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Detection and Characterization of a 3′ Untranslated Region Ribonucleoprotein Complex Associated with Human α-Globin mRNA Stability

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The highly stable nature of globin mRNA is of central importance to erythroid cell differentiation. We have previously identified cytidine-rich (C-rich) segments in the human α-globin mRNA 3′ untranslated region (α-3′UTR) which are critical in the maintenance of mRNA stability in transfected erythroid cells. In the present studies, we have detected *trans*-acting factors which interact with these *cis* elements to mediate this stabilizing function. A sequence-specific ribonucleoprotein (RNP) complex is assembled after incubation of the α-3′UTR with a variety of cytosolic extracts. This so-called α-complex is sequence specific and is not formed on the 3′UTR of either β-globin or growth hormone mRNAs. Furthermore, base substitutions within the C-rich stretches which destabilize α-globin mRNA in vivo result in a parallel disruption of the α-complex in vitro. Competition studies with a series of homoribopolymers reveals a striking sensitivity of α-complex formation to poly(C), suggesting the presence of a poly(C)-binding activity within the α-complex. Three predominant proteins are isolated by α-3′UTR affinity chromatography. One of these binds directly to poly(C). This cytosolic poly(C)-binding protein is distinct from previously described nuclear poly(C)-binding heterogeneous nuclear RNPs and is necessary but not sufficient for α-complex formation. These data suggest that a messenger RNP complex formed by interaction of defined segments within the α-3′UTR with a limited number of cytosolic proteins, including a potentially novel poly(C)-binding protein, is of functional importance in establishing high-level stability of α-globin mRNA.

Degradation of mature mRNA in eukaryotic cells is a regulated process that can be a significant determinant of gene expression. This regulatory process is seen in fertilized oocytes (5, 56) as well as in highly differentiated tissues (13, 55, 60). Multiple mechanisms underlie this regulation (12, 25, 50). *cis*-regulating elements can be found in the 5′ untranslated region (5′UTR) (43), the coding region (41, 52), and the 3′UTR (28, 60) and often affect the size of the poly(A) tract (5, 39, 56). *trans*-acting proteins which specifically bind to certain of these *cis* elements have been identified (29, 42, 50). An emerging theme is the paramount importance of the 3′UTR in control of mRNA stability and subsequent determination of gene expression (28).

Recent studies have emphasized a wide spectrum of functions which are mediated by the mRNA 3′UTR. This region can act as a *trans*-acting regulator of growth and differentiation in mouse myoblasts (45, 46), a localization determinant (21) and translational regulator in *Drosophila* embryos (22), a control element in sex determination of the germ cell precursors in *Caenorhabditis elegans* (3), and a translational regulator of cell cycle-related mRNAs in *Xenopus laevis* (56). However, the majority of studies on the 3′UTR focus on its role in determination of mRNA stability (15, 28, 56, 60). The most clearly defined example of this control is the iron-responsive element located within the 3′UTR of transferrin receptor mRNA (13, 14) and its interaction with a *trans*-acting factor, the iron regulator protein IRP (30, 49). Binding of IRP to the iron-responsive element stabilizes the transferrin receptor RNA. IRP is a monomeric protein of 90 kDa (26, 48), and its iron-responsive element-binding activity is controlled by the size of the free iron pool in the cell (29). In this way, levels of free cytoplasmic iron can be transduced to specific alterations in transferrin mRNA stability and to subsequent steady-state levels. A second well-characterized 3′UTR stability element, the adenosine-uridine-rich element (ARE), contains repeated copies of the motif AUUUA. This element is responsible to varying degrees for the highly labile nature of certain cytokine mRNAs (15, 50) including granulocyte-macrophage colony-stimulating factor (2, 55) lymphokine (9), c-*myc* (11, 57), and c-*fos* (16) mRNAs. Evidence suggests that these AREs may in some cases not be fully sufficient to destabilize mRNA (24, 36, 53, 63). Multiple proteins which bind to the ARE have been identified, and their individual effects are under study (8–10, 23, 51, 64). Several less-well-defined model mRNAs in which the 3′UTR is associated with mRNA stability have also been reported. These include mRNAs encoding erythropoietin (47), ribonucleotide reductase R1 (17), and m1 muscarinic acetylcholine receptor (32). Studies to further clarify relevant mRNA-protein interactions in each of these systems and the mechanisms by which they control mRNA stability are ongoing.

The α-globin gene is expressed exclusively in the erythroid lineage. While transcriptional control is central to this expression pattern, accumulation of globin mRNAs to well over 95% of total cellular mRNA during terminal erythroid differentiation relies heavily on their unusually long half-lives (6, 7, 31). The mechanism by which globin mRNA is stabilized in erythroid cells remains unknown. Previous studies from our laboratory suggest that in the case of human α-globin mRNA this property is conferred by the 3′UTR. Ribosome readthrough

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TABLE 1. Oligonucleotide sequences for PCR

Target template	Oligonucleotide sequence[a]	Orientation
α-3′UTR	CGTAATACGACTCACTATAGCTGGAGCCTCGGTATCCGT	Sense
	TGGAAGCTTGCTGGAGCCTCGGTAGCCGT[b]	Sense
	CCGAATTCGCCGCCCACTCAGACTTTAT[c]	Antisense
β-3′UTR	TAATACGACTCACTATAGGAGCTCGCTTTCTTGCTGTCCA	Sense
	GGCAGAATCCAGATGCTCAA	Antisense
GH-N-3′UTR	TAATACGACTCACTATAGGGATCTGCCCGGGTGGCATCC	Sense
	GGACAAGGCTGGTGGGCACT	Antisense

[a] Underlining indicates the T7 promoter.
[b] Contains a *Hin*dIII site for cloning.
[c] Contains an *Eco*RI site for cloning.

into the 3′UTR of α-globin mRNA (α-3′UTR), such as occurs in the common α-thalassemia mutation, αConstant Spring, results in α-globin mRNA destabilization and almost complete loss of function of the mutant α-globin allele (18, 27, 35, 60). The determinant(s) in the human α-3′UTR responsible for this phenotype has been mapped to three cytidine-rich (C-rich) regions. Mutations in these segments result in a direct, translationally independent, destabilization of α-globin mRNA (61). A working model in which one or more *trans*-acting factors specifically interact with these *cis* elements to form an mRNP complex which protects the mRNA from RNase attack has been proposed. In the present study, we have determined whether a *trans*-acting factor(s) specifically interacts with the *cis* elements within the α-3′UTR to form a messenger ribonucleoprotein (RNP) complex and whether the formation of such a complex is associated with α-globin mRNA stability, and we have initiated studies aimed at defining the individual *trans*-acting factor(s) involved.

MATERIALS AND METHODS

Cell culture and extract preparation. Mouse erythroleukemia (MEL) and fibroblast (C127) cells were cultured in minimal essential medium (MEM), human erythroleukemia (K562) cells were cultured in RPMI 1640, and HeLa cells were cultured in Dulbecco's modified Eagle's medium. Each medium was supplemented with 10% fetal bovine serum, 100 μg of streptomycin per ml, and 100 U of penicillin per ml. Cytoplasmic extracts were prepared from each cell line by a modification of the method of Dignam et al. (19). Cells were harvested at the exponential-growth phase, washed twice with phosphate-buffered saline (PBS), suspended at a concentration of 5×10^7 cells per ml in homogenization buffer (10 mM Tris-HCl [pH 7.4], 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM dithiothreitol), and lysed with 20 strokes in a Dounce homogenizer. The homogenate was adjusted to the RNA-protein-binding buffer conditions (150 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris HCl [pH 7.4], 0.5 mM dithiothreitol, 25 μM PNPG [*p*-nitrophenyl-β-D-glucoside], and 10 μg of leupeptin per ml) and centrifuged for 10 min at $2,000 \times g$ to pellet the nuclei. Supernatant from this step was harvested and centrifuged at $100,000 \times g$ for 60 min. The protein concentration of this high-speed S100 supernatant was determined by the bicinchoninic acid method (Pierce). The entire procedure was performed at 4°C, and the final protein concentration of the S100 from this procedure was adjusted to 8 to 10 mg/ml.

Cell nuclei were isolated by the method of Piñol-Roma et al. (44). HeLa cells and MEL cells were lysed in RSB100 buffer (10 mM Tris, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.5% Triton X-100, 2 μg of leupeptin per ml, 1 μg of pepstatin per ml, 1 U of aprotinin per ml), and the cell nuclei were washed twice in RSB100 and pelleted. The nuclei from 5×10^6 cells were lysed in 500 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer for SDS-PAGE analysis.

To generate radiolabeled proteins, MEL cells at the log phase of growth were metabolically labeled for 16 h with 20 μCi of [^{35}S]methionine (1,000 Ci/mmol; Amersham) per ml of methionine-free MEM containing 5% calf serum. S100 from the [^{35}S]methionine metabolically labeled MEL cells was prepared as described above.

RNA synthesis. DNA templates for the synthesis of 3′UTR fragments were generated by PCR (38) with the wild-type human α-globin gene, human α-globin genes containing specified 3′UTR mutations (61), the human β-globin gene, and the human growth hormone N gene as templates. The primers used for these PCRs are listed in Table 1. Transcription of each full-length 3′UTR was carried out with the amplified fragments as templates with the T7 promoter sequence incorporated in the 5′ primer. To generate a template for the α-3′UTR with 30 adenosines at the 3′ end, cDNA fragments were amplified with 5′ and 3′ primers containing *Eco*RI and *Hin*dIII sites, respectively. After amplification, the PCR product was digested with *Eco*RI and *Hin*dIII and the fragment was subcloned between the corresponding sites in the polylinker of plasmid pSP64(polyA) [construct pSP64(polyA)/α-3′UTR]. Transcripts from these subcloned templates contain a 30-nucleotide poly(A) 3′ tail originating from the pSP64(polyA) vector sequences. Unlabeled and ^{32}P-labeled 3′UTR probes were synthesized per the manufacturers' suggested conditions from the PCR products with T7 RNA polymerase (NEB) or from *Eco*RI-linearized pSP64(polyA)/α-3′UTR with SP6 RNA polymerase (Ambion). After the RNA polymerase reaction, the DNA templates were digested by RNase-free DNase I (Promega) and the RNA probes were desalted and separated from free nucleotides on a Sephadex G-50 mini-column. The specific activity of the RNA probes is approximately 50,000 cpm/ng.

RNA-binding and gel shift assay. The gel shift assay was carried out by the method of Leibold and Munro (33). In vitro-transcribed ^{32}P-labeled RNA (10,000 cpm/0.1 to 0.5 ng) was heated to 95°C for 5 min and subsequently allowed to cool to room temperature over 30 min. The RNA-protein-binding reaction was carried out at room temperature. RNA probe (10,000 cpm) was incubated with 40 μg of S100 proteins in a 10-μl total volume for 30 min. RNase mixture (1 μl) containing 1 U of RNase T_1 (Boehringer) per ml and 10 μg of RNase A (Sigma) per ml was then added and incubated at room temperature for 10 min, and heparin (Sigma) was then added to a final concentration of 5 mg/ml for an additional 10 min.

For gel shift studies in which unlabeled proteins were to be electrotransferred to nitrocellulose for Northwestern (NW) analysis (see below), 120 μg of MEL S100 proteins was used in the reaction mixture with or without 500 ng of unlabeled α-3′UTR.

For preparative gel shift studies in which the α-complex was to be excised from the gel for protein analysis, a total of 2 mg of MEL S100 extract was incubated with or without 18 μg of unlabeled α-3′UTR and each reaction mixture was loaded on a single 13-cm well of three native polyacrylamide gels. Each preparative gel included a single analytic lane containing an incubation mixture of S100 with ^{32}P-labeled α-3′UTR. This generated a labeled RNP complex position marker. The appropriate position of the preparative gel was excised, and the proteins were electroeluted (see below).

For competition assays, a 100-fold excess of unlabeled RNA competitors was mixed with ^{32}P-labeled probes prior to the addition of S100 proteins. Each of the four homoribopolymers (size range, 50 to 500 nucleotides; Sigma) was used as the competitor at a predetermined weight ratio to the ^{32}P-labeled probes. Subsequent to RNase digestion, samples were electrophoresed through a 5% nondenaturing polyacrylamide gel (acrylamide/bisacrylamide ratio, 60:1) in 0.5× Tris-borate-EDTA (TBE) buffer. Bands were visualized by autoradiography.

Electroelution of RNP complex proteins. Slices of preparative gel shift gels containing α-complex or the corresponding segments of control lanes containing uncomplexed MEL S100 extract were excised under autoradiographic guidance (see above). Each gel slice was electroeluted in 0.5× TBE at 4°C in a minielectroeluter (model 422; Bio-Rad) according to the manufacturer's suggested procedure. Eluted proteins were dialyzed against PBS at 4°C with three changes, precipitated with 4 volumes of acetone solution (80% acetone, 10 mM Tris-HCl [pH 8.0], and 30 mM NaCl) at −70°C overnight, and pelleted at $9,000 \times g$ at 4°C for 10 min. The eluates from each of three sets of large-scale gel shift preparations (total, 2 mg of MEL S100 proteins) were resuspended in 50 μl of SDS-PAGE loading buffer for subsequent NW analysis. A parallel incubation with S100 extract only and no RNA was carried out to isolate proteins which comigrate with the α-complex on the native gel.

Affinity chromatography. RNA affinity chromatography of cytosolic extracts was carried out by a modification of the procedure of Neupert et al. (40). ^{35}S-labeled S100 proteins were treated with 400 U of micrococcal nuclease per ml at 30°C for 30 min. EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; 5 mM] was then added to inactivate the enzyme. The sample was mixed with 40 μg of tRNA (Sigma) per ml, 5 mg of heparin per ml, and 2% mercaptoethanol and rocked at room temperature for 30 min. The treated sample was centrifuged at $10,000 \times g$ for 10 min to pellet the vanadyl

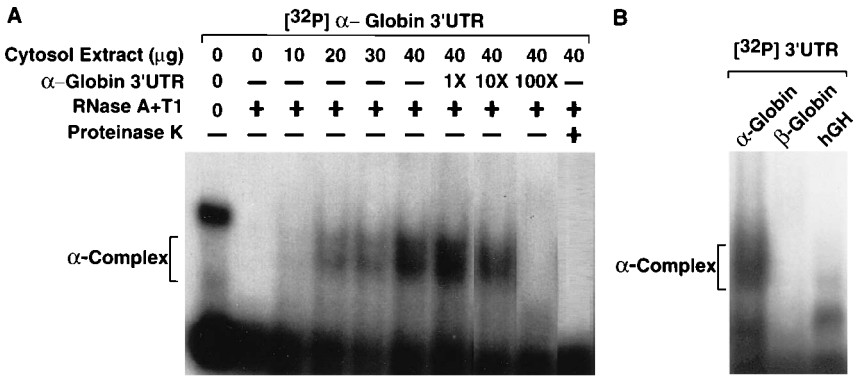

FIG. 1. Electrophoretic mobility gel shift assay demonstrating formation of an RNP complex on the α-globin 3′UTR. (A) RNP complex formation was assayed by incubation of a ^{32}P-α-globin mRNA 3′UTR fragment with S100 cytosol extract from MEL cells. An RNase-resistant complex (α-complex) formed upon addition of increasing amounts of protein is detected by nondenaturing PAGE. Formation of the α-complex is inhibited by unlabeled α-globin 3′UTR and is fully eliminated by addition of proteinase K to the S100 fraction prior to the binding reaction. (B) ^{32}P-labeled 3′UTRs of human α-globin, β-globin, and growth hormone (hGH) were incubated with cytosolic extract as for panel A. Both the β-globin and the hGH 3′UTRs fail to form the α-complex. The position of the α-complex is noted to the left of both autoradiographs.

elements, and the supernatant was treated with 1 U of inhibitor ACE (5 Prime-3 Prime) per 30 μl at room temperature for 15 min. To generate the α-3′UTR–poly(U) beads, α-3′UTR probes containing 30 adenosines at the 3′ end (see "RNA synthesis" above) were annealed to poly(U)-Sepharose 4B beads (Pharmacia) in RNA annealing buffer (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH7.5], 100 mM KCl). Poly(U), α-3′UTR–poly(U), and poly(C) agarose beads (Sigma) were washed in RNA-protein-binding buffer (see "Cell culture and extract preparation" above) before use. Treated ^{35}S-labeled S100 proteins (500 μl, 10 mg/ml) were incubated with 100 μl of RNA-linked beads, rocked at room temperature for 30 min, and washed five times with 10 volumes of RNA-protein-binding buffer containing 5 mg of heparin per ml. This procedure is referred to as single-step purification, since the crude protein sample was added directly to the affinity beads. For the electrophoretic mobility gel shift assay, affinity-purified proteins were released from the beads by incubation in 2 M KCl–25mM HEPES elution buffer [α-3′UTR–poly(U) and poly(U) beads] or in 4 M guanidine hydrochloride [poly(C) beads]. The samples were desalted on a Sephadex G-50 minicolumn and then incubated with 2% mercaptoethanol at room temperature for 15 min to renature the proteins. For SDS-PAGE analysis, affinity-purified proteins were released from the beads by boiling of the beads in SDS-PAGE loading buffer. To decrease background during the affinity purification, a two-step procedure was used. In this approach, the S100 proteins were initially loaded on poly(U) beads in heparin-free RNA-protein-binding buffer. Proteins were released by being washed with heparin-containing binding buffer (5 mg/ml). The eluate from this step was then reloaded on each of three RNA affinity beads, and then purification procedures were performed as described above.

Western and NW blot analysis. NW blot analysis from SDS-PAGE was carried out as described elsewhere (37) with modifications. Proteins were separated on SDS-PAGE and electrophoretically transferred to a nitrocellulose filter in a buffer of 25 mM Tris, 150 mM glycine, and 20% methanol. The filter was then preincubated in NW binding buffer (10 mM Tris [pH 7.4], 50 mM NaCl, 1 mM EDTA, 1× Denhardt's solution) containing 1 mg of heparin per ml and 1 mM dithiothreitol at room temperature for 1 h and washed for 15 min in NW binding buffer. The nitrocellulose membrane was probed with 10,000 cpm of 5′ end-labeled poly(C) (size range, 50 to 500 nucleotides; Sigma) per ml in NW binding buffer containing 20 μg of tRNA per ml at room temperature for 1 h. The membrane was given three 5-min washes in NW binding buffer, dried, and exposed to X-ray film for autoradiography. The NW blot was stripped by being washed in PBS containing 1% SDS at 37°C for 1 h to remove poly(C) probe and subsequently used for Western (immunoblot) analysis with monoclonal antibody 3C2 (a gift of G. Dreyfuss), which is specific for heterogeneous nuclear RNPs (hnRNPs) K and J (37). Enhanced chemiluminescence reagents (Amersham) were applied to the antibody-probed membrane, and signals were visualized by exposure to the X-ray film.

NW analysis of native gel shift was carried out by electrotransfer of proteins from the gel to a nitrocellulose membrane at 100 mA for 1.5 h in transfer buffer (25 mM Tris, 192 mM glycine) with a Semi-Phor blotter (model TE77; HSI Corp), and the filter was subsequently probed as detailed above.

RESULTS

A sequence-specific mRNP complex is formed on the α-3′UTR. C-rich *cis*-acting stability elements have been identified in the human α-globin 3′UTR (61). Clusters of base substitutions within these elements result in destabilization of human α-globin mRNA. We reasoned that these base substitutions might be disrupting RNA-protein complexes important to mRNA stability. To test this possibility, we utilized an electrophoretic mobility gel shift assay (Fig. 1A). ^{32}P-labeled α-3′UTR was incubated with MEL cell S100 cytosolic extracts, digested by RNase A and RNase T_1, and analyzed by native PAGE. The presence of multiple bands in the probe-only lane is most likely a function of alternative RNA secondary structures, since a single band is present on denaturing gels. In the absence of cytosolic extract, the RNA probe is completely degraded by RNase. With the addition of increasing concentrations of cytosolic protein, an RNase-resistant band appears on the gel. The formation of this complex is efficiently inhibited by unlabeled α-3′UTR and is destroyed by preincubation of cytosolic extract with proteinase K. The complex appears to be specific for α-3′UTR under the assay conditions used, as it fails to assemble on the 3′UTR of either human β-globin mRNA or human growth hormone mRNA, neither of which contains a C-rich sequence (Fig. 1B). These results demonstrate that a sequence-specific RNP complex, dependent on cytosolic protein(s), is formed on the α-globin 3′UTR. We refer to this as the α-complex.

Formation of the α-complex is associated with α-globin mRNA stability. A correlation between α-complex formation in vitro and α-globin mRNA stability in vivo was next tested (Fig. 2). Full-length α-3′UTR contains 109 nucleotides, extending from the first nucleotide after the translation termination codon to the poly(A) addition site (34). Defined clusters of base substitutions have been introduced into this region, and their impact on α-globin mRNA stability has been assessed (61). In the present study, nine of these sets of base substitutions (Fig. 2B) were introduced into an internally labeled ^{32}P-labeled α-3′UTR. Each ^{32}P-labeled RNA was used as a substrate for complex formation. Gel shift studies were carried out by incubation of MEL cell S100 extract with the wild type and each of the mutant α-3′UTRs (Fig. 2A). Four of the mutations either have no effect or have only a minimal adverse effect on α-globin mRNA stability (mutations 7 and 15 and mutations 11 and 23, respectively) (61). Each of these mutant RNAs is able to form a normally migrating α-complex (Fig. 2A). In contrast, the five remaining mutations fully destabilize the α-globin mRNA in vivo to the levels observed with the αConstant Spring thalassemic mutation and fail to form the α-complex in

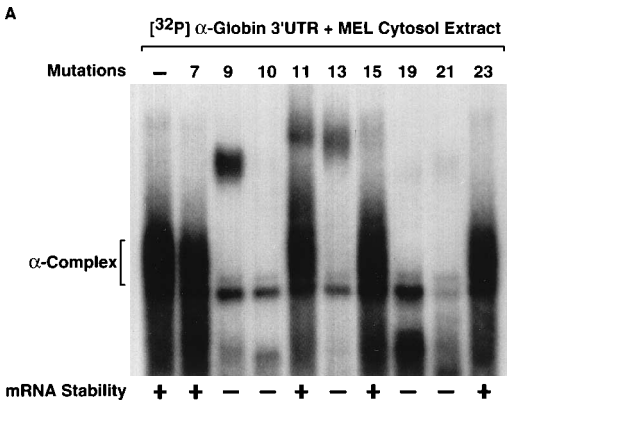

FIG. 2. Effects of defined base substitutions in the α-globin 3′UTR on α-complex formation. (A) Electrophoretic mobility gel shift assay. The number above each lane represents the position of each cluster of base substitutions as defined in panel B. The effect of each mutation on α-globin mRNA stability is indicated at the bottom of each respective lane (61); + indicates stable mRNA, and − indicates destabilized mRNA. An equal quantity of ^{32}P-labeled probe was used in each incubation. The position of the α-complex is labeled to the side of the autoradiograph. (B) Sequences of the wild-type and mutant α-3′UTRs used in complex formation. The number above the sequence is the number of base triplets 3′ to the normal termination codon. The shadowed boxes indicate the C-rich segments on the α-3′UTR which appear to play a role in α-globin mRNA stability (61).

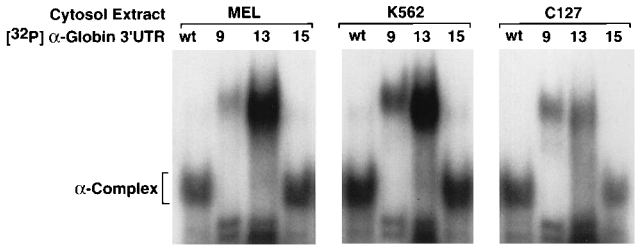

FIG. 4. Assessment of tissue and species specificities of α-complex formation. S100 cytosolic extracts of mouse erythroid (MEL), mouse fibroblast (C127), and human erythroid (K562) cells, labeled above the respective panels, were individually incubated with wild-type and mutant ^{32}P-labeled α-3′UTRs as noted above each respective lane. The position of the α-complex is noted to the left of the first autoradiograph.

vitro in a consistent manner (mutations 9, 10, 13, 19, and 21). Curiously, several of these destabilizing mutations form an aberrant, more-slowly moving complex. Interestingly, mutant 11, which results in a mild destabilization of the α-globin mRNA in vivo, forms both the normal α-complex and a slower aberrant complex. Taken together, these data reveal a remarkable coincidence between α-complex formation and α-globin mRNA stability.

α-Complex formation is sequence specific and is selectively inhibited by poly(C). Competition studies were performed to further characterize the sequence specificity of α-complex formation. In general, mutants which are competent in α-complex formation compete efficiently with wild-type α-3′UTR for α-complex formation while mutants unable to form the α-complex do not efficiently compete. An example is shown in Fig. 3. Mutant 23, which on its own can form the α-complex, competes efficiently with the wild-type α-3′UTR. In contrast, two representative destabilizing mutants, 10 and 19, which cannot form the α-complex, are unable to compete (Fig. 3A). These results suggest that the wild-type and nondestabilizing 3′UTR mutations are able to bind to the same set of factors responsible for α-complex formation while the destabilizing mutations interfere with appropriate factor binding.

The sequence specificity of α-complex formation was further characterized by testing of the abilities of tRNA and each of four homoribopolymers to compete with the wild-type α-globin 3′UTR. Only poly(C) selectively blocks α-complex assembly (Fig. 3B), and this competition is highly effective (Fig. 3C). The sensitivity of α-complex formation to poly(C) competition is of particular interest in light of the C-rich nature of α-3′UTR *cis*-acting stability elements (Fig. 2B). These data suggest that at least one of the proteins within the α-complex has direct poly(C)-binding activity.

Protein components of the α-complex lack tissue and species specificity. To assess the tissue and species specificity of the α-complex proteins, we compared cytosolic extracts of mouse erythroid (MEL) cells, human erythroid (K562) cells, and mouse fibroblasts (C127) by gel shift assay (Fig. 4). Wild-type and mutant α-3′UTRs show identical gel shift patterns

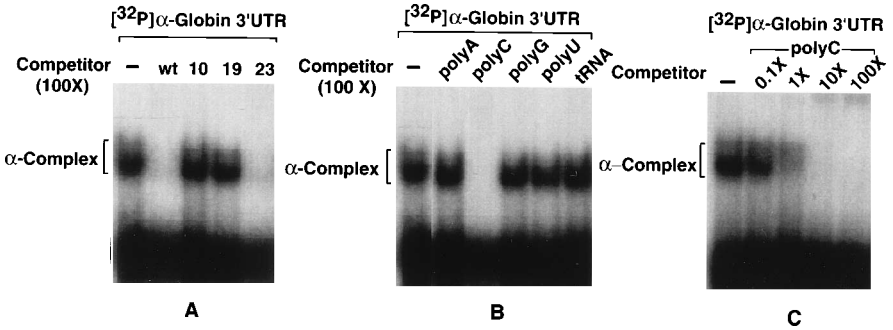

FIG. 3. Sequence-specific competition for α-complex formation. (A) The wild-type (wt) α-3′UTR and three derivative mutants (mutants 10, 19, and 23) were used as competitors for α-complex formation. Two of the mutations, 10 and 19, are destabilizing, and one mutation, 23, is nondestabilizing. A ^{32}P-labeled wild-type α-3′UTR was mixed with a 100-fold excess of each unlabeled competitor, and MEL S100 extract was then added. The binding reaction and gel shift assay were carried out as detailed in the text. (B) α-Complex competition by tRNA and each of four homoribopolymers. Each RNA was used at a 100-fold ratio to the [^{32}P]α-3′UTR (on a weight-weight basis). Poly(C) selectively competes with the wt α-globin 3′UTR for α-complex formation. (C) Effects of poly(C) concentration on α-complex competition. Ratios of competitor to probe are shown on a weight-weight basis. The position of the α-complex is indicated at the left of each autoradiograph.

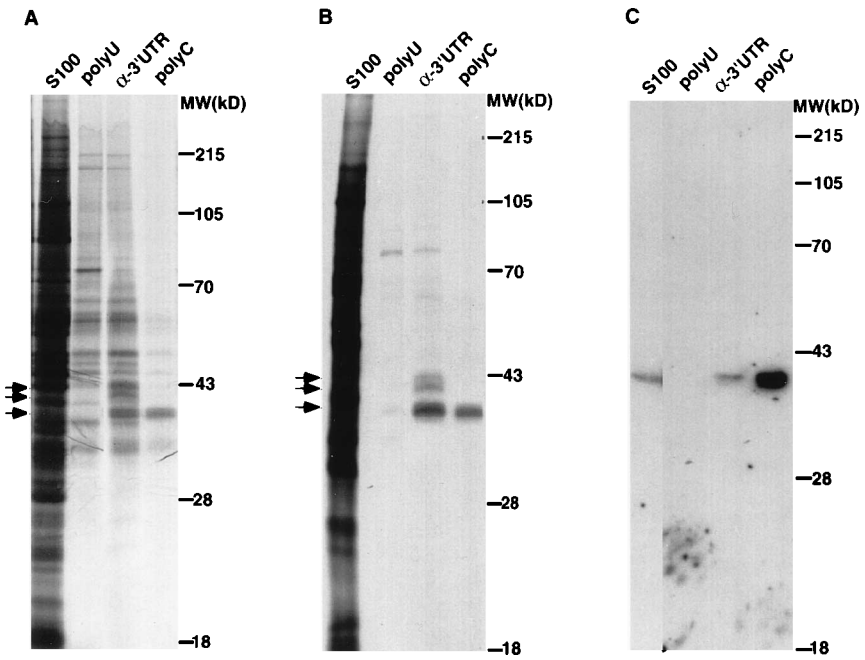

FIG. 5. RNA affinity purification of α-3′UTR-binding proteins from metabolically labeled cytosolic extract. (A) Single-step affinity purification. ^{35}S-labeled MEL S100 extract was bound to poly(U), α-3′UTR–poly(U), or poly(C) affinity beads and subsequently eluted as detailed in the text. The initial S100 preparation and eluted protein fractions from each matrix are shown in the respective lanes. The α-3′UTR affinity column contains the α-3′UTR hybridized to poly(U) beads. (B) Two-step affinity purification. ^{35}S-labeled S100 proteins were adsorbed with poly(U) beads prior to purification on each of the three affinity matrices as detailed in the text. Each lane is labeled as in panel A. (C) NW blot analysis of protein fractions with a ^{32}P-poly(C) probe. Unfractionated S100 MEL extract was loaded in the first lane, and protein fractions of one-step RNA affinity chromatography (panel A) were loaded in the subsequent three respectively labeled lanes of an SDS-PAGE gel. Proteins were electrotransferred to a nitrocellulose membrane, and the filter was incubated with the probe and exposed to film. Since multiple steps were taken to prepare the affinity-purified samples, the signals on the autoradiograph do not represent stoichiometric amounts of α-PCBP from lane to lane.

upon incubation with the S100 cytosolic proteins from each of the three cell lines. While the comigration of a complex on a native gel cannot be considered conclusive evidence that the complexes are identical in protein content, the identity of the migration pattern of the wild-type α-globin 3′UTR substrate and the identity of the alteration in complex migration with those of three mutant RNA substrates suggest that the proteins involved in the α-complex are conserved from mice to humans and are present in nonerythroid as well as erythroid cells.

The α-complex contains a poly(C)-binding protein which is necessary but not sufficient for complex formation. RNA and protein affinity chromatography was next performed to identify the *trans*-acting components of the α-complex. An affinity matrix was assembled by hybridization of a full-length α-3′UTR containing a 30-base adenosine 3′ tail to poly(U) Sepharose beads. Affinity chromatography was carried out in parallel with unhybridized poly(U) beads and with poly(C) agarose beads. A one-step affinity purification procedure (see Materials and Methods) revealed three proteins in the eluate of the α-3′UTR–poly(U) beads (43, 42, and 39 kDa) which were absent from the eluate of the poly(U) beads (Fig. 5A). The eluate from the poly(C) beads contained a single predominant 39-kDa protein band which comigrated with the smallest of the α-3′UTR-specific bands. As demonstrated in Fig. 5B, the same three proteins specifically interacting with the α-3′UTR are more easily detected when the S100 is preadsorbed to poly(U) beads prior to affinity chromatography. Again, the smallest band comigrated with the single predominant 39-kDa protein eluted from the poly(C) beads. The results of these studies suggest that at least three proteins, one of which directly binds poly(C) sequences, interact with the α-3′UTR. The presence of additional binding proteins in the complex, which may not be well visualized because of poor labeling, cannot be excluded by this result.

NW analysis with a ^{32}P-poly(C) probe was carried out with the affinity-purified fractions to determine if the 39-kDa protein isolated with the α-3′UTR contains poly(C)-binding activity. As expected, the 39-kDa band in the poly(C) affinity-purified preparation bound the poly(C) probe, as did the protein in the starting S100 extract. An identically migrating 39-kDa poly(C)-binding protein was present in the eluate of the α-3′UTR beads but was absent from the eluate of the poly(U) beads (Fig. 5C). Therefore, the same poly(C)-binding protein appears to be isolated by the α-3′UTR and by the poly(C) affinity probes.

The ability of the affinity-purified proteins to reconstitute the α-complex was tested (Fig. 6). Extracts from the single-step purification procedure (Fig. 5A) were used in these studies to maximize protein yield. Eluates from the α-3′UTR–poly(U) hybridization beads were competent to form an RNP complex that comigrated with the α-complex, while an eluate prepared in parallel from the poly(C) and poly(U) beads failed to form the α-complex. The complex formed by the proteins in the α-3′UTR eluate was sequence specific, as it formed on the wild-type α-3′UTR but not on a 3′UTR containing a destabilizing mutation (mutation 9). These data suggest that the three proteins isolated by the α-3′UTR are most likely sufficient for α-complex formation. However, as noted above, it cannot be ruled out that additional poorly visualized proteins in this fraction may also contribute. One of the prominent proteins identified in the complex is a 39-kDa poly(C)-binding protein. This protein is referred to as the α-globin 3′UTR poly(C)-binding protein or α-PCBP. Thus, α-PCBP is necessary but not sufficient for α-complex formation.

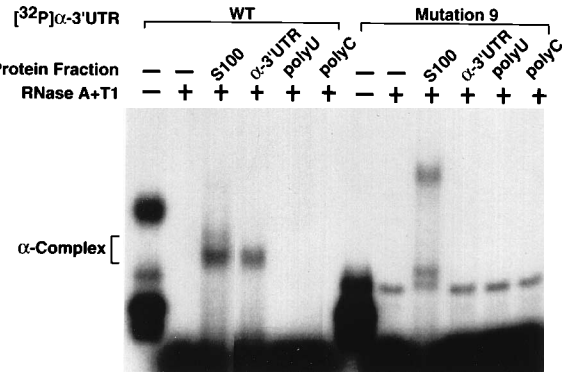

FIG. 6. Reconstitution of α-complex with RNA affinity-purified proteins. Samples whose analyses are shown in Fig. 5A were desalted and renatured prior to the RNA-protein-binding reaction (see Materials and Methods). ^{32}P-labeled wild-type (WT) α-3′UTR and mutation 9 RNAs were incubated with identical protein sample aliquots, labeled above each lane (Protein Fraction), RNase treated, and resolved on a nondenaturing PAGE gel.

To further substantiate the presence of α-PCBP in the α-complex, we carried out the experiment shown in Fig. 7. Three incubations with S100 extract were carried out in parallel and analyzed on a native gel. The first contained ^{32}P-labeled α-3′UTR (Fig. 7, lane 3), the second contained no RNA (lane 4), and the third contained an unlabeled α-3′UTR substrate (lane 5). An autoradiograph of the former reaction (Fig. 7, lane 3) marked the position of the α-complex. The latter (unlabeled) reactions (Fig. 7, lanes 4 and 5) were transferred to a membrane and probed with ^{32}P-poly(C). As can be seen in Fig. 7 (the middle panel), α-PCBP in the absence of the α-3′UTR (lane 4) migrates more slowly than the α-complex and shifts to a position identical to that of the α-complex upon addition of the RNA substrate (lane 5). Recruitment of α-PCBP to the α-complex was substantiated, and the size of the protein was confirmed by excision of a segment of the gel containing the α-complex and the corresponding segment of the adjacent lane (the reaction mixture lacking the α-3′UTR) and analysis of them by SDS-PAGE (Fig. 7, lanes 6 and 7, respectively). As is seen in the right panel of Fig. 7, the poly(C)-binding protein in the α-complex is identical in size to α-PCBP, and this protein is absent from the corresponding segment of the band shift gel when the α-3′UTR is not present in the incubation mixture. These data directly demonstrate the recruitment of α-PCBP to the α-complex.

α-PCBP is a novel cytoplasmic RNA-binding protein. Two nuclear poly(C)-binding proteins, a 68-kDa hnRNP K and a 66-kDa hnRNP J, have been previously characterized (37, 59). To distinguish the identity and cellular distribution of the 39-kDa α-PCBPs from those of hnRNPs K and J, we carried out an NW analysis of cytoplasmic and nuclear protein fractions prepared from MEL and HeLa cells with ^{32}P-poly(C) as a probe. Three proteins, of 39, 66, and 68 kDa, bound directly to ^{32}P-poly(C). The 39-kDa poly(C)-binding protein was predominantly cytoplasmic, while the 68- and 66-kDa proteins were, as expected, predominantly nuclear. The 39-kDa poly(C)-binding protein is present in both mouse and human cells and in nonerythroid as well as erythroid cells (Fig. 8A). To confirm the identities of the 66- and 68-kDa proteins, the MEL cell nuclear extract lane was stripped and subsequently probed with monoclonal antibody 3C2 (37), an antibody to hnRNPs K and J (Fig.

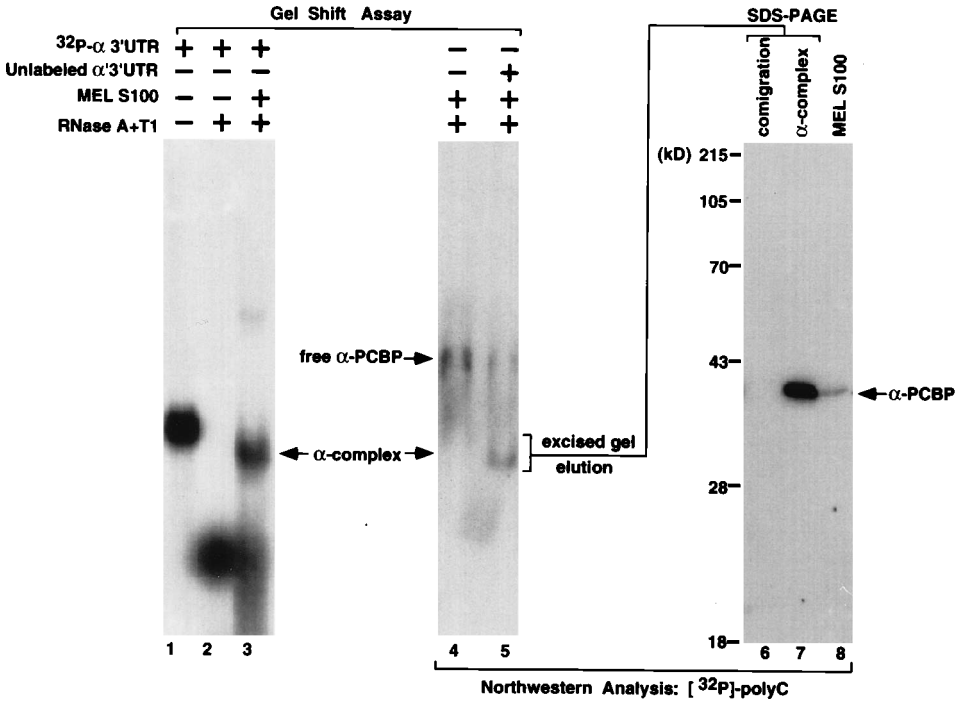

FIG. 7. α-PCBP is a component of the α-complex. The left panel shows an RNA gel shift analysis carried out as described in the Fig. 1 legend; the autoradiograph was developed by direct exposure of the gel to film. The middle panel is an NW analysis of a gel shift assay. The formation of the unlabeled mRNP complex (lane 5) was carried out in parallel with that of the labeled complex (lane 3), and electrophoresis was done on the same native gel. The migration position of the unlabeled α-complex (lane 5) is identified by the radioactive complex (lane 3). Proteins in lanes 4 and 5 were transferred to a nitrocellulose membrane and probed with ^{32}P-poly(C) to identify the positions of the poly(C)-binding protein in the absence and presence of the α-3′UTR, respectively. The right panel is an NW analysis of an SDS-PAGE gel. Lane 6, a control gel section taken from the lane containing MEL S100 extract but no mRNA excised at the position corresponding to the α-complex (as shown in lane 4); lane 7, the protein eluted from a gel slice of a native band shift gel containing the α-complex (as in lane 5); lane 8, total MEL S100 extract.

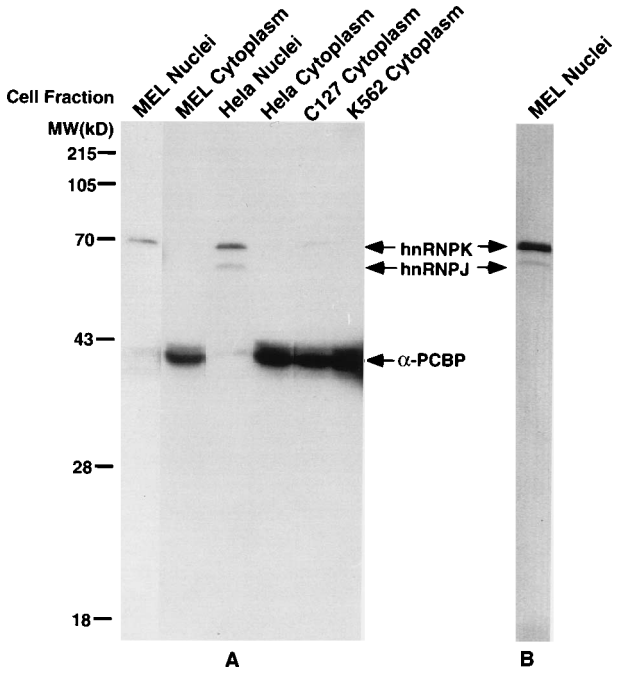

FIG. 8. The α-complex contains a novel cytoplasmic poly(C)-binding protein. (A) NW blot analysis with a ^{32}P-poly(C) probe demonstrates that the α-PCBP is predominantly cytoplasmic. Each cell fraction is labeled above the respective lane, and the positions of the protein molecular weight standards (not shown) are indicated to the left of the autoradiograph. (B) α-PCBP is distinct from hnRNP K and J. The MEL cell nuclear extract of the blot shown in panel A (after stripping) was incubated with monoclonal antibody 3C2, which is specific to hnRNP K and J. The signal was visualized with enhanced chemiluminescence reagents.

8B). Only the 68- and 66-kDa proteins were recognized by this antibody. There was no cross-reaction with a 39-kDa band when the remaining lanes were similarly tested (not shown). These data demonstrate that the poly(C)-binding protein component of the α-complex is neither hnRNP K nor hnRNP J but appears to represent a novel cytoplasmic RNA-binding protein.

DISCUSSION

The results presented in this report demonstrate that the 3′UTR of α-globin mRNA is able to interact with a subset of cytosolic proteins to form a sequence-specific RNP complex (Fig. 1). Formation of this complex parallels α-globin mRNA stability. All nine α-3′UTR mutations tested by gel shift assay showed a coincidence between α-complex formation in vitro and α-globin mRNA stability in vivo; mutations which destabilize the α-globin mRNA in vivo destabilize the complex in vitro while mutations which have minimal effect on stability in vivo have no significant effect on complex formation (Fig. 2). This suggests that α-complex formation on the α-globin 3′UTR may function to preserve the stability of the α-globin mRNA.

Our previous studies demonstrated that the α-globin mRNA stability element is composed at least in part of C-rich stretches in the α-3′UTR. The present studies support this finding. Base substitutions in three discontinuous C-rich stretches that destabilize mRNA also disrupt α-complex formation. It is clear that these C-rich stretches do not function independently, as mutation of any one of these segments is sufficient to destabilize the α-globin mRNA and to disassemble the α-complex (Fig. 2). This suggests that these C-rich stretches are not redundant but instead may each contribute to the formation of a higher-order mRNA structure that can be recognized by a set of *trans*-acting factors.

Formation of the α-complex appears to depend on the interaction of several proteins with discontinuous segments of the mRNA. The extreme sensitivity of the α-complex to poly(C) competition suggests that one of these proteins is a poly(C)-binding protein (Fig. 3). This is confirmed by the affinity chromatography studies. Identical 39-kDa bands are purified by both poly(C) and α-3′UTR–poly(U) affinity chromatography (Fig. 5). This protein can be directly identified in α-3′UTR affinity-purified protein by NW analysis with a ^{32}P-poly(C) probe (Fig. 5C). Two additional, closely grouped proteins, of 43 and 42 kDa, specifically bind as part of a complex to the α-3′UTR but not to poly(C). This suggests that these additional proteins are distinct non-poly(C)-binding proteins. The ability of the α-3′UTR–polyU-purified extract to reconstitute the α-complex (Fig. 6) suggests that the three α-3′UTR proteins identified by the two-step affinity purification may be sufficient for complex formation. Since the actual reconstitution experiment was carried out with the less pure single-step preparation, it is possible that additional minor proteins are also involved in complex formation. The demonstration that the 39-kDa poly(C)-binding protein is an integral part of the complex (Fig. 7) yet cannot by itself interact with the α-3′UTR to form the α-complex (Fig. 6) suggests that α-PCBP and one or more non-poly(C)-binding proteins are required for α-complex formation and α-globin mRNA stability.

The 39-kDa component of the α-complex, poly(C)-binding protein (α-PCBP), can be distinguished from the well-characterized poly(C)-binding proteins hnRNP K and J on the basis of size and cellular distribution (Fig. 8). In contrast to the nuclear localization of hnRNPs K and J, α-PCBP is present in the cytoplasm. The absence of poly(C)-binding proteins other than hnRNPs K and J in the nuclear extracts in prior studies (37, 59) is consistent with our findings. Furthermore, α-PCBP does not appear to contain the major epitope shared by hnRNPs K and J. Although it is possible that α-PCBP might represent a proteolytic product of the K or J protein, the difference in cellular distribution and the fact that even minor deletions in K and J result in a loss of poly(C)-binding activity (58) make this very unlikely. α-PCBP may be related to a recently described poly(C)-binding protein of undefined function (1) or may represent an as-yet-unidentified protein. The poly(C)-binding protein described in the present report is conserved from mice to humans and functionally contributes to α-globin mRNA stability.

Is the α-complex shared with other coregulated mRNAs? β-Globin is the major protein coexpressed with α-globin in adult erythroid cells. Our analysis failed to reveal any evidence for a shared mechanism for stabilization of both globin mRNA species. This lack of a conserved mechanism is surprising, as it suggests that two parallel stabilizing mechanisms may have evolved in the globin gene system. Consistent with the lack of a common messenger RNP complex (Fig. 2A) are the facts that the α- and β-globin 3′UTRs do not have any significant or recognizable sequence homology and the β-globin mRNA 3′UTR specifically lacks C-rich segments. This lack of a common posttranscriptional control mechanism for α- and β-globin mRNAs may parallel the transcriptional regulation of the two respective genes, which also appear to be quite different (54). Differences in apparent stability control between the closely related α- and β-tubulin mRNAs have also been noted (4), suggesting that in fact multiple parallel mechanisms may be at work for highly similar mRNAs in a single cell type. We

predict that different mechanisms will be found to control β-globin and α-globin mRNA degradation.

Despite the highly restricted nature of α-globin gene expression, the protein components of the α-complex appear to be ubiquitous (Fig. 4). As determined by gel shift analysis, an α-complex is formed from nonerythroid as well as erythroid cells. Consistently, a 39-kDa poly(C)-binding protein is detected in a wide range of cells by NW blot analysis (Fig. 8). This lack of tissue specificity for the proteins critical to the α-complex suggests that the tissue-specific nature of the α-3′UTR stability element (60) may reflect an additional component of mRNA decay such as a specific RNase. In addition, the ubiquitous nature of the α-complex proteins suggests that these proteins must have general and highly conserved functions in addition to the α-3′UTR binding.

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