

MINIREVIEW

Regulation of α -Globin mRNA Stability

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mRNA stability is a critical determinant of normal red blood cell development and function. The long half-life of globin mRNA is central to the continued synthesis of globin proteins throughout all stages of erythropoiesis, even as the cells undergo programmed transcriptional arrest during terminal differentiation. Studies of a naturally occurring α -thalassemic mutation that triggers marked destabilization of α -globin mRNA first led investigators to search for a stability determinant in the 3'-untranslated region (3'UTR). Analysis of this region identified three cytosine-rich (C-rich) segments that contributed to α -globin mRNA stability when studied in transfected erythroid cells. Subsequently, *in vitro* studies demonstrated assembly of a sequence-specific ribonucleic-protein (RNP) complex at this site. Mutations in the 3'UTR that blocked formation of this " α -complex" *in vitro* resulted in a parallel destabilization of α -globin mRNA in transfected cells. Members of the α -globin poly(C)-binding protein (α CP) subfamily of heteronuclear (hn) RNP K homology (KH) domain RNA-binding proteins have been identified as essential protein components of the α -complex. *In vitro* studies suggested that additional proteins may also contribute to α -complex structure and/or function. Surveys of additional highly stable mRNAs point to a general function for the α -complex in mRNA stabilization. *In vitro* and *in vivo* analyses indicated that the α -complex stabilizes α -globin mRNA by two mechanisms: control of 3'-terminal deadenylation and steric protection of an endoribonuclease-sensitive site. Confirmation of these pathways, determination of their relative importance, and generalization of these findings to additional systems await future studies. *Exp Biol Med* 228:387–395, 2003

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Overview of α -Globin mRNA Expression

Unique Characteristics of Erythroid Cells. The erythrocyte is a specialized cell that carries O₂ from the lungs to the tissues and helps to facilitate the transfer of CO₂ from the tissues to the lungs. There are two unique features of erythrocytes that are central to the present discussion. First, erythrocytes contain high levels of hemoglobin; up to 95% of the total soluble protein content is hemoglobin. Second, erythroid cells undergo an irreversible and global transcriptional arrest during terminal differentiation.

Distinct sets of globin genes are expressed during the various stages of human development, producing hemoglobin proteins with different subunit compositions (Fig. 1) (reviewed in Ref. 1). The major embryonic hemoglobin tetramers consist of $\zeta_2\varepsilon_2$ and $\zeta_2\gamma_2$, and major fetal hemoglobin comprises $\alpha_2\gamma_2$. Adult hemoglobin comprises two hemoglobin tetramers. $\alpha_2\beta_2$ (HbA) constitutes 97%–98% of total adult hemoglobin, and $\alpha_2\delta_2$ (HbA₂) is the minor adult hemoglobin constituting 2%–3% of total adult hemoglobin. The two α -globin genes ($\alpha 1$ and $\alpha 2$) encode an identical α -globin protein. However, the $\alpha 2$ -globin gene is expressed at 2- to 3-fold higher levels than the $\alpha 1$ -globin gene at both mRNA and protein levels (2, 3). Thus the $\alpha 2$ -globin gene is the dominant adult hemoglobin gene in the α -globin gene cluster (2). Transcription of this gene yields an mRNA with a length of 677 nucleotides (nt). The coding sequence is preceded by a 37-nt 5'-untranslated region (5'UTR) followed by a 109-nt 3'UTR. This dominant $\alpha 2$ -globin mRNA is the focus of this review and will be referred to as the α -globin mRNA.

The first five stages of red blood cell development occur in the bone marrow (Fig. 2). The basophilic erythroblast (BE) is the first committed erythroid precursor. This cell has a large nucleus containing transcriptionally active dispersed chromatin. When BEs are visualized by Wright/Giemsa staining, the cytoplasm appears blue, reflecting the presence of the cytoplasmic RNA. Transcription of the globin mRNAs occurs primarily at this stage and the following polychro-

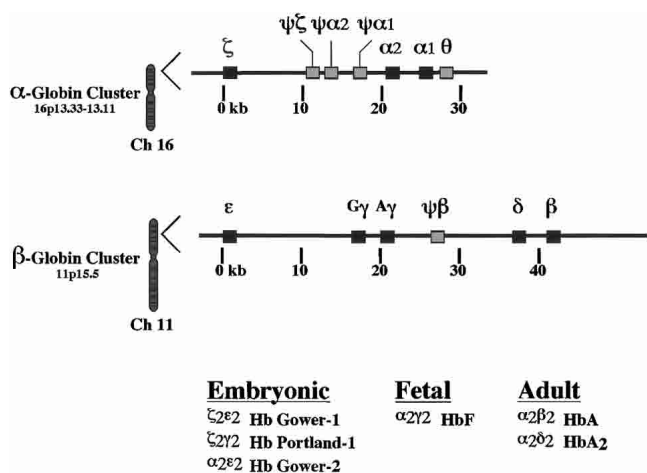


Figure 1. The human globin gene clusters and their developmental regulation. Shown are the α - and β -globin gene clusters and their positioning on chromosomes 16 and 11, respectively. The developmentally regulated switching from embryonic to fetal and fetal to adult hemoglobin tetramers is summarized below the cluster diagrams. ■, Genes; gray squares, pseudogenes.

matophilic erythroblast (PCE) cell stage. The PCE stains lighter blue, reflecting the increased levels of hemoglobin. This cell is smaller than the BE, and its chromatin condenses in parallel with the beginning of global transcriptional silencing. The chromatin is fully condensed in the even smaller orthochromatophilic erythroblast (OCE) cell, with increased transcriptional shut-off marked by a smaller nucleus. Further accumulation of hemoglobin causes this cell to stain pink. The normoblast (NB) stage is characterized by red staining of the cytoplasm due to large amounts of hemoglobin. This stage is distinguished by a fully condensed nucleus that is extruded from the cell. The final reticulocyte (retic) stage lacks a nucleus. The reticulocytes are then released from the bone marrow into the bloodstream. Synthesis of globin proteins continues for approximately 2–3 days in reticulocytes. The continued expression of globin proteins during this period, which is critical for normal red blood cell development, is achieved in part by the long half-life of the globin mRNAs. The half-life of the $\alpha 2$ -globin mRNA (>24 hr) in the developing erythroid cells,

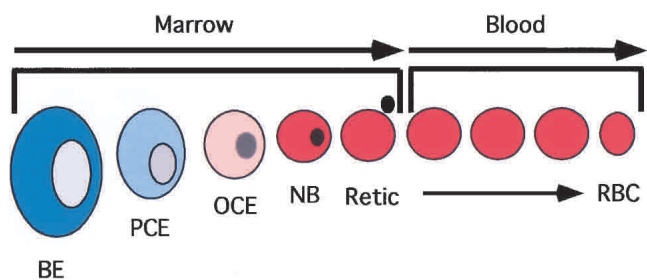


Figure 2. Stages of erythropoiesis. The name of each cell type is indicated by its abbreviation (see text for full names). The nucleus is shown as progressively darkened and condensed and is eventually extruded from the cell during the transition to the reticulocyte (retic) stage. The colors of the cells are shown as visualized by Wright/Giemsa staining.

is consistent with this time period of globin synthesis in the absence of transcription (4).

At the end of the final maturation period in the blood, all cellular organelles, membranes, and RNAs are cleared from the reticulocyte to form a mature, even smaller red blood cell (RBC). It has been postulated that the mechanism designed to maintain the long half-life of α -globin mRNA is functional at all stages of erythroid development before the reticulocyte stage. A global mechanism is then responsible for the final clearance of all mRNAs, including globin mRNAs, from terminally differentiated, circulating reticulocytes (5).

Evidence that the α -Globin mRNA is Regulated at the Level of mRNA Stability

α Constant Spring Thalassemia. The α Constant Spring (α^{CS}) mutation was first described in 1971. Its name was derived from the city in Jamaica, West Indies where it was first discovered in a family of Chinese ancestry (6). Several children in this family had a form of severe α -thalassemia characterized by accumulation of high levels of hemoglobin H in their red cells (HbH disease). Hemoglobin H is a tetramer of β -globin chains (β_4). Aside from not serving a physiologic function, this HbH homotetramer is unstable; β_4 aggregates form in the erythrocytes causing red cell damage, decreased red cell longevity, and a chronic hemolytic anemia. This pathologic accumulation of excess β -chains can be caused by a wide range of genetic defects (α -thalassemia mutations) that impair the expression of the complementing α -chains. The marked reduction in α -chain production in HbH disease is most commonly due to the loss of expression of both $\alpha 1$ - and $\alpha 2$ -globin genes on one chromosome ($-$) and the selective loss of the $\alpha 2$ -globin gene on the other chromosome ($-\alpha$). Thus, most individuals with HbH α -thalassemia have only a single (minor) functioning $\alpha 1$ -globin locus [genotype; ($-$ $-\alpha$)]. In this context, it was surprising to find that the index individual of the α^{CS} family had, in addition to the β_4 tetramers and $\alpha 2\beta 2$ (HbA), trace levels of an additional structurally abnormal α -globin hemoglobin, α^{CS} . Genetic and biochemical studies revealed the underlying genotype to be ($-$ $-\alpha^{CS}\alpha$) due to inheritance of a chromosome with the α^{CS} gene ($\alpha^{CS}\alpha$) from the father and the double deletion chromosome ($-$) from the mother. The α^{CS} mutation, which essentially destroys the function of the dominant $\alpha 2$ -globin gene, is now recognized as a leading cause of α -thalassemia worldwide with a gene frequency as high as 0.06 in Northeast Thailand (7). The α^{CS} mutation harbors a single nucleotide substitution at the wild-type α -globin mRNA (α^{WT}) translational stop codon (UAA to CAA). This mutation converts the termination codon to a glutamine codon and allows the ribosomes to translate for an additional 31 codons (93 nt) into the normally ribosome-free 3'UTR. The C-terminally extended α^{CS} protein is expressed at 3% of α^{WT} levels; thus, the CS mutation leads to a 97% loss of $\alpha 2$ -globin gene expression.

The cause of the decreased expression from the α^{CS} gene was initially unclear. Clegg *et al.* (6) showed that the α^{CS} mutant protein is stable. It was subsequently demonstrated that the transcription rate of α^{CS} mRNA is comparable with the α^{WT} mRNA. The data suggested that the α^{CS} mutation resulted in a posttranscriptional defect in α -globin gene expression. Consistent with this hypothesis, it was found that in an individual heterozygous for the α^{CS} mutation the levels of α^{CS} and α^{WT} mRNAs were similar in bone marrow erythroid cells, whereas α^{CS} mRNA was selectively absent in circulating reticulocytes (3). Because bone marrow contains transcriptionally active erythroid precursors, whereas the circulating reticulocytes are transcriptionally inactive, these results suggested that the defect in α^{CS} expression is due to destabilization of its RNA. Morales *et al.* (5) provided further *in vivo* evidence to support this hypothesis by demonstrating that α^{CS} mRNA was 4-fold less stable than α^{WT} mRNA when expressed in the erythroid cells of transgenic mice. Analysis of these mRNAs further revealed that the α^{CS} mRNA poly(A) tail was significantly shorter than the α^{WT} mRNA poly(A) tail. Therefore, destabilization of α^{CS} mRNA appeared to be linked to accelerated 3' terminal deadenylation.

The mechanism of α^{CS} mRNA destabilization was further defined using transfected murine erythroleukemia (MEL) cells as a model system (8). Equal amounts of the α^{WT} and α^{CS} genes were cotransfected into MEL cells, and the resulting mRNAs were monitored over time. The $\alpha^{CS}:\alpha^{WT}$ mRNA ratio was approximately 1.0 at 24 hr post-transfection, indicating that these genes were transcribed at equal rates. However, by 48 hr, the ratio had fallen to 0.25, and at 6 days, α^{WT} mRNA was still present, whereas the α^{CS} mRNA was no longer detected. These data demonstrated that the transfected MEL cell system mimics α^{CS} mRNA degradation observed in transgenic models and in humans carrying the α^{CS} mutation. Interestingly, when the same experiment was performed in several nonerythroid cell lines, it was found that the α^{WT} and α^{CS} mRNAs were equally stable. These studies suggested that the pathway responsible for α^{CS} mRNA decay is specific for erythroid cells.

The mechanism responsible for the accelerated decay of the α^{CS} mRNA was investigated by studying the expression of a series of α -globin mRNAs with site-specific mutations that controlled the site of translation termination (8). These studies revealed that all mutations that destroyed the termination codon destabilized the mRNA to the same extent as the naturally occurring α^{CS} mutation. In contrast, insertion of a stop codon into the α^{CS} gene immediately upstream of the α^{CS} mutation prevented ribosome entry into the 3'UTR and stabilized the α^{CS} mRNA. These results indicated that ribosomal entry into the 3'UTR of α -globin mRNA destabilized the message in erythroid cells. These data supported a model in which a stability determinant was present in this region of the α -globin mRNA and could be interfered with by the elongating 80S ribosome.

Mapping the α -Globin Stability Determinants.

The 3'UTR determinant(s) responsible for the longevity of α -globin mRNA were searched for by linker scanning mutagenesis and functional analysis in MEL cells (9). These studies revealed that three cytosine-rich (C-rich) regions, located at nt positions 25–31, 37–40, and 55–66 (the first nt 3' to the stop codon is 1) were required for α -globin mRNA stability in erythroid cells (Fig. 3; the C-rich regions are indicated in red). Mutation of any of these C-rich elements destabilized the α -globin mRNA, resulting in a 3- to 6-fold decrease in the levels 48 hr post-transfection. In contrast to the translationally dependent mRNA destabilization mediated by the α^{CS} mutation, the destabilization triggered by these 3'UTR base substitutions was direct and independent of ongoing translation. Furthermore, the stability of α -globin mRNA in nonerythroid cells appeared to be unaffected by these C-rich determinants. Based on these studies, it was proposed that the C-rich regions constitute an erythroid-specific determinant of α -globin mRNA stability.

A Specific Set of RNA-Binding Proteins Interact with the α -Globin mRNA Stability Determinant

The α -Complex. An electrophoretic mobility shift assay (EMSA) was used to detect RNA-protein (RNP) complexes that assembled at the C-rich determinants of the α -globin mRNA (10). A high-affinity and sequence-specific RNP complex was identified in this manner. Formation of this " α -complex" was dependent on the C-rich determinants; the α -complex was efficiently competed by addition of poly(C), but not with other homoribopolymers. Signifi-

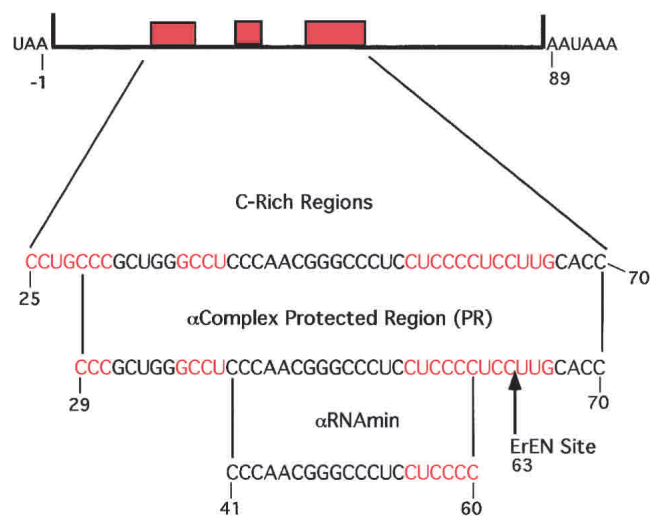


Figure 3. Important sequence determinants in the α -globin 3'-UTR. A segment of the α -globin 3'-UTR is illustrated, with the translational stop codon (UAA) and cleavage and polyadenylation signal (AAUAAA) indicated. The nucleotides are numbered in this and the following figures such that the first nucleotide 3' to the stop codon is nucleotide 1. The red boxes and red sequences represent the C-rich regions that comprise the stability determinant. Also shown are the sequences of the α -complex protected region (PR) that contains the ErEN Site and the minimal α -complex binding site (α RNAmin).

cantly, mutations in the C-rich regions that destabilized α -globin mRNA in transfected cells (9) (see above) blocked α -complex formation *in vitro*. This was consistent with a role of the α -complex in mRNA stabilization. Similar gel-shifted complexes were detected using MEL, K562 (human erythroleukemia), and C127 (mouse fibroblast) cells as sources of the cytoplasmic extract. These data indicated that the proteins responsible for forming the α -complex are conserved from humans to mouse and are not tissue specific. These data imply that the α -complex proteins may have different functions in different cell types and bind unique mRNA targets in nonerythroid cells.

The Minimal α -Complex Binding Site. RNase mapping was used to localize the minimal sequence in the α -globin 3'UTR required for α -complex assembly (11). The α -complex formed between the α -globin 3'UTR and extract derived from K562 cells was subjected to cleavage by RNases. An RNase-resistant 42 nt RNA fragment spanning nt 29–70 (α -complex protected region; PR) was mapped and localized within the 3'UTR (Fig. 3). The PR contains a portion of the first C-rich element and the complete second and third C-rich elements. To further localize the α -complex binding site, synthetic oligonucleotides were designed that spanned regions of the PR, and were tested by EMSA for their ability to form the α -complex. These experiments revealed that a 20-nt segment spanning nt 41–60 (termed α RNAmin; Fig. 3) was capable of forming the α -complex. Deletion of α RNAmin from the full-length α -globin 3'UTR abolished α -complex formation, and deletion of a single nt from either end of α RNAmin severely reduced α -complex formation. These results show that α RNAmin is necessary and sufficient for α -complex formation *in vitro*. A GenBank database search with the α RNAmin revealed three mRNAs with conserved sequences in their 3'UTRs: α (I)collagen, tyrosine hydroxylase, and 15-lipoxygenase (11). All of these mRNAs are highly stable, and their 3'UTRs have been linked to mRNA stability [α (I)collagen, Ref. 12; tyrosine hydroxylase; Ref. 13] or to translational control (15-lipoxygenase; Ref. 14). EMSAs demonstrated that each of the identified 3'UTRs formed a sequence-specific, gel-shifted complex that could be efficiently competed by each of the other 3'UTRs or by the α -globin 3'UTR. In contrast, mutant α -globin 3'UTRs that were unable to form the α -complex did not compete for the protein(s) bound to the other 3'UTRs. Furthermore, each complex was sensitive to poly(C) competition, but was insensitive to competition with other homoribopolymers. These results indicated that the RNA-protein complexes formed on each of these four 3'UTRs share one or more protein factors that have a characteristic poly(C)-binding specificity. Furthermore, the data suggested a model in which the α -complex constitutes a stability determinant common to a subset of highly stable mRNAs.

The α CP Family of KH Domain Proteins. The poly(C)-binding activity present in the α -complex was purified, and two proteins were identified by microsequencing,

α CP1 and α CP2 [α -globin poly(C)-binding proteins] (15). The α CPs, also known as hnRNP E (14) or as poly(C) binding proteins (PCBP) (16), comprise a subfamily of PCBP with a common evolutionary origin and a highly conserved structure (Fig. 4). The other major subfamily in the PCBP family are hnRNP K and J (see Ref. 17 for review). The α CPs are encoded by four unlinked genes in the human and mouse genomes (18–20). These genes encode six major α CP isoforms, α CP1, α CP2, α CP3, α CP4, and a major alternative splicing product of the α CP2 transcript, α CP2 KL (21) and a major alternative splicing product of the α CP4 transcript, α CP4A (20). Each of the α CPs is ubiquitously expressed in human and mouse tissues (16, 19, 20), and each binds poly(C) (Ref. 16 and our unpublished observations) and the human α -globin 3'UTR (Ref. 22 and our unpublished observations) with specificity and high affinity. α CP1 and α CP2 share the highest level of amino acid sequence similarity at 90% (18), whereas α CP2 and α CP4 are the least similar at 52% (20).

Each α CP contains three hnRNP KH domains that mediate binding to RNA; two KH repeats are located in the N terminus followed by a nonconserved region of variable length and the third KH domain is at the C terminus (Fig. 4). The KH domain is comprised of approximately 70 amino acid residues and is common to a wide spectrum of RNA-binding proteins (23). The structure of this domain has been solved by NMR and crystal analysis and has been defined as a triple β -sheet platform stacked against three α -helical segments (24). The KH domain can interact in a highly specific manner with four to five contiguous bases in a target RNA (25, 26).

Other Factors in the α -Complex. Kiledjian *et al.* (27), Wang *et al.* (28), and Henics (29) provided evidence that proteins in addition to α CP are present in the α -complex. Yeast two-hybrid screens using α CP1 and α CP2 as bait have detected a number of proteins. Of particular interest was the identification of the ARE binding/degradation

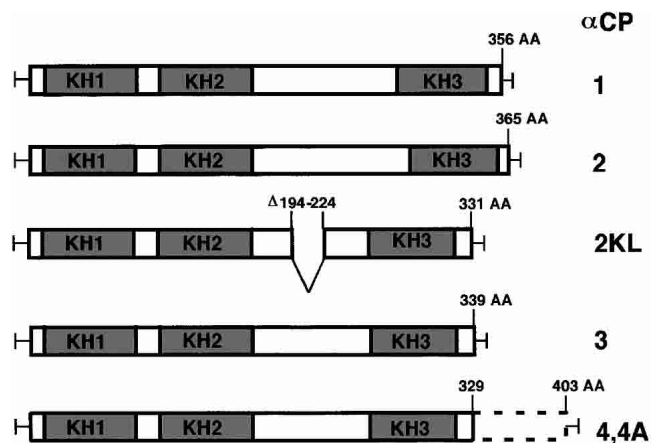


Figure 4. The six major isoforms of the α CP family. The proteins encoded by the major spliced forms of α CP1, α CP2, and α CP3 as well as the alternatively spliced α CP2KL and α CP4A are shown. The triple KH domains are highlighted as shaded rectangles. The full-length α CP4 is indicated by the dotted line.

factor (AUF-1, also known as hnRNP D) as an interacting partner of α CP1 and α CP2 (27). AUF-1 was named for its binding to AREs: AU-rich destabilizing elements located in the 3'UTRs of several unstable mRNAs (30). The two-hybrid screen also identified poly(A)-binding protein (PABP) as an interacting partner with both α CP1 and α CP2 (28). Both interactions were confirmed *in vitro* by copurification of PABP and AUF-1 with glutathione-S-transferase (GST)- α CP fusion proteins. Taken together, these yeast two-hybrid genetic screens identified two factors, AUF-1 and PABP, which may be components of the α -complex. In a complementing approach, Kiledjian *et al.* (27) excised the α -complex (derived from K562 cells) from a native gel for biochemical analysis. Six proteins were present in the gel slice containing the α -globin 3'UTR. Consistent with the yeast two-hybrid study, one of the proteins was identified as AUF-1 by Western blot analysis. The identity of the other proteins has remained elusive. It is noteworthy that none of these proteins were the same mass as PABP. Henics (29) also detected four distinct proteins (derived from MEL cells) that cross-linked to the α -globin 3'UTR. One of the proteins had a similar mass as PABP, although its identity (along with the other three proteins) remains unknown. Interestingly, three of these proteins are specifically competed by poly(C) and not poly(U), but it remains to be determined if any of these factors are α CP. Two of these proteins were reduced in α -globin 3'UTR-binding activity when assayed using extracts from MEL cells that have been induced to differentiate. Therefore, the RNA-binding activities of these proteins may be regulated by a differentiation-dependent signal. Even though the above evidence suggests that several factors are present in the α -complex, the precise composition of the α -complex remains controversial (see below).

The Binary Model of α -Complex Structure. Chkheidze *et al.* (22) showed by EMSA that recombinant α CP1, α CP2, and α CP2 KL were each capable of directly interacting with the α -globin 3'UTR and forming an α -complex. This complex had the same electrophoretic mobility as the α -complex formed using unfractionated cell extracts. This implied that each of the α CP isoforms is fully sufficient to form the α -complex. α CP2 KL, the most abundant α CP isoform in most cells, was studied in detail. Binding of recombinant α CP2 KL to the α -globin 3'UTR was selectively abolished by mutations that destabilized α -globin mRNA and blocked α -complex assembly in whole cell extracts. To determine the stoichiometry of the α -complex, the overall mass of the α -complex was determined by size-exclusion chromatography and by sucrose density gradient centrifugation. The data established that the α -complex comprised a binary assembly of a single molecule of α CP with the α -globin 3'UTR, and that α CP2 KL (and most likely α CP1 and α CP2) were fully sufficient to form the α -complex.

Functions of the Proteins That Interact with the α -Globin mRNA Stability Determinant

Protection from Deadenylation. Although determinants of the α -complex have been identified, the corresponding pathways involved in mRNA turnover and stabilization remain to be fully defined. Wang *et al.* (28) provided data to support the role of α CP1 and α CP2 in controlling the rate of deadenylation of α -globin mRNA. This group used a cell-free *in vitro* mRNA decay assay (IVDA) containing K562 cell extract to study the mechanism by which the α -complex regulates mRNA turnover. Capped and polyadenylated α -globin 3'UTR had a half-life of 5 hr in this system. In contrast, a mutant α -globin 3'UTR unable to assemble the α -complex had a half-life of 1.5 hr and its rate of deadenylation was higher than wild type. These data showed that the cell-free system recapitulates α -globin mRNA stability conferred by the α -complex *in vivo*, and suggested that the α -complex inhibits deadenylation. Disruption of the α -complex by addition of poly(C) to the cell-free extract also resulted in a 2-fold greater decay of the wild-type 3'UTR along with an accelerated deadenylation. Similar results were obtained when poly(C) binding activity was depleted from the extract using poly(C) agarose beads. Addition of purified fractions containing α CP1 and α CP2 to α CP depleted extracts resulted in a restabilization of the α -globin 3'UTR to approximately 85% of the input adenylated RNA. This restoration was specific; the addition of hnRNP U RNA binding domain had no effect on the deadenylation rate or on half-life of the α -globin 3'UTR. Because the authors detected an interaction between α CP1 and α CP2 with PABP using the yeast two-hybrid screen (see above), they hypothesized that both α CP1 and α CP2 slowed the deadenylation of the α -globin 3'UTR via interaction with PABP.

Protection from Endoribonucleolytic Cleavage.

By studying α -globin 3'UTR decay intermediates in a cell-free *in vitro* decay assay (IVDA) derived from MEL cells, Wang *et al.* (31) identified an endoribonuclease cleavage site in the α -globin 3'UTR. This site, mapped *in vitro* and *in vivo*, was localized 63 nt downstream from the translational stop codon (Fig. 3; the nt are numbered such that the first nucleotide 3' to the stop codon is nt 1). Interestingly, this cleavage site is within the α -complex protected region. Mutation of 3 nt on both sides of the cleavage site resulted in an α -globin 3' UTR that did not form the α -complex, and was resistant to cleavage. This showed that the endoribonuclease cleavage was sequence specific. When the cleavage site was inserted into an unrelated RNA, the resulting chimeric RNA was cleaved at the same site as in the α -globin 3'UTR. These data demonstrated that the endoribonuclease cleavage site could be transferred to a heterologous RNA. It was further demonstrated that nt 56–81 of the α -globin 3'UTR contains all the necessary sequence elements for cleavage when linked to a heterologous RNA (32). A series of linker scanning mutants were introduced by

substitution of the hexanucleotide GAGAGA at four different sites in the region spanning nt 56–81. Interestingly, all four of the substitution mutants inhibited cleavage by the endoribonuclease. In contrast, insertion of a second substitution sequence, AAGCUU, at the same positions did not inhibit endoribonucleolytic cleavage. It is possible that the region spanning 56–81 fold into a distinct structure recognized by the endoribonuclease, and that insertion of GAGAGA is more detrimental to the structure than insertion of the sequence AAGCUU. The secondary structure of this region of the α -globin 3'UTR has been reported by Thisted *et al.* (33) (Fig. 5). In this structure, the 51–63 region consists of a single-stranded C-rich region, whereas the adjacent 64–81 region folds into one-half of a stem with two bulges. Interestingly, the endoribonuclease cleavage site (nt 63) is located just 5' to this double-stranded region. Thus, the single-stranded C-rich region may be the core endoribonuclease recognition site because insertion of either GAGAGA or AAGCUU abolishes, or alters cleavage specificity, respectively (32). Likewise, the surrounding stem structure may be important to display the core endoribonuclease recognition site in a single-stranded conforma-

tion. It would be informative to test this hypothesis by determining the structure of the various insertion mutants.

Comparison of nonerythroid versus erythroid extracts for cleavage activity in the IVDA suggested that the endoribonuclease activity was enriched in erythroid cells (31). Therefore, this activity was referred to as erythroid enriched endoribonuclease (ErEN). Fractionation of the cell-free extract revealed that the ErEN is found in the soluble cytoplasmic fraction, and is not associated with polysomes or with ribosomal salt wash fractions. The activity does not require ATP hydrolysis. Because the addition of oligo(dC) to the cell-free decay reaction sequesters the α -complex away from the α -globin 3'UTR and results in enhanced detection of the decay intermediates, the authors conclude that the α -complex functions to protect the α -globin mRNA from nucleolytic attack by the ErEN.

Wang *et al.* (34) used the IVDA to further characterize the ErEN activity present in MEL cells. Radiolabeled unadenylated and polyadenylated α -globin 3'UTRs were incubated in the IVDA, and the resulting cleavage products were analyzed over time. After 10 min of incubation of the unadenylated substrate in the IVDA, ErEN cleavage prod-

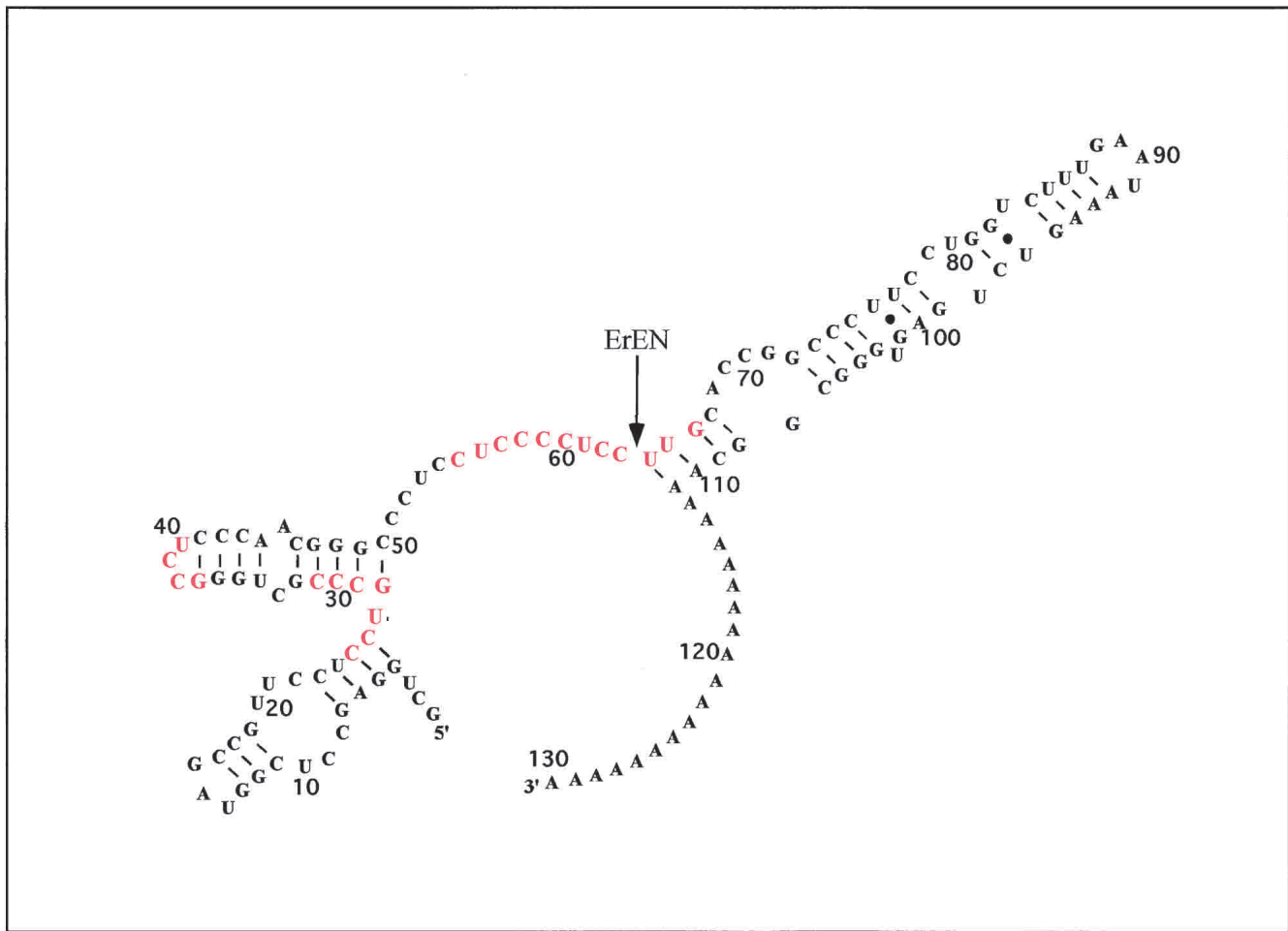


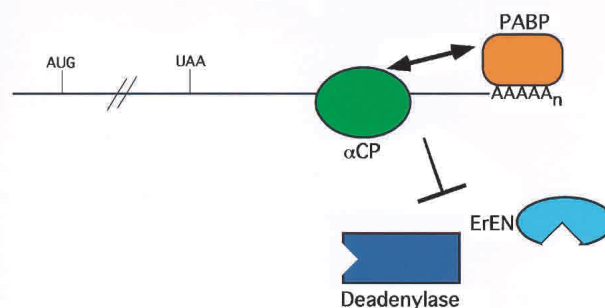
Figure 5. The structure of the wild-type α -globin 3'UTR. The three C-rich elements that stabilize the α -globin mRNA and mediate formation of the α -complex are indicated in red. The ErEN cleavage site at position 63 is shown by the arrow. Watson-Crick base pairs are indicated by the dashes, and non-Watson-Crick base pairs are indicated by the dots.

ucts were detected and continued to accumulate over the 60-min course of the experiment. In contrast, no ErEN cleavage products were detected using the adenylated substrate, even after 60 min of incubation in the IVDA. This experiment demonstrated that the poly(A) tail can prevent the cleavage of the α -globin 3'UTR by ErEN. The authors then determined whether the poly(A) effect on ErEN activity was due to the poly(A) tail itself, or due to a *trans*-acting factor. Because PABP is most likely the major cytoplasmic poly(A) binding activity, the authors depleted this protein from the extract by incubation with poly(A) agarose beads, and analyzed the ErEN cleavage products upon incubation with adenylated α -globin 3'UTR. In the absence of PABP, ErEN cleavage products were detected by 15 min of incubation. Curiously, these ErEN cleavage products were still adenylated even in the absence of PABP. Although this may indicate that the deadenylase is inactive in the *in vitro* decay reaction, a previous report by the same authors (28) showed that deadenylation of the α -globin 3'UTR occurred upon PABP depletion. The reason for these conflicting results may be the longer incubation time (2 hr) in the latter experiment. If this is the case, then it is possible that the ErEN cleaves the α -globin 3'UTR more rapidly than the deadenylase that removes the poly(A) tail. Addition of recombinant PABP to the PABP-depleted IVDA fully restored the resistance of the RNA to ErEN cleavage. This restoration was specific; the addition of hnRNP U RNA-binding domain to PABP-depleted IVDA had no effect on ErEN-mediated cleavage. These results confirmed that the inhibitory effect on ErEN activity is mediated by PABP (34).

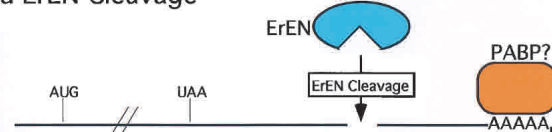
Depletion of α CP from the IVDA accelerated ErEN-mediated cleavage of a polyadenylated α -globin 3'UTR, even though PABP was likely still present (34). Because the inhibitory effect of PABP on ErEN activity was abolished when α CP was removed, the authors conclude that the inhibitory activity of PABP is mediated by α CP. Reconstitution experiments involving addition of recombinant α CP to α CP-depleted IVDA to confirm these findings were not reported. However, supporting evidence for PABP- α CP-mediated regulation of ErEN activity was suggested by EMSA studies. It was found that PABP increased the binding affinity of α CP1 to the α -globin 3'UTR by approximately 3-fold. Similar results were obtained with α CP2. Conversely, it was also shown by EMSA that α CP1 or α CP2 enhanced the binding of PABP to the poly(A) tail. These binding studies suggest that the interactions of PABP and α CP with the α -globin 3'UTR function to regulate ErEN activity by enhancing the binding affinities of each protein to their respective binding sites on the α -globin mRNA.

The above studies suggest that there may be two distinct pathways of α -globin mRNA decay: a deadenylation-dependent 3' exonuclease pathway and an endoribonucleolytic pathway. In both of these pathways, α CP protects the α -globin mRNA from decay (see model in Fig. 6).

Stable α -globin mRNA



Decay of α -globin mRNA via ErEN Cleavage



Decay of α -globin mRNA via Deadenylation



Figure 6. Model of the regulation of α -globin mRNA stability. The translational start codon (AUG), stop codon (UAA), and poly(A) tail ($AAAAA_n$) are indicated. The top panel shows conditions where the α -globin mRNA is stable, whereas the next two panels depict two pathways that lead to α -globin mRNA decay. The double arrow indicates the interaction of α CP with PABP.

Model of α -Globin mRNA Stability. The model illustrated in Figure 6 summarizes the current understanding of the mechanisms involved in stabilization of the α -globin mRNA. Under conditions where the α -globin mRNA is stable (Fig. 6, top panel), α CP binds to the α RNAmin site on the α -globin 3' UTR. The region protected by α CP binding (PR; see Fig. 3) also contains the ErEN cleavage site (ErEN site). Thus, binding of α CP prevents access of ErEN to the site and protects the RNA from endonucleolytic cleavage. α CP also contacts PABP (as indicated by the double arrow), which is bound to the poly(A) tail. Because this protein-protein interaction increases the binding affinity of α CP to the α RNAmin site, the mRNA is further protected from ErEN-mediated cleavage. Additionally, the α CP-PABP interaction increases the affinity of PABP for the poly(A) tail, which may explain the findings by several groups (see above) that α CP can hinder deadenylation of the α -globin mRNA. In the absence of α CP (Fig. 6, middle panel), ErEN has access to the ErEN site, and the RNA is cleaved. It is unclear whether deadenylation occurs prior to ErEN cleavage (see discussion above), and it is therefore unknown whether PABP is still bound to the mRNA. The factors that degrade the remaining cleaved mRNA remain to be identified. In the absence of PABP (Fig. 6, bottom

panel), deadenylation and decay of the α -globin mRNA occurs. It remains unknown whether α CP is still bound to the α -globin mRNA in the absence of PABP and the poly(A) tail.

Although the foregoing studies illuminate many aspects of the α -globin mRNA stabilization model, many questions remain. One question is whether the decay pathways defined primarily in *in vitro* decay systems are of rate-limiting importance *in vivo*. In addition, it is unknown whether the two degradation pathways function independently of one another or are they functionally linked. If they are independent, why does the cell have two degradation pathways? It is possible that the constitutive half-life of α -globin mRNA is mediated by the deadenylation-dependent pathway, whereas regulated stability of α -globin mRNA is controlled by ErEN. This would suggest that the binding of α CP to the ErEN site is regulated. Perhaps the ErEN-dependent degradation pathway is controlled by a cellular signaling cascade in erythroid cells, which ultimately ends in altering the α -globin RNA binding activity of α CP. Defining these signals and their underlying pathways will be of major importance when considering dynamic alterations in α CP function and alterations in stability of specific mRNAs. Answers to these and additional questions relating to the broader role of the α -complex and α CP proteins in mRNA structure and function can be addressed in future studies.

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1. Russell JE, Morales J, Liebhaber SA. The role of mRNA stability in the control of globin gene expression. New York: Academic Press, pp249–287, 1997.
2. Liebhaber SA, Cash FE, Ballas SK. Human α -globin gene expression: the dominant role of the α 2-locus in mRNA and protein synthesis. *J Biol Chem* **261**:15327–15333, 1986.
3. Liebhaber SA, Kan YW. Differentiation of the mRNA transcripts originating from the α 1- and α 2-globin loci in normals and α -thalassemics. *J Clin Invest* **68**:439–446, 1981.
4. Aviv H, Voloch Z, Bastos R, Levy S. Biosynthesis and stability of globin mRNA in cultured erythroleukemic Friend cells. *Cell* **8**:495–503, 1976.
5. Morales J, Russell JE, Liebhaber SA. Destabilization of human α -globin mRNA by translation anti-termination is controlled during erythroid differentiation and is paralleled by phased shortening of the poly(A) tail. *J Biol Chem* **272**:6607–6613, 1997.
6. Clegg JB, Weatherall DJ, Milner PF. Haemoglobin Constant Spring: a chain termination mutant? *Nature* **234**:337–340, 1971.
7. Laig M, Pape M, Hundrieser J, Flatz G, Sanguansermsri T, Das BM, Deka R, Yongvanit P, Mularlee N. The distribution of the Hb Constant Spring gene in Southeast Asian populations. *Hum Genet* **84**:188–190, 1990.
8. Weiss IM, Liebhaber SA. Erythroid cell-specific determinants of α -globin mRNA stability. *Mol Cell Biol* **14**:8123–8132, 1994.
9. Weiss IM, Liebhaber SA. Erythroid cell-specific mRNA stability elements in the α 2-globin 3'-nontranslated region. *Mol Cell Biol* **15**:2457–2465, 1995.
10. Wang X, Kiledjian M, Weiss IM, Liebhaber SA. Detection and characterization of a 3'-untranslated region ribonucleoprotein complex associated with human α -globin mRNA stability. *Mol Cell Biol* **15**:1769–1777, 1995.
11. Holcik M, Liebhaber SA. Four highly stable eukaryotic mRNAs assemble 3'-untranslated region RNA-protein complexes sharing cis and trans components. *Proc Natl Acad Sci USA* **94**:2410–2414, 1997.
12. Stefanovic B, Hellerbrand C, Holcik M, Briendl M, Liebhaber SA, Brenner DA. Posttranscriptional regulation of collagen α 1(I) mRNA in hepatic stellate cells. *Mol Cell Biol* **17**:5201–5209, 1997.
13. Paulding WR, Czyzyk-Krzeska MF. Regulation of tyrosine hydroxylase mRNA stability by protein-binding, pyrimidine-rich sequence in the 3'-untranslated region. *J Biol Chem* **274**:2532–2538, 1999.
14. Ostareck DH, Ostareck-Lederer A, Wilm M, Thiele BJ, Mann M, Hentze MW. mRNA silencing in erythroid differentiation: hnRNP K and hnRNP E1 regulate 15-lipoxygenase translation from the 3' end. *Cell* **89**:597–606, 1997.
15. Kiledjian M, Wang X, Liebhaber SA. Identification of two KH domain proteins in the α -globin mRNP stability complex. *EMBO J* **14**:4357–4364, 1995.
16. Leffers H, Dejgaard K, Celis JE. Characterization of two major cellular poly (rC)-binding human proteins, each containing three K-homologous (KH) domains. *Eur J Biochem* **230**:447–453, 1995.
17. Makeyev AV, Liebhaber SA. The poly(C)-binding proteins: a multiplicity of functions and a search for mechanisms. *RNA* **8**:265–278, 2002.
18. Tommerup N, Leffers H. Assignment of human KH-box-containing genes by in situ hybridization: hnRNPK maps to 9q21.32-q21.33, PCBP1 to 2p12-p13, and PCBP2 to 12q13.12-q13.13, distal to FRA12A. *Genomics* **32**:297–298, 1996.
19. Makeyev AV, Chkheidze AN, Liebhaber SA. A set of highly conserved RNA-binding proteins, α CP-1 and α CP-2, implicated in mRNA stabilization, are coexpressed from an intronless gene and its intron-containing paralog. *J Biol Chem* **274**:24849–24857, 1999.
20. Makeyev AV, Liebhaber SA. Identification of two novel mammalian genes establishes a subfamily of KH-domain RNA-binding proteins. *Genomics* **67**:301–316, 2000.
21. Funke B, Zuleger R, Benavente R, Schuster T, Goller M, Stevenin J, Horak I. The mouse poly(C)-binding protein exists in multiple isoforms and interacts with several RNA-binding proteins. *Nucleic Acids Res* **24**:3821–3828, 1996.
22. Chkheidze AN, Lyakhov DL, Makeyev AV, Morales J, Kong J, Liebhaber SA. Assembly of the α -globin mRNA stability complex reflects binary interaction between the pyrimidine-rich 3'-untranslated region determinant and poly(C) binding protein α CP. *Mol Cell Biol* **19**:4572–4581, 1999.
23. Gibson TJ, Thompson JT, Heringa J. The KH domain occurs in a diverse set of RNA-binding proteins that include the antiterminator NusA and is probably involved in binding to nucleic acid. *FEBS Lett* **324**:361–366, 1993.
24. Musco G, Stier G, Joesph C, Morelli MA, Nilges M, Gibson TJ, Pastore A. Three-dimensional structure and stability of the KH domain: molecular insights into the fragile X syndrome. *Cell* **85**:237–245, 1996.
25. Lewis HA, Chen H, Edo C, Buckanovich RJ, Yang YYL, Musunuru K, Zhong R, Darnell RB, Burley SK. Crystal structures of nova-1 and nova-2 K-homology RNA-binding domains. *Structure* **7**:191–203, 1999.
26. Lewis HA, Musunuru K, Jensen KB, Edo C, Chen H, Darnell RB, Burley SK. Sequence-specific RNA binding by a nova KH domain: implications for paraneoplastic disease and the fragile X syndrome. *Cell* **100**:323–332, 2000.
27. Kiledjian M, DeMaria CT, Brewer G, Novick K. Identification of AUF1 (heterogeneous nuclear ribonucleoprotein D) as a component of the α -globin mRNA stability complex. *Mol Cell Biol* **17**:4870–4876, 1997.
28. Wang Z, Day N, Trifillis P, Kiledjian M. An mRNA stability complex functions with poly(A)-binding protein to stabilize mRNA in vitro. *Mol Cell Biol* **19**:4552–4560, 1999.
29. Henics T. Differentiation-dependent cytoplasmic distribution and in vivo RNA association of proteins recognized by the 3'-UTR stability

- element of α -globin mRNA in erythroleukemic cells. *Biochem Biophys Res Commun* **279**:40–46, 2000.
30. Shyu AB, Greenberg ME, Belasco JG. The c-fos transcript is targeted for rapid decay by two distinct mRNA degradation pathways. *Genes Dev* **3**:60–72, 1989.
 31. Wang Z, Kiledjian M. Identification of an erythroid-enriched endoribonuclease activity involved in specific mRNA cleavage. *EMBO J* **19**:295–305, 2000.
 32. Rodgers ND, Wang Z, Kiledjian M. Characterization and purification of a mammalian endoribonuclease specific for the α -globin mRNA. *J Biol Chem* **277**:2597–2604, 2002.
 33. Thisted T, Lyakhov DL, Liebhaber SA. Optimized targets of two closely related triple KH domain proteins, heterogeneous nuclear ribonucleoprotein K and α CP-2KL, suggest distinct modes of RNA recognition. *J Biol Chem* **20**:17484–17496, 2001.
 34. Wang Z, Kiledjian M. The poly(A)-binding protein and an mRNA stability protein jointly regulate an endoribonuclease activity. *Mol Cell Biol* **20**:6334–6341, 2000.