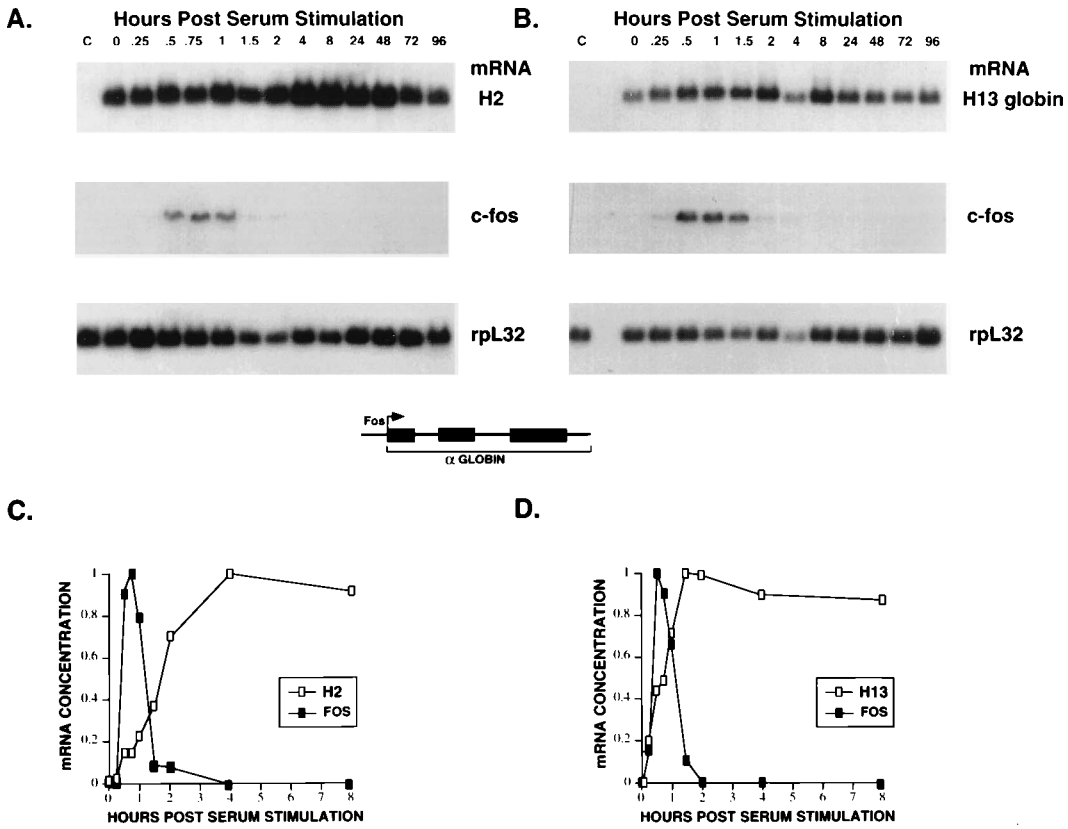

FIG. 7. Wild-type and mutant α2-globin mRNAs are stable in the nonerythroid C127 cells. Pools of C127 cells stably transformed with the chimeric *fos*/H2 globin gene (A) or the chimeric *fos*/H13 gene (B) were cultured for 3 days at 0.5% FBS and induced with 20% FBS. At the indicated times after serum induction, the cells were harvested for RNA, and 10 mg of RNA was analyzed by Northern blot hybridization. The serum starvation-induction was performed twice on the same cell line with similar findings, and Northern blot data from one set of experiments are shown. A nontransfected C127 control RNA (lane C) is at the left. Each blot was hybridized sequentially with random primer-labeled probes to detect mRNAs for α-globin, rpL32, and c-*fos* as noted. RNA levels were determined by quantitating the signals from the Northern blots with a PhosphorImager (Molecular Dynamics). Samples were normalized for loading, and the highest value postinduction was defined as 1.0. To plot the concentrations for the H2 (C) and H13 (D) globin mRNAs, the amount of signal present at T_0 was subtracted from each time point.

mine if translation was also part of the mechanism mediating turnover of mRNAs containing mutations in the C-rich elements, translation of the unstable U9 message was inhibited by mutating the initiation codon (AUG→ACG) of the U9 gene (ACG.U9). Inhibiting translation of the U9 gene in this manner did not increase message stability (Fig. 8, ACG.U9). These results indicate the α2-globin mRNA can be targeted for accelerated degradation by two different modes, a translation-dependent mechanism (CS mRNAs) and a translation-independent mechanism (mutations in the C-rich regions).

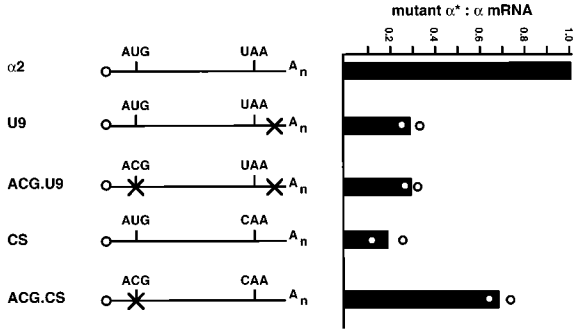

FIG. 8. Translation-independent destabilization. MEL cells were coelectroporated with the mutant (α*) and wild-type (α) α2-globin genes as shown. Two days later, the mutant/normal α2-globin mRNA ratios were determined by the RT-PCR cloning assay as described previously (52, 53). Values for α2-globin mRNAs were defined as 1.0, and values for mutant mRNAs were expressed relative to this value. Identities of the mRNAs are shown on the left; the cap site (○), translation start site (AUG) or mutation (ACG), stop codon (UAA) or mutation (CAA), U9 mutation (×), and polyadenylation site (A_n) are labeled.

DISCUSSION

Currently, very little is known about the mechanisms or features of an mRNA which mediate its stability. To identify potential *cis*-acting sequences and *trans*-acting factors which might contribute to the longevity of an mRNA, we have begun a detailed analysis of mutations that decrease stability of the normally long-lived α-globin mRNA. The initial studies focused on a subset of naturally occurring and site-directed mutations in which the ribosome reads through into the α2-globin 3′ NTR. Using this type of ribosome readthrough assay, we demonstrated an accelerated turnover of the mutant mRNAs in MEL cells (53). The mechanism mediating mRNA degradation was translation dependent and required that the ribosome translate between one and four codons into the 3′ NTR (53). These results suggested that the movement of a translating ribosome into this region disturbed a stability determinant and targeted the mRNAs for accelerated degradation. In contrast, translational readthrough did not destabilize the α2-glo-

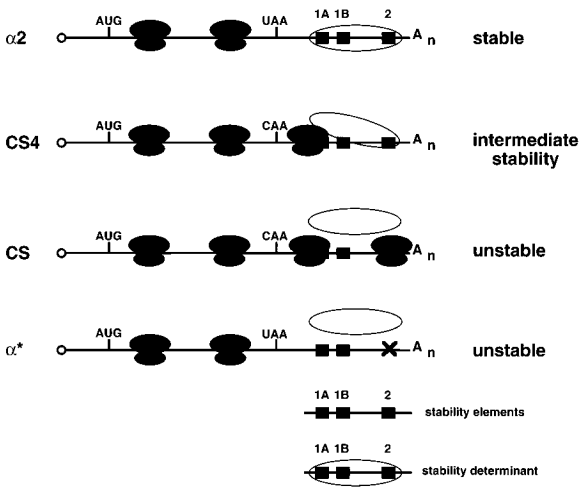

FIG. 9. Model. Stability of α2-globin mRNAs in erythroid cells requires a determinant or complex in the 3′ NTR formed by the interaction of the three C-rich elements 1A, 1B, and 2 (grey boxes). During normal translation, the ribosome terminates and the 3′ NTR determinant remains intact (α2-globin mRNA). In the CS4 readthrough mutant, the ribosome translates four codons into the 3′ NTR and begins to encroach upon the stability complex. This results in a loss of the functional determinant and targets the mRNA for accelerated turnover. Some percentage of these mRNAs re-form the complex, accounting for the intermediate levels of CS4 mRNAs. When the ribosome translates 31 codons into the 3′ NTR (CS), all three stability elements are disrupted, causing substantial levels of degradation. For the *Hin*dIII mutants (α*) used in the scanning mutagenesis assay, the stop codon was left intact and the ribosome would not advance into the 3′ NTR and therefore would not function in this type of disruptive capacity. Instead, base substitutions in a C-rich element may inhibit or abolish formation of the determinant. This uncouples the requirement for the ribosome in the degradation process and allows for accelerated turnover of the mRNA in erythroid cells through a translation-independent mechanism.

bin mRNA in nonerythroid cells, indicating that the determinant functioned in an erythroid cell-specific manner to modulate message stability (53).

To better understand how the α2-globin 3′ NTR mediates stability and the basis for the cell-type-specific differences in mRNA metabolism, the *cis*-acting elements that comprise the stability determinant were defined in detail by using a *Hin*dIII scanning mutagenesis assay (Fig. 2). Mutations which destabilized the α-globin mRNA in erythroid cells mapped to three C-rich segments within the 3′ NTR. None of these mutant mRNAs were unstable in nonerythroid cells, confirming that these C-rich segments define the erythroid cell-specific stability determinant. Since mutations in single elements were sufficient to destabilize the message in erythroid cells, these regions probably interact to form the functional determinant. However, in contrast to the naturally occurring and site-directed translational readthrough mutants, the mechanism mediating turnover of α2-globin mRNAs with mutations in the C-rich elements is translation independent.

Data from the ribosome readthrough and the *Hin*dIII scanning mutagenesis assays have been integrated into a model which accommodates the translation-dependent (ribosome readthrough mutants) and translation-independent (C-rich mutants) mechanisms of mRNA turnover (Fig. 9). We propose that the C-rich elements (1A, 1B, and 2) in the α2-globin 3′ NTR interact to form a complex or determinant which is required for mRNA stability in erythroid cells. During normal translation, the ribosome terminates and presumably dissociates from the mRNA, leaving the 3′ NTR determinant intact (α2 mRNA; Fig. 9). In the CS4 readthrough mutant, the ribosome translates four codons into the 3′ NTR and begins to encroach upon the stability complex. This results in a loss of the functional determinant and targets the mRNA for accelerated turnover. After translation termination, some percentage of these mRNAs may be capable of re-forming the complex, thus accounting for the intermediate levels of CS4 mRNAs. However, when the ribosome translates 31 codons into the 3′ NTR, as occurs in the mutant CS mRNA, all three stability elements in the complex are disrupted, causing substantial levels of degradation. For the *Hin*dIII mutants (α*) used in the scanning mutagenesis assay, the stop codon was left intact and the ribosome would not advance into the 3′ NTR and therefore would not function in this type of disruptive capacity. Instead, base substitutions in a C-rich element may inhibit or abolish formation of the determinant. This would uncouple the requirement for the ribosome in the degradation process and would allow for accelerated turnover of the mRNA in erythroid cells through a translation-independent mechanism.

Consistent with this model, both the ribosome readthrough and *Hin*dIII scanning mutagenesis assays map the stability determinant to roughly the same position within the α2-globin 3′ NTR. Data from the ribosome readthrough assay demonstrated that mRNAs in which the ribosome translates one codon into the 3′ NTR are stable (CS1 [53]). On the basis of a translating ribosome spanning an average of 25 to 30 bases of a mRNA (28, 55), the first 12 to 15 bases 3′ to the CS1 stop codon (or 15 to 18 bases 3′ to the normal stop codon) would be disrupted by ribosome. As this degree of translational readthrough has no effect on message stability, mutations located within the first 18 bases 3′ to the normal stop codon also should not be involved in mediating stability. Consistent with this hypothesis, mutations in the first 21 bases 3′ to the stop codon (Fig. 3) do not affect mRNA stability.

The finding that mutations in individual C-rich elements were sufficient to destabilize the α2-globin mRNA in erythroid cells (Fig. 3 and 5) suggests that these segments interact to create a functional stability determinant. Such interactions could occur through the formation of stem-loop structures or the binding of *trans*-acting factors. Although computer-generated secondary structure models (60) for the wild-type and mutant α2-globin 3′ NTRs indicate the potential to form a number of base-paired regions (data not shown), there is no apparent correlation between the stability of the mutant mRNA in MEL cells and the extent to which its RNA folding pattern resembles that of the wild type. In addition, if secondary structures alone regulated mRNA turnover, these mutant mRNAs should be unstable in nonerythroid cells as well. Our findings demonstrate that this is not the case (Fig. 6 and 7) (53). Substantial data from our laboratory (51) support the alternative model, that the C-rich elements either directly bind or contribute to the formation of structures which are capable of binding *trans*-acting factors. The formation of this mRNA-protein complex would constitute the erythroid cell-specific determinant which confers stability to the α2-globin mRNA in erythroid cells.

Perhaps the most interesting aspect of this determinant is that it mediates α2-globin mRNA stability in a tissue-specific manner. Apparently, it is the loss of function when an element is mutated or disrupted by translational readthrough which leads to accelerated degradation in MEL cells and RBCs. The determinant is not needed in nonerythroid cells, as mutant and normal α2-globin mRNAs are stable (this report and reference 53). Thus, the determinant may function to protect the message from an erythroid cell-specific RNase. Recognition of an intact determinant may be one way the α2-globin mRNAs

avoid the massive degradation of nonglobin mRNAs which accompanies RBC differentiation. Conceivably, subsets of mRNAs in RBCs could contain a similar complex and be protected from degradation. It is of note that the β-globin mRNA, which accumulates to very high levels during RBC differentiation, does not contain these C-rich elements, nor does translational readthrough appear to destabilize the mRNA (10, 16). Instead, the turnover of this mRNA in vitro occurs at AU sites (3). This raises the intriguing possibility that multiple mechanisms are responsible for mediating the stability of globin mRNAs in erythroid cells and that tissue-specific RNases may also regulate levels of gene expression.

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