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The KH-Domain Protein αCP Has a Direct Role in mRNA Stabilization Independent of Its Cognate Binding Site

Jian Kong, Xinjun Ji, and Stephen A. Liebhaber*

Departments of Genetics and Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

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Previous studies suggest that high-level stability of a subset of mammalian mRNAs is linked to a C-rich motif in the 3′ untranslated region (3′UTR). High-level expression of human α-globin mRNA (hα-globin mRNA) in erythroid cells has been specifically attributed to formation of an RNA-protein complex comprised of a 3′UTR C-rich motif and an associated 39-kDa poly(C) binding protein, αCP. Documentation of this RNA-protein α-complex has been limited to in vitro binding studies, and its impact has been monitored by alterations in steady-state mRNA. Here we demonstrate that αCP is stably bound to hα-globin mRNA in vivo, that α-complex assembly on the hα-globin mRNA is restricted to the 3′UTR C-rich motif, and that α-complex assembly extends the physical half-life of hα-globin mRNA selectively in erythroid cells. Significantly, these studies also reveal that an artificially tethered αCP has the same mRNA-stabilizing activity as the native α-complex. These data demonstrate a unique contribution of the α-complex to hα-globin mRNA stability and support a model in which the sole function of the C-rich motif is to selectively tether αCP to a subset of mRNAs. Once bound, αCP appears to be fully sufficient to trigger downstream events in the stabilization pathway.

The complicated and highly adaptable functions of eukaryotic cells reflect multiple and interrelated levels of gene regulation. A major component of many gene regulatory pathways is exerted at the level of mRNA stability. For example, potent regulators of cell growth, such as proto-oncogenes, cytokines, and cell cycle control proteins, are encoded by mRNAs with very short half-lives ($t_{1/2}$). This property allows for rapid upswings in mRNA representation at the time of their induction and equally rapid clearance of their mRNAs once the stimulus passes. At the opposite end of the stability spectrum are mRNAs encoding abundant proteins expressed in terminally differentiated cells. Here high-level stability matches the dictates of economy of scale rather than a need for a rapid response. Pathways that determine these substantial differences in mRNA $t_{1/2}$, which can differ by over 100-fold, are thus central to gene expression profiles and are of significant interest for detailed study.

Factors that mediate mRNA turnover and determine individual decay rates must recognize structures that define and distinguish each species of mRNA or subsets of mRNAs. This recognition process is based on sequence- and structure-specific interactions with corresponding sets of RNA binding proteins. RNP complexes central to stability control can be considered in two categories, those common to all mRNAs and those that are mRNA specific and define individual stability profiles. Two structures shared by almost all eukaryotic mRNAs are a 5′ m^7Gppp cap and 3′ poly(A) tail. The 5′cap structure is bound in the nucleus by the nuclear cap binding complex, comprising CBP80 and CBP20 (18), and is bound in the cytoplasm by the eIF4F complex (for a review see reference 14). At the opposite end of the mRNA, the 3′ poly(A) tail is associated with poly(A) binding protein (PABP) (14). These 5′- and 3′-terminal RNP structures contribute to mRNA stability, as they protect the termini from pervasive exonuclease activities. They also facilitate translation and may interact with each other to assemble the mRNA in a closed loop that serves to monitor the structural integrity of an mRNA prior to translation. Removal or decay of either of the terminal RNP complexes can constitute a rate-limiting event in mRNA decay.

Determinants that specify stability profiles of individual mRNA species are most often located in the 3′ untranslated region (3′UTR) (16, 29, 35, 37, 38, 45). This positioning is thought to protect critical RNP structures from displacement by scanning or translating ribosomes. The first category of stability elements to be described in detail encompasses the AU-rich elements (AREs) that are found in multiple hyperunstable mRNAs (for a review, see reference 4). AREs are recognized by a group of binding proteins that trigger accelerated shortening of the poly(A) tail (1, 22, 31, 44), decapping (13), and selective recruitment of an exosome complex (7). In contrast to this well-defined pathway of accelerated mRNA decay, the molecular mechanism(s) that supports high-level mRNA stabilization is less well understood. Due to their unusual stability, robust expression, and wealth of naturally occurring mutations, the globin mRNAs offer useful models for the study of this category of mRNAs. The high-level stability of globin mRNAs is central to the continued production of globin proteins for several days after global transcriptional silencing in terminally differentiating red cells.

Prior studies support a model in which the stability of the human α2-globin (hα2-globin) mRNA is dependent upon the formation of a sequence-specific mRNP complex in its 3′UTR (39, 43). This α-complex comprises a C-rich motif bound by one or more isoforms of the KH-domain RNA binding protein αCP [for α-globin poly(C) binding protein (17, 21)]. The αCP proteins, also known as poly(C) binding proteins (23) and

* Corresponding author. Mailing address: Departments of Genetics and Medicine, University of Pennsylvania School of Medicine, Room 428 CRB, 415 Curie Blvd., Philadelphia, PA 19104. Phone: (215) 898-7834. Fax: (215) 573-5157. E-mail: liebhabe@mail.med.upenn.edu.

hnRNP Es (30), are ubiquitously expressed. These proteins contain a highly conserved triple repeat of the KH domain found in a wide spectrum of RNA binding proteins. αCPs and the structurally related nuclear protein hnRNP K comprise the major poly(C) binding proteins in eukaryotic cells (25). αCPs have been implicated in a wide range of posttranscriptional regulatory pathways, including mRNA stabilization (17, 39), translation activation (3, 12), and translation silencing (11, 30, 32).

The α-complex has been linked to α-globin mRNA stabilization on the basis of in vitro and cell-based studies. Mutations within the C-rich segments of α-globin mRNA that block α-complex assembly in vitro result in a parallel decrease in steady-state levels of α-globin mRNA as assessed in transfected erythroid cells in culture (39, 43). The minimal *cis*-acting stability determinant of the α-complex in vitro is encompassed in a 42-nucleotide (nt) segment that is protected by αCP from RNase I digestion. This α-complex protected region (PR) is sufficient for αCP assembly (17). Whether αCP binds to the PR region of α-globin mRNA in vivo and whether its positive impact on α-globin mRNA levels is mediated by prolongation of the mRNA $t_{1/2}$ remain to be directly established.

The mechanism by which the α-complex alters mRNA has been studied in some detail. By using an in vitro RNA decay system, αCP has been shown to have two potentially independent roles in α-globin mRNA stabilization. In vitro studies have demonstrated that αCP can bind to PABP and lower rates of poly(A) shortening (40). The importance of poly(A) shortening in α-globin mRNA stability is supported by analysis of poly(A) tail size on wild-type and mutant hα-globin mRNAs in transgenic mice (28). The poly(C)-rich motif may also have intrinsic importance to a second pathway of mRNA decay. A sequence-specific erythroid cell-enriched endoribonuclease activity that selectively cleaves within the C-rich αCP binding site of the hα-globin 3′UTR has been described (33, 41, 42). In this case, studies suggest a model in which the bound αCP specifically protects this endosensitive site from attack by erythroid cell-enriched endoribonuclease cleavage. The relative contributions and potential interactions of these two pathways in vivo remain to be documented and explored.

In addition to its assembly on α-globin mRNA, the α-complex assembles at C-rich regions within 3′UTRs of additional highly stable mRNAs (17, 25). This has led to the suggestion that the α-complex represents a general determinant of mRNA stabilization. The α-complex may thus play a role in a wide range of cells and gene expression systems. In the present report we establish a series of erythroid and nonerythroid cell lines that support conditional expression of α-globin mRNA. We used these cell lines to establish the contribution of the α-complex to the stability of hα-globin mRNA and to distinguish the roles of the *cis*-acting pyrimidine-rich determinant and the targeted αCP binding protein in this process. The observations firmly establish an in vivo and direct role for the αCP protein in stabilization of α-globin mRNA and establish a model system for the further dissection of downstream events in this pathway.

MATERIALS AND METHODS

Generation and analysis of cell clones stably expressing tTA. Mouse erythroleukemia (MEL), C127, and HeLa cells growing under standard conditions were transfected with linearized pTet-TAk DNA (Life Technologies) containing a tetracycline transactivator (tTA) and pcDNA3 DNA (Invitrogen) carrying the neomycin resistance gene (19:1 molar ratio of the two plasmids). Tetracycline was added at 500 ng/ml immediately after transfection. Cells were maintained in the medium with tetracycline (500 ng/ml for MEL cells and 1 μg/ml for C127 and HeLa cells) and placed under G418 selection (500 μg/ml) for 2 weeks. G418-resistant clones from each cell line were isolated, expanded, and subjected to a transcription induction assay by transfection with a reporter plasmid containing the luciferase (Luc) open reading frame (ORF) under tetracycline operator control (pUHC13-3 DNA; Life Technologies). The transfected cells were maintained for 40 h in medium containing 10% fetal bovine serum, with or without tetracycline at the concentration indicated above. Cells were then lysed and assayed for luciferase activity (in relative light units) by using a E1500 detection system (Promega). The histogram (see Fig. 2) shows the Luc expression levels from some of the responsive lines in the presence or absence of tetracycline. The ratio of the two values (induction ratio) is shown above each of the sets of bars. MEL-P1B2, C127-25, and HeLa-4, which had the best combination of low background expression (leakiness) and high induction potential, were used in the studies.

Plasmid construction. The hα2-globin gene extending from the transcriptional initiation site to 87 bases 3′ to polyadenylation site was amplified by PCR from pSV2-α2^{WT} (wild type) DNA. *Eco*RI and *Not*I recognition sites were included within the 5′ and 3′ primers, respectively. The 926-bp *Eco*RI-*Not*I fragment was subcloned into pTet-Splice (Life Technologies). The new plasmid, pTet-α^{WT}, was used as the parent plasmid for various mutant constructs. pTet-α^{H19} was generated by replacement of the 304-bp wild-type *Bst*EII-*Not*I fragment with that derived from pSV2-α2^{H19} DNA. To construct plasmids pTet-α^{ARE}, pTet-α^{Neut}, pTet-α^{MS2}, and pTet-α^{R7α1}, the 42-bp PR was replaced by the following: a 53-bp ARE from the granulocyte-macrophage colony-stimulating factor gene, a 42-bp "neutral" sequence from the coding region of hα-globin gene, a 26-bp MS2 sequence from bacterial phage DNA, and a 50-bp SELEX sequence. Each replacement was carried out by PCR-based splice overlap extension. The 53-bp ARE and 42-bp neutral sequence were also inserted in *cis* to the PR to generate pTet-α^{ARE-PR}, pTet-α^{PR-ARE}, and pTet-α$^{Neut-PR}$ by using the same strategy. There was a 6-bp spacer between the ARE and PR or Neut and PR.

The MS2- and MS2-αCP-encoding plasmids were constructed as follows: a DNA fragment encoding the 132-amino-acid N-terminal portion of the MS2 coat protein followed by a linker sequence (PRGSH6PN, termed RGSH linker) and carrying a translational stop codon was inserted between the *Hin*dIII and *Bam*HI sites of pcDNA3.1 (Invitrogen), downstream of a cytomegalovirus promoter, to form pcDNA3-MS2. pcDNA3-MS2-αCP was created by replacing a 63-bp *Mfe*I-*Xho*I fragment in pcDNA3-MS2 with a 1.1-kb *Eco*RI-*Xho*I fragment encoding the mouse αCP-KL, resulting in an in-frame MS2-αCP fusion protein-expressing plasmid.

EMSA. Electrophoretic mobility shift assay (EMSA) was carried out as described previously (39) with minor modifications. MEL/tTA cells were transiently transfected with pcDNA3-MS2 or pcDNA3-MS2-αCP DNA, and S10 extracts were prepared after 24 h. In vitro-transcribed RNA probe (~20,000cpm) was incubated with 10 to 15 μg of S10 extract from mock-transfected or transfected MEL/tTA cells. The incubation was in 20 μl of binding buffer (10 mM Tris-HCl [pH 7.4], 150 mM KCl, 1.5 mM MgCl, and 0.5 mM dithiothreitol) at room temperature for 20 min. The binding samples were subsequently incubated with RNase T_1 (20 U; Roche) at room temperature for 10 min. One microliter of heparin (50 mg/ml) was added to each reaction mixture prior to loading. Samples were resolved on a 5% native polyacrylamide gel.

Cell transfection and RNA purification. MEL/tTA cells were transfected with pTet-α^{WT} and various mutant pTet plasmids DNA by electroporation. Cells were split 1 day before transfection and cultured in minimal essential medium supplemented with 10% fetal bovine serum, 1× Antibiotic-Antimycotic (Invitrogen), and 100 ng of tetracycline per ml. Cells were washed three times in cold Dulbecco phosphate-buffered saline (PBS) and resuspended in cold serum-free minimal essential medium at a concentration of 3×10^7 to 5×10^7 per 0.5 ml. Two micrograms of pTet DNA and 18 μg of carrier DNA were added to the cell suspension and mixed thoroughly. Electroporation was performed at 250 V, 1,180 μF, and low resistance in a BRL Cell-Porator system. Tetracycline was present in the medium at 50 ng/ml during the first 16 to 24 h after transfection. Cells were then washed with Dulbecco PBS and replated into several smaller dishes (60-mm diameter) for time course chase. A 4-h transcription pulse driven by the *tet* promoter was induced in the absent of tetracycline, after which tetracycline was added back at 500 ng/ml.

The adherent cell lines, C127tTA and HeLa/tTA, were transfected by using liposomal reagent Trans-IT (Mirus). Cells were split and plated into 60-mm-diameter culture dishes 1 day before transfection in regular medium with 1 μg of

tetracycline per ml; 60 to 80% confluence was achieved at time of transfection. pTet plasmid DNA (0.2 μg) and carrier DNA (5.8 μg) were mixed and coated with 12 μl of Trans-IT before they were added to cells. The 4-h transcriptional pulse was terminated by addition of 1 μg of tetracycline per ml. Total RNA was isolated at the indicated time points during the subsequent chase with Trizol reagent (Invitrogen). All RNA samples were treated with RNase-free DNase I (Ambion) before being subjected to RNase protection assay (RPA).

IP. HeLa/tTA cells were transfected with pTet-α^{WT} or pTet-α^{Neut} DNA, and transcription was driven by the *tet* promoter for a continuous 24 h. The cells were washed with ice-cold PBS twice and lysed by repeated pipetting in 500 μl of ice-cold TMK100 lysis buffer (10 mM Tris-HCl[pH 7.4], 5 mM $MgCl_2$, 100 mM KCl, 2 mM dithiothreitol, 1% Triton X-100, and 100 U of RNase inhibitor [Promega] per ml in diethyl pyrocarbonate-treated water) for 5 min. Nuclei were cleared at 10,000 × *g* for 10 min at 4°C, and the supernatant (S10) was used for immunoprecipitation (IP). Ten microliters of anti-CP2 rabbit serum (FF3) or preimmune rabbit serum was incubated with 200 μl of a 50% protein A-Sepharose slurry in 600 μl of PBS for 1 h at 4°C with gentle rocking. S10 extract (50 μl) was diluted in 600 μl of IP binding buffer (20 mM HEPES [pH 7.9], 150 mM NaCl, 0.05% Triton X-100, 100 U of RNase inhibitor per ml) and incubated with antibody-coupled protein A-Sepharose for 2 h at 4°C. The immunoprecipitated complexes were washed four times with IP binding buffer, and then 400 μl of IP elution buffer (0.1 M Tris-HCl [pH 7.5], 12.5 mM EDTA, 0.15 M NaCl, 1% sodium dodecyl sulfate [SDS]) was added to the protein A-Sepharose–RNP complex pellet and the complex was disrupted by boiling for 3 min. RNA was then extracted and ethanol precipitated prior to RPA analysis. For protein analysis, 100 μl of 2× SDS loading buffer was added to the beads and boiled for 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis and probed with rabbit anti-αCP2 (laboratory designation, FF3 antibody).

Polysome analysis. Ten to 50% linear sucrose gradients containing 100 mM KCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, and 20 mM HEPES (pH 7.4) were prepared in 5-ml Beckman ultracentrifuge tubes with a two-chamber gradient mixer. MEL/tTA or HeLa/tTA cells were incubated with cycloheximide (100 μg/ml, freshly prepared in ethanol) for 15 min prior to harvesting. The MEL/tTA cells were then transferred to a 50-ml tube containing 20 ml of frozen crushed PBS and 100 μg of cycloheximide per ml and centrifuged at 1,000 × *g* for 5 min at 4°C. The cell pellet was washed twice with ice-cold PBS and lysed by repeated pipetting in 500 μl of ice-cold TMK100 lysis buffer (10 mM Tris-HCl [pH 7.4], 5 mM $MgCl_2$, 100 mM KCl, 2 mM dithiothreitol, 1% Triton X-100, and 100 U of RNase inhibitor [Promega] per ml in diethyl pyrocarbonate-treated water) for 5 min. HeLa/tTA cells were washed and then lysed on ice by treatment with 500 μl of ice-cold TMK100 lysis buffer for 5 min. The nuclei were cleared at 10,000 × *g* for 10 min at 4°C, and the supernatants were loaded over the top of sucrose gradients. For EDTA treatment, one aliquot of S10 was brought to a final concentration of 20 mM EDTA and incubated for 15 min at 4°C prior to being loaded onto the sucrose gradient. These gradients were ultracentrifuged at 40,000 rpm for 85 min at 4°C (Beckman SW41 rotor). Sixteen fractions (650 μl per fraction) were collected into 1.5-ml microcentrifuge tubes containing 70 μl of 10% SDS, and the gradient profile was monitored via UV absorbance at 254 nm with a UA-5 detector (ISCO, Lincoln, Nebr.). Each sample was digested with 8 μl of protease K (20 mg/ml) solution at 37°C for 30 min and stored at −80°C prior to RNA extraction.

RPA. RPA was performed as described previously (24) with some minor modifications. Briefly, a [^{32}P]CTP-labeled 244-nt antisense hα-globin RNA probe was synthesized in vitro with SP6 RNA polymerase (Ambion) from a DNA template spanning the 132-nt first exon of the hα-globin gene. A mouse or human GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antisense RNA probe was used as an internal control. Ten micrograms of total RNA from each time point sample (for mRNA $t_{1/2}$ analysis) or an equal portion from each of the fractions from the sucrose gradient was cohybridized with the two probes in 20 μl of hybridization buffer {40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] [pH 6.4], 1 mM EDTA [pH 8.0], 0.4 M NaCl, and 80% formamide} at 51°C overnight. The excess probes and unhybridized RNA were eliminated by treatment with 0.5 U of RNase A and 20 U of RNase T_1 (RNases Cocktail; Ambion) in 200 μl of digestion buffer (300 mM NaCl, 10 mM Tris-HCl [pH 7.4], 5 mM EDTA [pH 8.0]) at room temperature for 15 min. Each reaction was terminated by addition of 17 μl of 2-mg/ml proteinase K in 8% SDS at 37°C for 20 min. The precipitated protected RNA fragments were resolved on a 6% polyacrylamide gel containing 8 M urea. Quantitation was performed with PhosphoImage 1.1 software. The mRNA concentration at each time point was normalized to the cell number in the sample to account for cell proliferation (accumulation of GAPDH and dilution of the pulsed α-globin mRNA) during the chase period.

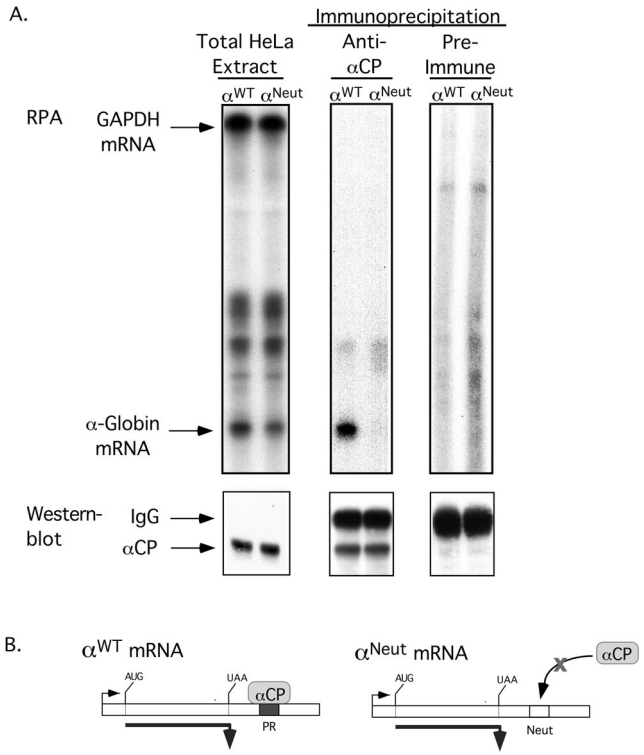

FIG. 1. The α-complex assembles at the 3′UTR of α-globin mRNA in vivo and is unique to the 3′UTR C-rich motif. (A) α-complex assembly at the 3′UTR of wild-type α-globin (α^{WT}) mRNA. HeLa cells were transiently transfected with pTet-hα^{WT} or pTet-α^{Neut} DNA, and the transcription of target mRNA was induced for 24 h. S10 cytoplasmic extract from each set of transfected cells was incubated with purified anti-αCP antiserum FF3 (middle panels) or preimmune serum (right panels). The immunoprecipitate was isolated and analyzed for GAPDH and hα-globin mRNAs by RPA (upper panels). RNA from total cell extract was used as a control (upper left panel). The positions of the α-globin mRNA and GAPDH mRNA signals are indicated to the left. The lower panels (Western blots with anti-αCP2) confirm the specific presence of αCP in the immunoprecipitates. The selective precipitation of the α^{WT} mRNA was confirmed in multiple ($n = 4$) independent studies. (B) Diagrams of the α^{WT} and α^{Neut} mRNAs. The ability of the two mRNAs to bind αCP is indicated.

RESULTS

αCP binds to α-globin mRNA in vivo and is restricted to the 3′UTR C-rich motif. In vivo interaction of αCP with the target α-globin mRNA was assessed by co-IP. Clarified (S10) cytoplasmic extracts were prepared from HeLa cells transfected with a plasmid expressing either the wild-type hα-globin mRNA (α^{WT}) or a mutant form of the mRNA in which the αCP binding site (PR motif; see the introduction) was replaced by a neutral sequence (α^{Neut}) (Fig. 1B). Both mRNAs could be detected in the respective extracts by RPA (Fig. 1A, upper left panel). αCP-containing complexes were immunoprecipitated with an affinity-purified antiserum that recognizes an epitope common to the two major αCP isoforms (10). Control IP with preimmune serum was carried out in parallel. The anti-αCP serum selectively coprecipitated the α^{WT} mRNA but failed to bring down the α^{Neut} mRNA (Fig. 1A, upper center panel). The presence of αCP in parallel IPs was shown by Western blotting (lower panels). These data demonstrate that αCP

binds to hα-globin mRNA in vivo and reveals that this binding is restricted to the C-rich motif in the 3′UTR.

Generation of erythroid and nonerythroid tTA cell lines. The development of conditional expression systems has facilitated approaches to the analysis of mRNA decay in intact cells (15, 19). By placing a gene of interest under the control of the procaryotic *tet* operator, a cell can be pulsed with the mRNA of interest by controlling the presence of tetracycline in the medium. The decay of the encoded mRNA can then be monitored over time to arrive at an accurate $t_{1/2}$ determination (15, 47). In contrast to other approaches, the tetracycline-controlled system can be carried out in a cell that is otherwise unperturbed with regard to cell growth and gene expression profile.

MEL cells and two widely used nonerythroid cell lines, C127 and HeLa, were each stably transfected with a plasmid (pTet-tTA) encoding a *tet* repressor protein fused to a generally acting transcription-activation domain (tTA). In the absence of tetracycline, the tTA protein binds to a multimerized *tet* operator and activates transcription of a linked target gene containing minimal promoter elements. Addition of tetracycline blocks this interaction and silences transcription. This constitutes a "Tet-off" system. The tTA plasmid was cotransfected at a 19:1 molar ratio with a plasmid encoding neomycin resistance (pcDNA3), and a series of 50 individual neomycin-resistant clones were selected from each of the three cotransfected cell lines. The effectiveness of tTA control was characterized for each clone by assessing the transcriptional control of a transiently transfected luciferase reporter under *tet* control (pTet-Luc). Luciferase activity was determined in medium containing tetracycline to assess background or leaky expression of the target gene and after 40 h in medium without tetracycline to assess the level of transcriptional induction (Fig. 2). A clone from each of the three sets of transfections that combined an optimal combination of low background expression and robust induced expression was selected for subsequent studies. These selected clones, MEL-P1B2, C127-25, and HeLa-4, will be referred to as MEL/tTA, C127/tTA, and HeLa/tTA, respectively.

Analysis of hα-globin mRNA decay rates in MEL/tTA cells. MEL/tTA cells were transfected with a hα-globin gene positioned 3′ to the *tet* operator/cytomegalovirus minimal promoter (pTet-α^{WT}). The gene contained 87 bases of 3′ flanking region to assure efficient and accurate 3′ processing. Cells were maintained in medium containing tetracycline overnight, and the next day they were switched to medium without tetracycline for a 4-h transcriptional induction. Cells were then returned to medium containing tetracycline, and decay of α-globin mRNA was monitored over time by RPA. The accurate 3′ processing of the newly transcribed hα-globin mRNA in the MEL cells was confirmed by high-resolution Northern blotting of deadenylated RNA samples (data not shown). The level of α-globin mRNA at each time point was normalized to that of the endogenous GAPDH mRNA, and the $t_{1/2}$ was determined by a best-fit analysis of the data points (see Materials and Methods). The decay curve revealed that wild-type α-globin mRNA had a $t_{1/2}$ of 10.5 h (Fig. 3).

Assembly of the α-complex predicts stabilization of the α-globin mRNA in MEL cells. The correlation between α-complex formation and α-globin mRNA stability was directly

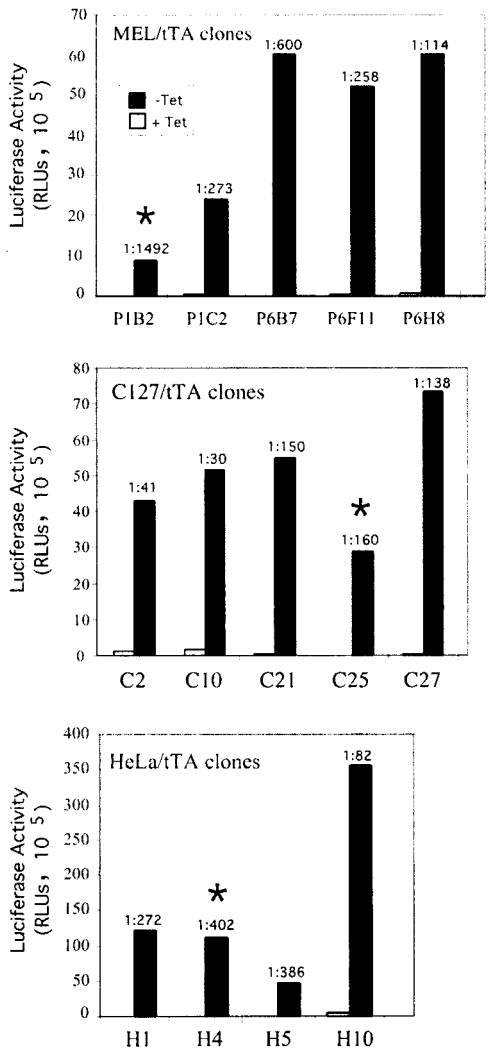

FIG. 2. Generation of MEL, C127, and HeLa cell lines with an optimally expressed tTA. Each cell line was stably transfected with a tTA-encoding plasmid (pTet-TAk). Individual clones from each transfection (a subset is shown) were transiently transfected with 0.1 μg of a reporter plasmid containing the luciferase ORF under *tet* operator control (pUHC13-3 DNA). After a 40-h induction in medium without tetracycline, the transfected cells were lysed and assayed for luciferase activity (relative light units [RLUs]). The histogram shows the luciferase expression levels for each line in the presence or absence of tetracycline. The ratio of the two values (induction ratio) is shown above each of the sets of bars. A single optimal clone from each cell line was chosen for subsequent studies (asterisks).

tested in the MEL/tTA model. A set of four α-globin genes with altered 3′UTRs was generated for analysis (Fig. 3A). The encoded mRNAs contain base substitutions and/or deletions within the 3′UTR that eliminate αCP binding ($\alpha2^{\Delta PR}$, $\alpha2^{Neut}$, and $\alpha2^{H19}$) or an insertion that alters the distance between the αCP binding site (PR motif) and the translation termination codon ($\alpha2^{Neut\text{-}PR}$). Each of these genes was placed under tTA control, and the decay curves were determined in MEL/tTA cells (Fig. 3B). The stability of the hα-globin mRNA was not affected by insertion of the neutral sequence between the translation termination codon and the PR ($\alpha^{Neut\text{-}PR}$ $t_{1/2}$ =

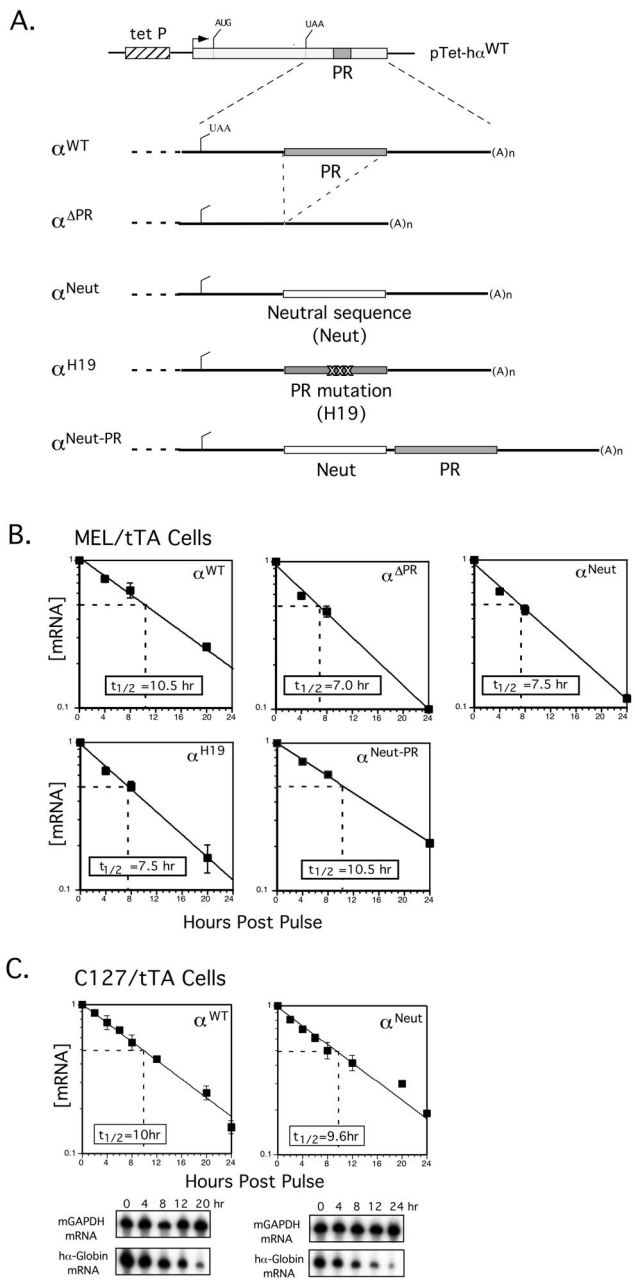

FIG. 3. hα-globin mRNAs lacking a functional αCP binding site within the 3′UTR are destabilized. (A) Diagrams of a series of hα-globin mRNAs containing defined mutations of the αCP binding site. Only the structure of the 3′UTR is shown. The mutations were introduced by deletion of the entire PR ($\alpha^{\Delta PR}$), by replacing the PR with a neutral sequence (α^{Neut}), or by disrupting the third major C-rich segment with a series of base substitutions (α^{H19}). Also shown is a control construct with the neutral sequence inserted in *cis* to the intact PR ($\alpha^{Neut\text{-}PR}$). (B) Decay curves of each hα-globin derivative mRNA in MEL/tTA cells. mRNA was pulsed from each of the indicated genes by tetracycline induction and quantified by RPA as detailed in Materials and Methods. Error bars indicate standard deviations. (C) Decay of hα-globin mRNAs with and without the αCP binding site in C127/tTA cells. Stabilities of α^{WT} and α^{Neut} mRNAs in C127/tTA cells were assessed. A representative autoradiograph of the gel is shown for each RNA. The procedure was as for panel B. A similar set of data was obtained from expression in HeLa/tTA cells (not shown).

10.5 h). In contrast, all three of the α-globin mRNAs lacking a functional αCP binding site decayed at an accelerated rate ($t_{1/2}$ = 7.0 to 7.5 h) (Fig. 3B). The stability of the $\alpha^{Neut\text{-}PR}$ indicates that the distance between the αCP binding site and the translation termination codon is not critical to α-complex function. The instability of the remaining mutant mRNAs establishes a tight linkage between assembly of the α-complex and full stabilization of the α-globin RNA in erythroid cells.

Stability of α-globin mRNA in nonerythroid cells is maintained independent of the α-complex. The stability of each of the α-globin mRNAs analyzed in the MEL/tTA cells was reanalyzed in mouse fibroblasts (C127/tTA) (Fig. 3C). The stability of the α^{WT} mRNA was identical to that measured in MEL cells ($t_{1/2}$ = 10 h). However, in marked contrast to the observation in MEL cells, removal of the αCP binding site (α^{Neut}) failed to destabilize the α-globin mRNA. A parallel set of studies with the HeLa/tTA cells gave the same pattern of results as for the C127/tTA cells (data not shown). Thus, mutations that interfere with α-complex formation and/or function result in a selective destabilization of α-globin mRNA in erythroid cells.

mRNA stability and translational activity can be intimately linked (26, 27). Thus, the apparent erythroid specificity of the α-complex might reflect selective translation of α-globin mRNA in erythroid cells. To assess this possibility, polysome analysis was carried out on MEL/tTA and HeLa/tTA cells transfected with the pTet-hα^{WT} plasmid (Fig. 4). Clarified S20 cytoplasmic extracts prepared after a 24-h induction of mRNA expression in medium without tetracycline were sedimented through a 10 to 50% sucrose gradient, and the gradient fractions were assayed for hα-globin mRNA and for mouse GAPDH mRNA (as a control). α^{WT} mRNA in the MEL/tTA cells was fully loaded on polysomes, and its distribution peaked at a polysome size of three to four ribosomes. The GAPDH mRNA, which has an ORF significantly larger than that of α-globin (335 and 141 codons, respectively), was appropriately distributed on heavier polysomes (Fig. 4A). In HeLa/tTA cells there was slightly more α-globin mRNA in the prepolysomal region, and the mean polysome size was slightly smaller than that seen in the MEL/tTA cells, but the great majority of the α-globin mRNA was polysome associated and therefore translationally active (Fig. 4B). The association of α-globin mRNA with polysomes was confirmed in both cases by EDTA release. Ribosomes were dissociated in the presence of EDTA, and as a result, mRNAs shifted to the prepolysomal fractions (Fig. 4C and D). Thus, α-globin is associated with ribosomes in MEL and HeLa cells. The stability of the α-globin mRNAs lacking αCP binding sites in nonerythroid cells therefore does not appear to reflect sequestration of α-globin mRNA in translationally inert mRNPs.

Stabilization by the α-complex can be overridden by a strong destabilizing determinant. A subset of mRNAs are actively destabilized via action of an ARE in their 3′UTRs (8). One aspect of ARE function involves accelerated shortening of the poly(A) tail (34, 46). In a reciprocal fashion, stabilization of α-globin mRNA by the α-complex appears to be mediated via stabilization of the poly(A) tail (28, 40). Thus, the ARE and the α-complex appear to function through opposing effects on a common poly(A)-related decay pathway. To establish whether either of these determinants mediates a dominant

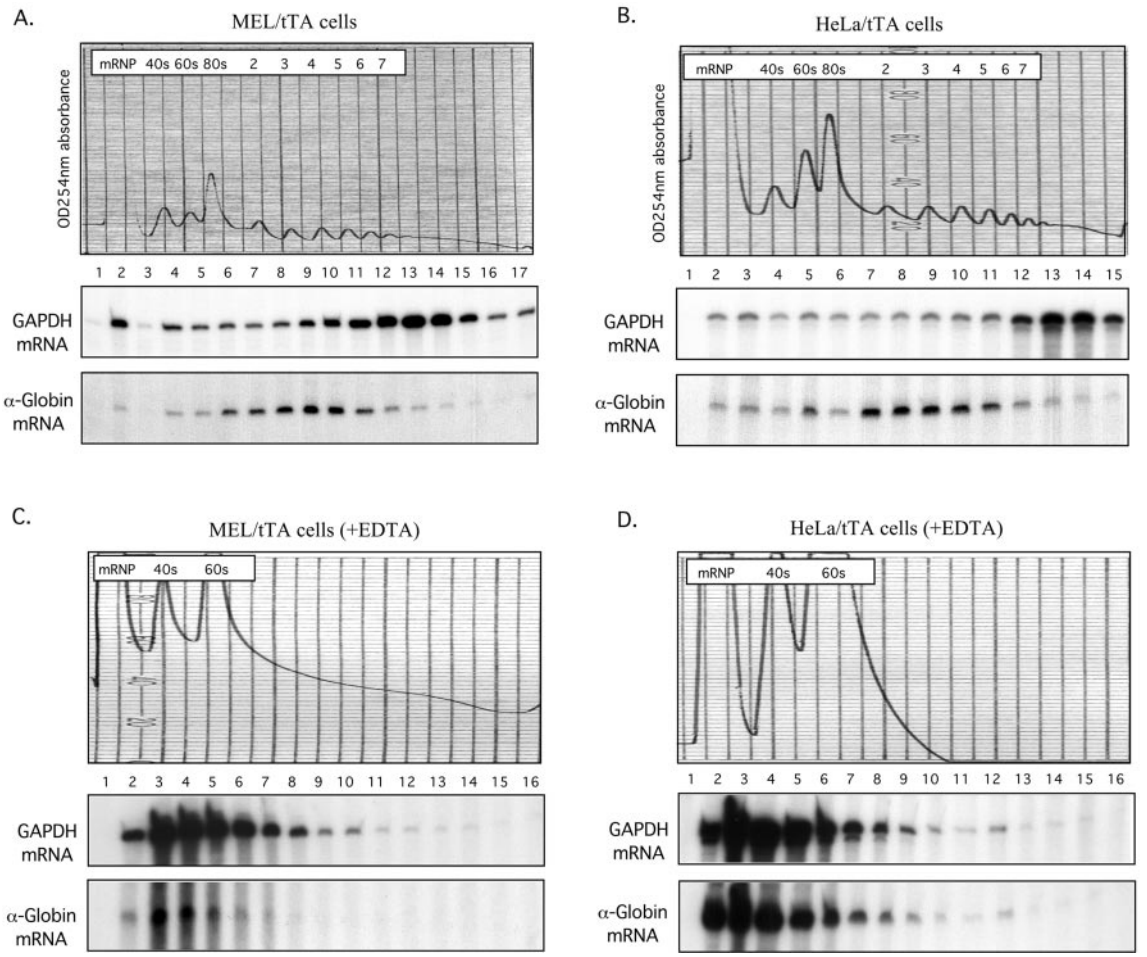

FIG. 4. hα-globin mRNA is actively translated in both erythroid MEL cells and nonerythroid HeLa cells. (A and B) MEL/tTA or HeLa/tTA cells were transfected with pTet-hα^{WT}, and the expression of the transgene was induced for a continuous 24 h. Cells were then lysed and fractionated on a sucrose gradient. Absorbance profiles at 254 nm of sucrose-fractionated MEL/tTA and HeLa/tTA cytoplasmic extracts are shown (upper panels). Positions of mRNP; 40s, 60s, and 80s monosomes; disamers; and polysome multimers are indicated above the peaks. The middle and lower panels show the distribution of α^{WT} mRNA and endogenous GAPDH mRNAs across the gradient. Analysis was by RPA as detailed in Materials and Methods. (C and D) The addition of EDTA to the cell lysates dissociates ribosomes and results in the shift of mRNAs to the prepolysomal fractions.

effect on stability, they were juxtaposed within the 3′UTR of α^{WT} mRNA. To test for a possible effect of polarity on their relative contributions, the ARE was placed both 5′ and 3′ to the PR (α2$^{ARE\text{-}PR}$ and α2$^{PR\text{-}ARE}$) (Fig. 5A). Analysis of decay rates in MEL cells revealed that the relative positioning of the αCP binding site and the ARE may have some effect on the stability, but compared to the α^{WT} $t_{1/2}$ of 10 to 10.5 h, the stabilities of all three ARE-containing derivative mRNAs were quite low (≤1 h) (Fig. 5B). Thus, the effect of the ARE on mRNA decay was dominant to the stabilizing effect of the α-complex.

αCP can stabilize α-globin mRNA independent of its native binding site. At a minimum, the α-complex is comprised of the αCP protein and its C-rich binding site (10). Three models of αCP function in stabilization of hα-globin mRNA can be considered. (i) Stabilization of target mRNAs may be mediated by some property of the α-complex as a whole. (ii) The binding site per se may play a specific and active role in the α-globin mRNA decay pathway by serving as a cleavage site for an erythroid-enriched endonuclease; in the model, αCP serves as a steric blocker of cleavage at this site (3, 33, 41). (iii) Stabilization may be entirely mediated by αCP, with the binding site simply serving a tethering role. To distinguish among these models, the native αCP binding site was removed from the α-globin mRNA and was replaced by a neutral sequence (as described above) or by a high-affinity αCP-binding site previously identified by RNA SELEX (R7α1 aptemer) (36) (Fig. 6). The stability of the α^{WT} mRNA was compared to those of the derivative α^{Neut} and α2$^{R7\alpha1}$ RNA in MEL/tTA cells. While replacement of the αCP binding site with the neutral sequence destabilized the mRNA, replacement with the artificial αCP binding motif resulted in full restabilization ($t_{1/2}$ = 10.5 h) (Fig. 6). Thus, αCP was able to fully maintain the stability of hα-globin mRNA by docking to a nonnative RNA structure.

α-globin RNA stability is maintained by indirect tethering of an αCP fusion protein to the 3′UTR. The critical determinant(s) of the α-complex involved mRNA stabilization was further explored by using a second tethering approach. In this

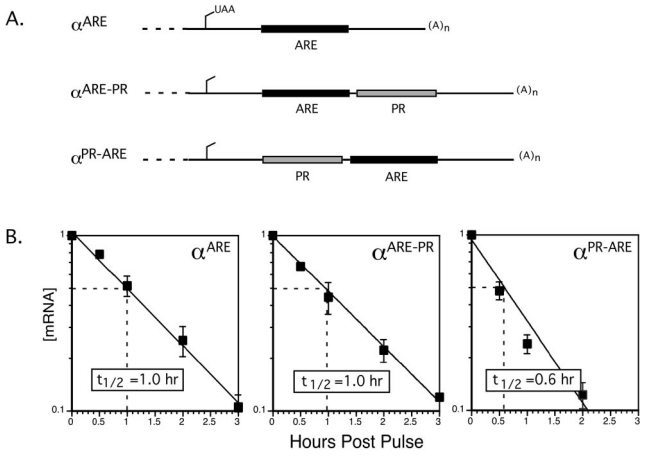

FIG. 5. Stabilization of α-globin mRNA by the α-complex in MEL cells can be overridden by placing the ARE destabilizing element in *cis*. (A) Diagrams of the set of three ARE-containing mRNAs analyzed for stability. In each mRNA a 53-nt ARE was inserted into 3′UTR of α-globin RNA either in place of PR (α^{ARE}) or in *cis* to the PR sequence ($\alpha^{ARE\text{-}PR}$ and $\alpha^{PR\text{-}ARE}$). (B) Decay curves showing the $t_{1/2}$ of each mRNA analyzed by RPA in MEL/tTA cells. Error bars indicate standard deviations.

approach, direct binding of αCP with the target mRNA was entirely eliminated and αCP was tethered to the 3′UTR of the α-globin mRNA via a foreign RNA-protein interaction. The prokaryotic MS2 protein and its cognate RNA binding structure were used for this purpose (2, 6) (Fig. 7A). To achieve the desired tethering, the major αCP isoform, αCP2-KL (10), was fused in frame to the phage RNA binding protein MS2 (MS2-αCP). An RNA stem-loop motif that serves as a high-affinity binding site for MS2 was inserted in the α-globin mRNA 3′UTR in place of the αCP binding site. The resultant plasmid, pTet-$\alpha 2^{MS2}$, was transfected into MEL/tTA cells, either alone or along with expression plasmids encoding either MS2 or the MS2-αCP fusion protein. EMSA confirmed expression of the

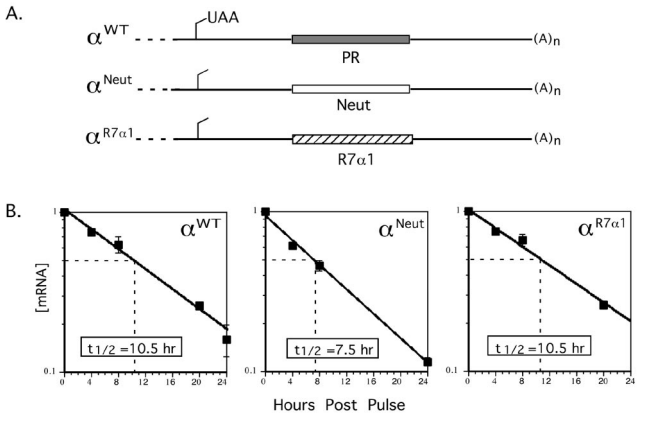

FIG. 6. Stability of α-globin mRNA can be maintained via an artificial αCP binding motif. (A) Diagrams showing the structures of α-globin mRNA (α^{WT}), the mutant lacking αCP binding motif (α^{Neut}), and the mutant bearing a SELEX-derived artificial αCP docking site R7α1 ($\alpha^{R7\alpha1}$). (B) The $t_{1/2}$ of $\alpha^{R7\alpha1}$ mRNA in MEL/tTA cells analyzed by RPA and compared to those of α^{WT} and α^{Neut}. Error bars indicate standard deviations.

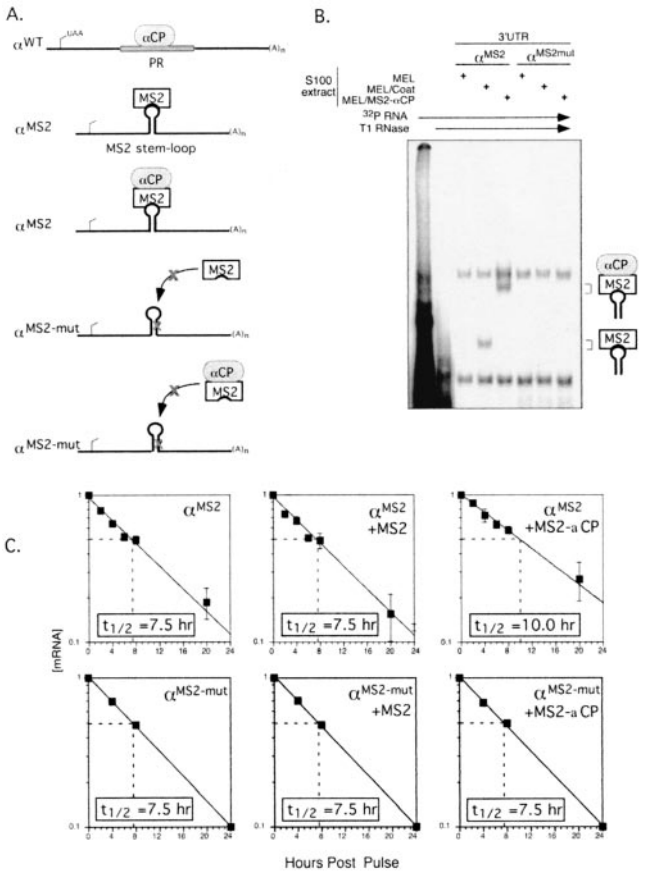

FIG. 7. Stability of α-globin mRNA can be mediated by a tethered αCP fusion protein. (A) Diagrams showing the approach for tethering αCP to the 3′UTR to hα-globin mRNA. The targeted α^{MS2} mRNA has had the αCP binding site replaced by an MS2 binding motif. See text for details. (B) EMSA analysis confirming the expression of the MS2 and MS2-αCP fusion proteins and their abilities to bind to the MS2 binding motif in the α^{MS2} 3′UTR. Cytosolic extracts from MEL cells transfected with MS2- or MS2-αCP fusion protein-encoding plasmid was incubated with ^{32}P-labeled α^{MS2} or $\alpha^{MS2\text{-}mut}$ 3′UTR. The mRNPs formed are indicated at the right. The signals crossing all of the lanes with cell extracts (top and bottom bands) reflect background interactions. (C) Decay curves showing the specificity and sufficiency of the MS2-αCP fusion protein to restore stability of α-globin mRNA in MEL/tTA cells. The stability can be fully restored only when the MS2-αCP fusion protein is targeted to the wild-type MS2 binding site. Error bars indicate standard deviations.

MS2 and MS2-αCP proteins in the transfected cells and documented the ability of these proteins to bind to the 3′UTR of $\alpha 2^{MS2}$ RNA (Fig. 7B). The $t_{1/2}$ of the $\alpha 2^{MS2}$ RNA in the MEL/tTA cells was determined (Fig. 7C). Replacement of the PR with the MS2 binding site destabilized the α-globin mRNA. The shortened $t_{1/2}$ of the $\alpha 2^{MS2}$ mRNA was unaffected by cotransfection with the MS2 expression plasmid. However, the stability was fully restored to wild-type α-globin mRNA levels when this same $\alpha 2^{MS2}$ mRNA was coexpressed with the MS2-αCP fusion protein. As an additional control, a set of mutant $\alpha 2^{MS2}$ mRNAs in which the MS2 motif contained a point mutation within the stem-loop that abolished MS2 binding was assembled ($\alpha 2^{MS2\text{-}mut}$). Neither the MS2 nor the MS2-αCP fusion protein expressed in the transfected cells could bind to

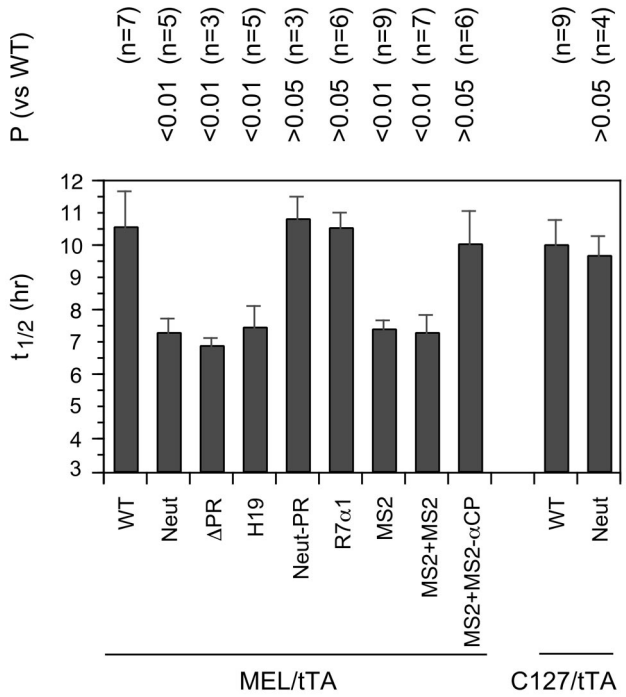

FIG. 8. αCP binding is necessary and sufficient for the erythroid-specific stabilization of human α-globin mRNA. The histogram summarizes all mRNA stability data in this report. The $t_{1/2}$ of the wild-type and mutant α-globin mRNAs are shown (average ± standard deviation). The numbers of independent experiments done with each mRNA and the corresponding *P* value (Student *t* test) of each mutant mRNA versus α^{WT} are shown above the respective bars.

the mutant motif (Fig. 7B), and neither was able to stabilize the $\alpha2^{MS2\text{-}mut}$ mRNA (Fig. 7C). These data indicate that the stabilization function of the native α-complex can be completely replaced by tethering of the αCP protein to an artificial site lacking a poly(C) motif.

DISCUSSION

The studies reported here are summarized in Fig. 8. These data lead us to conclude that αCP binds to α-globin mRNA in vivo, that the resulting α-complex is essential for full levels of mRNA stabilization in erythroid cells, and that αCP stabilizes α-globin mRNA independent of its native binding site. Of additional importance is the observation that α-globin mRNA stability in erythroid cells can be maintained by an artificial αCP docking site as well as by an artificially tethered αCP fusion protein. These results support the paramount importance of αCP in hα-globin mRNA stabilization and set the stage for the analysis of downstream interactions and underlying mechanisms of action.

Previous studies from our laboratory and others suggest that binding of αCP to a 3′UTR C-rich region (PR) of hα-globin mRNA plays an important role in α-globin gene expression (39–43). Direct interactions between the 3′UTR of hα-globin mRNA and each of the three major αCP isoforms (αCP1, αCP2, and αCP2-KL) have been documented by in vitro binding studies (10). Structural analysis of the resultant α-complex suggests that it comprises a 1:1 stoichiometry of αCP and the RNA target sequence. Additional information has come from affinity purification of the α-complex (20). These studies support a model in which the α-complex is a multiprotein complex containing AUF1/hnRNP D, in addition to αCP. Of particular interest is that AUF1/hnRNP D is also associated with the ARE that mediates rapid mRNA turnover. The simple binary model of the α-complex and the more complex multiprotein α-complex model have in common a central and essential role of αCP in recognizing and binding to a C-rich motif in target mRNAs. The present study attempts to document the association between αCP and α-globin mRNA in vivo and further characterize the role of αCP in mRNA stabilization.

The contribution of αCP binding to hα-globin mRNA stability was initially inferred from a linkage between 3′UTR mutations that block α-complex assembly and alterations in the steady-state α-globin mRNA (43). Although stability is an important determinant of mRNA accumulation, the steady-state concentration of an mRNA can reflect alterations in additional steps in gene expression. This caveat is of particular importance in the case of mRNAs targeted by αCP proteins, because αCPs are present in both the nucleus and cytoplasm and shuttle between the two compartments. Thus, the direct determination of mRNA structural $t_{1/2}$ is essential to conclusions regarding mRNA stabilization.

In the present report we address the in vivo association of αCP with α-globin mRNA and attempt to directly link this association with alterations in mRNA stability. In vivo association of αCP with hα-globin mRNA was documented by IP of αCP-containing RNP complexes (α-complexes) from cell extracts (Fig. 1). These purified complexes contained α-globin mRNA and selectively excluded a mutant form of this mRNA, α^{Neut}, that lacked a functional αCP binding site. These studies indicate that the association of αCP with α-globin mRNA occurs in vivo and further demonstrates that this association is specific and exclusive to the C-rich determinant in the 3′UTR.

Directly linkage of α-complex formation and hα-globin mRNA stability was established by using a tetracycline-dependent conditional transcription system (15, 47). To maximize the utility of this approach, it was necessary to identify tTA-expressing cell lines that had minimal background (leaky) expression in the "off" state (medium containing tetracycline) (Fig. 2). This is of particular importance when working with highly stable mRNAs; low-level leaky expression can result in accumulation of substantial background levels of the mRNA that obscure the signal from the mRNAs newly synthesized during the short induction period.

The stabilizing role of the C-rich element in the 3′UTR of hα-globin mRNA was directly established by using the tetracycline-induction model. Mutations that either deleted this determinant (ΔPR), replaced it with a neutral sequence (Neut), or introduced a sequence substitution that interfered with αCP binding (H19) were introduced (Fig. 3A and B). In contrast to the 10- to 10.5-h $t_{1/2}$ of the α^{WT} RNA, each of the mutations that abolished α-complex formation destabilized the α-globin mRNA to a $t_{1/2}$ of 7.5 h (Fig. 8). An additional mutation, in which a neutral sequence was inserted between the stop codon and the αCP binding site, had no adverse effect on mRNA stability. Analysis of this mutation also indicated that the limited change in the spacing between the αCP binding site and the translation termination codon of the mRNA was not

critical to the stabilizing function of the α-complex. These data, taken together, suggest a direct association between α-complex formation in the 3′UTR and the α-globin mRNA stability. They demonstrate that elimination of the αCP binding destabilizes α-globin mRNA in erythroid cells.

The role of the α-complex appears to differ among cell types. Analyses of α-globin mRNA in two nonerythroid cell lines, C127/tTA and HeLa/tTA, were remarkable in that neither ribosome extension into the 3′UTR (data not shown) nor mutation of the αCP binding site adversely affected the α-globin mRNA stability (Fig. 3C). Thus, within the limits of this comparison, the dependence on the α-complex for stabilization of α-globin mRNA was selective to erythroid cells. This difference does not appear to reflect major differences in the translational activities of the target mRNAs (Fig. 4) but instead may reflect cell type-specific mRNA decay pathways.

The function of the α-complex was characterized by comparing its activity to that of a well-defined destabilizing determinant. AREs direct a deadenylation-dependent decay pathway (5, 9, 34, 46). In the case of the α-complex, in vitro decay studies and in vivo analyses both indicate that stabilization of α-globin mRNA correlates with stabilization of its 3′ poly(A) tail (28, 40). In vitro decay studies further suggest that this action of the α-complex may reflect direct interaction between the αCP protein bound to the poly(C)-rich determinant and PABP bound to the poly(A) tail (42). Placing the ARE in *cis* to the PR motif in the α-globin 3′UTR dramatically destabilizes the mRNA stability, regardless the orientation of the two elements relative to each other (Fig. 5). Thus, the destabilizing function conferred by ARE is dominant over the stabilizing function of α-complex. The basis for this dominance will be of interest for future investigation.

The structural basis for α-complex activity was investigated. The stabilizing effect of the α-complex can reflect distinct aspects of α-complex structure: the structure of the αCP protein itself, specific properties of the RNA binding site, or the overall higher-order structure of the αCP/RNA (RNP) complex. To address these models, the role of αCP was segregated from that of the binding site by two recruiting approaches. In the first, the native αCP binding site was replaced by an artificial sequence (R7α1) isolated by SELEX technology. This R7α1 aptemer contains highly exposed poly(C) motifs and binds αCP with high specificity and affinity (36). Replacement of the native αCP binding site with the R7α1 sequence maintained the $t_{1/2}$ to the α^{WT} value. (Fig. 6). This suggested that the native binding site per se was not essential for α-complex function. Instead, the αCP binding ability of this site is of primary importance. In the second approach, the sufficiency of αCP binding in the stabilization of α-globin mRNA was more rigorously tested by tethering αCP to the 3′UTR in the total absence of its own RNA binding activity. This was accomplished by replacing the αCP binding site with the binding motif for the MS2 phage coat protein (Fig. 7A and B). While this replacement destabilized the α-globin mRNA ($t_{1/2}$ = 7.5 h), coexpression of an MS2-αCP fusion protein, but not MS2 protein, fully restored its stability ($t_{1/2}$ = 10 h). Additional controls confirmed that the restabilization was dependent on fusion protein binding to the target mRNA (Fig. 7C). The two sets of tethering experiments are consistent in indicating that αCP binding to the target mRNA is the critical event in α-globin mRNA stabilization.

The basis for the selective dependence on αCP binding for stabilization of α-globin mRNA in erythroid cells (Fig. 3B and C) remains to be defined. Since αCPs are widely expressed, it is difficult to attribute the apparent erythroid specificity to this protein alone. The role of an erythroid-enriched RNA endonuclease such as the previously reported ErEN (33, 41) could explain this finding, as the α-complex would then be needed only to protect the α-globin mRNA in erythroid cells harboring this erythroid RNase. However, our data do not fully support such a model. Replacement of the native binding site with a totally unrelated prokaryotic binding site (MS2) destabilizes the α-globin mRNA, and it is restabilized by coexpression of a tethered αCP fusion protein to the same extent as seen for the wild-type α-globin mRNA. This suggests that destabilization need not be triggered by site-specific cleavage and that stabilization by αCP may not reflect protection of such an endonuclease-sensitive site from an erythroid-enriched endonuclease. It appears more likely that the primary stabilizing function of αCP to α-globin mRNA involves interactions with distal RNA structures and/or protein factors. Thus, the basis for the erythroid specificity remains to be further explored.

The data from the present study support a direct role of αCP in mRNA stabilization. The mechanisms involved in this stabilization can now be further explored. Additional proteins, possibly including some of those identified as αCP associated by affinity purification (20), may be involved in this process. The ability to stabilize the α-globin mRNA via a tethered fusion protein will facilitate subsequent studies in this area. Such an approach will specifically facilitate detailed mapping of the determinants in αCP that are important in downstream stabilizing events independent of its RNA binding activity.

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