

A Nucleolin-Binding 3' Untranslated Region Element Stabilizes β -Globin mRNA In Vivo

Yong Jiang,¹ Xiang-Sheng Xu,¹ and J. Eric Russell^{1,2*}

Departments of Medicine (Hematology/Oncology)¹ and Pediatrics (Hematology),² University of Pennsylvania School of Medicine and The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania

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The normal expression of human β globin is critically dependent upon the constitutively high stability of its encoding mRNA. Unlike with α -globin mRNA, the specific *cis*-acting determinants and *trans*-acting factors that participate in stabilizing β -globin mRNA are poorly described. The current work uses a linker-scanning strategy to identify a previously unknown determinant of mRNA stability within the β -globin 3' untranslated region (3'UTR). The new determinant is positioned on an mRNA half-stem opposite a pyrimidine-rich sequence targeted by α CP/hnRNP-E, a factor that plays a critical role in stabilizing human α -globin mRNA. Mutations within the new determinant destabilize β -globin mRNA in intact cells while also ablating its 3'UTR-specific interaction with the polyfunctional RNA-binding factor nucleolin. We speculate that 3'UTR-bound nucleolin enhances mRNA stability by optimizing α CP access to its functional binding site. This model is favored by in vitro evidence that α CP binding is enhanced both by *cis*-acting stem-destabilizing mutations and by the *trans*-acting effects of supplemental nucleolin. These studies suggest a mechanism for β -globin mRNA stability that is related to, but distinct from, the mechanism that stabilizes human α -globin mRNA.

Erythroid cells accumulate hemoglobin through a process that is critically dependent upon the high stabilities of mRNAs that encode their constituent α - and β -globin subunits (10, 64). In vivo analyses estimate a half-life for human α -globin mRNA of between 24 and 60 h (47, 62, 63, 74), while similar studies with cultured NIH 3T3 and murine erythroleukemia (MEL) cells (2, 36, 42), primary mouse hematopoietic cells (4), and human erythroid progenitors (62, 63) suggest a half-life value for human β -globin mRNA that exceeds 16 to 20 h. Globin mRNAs survive, and continue to translate at high levels, for as long as a week following nuclear condensation and extrusion in transcriptionally silent erythroid progenitor cells. As might be anticipated, mutations that impair the normal stabilities of globin mRNAs can severely impact the levels of their encoded proteins. For example, an mRNA-destabilizing mutation reduces the expression of $\alpha^{\text{Constant Spring}}$ to less than 2% of normal levels (45, 51, 80), resulting in a clinically important form of thalassemia characterized by a substantial imbalance in α - and β -globin chain accumulation (10, 64).

The *cis*-acting determinants and *trans*-acting factors that participate in regulating α -globin mRNA stability have recently been identified, and the relevant molecular mechanisms have been described in detail. Mutational analyses carried out with cultured cells (80, 81) and with animal models (52, 66) clearly demonstrate the importance of the 3' untranslated region (3'UTR) to the constitutively high stability of α -globin mRNA (45). Other studies have mapped this characteristic to a phylogenically conserved, 16-nucleotide (nt) C/U-rich element in this region (39, 75, 76). The *cis*-acting pyrimidine-rich

element (PRE) assembles an mRNP " α -complex" that comprises a member of the α CP/hnRNP-E family of mRNA-binding proteins (37, 39, 48, 75) and possibly one or more additional *trans*-acting factors (17, 38, 77). The α -complex may slow α -globin mRNA decay by enhancing the binding of poly(A)-binding protein to the poly(A) tail (77, 79). The α -complex may also prevent the access of an erythroid-cell-specific endoribonuclease to the α -PRE (61, 78, 79), mimicking mechanisms through which several nonglobin mRNAs evade endonucleolytic cleavage (5, 6, 8).

Unlike with α -globin mRNA, neither the *cis* elements nor the *trans*-acting factors that specify the constitutively high stability of human β -globin mRNA have been fully described. Although several hundred mutations are known to affect β -globin gene expression, few offer any insight into the position of a specific β -globin mRNA stability-enhancing region or its likely mechanism (10). Common mutations that encode premature translation termination codons or adversely affect processing of β -globin pre-mRNA, though accelerating its degradation, utilize mRNA-indifferent decay pathways (10, 26, 49) and consequently do not illuminate the putative β -globin mRNA-restricted mechanism(s) that defines its high baseline stability.

The search for a discrete β -globin mRNA stability element has focused largely on its 3'UTR, since sequences dictating the stabilities of mRNAs encoding α globin (80, 81), transferrin receptor (40), histones (57), and a variety of cytokines and lymphokines (70) are commonly positioned in this ribosome-free region. This expectation has been fulfilled by studies of chimeric *fos*- β -globin mRNA in cultured cells (36) and full-length β -globin mRNA in MEL cells and in whole-animal models (65, 84). The latter studies were particularly informative, demonstrating that β -globin mRNA can be destabilized by engineered mutations that permit ribosomes to read past its native translational terminal codon and through the entire 3'UTR, disrupting hypothetical mRNP structures positioned

* Corresponding author. Mailing address: Abramson Research Building, Room 316F, The Children's Hospital of Philadelphia, 34th Street and Civic Center Boulevard, Philadelphia, PA 19104. Phone: (215) 590-3880. Fax: (215) 590-4834. E-mail: jeruss@mail.med.upenn.edu.

in that region. In contrast, β -globin expression is minimally impacted by naturally occurring and synthetic frameshift mutations that restrict ribosomal readthrough to the proximal one-third of the 3'UTR (11, 20, 25, 65), suggesting that critical determinants of mRNA stability are likely to be positioned further downstream. Scattered, naturally occurring single-nucleotide substitutions and small deletions in the terminal portion of the 3'UTR do not materially affect β -globin mRNA stability (3, 12, 34) and are consequently of little help in mapping important *cis*-acting stability elements. Recent interest has focused on a 14-nt pyrimidine-rich element in the β -globin 3'UTR (the β -PRE) because of its obvious similarity to the α -PRE stability determinant. Deletion or purine substitution of the β -PRE reduces the stability of human β -globin mRNA in transgenic mice by approximately one-half (84), indicating the importance of this structure to β -globin gene expression.

Although the mechanism through which the β -PRE confers stability to the β -globin mRNA is not known, several observations suggest that it, like the α -PRE, may be functionally targeted by α CP. First, the human α - and β -globin genes evolved from a single ancestral globin gene (28, 29), raising the possibility of a primitive, evolutionally conserved mechanism for stabilizing their mRNAs. Second, α CP is known to play an important role in stabilizing several nonglobin mRNAs that contain pyrimidine-rich 3'UTR motifs [e.g., 15-lipoxygenase (55), tyrosine hydroxylase (18), and α 1(I) collagen (73)] and may participate in stabilizing other globin mRNAs (66). Third, electrophoretic mobility studies demonstrate that the β -globin 3'UTR assembles an mRNP structure in vitro that may contain α CP (84). Formal attempts to directly link α CP to β -globin mRNA stability, however, have been unsuccessful, suggesting that the constitutive stability of β -globin mRNA in vivo requires mRNA structural conformations or additional *trans*-acting factors that are poorly recapitulated in vitro. Consequently, the posttranscriptional mechanisms that maintain β -globin mRNA levels in vivo remain poorly understood, despite their obvious relevance to common disorders of globin gene expression.

The current article provides new insights into the structural basis for β -globin mRNA stability, suggesting an underlying mechanism that differs in several fundamental respects from the mechanism dictating the stability of human α -globin mRNA. A previously unrecognized *cis* determinant of β -globin mRNA stability is identified by assessing the survival of derivative mRNAs containing defined site-specific mutations in vivo. Independent experiments in vitro, in cultured cells, and in primary human erythroid progenitors identify nucleolin, a ubiquitous, structurally heterogeneous, polyfunctional RNA-binding protein, as a cytoplasmic factor that binds to the β -globin 3'UTR in a sequence-specific manner. A link between nucleolin binding and mRNA stability is provided by subsequent in vitro and in vivo analyses demonstrating that functional mutations within the stability determinant also interfere with nucleolin binding. These data, and previous experimental evidence favoring an mRNA-stabilizing role for α CP, are accommodated by a model in which nucleolin facilitates the access of α CP to its functional β -globin 3'UTR target site. We demonstrate a key aspect of this model by showing that disruption of a high-order structure within the β -globin 3'UTR facilitates α CP binding in vitro. These studies suggest a mecha-

nism for β -globin mRNA stability that is related to, but distinct from, the mechanism that stabilizes human α -globin mRNA.

MATERIALS AND METHODS

Cell culture. HeLa cells expressing the tetracycline-regulated transactivator (tTA) fusion protein (BD Biosciences) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in a humidified 5% CO₂ environment. Suspension MEL cells were cultured under similar conditions, while human K562 cells were grown in Iscove's modified Dulbecco's medium containing 4 mM glutamine and 1.5 g/liter sodium bicarbonate and supplemented with 10% fetal bovine serum. Cells ($\sim 5 \times 10^5$) were transfected with 5 μ g supercoiled DNA using Superfect reagent as recommended by the manufacturer (QIAGEN). Doxycycline was added to a final concentration of 1 μ g/ml when required.

Gene cloning. pTRE- β^{WT} was constructed from a 3.3-kb fragment of human genomic DNA containing the intact β -globin gene and contiguous 3' flanking region, inserted into the SacII-ClaI polylinker site of pTRE2 (BD Biosciences). Linker-scanning mutations were introduced into the human β -globin gene by a splice overlap extension-PCR method (65, 84) using paired, complementary 30-nt primers containing the desired HindIII mutation (5'AAGCTT3'). The resulting mutated 904-bp cDNAs were then substituted for the cognate EcoRI-EcoNI fragment of pTRE- β^{WT} . Chemically competent DH5 α *Escherichia coli* cells were transformed (Invitrogen), mini-prep DNA was prepared from individual colonies (QIAGEN), and the structures of the variant β -globin genes were subsequently validated by HindIII digestion and by automated dideoxy sequencing. pTRE- β^{ARE104} and pTRE- β^{ARE130} were constructed by introducing a 59-bp A/U-rich mRNA instability element (70) into the HindIII sites of pTRE- β^{H104} and pTRE- β^{H130} , respectively.

RNAse protection analysis. Cellular RNAs prepared from cultured cells using TRIzol reagent (Gibco-BRL) were analyzed as described previously (66, 84). ³²P-labeled β -globin and β -actin probes were prepared by in vitro transcription of DNA templates using SP6 RNA polymerase (Ambion). The 287-nt β -globin probe protects a 199-nt sequence of human β -globin mRNA exon II, while the 313-nt β -actin probe protects a 160-nt exonic fragment of human β -actin mRNA (84). Band intensities were quantitated from PhosphorImager files using ImageQuant software (Amersham Biosciences).

RT-PCR⁺ analysis (65). Purified RNAs (~ 500 ng) were reverse transcribed and thermally amplified using Superscript one-step reagents under conditions recommended by the manufacturer (Invitrogen) and then amplified for 40 cycles using exon II (5'ACCTGGACAACCTCAAGG3') and exon III (5'TTTTTTTT TTTGCAATGAAAATAAATG3') primers that generate a 355-bp cDNA product encompassing the full β -globin 3'UTR. Reaction mixtures were subsequently augmented with 100 μ mol of a nested ³²P-labeled exon II primer (5'CCACAC TGAGTGAGCTGC3') and 0.5 μ l Platinum *Taq* (Invitrogen) and product DNA amplified for one additional cycle. This method generates 328-nt ³²P-labeled homodimeric DNAs that fully digest with HindIII to generate ³²P-labeled products between 189 and 285 bp in length.

Proteomics. Analyses were carried out by the University of Pennsylvania Proteomics Facility. Tryptic digests were resolved on a Voyager DE Pro (Applied Biosystems), and protein identities were deduced from MS-Fit (University of California) analysis of peptide fragments using the NCBInr database. Time-of-flight (TOF)-TOF analysis was carried out using a 4700 proteomics analyzer (Applied Biosystems) equipped with Global Proteomics Server analytical software.

Cytosolic extract. Extracts were prepared as previously described (19, 21). Briefly, phosphate-buffered saline (PBS)-washed cells were incubated for 20 min at 4°C in RNA immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM Na₃VO₄, 1 mM NaF, and 1 \times protease inhibitor cocktail [BD Biosciences]). The lysate was centrifuged at 13,000 \times g for 15 min, and the supernatant was collected and stored at -80° C. For cross-linking studies, in vitro-transcribed, ³²P-labeled RNAs were incubated with cytoplasmic extract and exposed to UV light (3,000 mJ/cm²) for 5 min.

Fluorescence-activated cell sorter (FACS) analysis. A protocol for all animal work was approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania School of Medicine. EDTA-anticoagulated whole blood was stained with thiazole orange as directed by the manufacturer (Sigma) (31). Erythroid cells were identified by their characteristic forward- and side-scatter properties using a FACSVantage cell sorter equipped with Digital Vantage options (Becton-Dickinson). Thiazole orange-staining cells (reticulocytes) were collected, excluding a small population of hyper-staining nucleated erythroid progenitor cells.

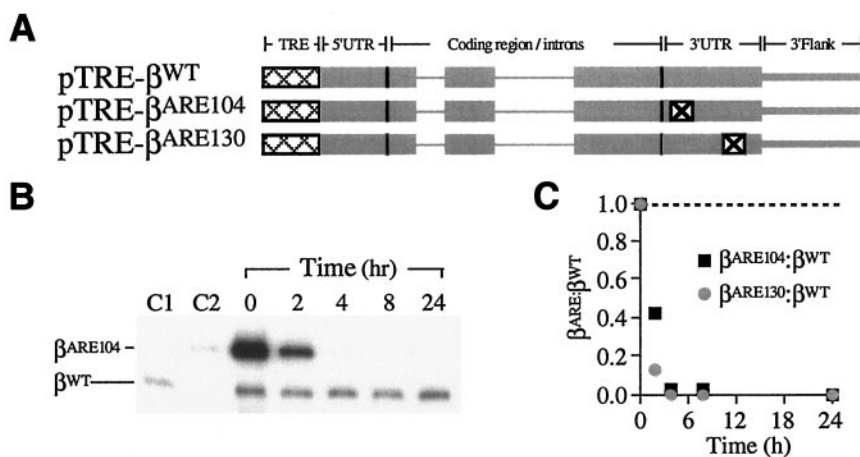


FIG. 1. Unstable and stable variant β -globin mRNAs can be distinguished in intact cells. (A) Structures of conditionally expressed reporter genes encoding variant β -globin mRNAs. pTRE- β^{WT} contains the full-length human β -globin gene, including native intronic, exonic, and 3'-flanking sequences (thin, thick, and intermediate gray lines, respectively), downstream of a Tet-conditional TRE promoter (dotted cross-hatching). pTRE- β^{ARE104} and pTRE- β^{ARE130} are identical to pTRE- β^{WT} except for a 59-bp ARE instability element (☒) at either of two 3'UTR positions. (B) Variant β^{ARE104} mRNA is unstable in cultured cells. The levels of β^{WT} and β^{ARE104} mRNAs in transiently transfected HeLa^{tTA} cells were assessed by RT-PCR⁺ at defined intervals following Dox exposure. The intensities of the β^{WT} bands were balanced by adjusting sample loading. C1 and C2 contain RNA from cells transfected singly with pTRE- β^{WT} and pTRE- β^{ARE104} , respectively. (C) ARE-mediated destabilization of β -globin mRNA in cultured cells. The autoradiograph in panel B was analyzed by PhosphorImager densitometry and the $\beta^{ARE104}/\beta^{WT}$ ratio plotted (black squares). Results from a parallel study of β^{ARE130} are also plotted (gray circles). The dashed line indicates the β^{ARE}/β^{WT} ratio that would be observed if the two mRNAs were equally stable.

Affinity enrichment studies. Custom 5'-terminal biotinylated single-stranded DNAs (ssDNAs) were purchased from Integrated DNA Technologies (Coralville, IA). Molar equivalents of each ssDNA (3 pmol) were incubated for 1 h at 4°C in PBS (pH 7.2) along with 100 μ l of preequilibrated ImmunoPure immobilized avidin agarose beads (Pierce Biotechnology). The pelleted beads were washed four times with PBS, incubated at 4°C for 1 h with 1 ml cytoplasmic extract, and then washed five times with PBS. Bound proteins were eluted with loading buffer and resolved on a precast 4 to 12% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels as recommended by the manufacturer (Invitrogen). A parental ssDNA corresponding to the β -globin 3'UTR stem-loop structure (5'ATTCTATTAAAGGTTTCCTTTGTTCCCTAA GTCCAACACTAAACTGGGGATATATGAAGGCGCTTGAGCATC3') was modified by the deletion of an internal 18-nt sequence (5'GGGGATAT TATGAAGGG3') and by the substitution of an unrelated 18-nt sequence (5'ATGCCGTAATGCCGTAAT3') or a sequence encompassing the β -PRE (5'TTCCTTGTTCCTAAGT3') at the same site.

Western blotting. Antibodies purchased from Santa Cruz Biotechnology included mouse monoclonal anti-human nucleolin (MS-3), rabbit polyclonal anti-human nucleolin (H-250), goat polyclonal anti-human HDAC-2 (C-19), rabbit polyclonal anti-human tumor necrosis factor alpha, and goat polyclonal anti-human hnRNP-E1 (T-18). Rabbit polyclonal anti-human actin antibodies were purchased from Sigma (A-2066). Protein samples in loading buffer were denatured at 100°C for 5 min, resolved on a precast 4 to 12% gradient SDS-PAGE gel, and transferred to a nitrocellulose membrane using an XCell II blot module according to the manufacturer's instructions (Invitrogen). Blots were blocked for 1 h at room temperature in PBS containing 0.1% Tween 20, supplemented with 3% dried milk, and then incubated for an additional hour following antibody addition. Membranes washed with the Tween 20-PBS mixture were subsequently incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) and analyzed using a chemiluminescence method (ECL kit; Amersham).

RNA immunoprecipitation. HeLa cell extracts were prepared as previously described (15, 16). PBS-washed erythrocytes were isolated from EDTA-anticoagulated whole blood by fractionation over a Histopaque 1.077/1.119 bilayer cushion (Sigma). Extracts prepared in RIPA buffer (1 ml) were precleared with 60 μ l protein A-agarose beads (Invitrogen) and then incubated at 4°C for 3 h with nucleolin H-250 antibodies. Fresh protein A-agarose beads (60 μ l) were then added, and the incubation continued for another 2 h. Immunoprecipitates were washed three times in RIPA buffer, and bound RNAs were collected by TRIzol extraction and ethanol precipitation for subsequent analysis. Control 18S pre-RNAs were RT-PCR amplified using oligomers 5'GTTCGTGCGACGTG

GGCGTGG3' and 5'CAGACCCGCGACGCTTCTTCGT3', producing a 501-bp cDNA fragment (1).

Preparation of recombinant α CP and purification of nucleolin. A glutathione S-transferase- α CP1 fusion protein was purified from DH5 α cells transfected with pEGX-6P- α CP1 (kind gift of M. Kiledjian, Rutgers University); the glutathione S-transferase domain was subsequently cleaved with PreScission proteinase (Pharmacia Biotech). Human nucleolin was affinity enriched from HeLa and/or K562 cell extract using an agarose-immobilized 2'-O-methyl RNA sequence (5'UAAUAAAGGUUCCUUGUCCCUAAGUCCAAC3'). A related method was used to prepare nucleolin-depleted extract.

RESULTS

Validation of a method for analyzing the stability of β -globin mRNA in intact cells. To facilitate our studies of β -globin mRNA stability, we developed a system in which a single defined gene can be transcriptionally silenced in intact, translationally competent cells. This approach permits mRNA decay to be assessed in vivo using a transcriptional chase approach that does not compromise cell viability (41, 82). The method requires cells that constitutively express a tTA fusion protein that activates genes linked to a recombinant hybrid tetracycline response element (TRE). tTA activity is rapidly and efficiently inhibited in the presence of tetracycline or doxycycline (Dox), which does not affect the expression of other, constitutively expressed eukaryotic genes. Consequently, the stabilities of mRNAs encoded by TRE-linked genes can be estimated by assessing their rate of disappearance from Dox-treated cells.

The proposed use of tTA-expressing HeLa cells was tested by assessing the fate of mRNAs carrying a known mRNA-destabilizing determinant, the 3'UTR A/U-rich element (ARE) derived from human granulocyte-macrophage colony-stimulating factor mRNA (70) (Fig. 1A). TRE-linked β -globin genes were constructed to contain either the native 3'UTR (pTRE- β^{WT}) (84) or 3'UTRs engineered to contain single-copy ARE

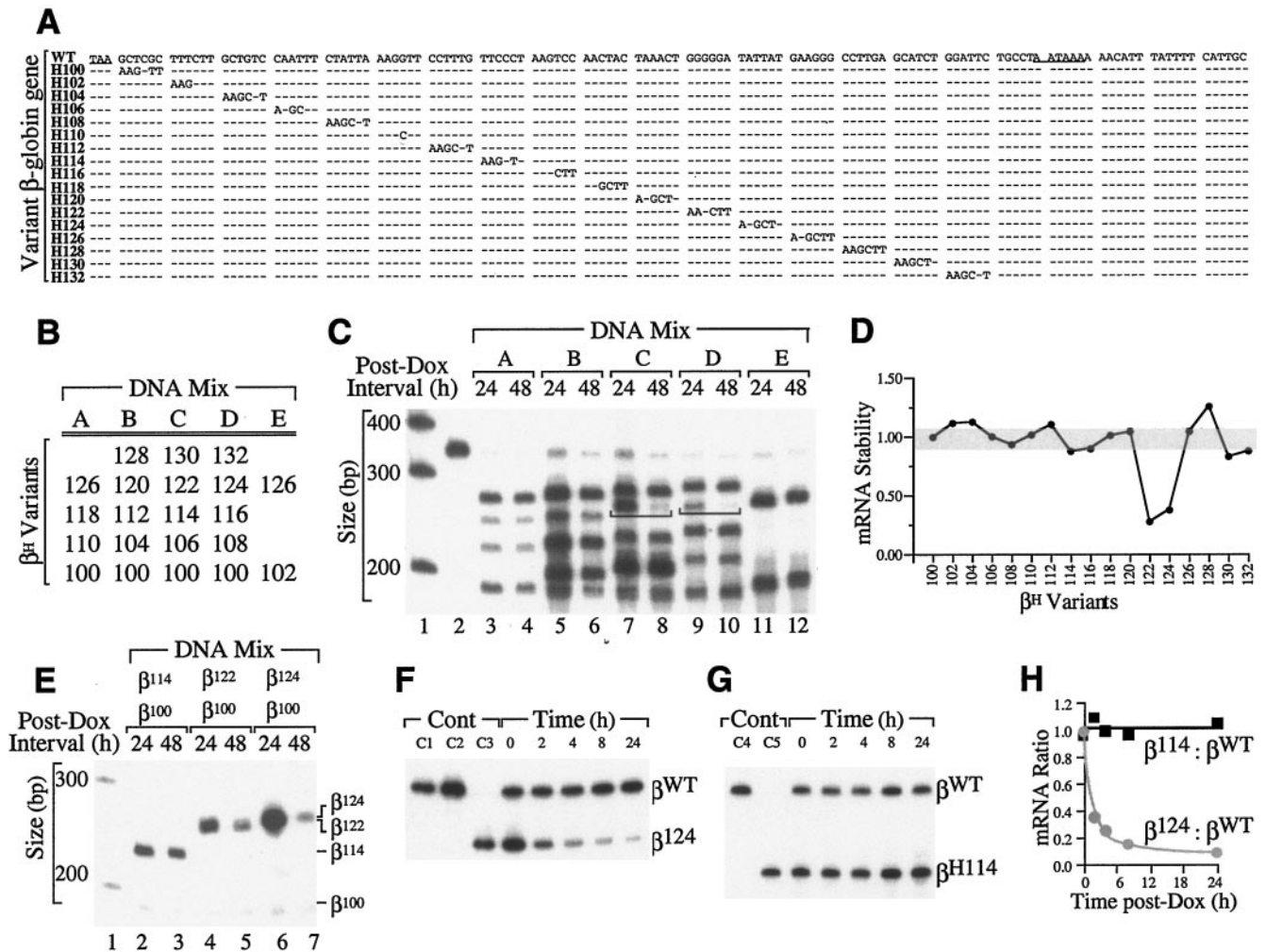


FIG. 2. Two adjacent hexanucleotide mutations destabilize β -globin mRNA in intact cultured cells. (A) Structures of variant β -globin genes. The 3'UTR of the wild-type β -globin gene (WT) is illustrated, with the TAA termination codon and AATAAA polyadenylation signal underlined. Each variant β -globin gene (designated H100, H102, and H104, etc.) contains a site-specific AAGCTT hexanucleotide substitution encoding a HindIII recognition site. Dashes indicate identity with the WT sequence. (B) Composition of DNA mixes used for mRNA stability studies in cultured cells. Mixes A to D each contain four or five variant TRE-linked β^H -globin genes, including one (β^{H100}) whose mRNA is used as a normalization control in subsequent analyses. Mix E contains a control variant β^{H126} gene for the same purpose. (C) Relative stabilities of variant β -globin mRNAs following transcriptional silencing of their encoding genes. HeLa^{tTA} cells transfected with DNA mixes A to E were exposed to Dox, and total RNA was recovered from aliquots following an additional 24 or 48 h of culture. RT-PCR⁺-amplified products were restricted with HindIII to generate differently sized DNA fragments whose quantities correspond to the levels of individual variant β^H mRNAs in the original sample. Brackets emphasize the rapid interval decline in β^{H122} mRNA (lanes 7 and 8) and β^{H124} mRNA (lanes 9 and 10), relative to levels of other variant β^H mRNAs. Lanes 1 and 2 contain ³²P-labeled size markers and the undigested PCR product from mix A, respectively. (D) Relative stabilities of variant β^H mRNAs. The stabilities of individual variant β^H mRNAs are plotted. Stability is defined as $[(\beta^H)_{48}/(\beta^H)_{24}]/[(\beta^{H100})_{48}/(\beta^{H100})_{24}]$, with the stability of β^{H100} arbitrarily assigned unit value (subscript values represent the post-Dox intervals in hours). (E) Accelerated decay of variant β^H mRNAs in intact cultured cells. The stabilities of mRNAs encoded by variant β^{H114} , β^{H122} , and β^{H124} genes (top) were established singly, relative to that of internal control β^{H100} mRNA, as described for panel C. The positions of individual HindIII-restricted RT-PCR⁺ products are indicated to the right. Lane 1 contains a DNA size marker. (F and G) Formal decay analyses of β^{H124} and control β^{H114} mRNAs. Mixes containing pTRE- β^{WT} and either pTRE- β^{H124} (F) or pTRE- β^{H114} (G) were transfected into HeLa^{tTA} cells, and relative mRNA levels were established by RT-PCR⁺ at defined intervals following Dox exposure. Controls (Cont) include undigested β^{WT} (C1), HindIII-digested β^{WT} (C2), HindIII-digested β^{H124} (C3), undigested β^{H114} (C4), and HindIII-digested β^{H114} (C5). (H) Relative stabilities of β^{H124} and control β^{H114} mRNAs. Band intensities were established from the autoradiographs in panels F and G by PhosphorImager densitometry. Levels of β^{H124} and β^{H114} mRNAs, relative to levels of coexpressed β^{WT} mRNA and normalized to the corresponding ratio at time zero, are plotted in gray and black, respectively.

inserts (pTRE- β^{ARE104} and pTRE- β^{ARE130}). pTRE- β^{WT} was cotransfected into HeLa^{tTA} cells with either pTRE- β^{ARE104} or pTRE- β^{ARE130} , and the levels of their encoded mRNAs were established at defined intervals following Dox exposure. Unlike with β^{WT} mRNA, the level of each β^{ARE} mRNA fell rapidly

(Fig. 1B and C), confirming the utility of the tTA-TRE system for differentiating unstable and stable mRNAs in intact, cultured cells.

Human β -globin mRNA is destabilized by either of two adjacent site-specific 3'UTR mutations. To map critical *cis*

determinants of β -globin mRNA stability, 17 full-length β -globin genes were constructed, each containing a hexanucleotide substitution at a unique 3'UTR position (Fig. 2A). Collectively, the mutations saturate 102 nt of the 107-nt sequence of β -globin 3'UTR between the native TAA translational termination codon and the AATAAA polyadenylation signal. HeLa^{tTA} cells were cotransfected with DNA mixes comprising different combinations of TRE-linked, variant β^H -globin genes, including one (β^{H100}) that was arbitrarily selected as an internal control (Fig. 2B). The level of each variant β^H mRNA, relative to that of β^{H100} mRNA, was subsequently determined by RT-PCR⁺ following 24- and 48-hour exposures to Dox (65). Two of the variant β^H mRNAs containing hexanucleotide substitutions at 3'UTR positions 122 and 124 displayed levels that fell four- to fivefold faster than those of other variant β^H mRNAs (Fig. 2C and D). These results were confirmed in a duplicate analysis utilizing a different post-Dox interval (not shown) and in related experiments in which genes encoding unstable variant β^{H122} and β^{H124} mRNAs and stable variant β^{H114} mRNA were separately transfected into HeLa^{tTA} cells along with internal control pTRE- β^{H100} (Fig. 2E). Formal mRNA stability studies were subsequently carried out using Dox-exposed HeLa^{tTA} cells that had been cotransfected with TRE-linked genes encoding β^{WT} and either β^{H114} or β^{H124} mRNA (Fig. 2F to H). By comparison to the level of β^{WT} mRNA, that of β^{H124} mRNA fell rapidly (Fig. 2F and H), while that of control β^{H114} mRNA remained stable (Fig. 2G and H). The combined results of screening and formal mRNA stability analyses confirm the importance of the 12-nt H122/H124 sequence to the intrinsically high stability of β -globin mRNA.

Nucleolin binds to the β -globin 3'UTR in intact cultured cells and primary erythroid cells. The stabilities of many mRNAs, including those encoding α globin (75), $\alpha 1(I)$ collagen (73), tyrosine hydroxylase (18), histone (57), and the transferrin receptor (53), require the assembly of defined mRNP effector complexes on specific determinants within their 3'UTRs. To identify candidate *trans*-acting factors that might functionally interact with the β -globin 3'UTR, agarose-immobilized ssDNAs corresponding to the β^{WT} 3'UTR and to negative-control poly(dI · dC) were separately incubated with cytoplasmic extract prepared from cultured human erythroid K562 cells. Three bands that displayed relative specificities for the β^{WT} 3'UTR were subsequently excised and subjected to matrix-assisted laser desorption ionization (MALDI)-TOF analysis (Fig. 3A). The ~100-kDa band was unambiguously identified as nucleolin from 14 tryptic peptide fragments representing 22% coverage (molecular weight search, 1.469×10^4) (Fig. 3B); the identities of the remaining two bands could not be established with certainty. Companion experiments indicated that nucleolin binds equally well to related full-length and truncated agarose-immobilized RNAs and 2'-O-methylated RNAs, respectively (Fig. 3C). These results were corroborated by parallel TOF-TOF analyses of affinity-enriched erythroid MEL cell extract that also unequivocally identified nucleolin (data not shown). This dual preliminary identification was subsequently confirmed by Western blot analysis of affinity-enriched proteins using a polyclonal nucleolin antibody (Fig. 3D). Nucleolin appears to bind to the β -globin 3'UTR in a sequence-specific manner, as increasing quantities of an unrelated soluble competitor ssDNA effectively compete back-

ground proteins from an agarose-immobilized ssDNA β -globin 3'UTR ligand but do not affect nucleolin binding (Fig. 3E). In addition, UV-cross-linked nucleolin- β -3'UTR mRNPs assemble in K562 cytoplasmic extract but not in extracts that are affinity depleted of nucleolin, confirming that nucleolin also binds to β^{WT} RNA (Fig. 3F, lanes T and D, respectively). These results document the sequence-specific binding of nucleolin to the β -globin mRNA 3'UTR *in vitro* and suggest that this interaction may subserve a critical function *in vivo*.

Nucleolin localizes to the cytoplasm of intact erythroid progenitor cells. Although nucleolin has been identified in the cytoplasm of nonerythroid cells (7, 68), its presence in erythroid cytoplasm has never been formally established. Two methodologically independent approaches were used to demonstrate that nucleolin can be found in the cytoplasm of erythroid cells representing temporally distinct stages of terminal differentiation. Nucleolin was easily detected by Western analysis of cytoplasm prepared from murine erythroid MEL cells (Fig. 4A) and was also identified in extract prepared from FACS-sorted murine reticulocytes (Fig. 4B). These results confirm that nucleolin is abundant in erythroid cytoplasm, permitting consideration of its potential role in stabilizing the relatively pure population of globin mRNAs that also populate these cells.

Nucleolin binds human β -globin mRNA in both cultured cells and primary human erythroid progenitors. The demonstration that nucleolin binds to ssDNA and RNA corresponding to the β -globin 3'UTR *in vitro* predicted its capacity to interact with full-length β -globin mRNA transcripts *in vivo* in intact cells. This hypothesis was subsequently tested using an RNA-immunoprecipitation (RIP) method (15, 16). Human β -globin mRNA was detected in cell extract as well as in a nucleolin immunoprecipitate prepared from cells transfected with pTRE- β^{WT} (Fig. 5A, lanes 3 and 5) but not in fractions prepared from cells transfected with an empty pTRE control vector (lanes 2 and 4). The specificity of the nucleolin-globin mRNA interaction was indicated by control experiments in which constitutively expressed GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA was observed in cell extract (Fig. 5A, lanes 6 and 7) but not in the nucleolin immunoprecipitate (Fig. 5A, lanes 8 and 9). Human β -globin mRNA was not identified in immunoprecipitate prepared with an unrelated antibody (Fig. 5B, compare lanes 4 and 5), demonstrating that the results do not arise from artifactual binding of β -globin mRNA to immunoglobulin. The likely physiological importance of the interaction between nucleolin and the β -globin mRNA was indicated by RIP analyses of lysate prepared from density-fractionated human erythroid progenitors. Both β -globin mRNA and control GAPDH mRNA were observed in the unfractionated lysate (Fig. 5C, lanes 2 and 4), while β -globin mRNA, but not GAPDH mRNA, was detected in immunoprecipitate prepared using nucleolin antibody (Fig. 5C, compare lanes 3 and 5). These experiments confirm that β -globin mRNA and nucleolin interact with high mutual specificity in intact cultured cells as well as in primary human erythrocytes.

An mRNA-destabilizing mutation in the β -globin 3'UTR reduces nucleolin binding *in vitro* and *in vivo*. The proposed functional linkage between nucleolin binding and β -globin mRNA stability was subsequently investigated by assessing the affinity of nucleolin for variant β^H -globin mRNAs containing

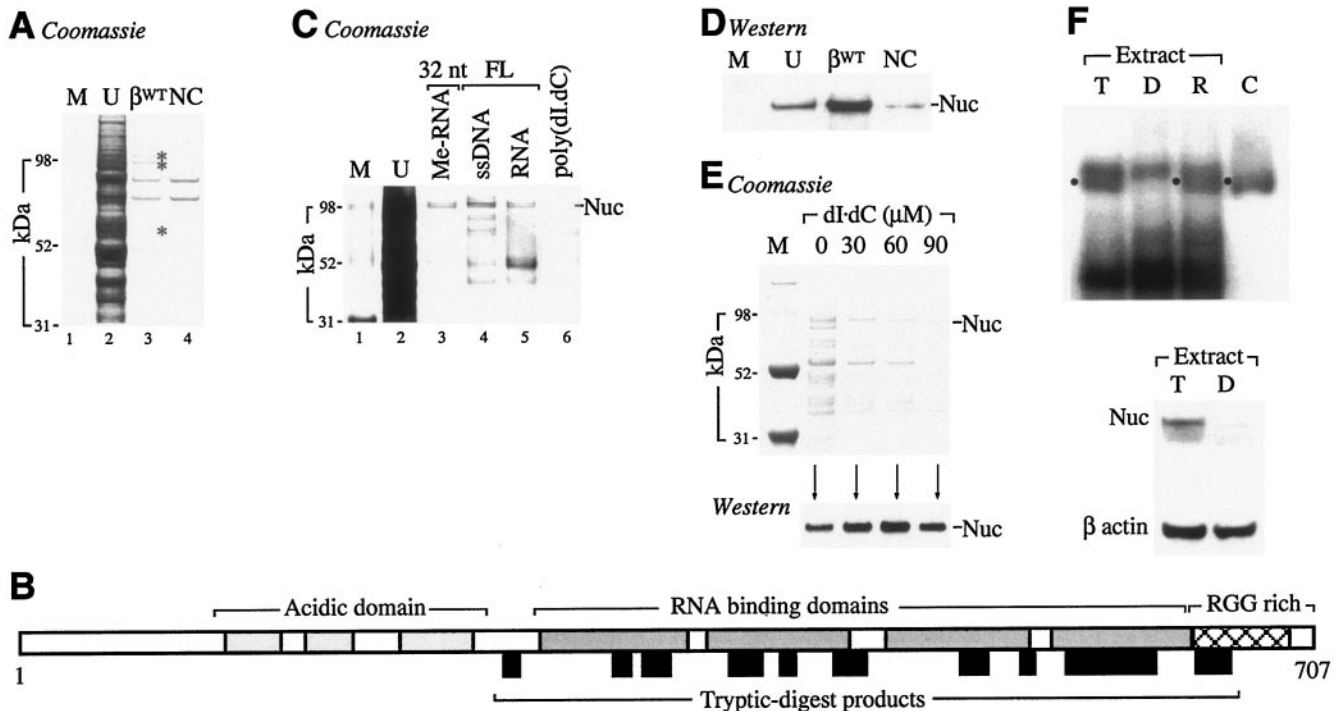


FIG. 3. Identification of a cytoplasmic factor that exhibits binding specificity for the β^{WT} 3'UTR. (A) Affinity enrichment of candidate β -globin 3'UTR-binding factors. Agarose-immobilized ssDNAs corresponding to the 132-nt full-length β -globin 3'UTR (β^{WT}) or to a poly(dI · dC) negative control (NC) were incubated with K562 cytoplasmic extract, and adherent factors were resolved by SDS-PAGE. Three bands were analyzed by MALDI-TOF (asterisks). Lanes M and U contain protein size markers and unfractionated extract, respectively. (B) Identification of nucleolin as a β -globin 3'UTR-binding factor. A diagram illustrates key structural features of full-length human nucleolin, including amino-terminal acidic domains (light shading), RNA-binding domains (dark shading), and a carboxy-terminal, RGG-rich domain (crosshatched). The sizes and positions of tryptic-digest fragments, identified by MALDI-TOF analysis of affinity-enriched K562 cell extract, are indicated as black boxes below the diagram. (C) Nucleolin (Nuc) binds liganded ssDNAs and RNAs corresponding to the β -globin 3'UTR. K562 extract was affinity enriched using a 32-nt ligand corresponding to the H122/H124 site (32 nt) or ligands comprising the full-length (FL) β -globin 3'UTR. Ligands comprised ssDNA, in vitro-transcribed RNA (RNA), or 2'-O-methyl RNA (Me-RNA). Poly(dI · dC) was assessed in parallel as a negative control. Lanes M and U contain protein size markers and unfractionated extract, respectively. (D) Immunological confirmation of nucleolin as a β -globin 3'UTR-binding factor. Affinity-enriched lysate from panel A was analyzed by Western transfer analysis using nucleolin antibody MS-3. Lane U contains unfractionated extract analyzed in parallel as a migration control. (E) Sequence-specific binding of nucleolin to the β -globin 3'UTR. Agarose-immobilized ssDNAs corresponding to the β^{WT} 3'UTR were incubated with MEL cytoplasmic extract in the presence of defined quantities of competitor poly(dI · dC). Adherent proteins were resolved on a Coomassie blue-stained SDS-polyacrylamide gel (top) and subjected to Western blot analysis using nucleolin antibody MS-3 (bottom). (F) Nucleolin binds to the 3'UTR of β -globin mRNA. In vitro-transcribed, ^{32}P -labeled RNAs corresponding to the β^{WT} 3'UTR were incubated with total (lane T) or nucleolin-depleted (lane D) K562 extract and cross-linked with UV light, and mRNPs were resolved on a nondenaturing acrylamide gel. RNAs incubated in reconstituted lysate (lane R) and with affinity-purified nucleolin (lane C) were analyzed in parallel as controls. Bands corresponding to nucleolin- β 3'UTR mRNPs are indicated (black spots). (Bottom) The efficiency of nucleolin depletion was assessed by Western blot analysis of reagent extracts using nucleolin antibodies (bottom). The stripped blot was rehybridized with a β -actin antibody to control for variations in sample loading.

destabilizing and control nondestabilizing 3'UTR hexanucleotide linker-scanning substitutions. The affinity of purified nucleolin for ssDNAs corresponding to the β -globin 3'UTR was substantially reduced by the mRNA-destabilizing H124 mutation but not by flanking mutations at position H120 or H126 that had had no discernible effect on β -globin mRNA stability in earlier *in vivo* studies (Fig. 6A). The adverse effect of the H124 mutation on nucleolin binding was also demonstrated *in vivo* using RIP analyses of HeLa^{IT_A} cells expressing β^{WT} , β^{H112} , and β^{H124} mRNAs (Fig. 6B). Each mRNA was easily detected in the cell extract (Fig. 6B, lanes 2, 4, and 6), while only the stable β^{WT} and β^{H112} mRNAs—but not the unstable β^{H124} mRNA—were present in the nucleolin immunoprecipitate (Fig. 6B, lanes 3, 5, and 7). Pre-rRNA, which is known to bind nucleolin strongly (1), was observed in all sam-

ples, confirming the quality of the mRNAs and controlling for other aspects of the experimental method. These results were corroborated by parallel analyses of β^{WT} , β^{H112} , and β^{H124} mRNAs using an independent RNase protection approach (Fig. 6C) and confirmed in repeat analyses (data not shown). Consequently, the native sequence targeted by the H124 mutation appears to function both as a determinant of β -globin mRNA stability and as a binding site for nucleolin, providing a critical link between these two processes.

A model for β -globin mRNA stability. Although the β -PRE appears to be a determinant of β -globin mRNA stability *in vivo* (84), its anticipated role as a target for α CP binding has been difficult to recapitulate *in vitro*. We propose a model for β -globin mRNA stability that incorporates the findings presented here and, in addition, accounts for previous experimental evi-

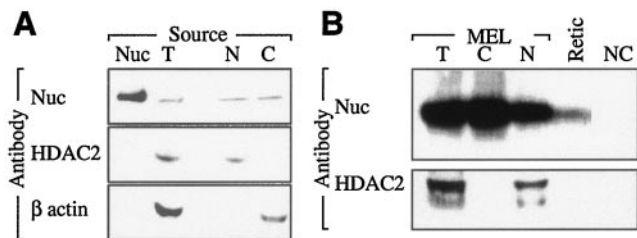


FIG. 4. Nucleolin is present in the cytoplasm of differentiating erythroid cells. (A) Nucleated erythroid progenitors contain cytoplasmic nucleolin. Western blot analysis was performed on total (T), nuclear (N), and cytoplasmic (C) extracts prepared from MEL cells using nucleolin (Nuc) antibody. The blot was stripped and rehybridized with antibodies directed against nucleus- and cytoplasm-specific histone deacetylase-2 (HDAC-2) and β actin, respectively. Affinity-purified nucleolin was analyzed in parallel as a positive control. (B) Anucleate erythroid progenitors (reticulocytes) contain cytoplasmic nucleolin. Hemolysate prepared from FACS-sorted murine reticulocytes (Retic) was analyzed by Western transfer analysis using nucleolin antibody. Total, cytoplasmic, and nuclear extracts prepared from MEL cells were analyzed in parallel as positive controls, and recombinant α CP was run as a negative control (NC). The blot was stripped and rehybridized with HDAC-2 antibody to confirm the absence of contaminating nucleoplasm in the Retic sample.

dence that indirectly implicates α CP in this process. In this model, the β -globin 3'UTR has the potential to assume a highly stable stem-loop structure that incorporates the β -PRE and nucleolin-binding sites into its left and right half-stems, respectively (Fig. 7A). If secondary structure were to inhibit the access of α CP to the β -PRE-binding site, then any process

that weakens the stem structure would be predicted to facilitate α CP binding (Fig. 7B).

The possibility that native secondary structure inhibits α CP binding was tested in three independent affinity-binding studies. Results from the first study suggest that α CP access to the β -PRE is highly dependent upon its mRNA context: recombinant α CP (α CP) binds poorly to an ssDNA corresponding to the full-length β -3'UTR (Fig. 7C, lane 5), while binding avidly to ssDNAs corresponding to the β -PRE either in isolation (Fig. 7C, lane 6) or when inserted into a different 3'UTR (Fig. 7C, lane 7). In a second study, baseline interaction of α CP with the left-half-stem β -PRE was ablated by its preincubation with an ssDNA corresponding to the right half-stem (Fig. 7D). A third study demonstrated that α CP binds poorly to the intact 3'UTR stem-loop structure (Fig. 7E, lane 2) while, in agreement with our predictions, binding strongly to 3'UTRs that contain stem-destabilizing substitutions (Fig. 7E, lanes 3 and 6) or deletions (Fig. 7E, lane 5). The results of all three experiments are consistent with a model in which native structure within the β -globin 3'UTR must be remodeled as a precondition for α CP interaction with the β -PRE.

The potential role that nucleolin may play in remodeling the 3'UTR stem-loop structure in vivo was investigated by assessing the binding of α CP to agarose-immobilized β -globin 3'UTRs in vitro under different conditions. The poor baseline affinity of α CP for the naked probe is significantly enhanced by preincubating the β -globin 3'UTR with affinity-purified nucleolin (Fig. 7F, compare lanes 2 and 4). Although this result does not favor any specific mechanism, the possibility that nucleolin facilitates α CP binding through its effect on mRNA

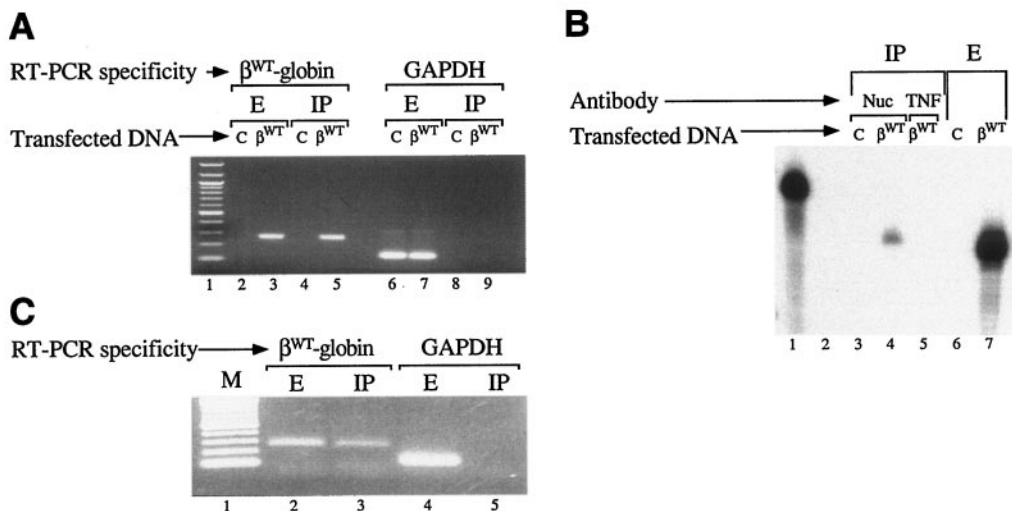


FIG. 5. Nucleolin binds to β -globin mRNA in intact cells. (A, B) Specificity of nucleolin- β -globin mRNA interaction in vivo. (A) HeLa^{TA} cells were transfected with pTRE- β^{WT} (β^{WT}) or with an empty pTRE vector control (C). Total RNA recovered from cell extract (E) or nucleolin immunoprecipitate (IP) was RT-PCR amplified using β^{WT} sequence-specific oligomers, generating a 261-bp product (lanes 2 to 5), or with GAPDH mRNA-specific oligomers, producing a 116-bp product (lanes 6 to 9). Lane 1 contains a 100-bp DNA ladder. (B) Total RNA was recovered from immunoprecipitate (lanes 3 to 5) or extract (lanes 6 and 7) prepared from cells transfected with pTRE- β^{WT} (β^{WT}) or with the empty pTRE vector control (C). Immunoprecipitates were prepared using nucleolin- or tumor necrosis factor-specific antibodies (Nuc or TNF, respectively). RNAs were analyzed by RNase protection using in vitro-transcribed, ³²P-labeled RNA probes (84). Intact and RNase-digested ³²P-labeled probes were run in lanes 1 and 2, respectively. (C) Nucleolin binds β -globin mRNA in intact human erythroid cells. Purified RNA prepared from the extract or nucleolin immunoprecipitate of density-fractionated human erythroid cells was RT-PCR amplified using human β -globin- and GAPDH-specific oligomers. M, DNA size markers.

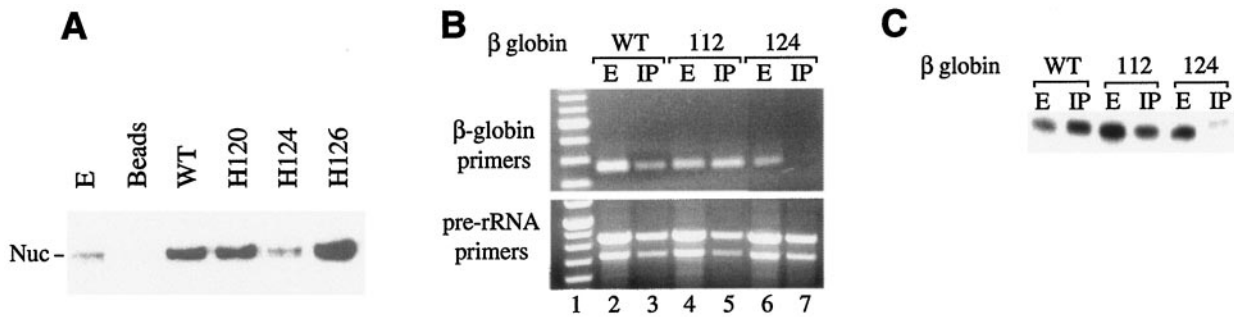


FIG. 6. Differential binding of nucleolin to mRNA-stabilizing and -destabilizing 3'UTR determinants. (A) β -Globin mRNA-destabilizing linker-scanning mutations reduce nucleolin binding in vitro. Agarose-immobilized, 59-nt ssDNAs corresponding to the proposed 3'UTR nucleolin-binding region of β -globin mRNA were incubated in cytoplasmic extract, and adherent proteins were assessed by Western transfer analysis using nucleolin antibody. The wild-type sequence (WT) as well as sequences containing destabilizing (H124) and nondestabilizing (H120 and H126) HindIII mutations were assessed. Unfractionated extract (E) and extract adhering to unliganded agarose beads were run in the first two lanes as controls. (B, C) Full-length, unstable β^{H124} mRNA binds nucleolin poorly in vivo in intact, cultured cells. Unfractionated cell extract or nucleolin immunoprecipitate (IP) was prepared from cultured cells transfected with genes encoding β^{WT} , β^{H112} , and β^{H124} mRNAs. (B) Recovered RNAs were RT-PCR amplified using primers specific to β -globin mRNA (top) or to internal control pre-rRNA (bottom). The reaction products were resolved on an ethidium bromide-stained, nondenaturing polyacrylamide gel. Lane 1 contains a 100-bp DNA ladder. (C) Recovered RNAs were assessed by RNase protection using an in vitro-transcribed, ^{32}P -labeled β -globin RNA probe.

structure is suggested by the observation that α CP binding is also enhanced, in the absence of nucleolin, by prior heat denaturation of the agarose-immobilized β -3'UTR ligand (Fig. 7F, lane 3). In the aggregate, the results of these in vitro analyses are consistent with the assembly of a stable structure within the β -globin 3'UTR that inhibits α CP binding and suggest that nucleolin facilitates α CP access through interaction with this structure.

DISCUSSION

Although the high stability of human β -globin mRNA has been repeatedly demonstrated (2, 4, 36, 42, 62, 63) and the importance of this characteristic to normal β -globin expression firmly established (10, 64), the relevant molecular mechanisms have yet to be defined. The current article provides original evidence that this process is likely to require *cis* determinants and *trans*-acting factors that have not previously been appreciated. We initially designed and validated an important method for assessing the stability of an mRNA in vivo in intact cultured cells without affecting the expression or function of other cellular mRNAs (Fig. 1). Using this approach, we identified a defined 3'UTR region that is critical to normal β -globin mRNA stability (Fig. 2), thus linking this important functional characteristic to a discrete, previously unrecognized structural determinant. This evidence does not rule out the possibility that other *cis* elements may participate in this process, since the experimental method is likely to overlook extensive and/or highly degenerate structural determinants. This technical limitation may explain why the β -PRE element, whose importance has previously been implicated in vitro and in vivo (84), was not functionally identified. Nevertheless, the critical nature of the H122-H124 region to β -globin mRNA stability is clear.

Among three candidate 3'UTR-binding factors, nucleolin appears most likely to play a central role in stabilizing β -globin mRNA in vivo. Nucleolin displays a relative specificity for ssDNAs corresponding to the β -globin 3'UTR in vitro (Fig. 3) and interacts with full-length β -globin mRNA both in intact

cultured cells and in primary human erythroid progenitors (Fig. 5). Moreover, binding is ablated in vivo by mRNA-destabilizing mutations but preserved in β -globin mRNAs carrying control nondestabilizing mutations, firmly linking nucleolin binding to its proposed mRNA-stabilizing function (Fig. 6).

Although it is tempting to speculate that nucleolin directly stabilizes β -globin mRNA, we think it more likely that this highly abundant factor facilitates functional interaction of other, known globin mRNA-stabilizing factors, such as α CP. Our structural analyses are consistent with this possibility; nucleolin binds to the right half-stem of a stable 3'UTR stem-loop structure, directly opposite to the β -PRE (Fig. 7A). Among several mechanistic possibilities, we favor one in which nucleolin binding is required to relax a stem-loop structure that is predicted to interfere with α CP binding (Fig. 7B). In vitro studies show enhanced α CP binding to 3'UTRs in which the stem-loop structure is disrupted (Fig. 7C to E), consistent with the proposed mechanism. A specific role for nucleolin in this process is suggested by the demonstration that α CP binding to the β -globin 3'UTR can be enhanced either by heat denaturation or by preincubation with immunopurified nucleolin (Fig. 7F). Though potentially informative, these studies do not resolve the final common pathway through which the combined activities of nucleolin and α CP effect mRNA stabilization, including potential effects on β -globin mRNA polyadenylation and/or translational efficiency. Nevertheless, we find the proposed model to be particularly attractive because it accommodates both our current data and evidence from previous studies favoring a critical role for α CP in stabilizing the β -globin mRNA.

The apparent role that nucleolin plays in stabilizing β -globin mRNA is consistent with its participation in a wide range of molecular processes. In the nucleus, nucleolin is associated with ribosome biogenesis (9, 35), chromatin remodeling (23), immunoglobulin isotype switching (30), telomere formatting (33), and posttranscriptional processing of nascent mRNAs (33). In the cytoplasm, nucleolin binds to the 5' and 3'UTRs of specific mRNAs, enhancing both their stabilities and their translational efficiencies (15, 56, 58, 69, 71, 72, 85, 86). This

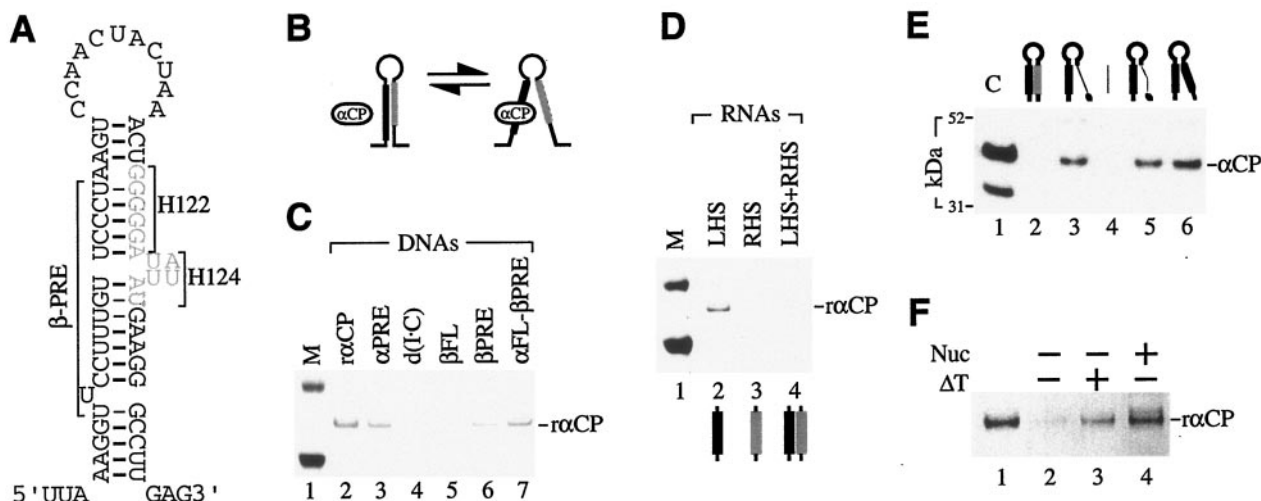


FIG. 7. Model for regulated β -globin mRNA stability. (A) A secondary structure exists within the β -globin 3'UTR. A stable stem-loop structure within the β -globin 3'UTR is predicted by the Zuker algorithm using default parameters (50, 87). The positions of the β -PRE and the two previously identified mRNA-destabilizing hexanucleotide mutations (H122 and H124) (gray) are indicated. (B) Predicted effect of the secondary structure on α CP binding. The access of α CP to its functional β -PRE-binding site (black) is favored by the relaxation of a native β -globin mRNA stem-loop motif. The positioning of a binding site for nucleolin on the opposite (right) half-stem suggests a role for nucleolin in shaping the high-order 3'UTR structure. (C) RNA context-dependent binding of α CP to the β -PRE. ssDNA ligand-bound α CP was resolved by Coomassie blue staining after SDS-PAGE. Agarose-immobilized ligands (top), including the α -PRE and β -PRE (lanes 3 and 6), the full-length β -3'UTR (lane 5), a full-length α -globin 3'UTR in which the β -PRE is substituted for the α -PRE (lane 7), and a negative-control poly(dI · dC) (lane 4), are identified. Lanes 1 and 2 contain protein standards (M) and α CP, respectively. (D) α CP binding to the β -PRE is inhibited by its participation in a stable stem structure. Agarose-immobilized 2'-O-methylated RNAs corresponding to the predicted left and right half-stems (LHS and RHS, respectively) of the 3'UTR structure (32 nt each) were incubated with α CP either singly (lanes 2 and 3) or in combination (lane 4), and adherent α CP was resolved by Coomassie blue staining of SDS-PAGE gels. The LHS (black) and RHS (gray) contain the β -PRE and the H122/H124 nucleolin-binding sites, respectively. M, protein size markers. (E) Mutations that disrupt the 3'UTR secondary structure enhance α CP binding to β -globin mRNA. Agarose-immobilized ssDNAs were incubated with HeLa cell extract, and adherent factor was analyzed by Western blot analysis using α CP antibody. The predicted structures of individual ssDNAs are schematically illustrated (top). The β -PRE and proposed nucleolin-binding sites are represented as thick black and gray lines. Right-half-stem modifications include the deletion of a native 18-nt sequence (broken thin black line) (lane 5), the substitution of an unrelated 18-nt sequence (thin gray line) (lane 3), and the substitution of a stem-destabilizing 18-nt region containing the β -PRE (lane 6). The unrelated stem-destabilizing sequence was analyzed as a control (lane 4). Lane 1 contains recombinant α CP as a migration control (C). See Materials and Methods for details of each ssDNA sequence. (F) Nucleolin (Nuc) enhances α CP binding to the β -globin 3'UTR in vitro. Agarose-immobilized ssDNAs corresponding to the β -globin 3'UTR were incubated with α CP following no pretreatment (lane 2), heat denaturation at 95°C for 5 min (Δ T) (lane 3), or preincubation with affinity-purified nucleolin (lane 4). Ligand-bound α CP was analyzed by SDS-PAGE. Lane 1 contains α CP as a migration control.

functional diversity reflects both the complexity of the nucleolin core structure and the heterogeneity of isoforms that it can assume. The core structure, which comprises acidic and glycine-rich domains as well as four RNA-binding domains (RBDs) (43), is extensively modified by targeted proteolysis (14, 24), phosphorylation (13, 54, 59, 60, 67), ADP-ribosylation (44), and methylation (46), resulting in combinatorial structural complexity that may form the basis for its observed functional heterogeneity.

The four centrally positioned RBDs of nucleolin mediate its interaction with RNA both in the nucleus (32, 33) and in the cytoplasm (15, 56, 58, 69, 71, 72, 85, 86). These domains, which are structurally similar to RBDs in protein factors that regulate the stabilities and translational efficiencies of other mRNAs (22), appear to subservise a parallel spectrum of functions in nucleolin. Nucleolin has been reported to stabilize mRNAs encoding amyloid precursor protein (85, 86), renin (58, 72), CD154 (71), and Bcl-2 (56, 69) by binding to structurally distinct *cis* elements within their 3'UTRs. The heterogeneity in its post-translational modification may account for nucleolin's equally heterogeneous mRNA-binding specificities. The nucleolin-binding sites of interleukin 2 and amyloid precursor protein mRNAs,

which share a common 5'CUCUCUUUA3' target sequence (15, 85, 86), differ from the A/U-rich nucleolin-binding site in the 3'UTR of Bcl-2 mRNA (56, 69) and from the 5'UCC CGA3' motif mediating its binding to rRNA (1, 27). Nucleolin may also bind to motifs corresponding to splice acceptor sequences (5'UUAGG3') (33) and to G-quartet and other related nonlinear, thermodynamically favorable nucleic acid structures that are not predicted by common mRNA-folding algorithms (83). The β -globin mRNA nucleolin-binding determinant that we describe (Fig. 2) is dissimilar to each of these linear elements, possibly reflecting interaction with a subset of nucleolin structural isoforms that carry specific phosphoryl, ADP-ribosyl, or methyl modifications.

The wide variety of molecular processes that require nucleolin suggests that it may serve a general role—as a molecular scaffold or perhaps a substrate-remodeling factor—acting in concert with other proteins that provide the required functional specificity. We suggest that a specific nucleolin- β -globin mRNP may have to assemble before α CP can bind, and subsequently stabilize, the full-length β -globin mRNA. This hypothesis may explain the difficulties that we and others have encountered in attempting to demonstrate bimolecular inter-

action between the β -globin 3'UTR and α CP in vitro, despite the clear importance of the proposed α CP-binding site to human transgenic β -globin mRNA stability observed in vivo in mice (84). The potential importance of cooperative interactions between these two protein factors is additionally indicated by analyses of renin mRNA, which, like β -globin mRNA, displays nucleolin-dependent stability and binds both nucleolin and α CP to the same 3'UTR region (58, 72). These aspects of nucleolin function merit close scrutiny as they may provide opportunities for novel therapeutic approaches to the treatment of common thalassemias and hemoglobinopathies.

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