Stabilization and cytoskeletal-association of LDL receptor mRNA are mediated by distinct domains in its 3′ untranslated region

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Abstract **The mRNA encoding the human low density lipoprotein (LDL) receptor is transiently stabilized after phorbol ester treatment of HepG2 cells and has been shown to associate with components of the cytoskeleton in this cell line (G. M. Wilson, E. A. Roberts, and R. G. Deeley, *J. Lipid Res.* 1997.** 38: **437–446). Using an episomal expression system, fragments of the 3′ untranslated region (3′UTR) of LDL receptor mRNA were transcribed in fusion with the coding region of β-globin mRNA in HepG2 cells. Analyses of the decay kinetics of these β-globin-LDL receptor fusion mRNA deletion mutants showed that sequences in the proximal 3′UTR of LDL receptor mRNA including several AU-rich elements (AREs) were sufficient to confer short constitutive mRNA half-life in the heterologous system. Stabilization of LDL receptor mRNA in the presence of PMA required sequences in the distal 3′UTR, at or near three *Alu*-like repetitive elements. Furthermore, the 3′UTR of LDL receptor mRNA conferred cytoskeletal association on the otherwise unassociated β-globin mRNA, by a mechanism involving at least two distinct RNA elements. Comparisons of decay kinetics and subcellular localization of endogenous LDL receptor mRNA and β-globin-LDL receptor mRNA fusions in HepG2 cells have demonstrated that several *cis*-acting elements in the receptor 3′UTR contribute to post-transcriptional regulation of receptor expression, and provide further support for involvement of the cytoskeleton in the regulation of LDL receptor mRNA turnover.—** Wilson, G. M., M. Z. Vasa, and R. G. Deeley. **Stabilization and cytoskeletal-association of LDL receptor mRNA are mediated by distinct domains in its 3′ untranslated region. *J. Lipid Res.* 1998.** 39: **1025–1032.**

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The liver plays a critical role in the maintenance of cholesterol and serum lipoprotein homeostasis. It is the major site of de novo synthesis of cholesterol (1), as well as the production of lipoprotein particles required for transport of cholesterol, triglycerides, and fatty acids to peripheral tissues (2, 3). In addition, the liver plays an important role in lipoprotein efflux from the circulation and is responsible for the excretion of cholesterol via bile salt formation (4, 5). Regulation of the expression of hepatic low density lipoprotein (LDL) receptors, which are responsible for binding and internalization of cholesterol-rich LDL particles from the plasma, is a key component of this process. The importance of hepatic LDL receptor expression is clearly demonstrated by the elevated plasma cholesterol levels seen in individuals with familial hypercholesterolemia (FH), in which one or both alleles of the LDL receptor gene locus do not encode functional receptors (reviewed in 2, 6, 7). Introduction of functional hepatic LDL receptors to homozygous FH patients by liver transplantation (8) or ex vivo gene therapy (9) has been shown to significantly lower plasma cholesterol in these individuals.

The regulation of hepatic LDL receptor expression has been extensively studied using the highly differentiated human hepatoblastoma cell line HepG2 (10, 11). In this cell line, LDL receptor mRNA levels have been shown to be repressed in response to cholesterol and lipoprotein loading (12, 13), resulting in decreases in LDL binding (14) and degradation (15). In addition, several signal transduction pathways, including the cyclic AMP, inositol 1,4,5-trisphosphate-Ca^{2+}, and diacylglycerol-protein kinase C (PKC) systems (16, 17), have been shown to mediate receptor mRNA induction in HepG2 cells, and activation of PKC by treatment with phorbol esters results in increased LDL-binding and catabolism (18). Activation of these signal transduction pathways is known to promote intracellular events required for cell proliferation or differentiation in many systems (reviewed in 19–21). Thus, induction of LDL receptor expression by these stimuli

Abbreviations: AER, AU-rich element; DEPC, diethylpyrocarbonate; DMSO, dimethylsulfoxide; FCS, fetal calf serum; FH, familial hypercholesterolemia; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDL, low density lipoprotein; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; UTR, untranslated region.

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could be involved in the recruitment of cholesterol for new membrane synthesis in both hepatic and non-hepatic tissues.

Previously, we demonstrated that treatment of HepG2 cells with the phorbol ester, phorbol-12-myristate-13-acetate (PMA), resulted in an induction of LDL receptor mRNA involving both transcriptional activation and transient stabilization of receptor mRNA (22). In addition, we provided evidence that LDL receptor mRNA may associate with components of the cytoskeleton in HepG2 cells, and that the integrity of the actin cytoskeleton influences the rate of LDL receptor mRNA degradation. In particular, disruption of the actin cytoskeleton with cytochalasin D increased the constitutive stability of LDL receptor mRNA, but abrogated further stabilization by phorbol esters. These results suggested that components of the mRNA degradative machinery may be associated with cytoskeletal structures in HepG2 cells, raising the possibility that subcellular localization of some mRNAs directly contributes to their turnover rates. In this study, we have made use of an episomal expression vector system that allows the effects of specific sequences on the decay and subcellular localization of β-globin-fusion mRNAs to be monitored (23). This plasmid, termed pC7βG, was used to identify *cis*-acting regions of LDL receptor mRNA contributing to its stability and cytoskeletal interaction. We demonstrate that the 3′ untranslated region (3′UTR) of receptor mRNA is capable of conferring short constitutive half-life, stabilization in response to PMA treatment, and association with components of the cytoskeleton when fused to the coding region of β-globin mRNA. Analyses of deletion mutants have identified distinct regions of LDL receptor mRNA involved in each of these processes.

MATERIALS AND METHODS

Materials

[^{32}P-α]dATP (3000 Ci/mmol), [^{32}P-α]UTP (3000 Ci/mmol), and Reflection autoradiography film were purchased from NEN (Mississauga, ON); MagnaCharge nylon membranes were from MSI (Westborough, MA); formaldehyde solution (37% w/w) was from Fisher (Nepean, ON); electrophoresis grade agarose was from ICN Canada (Montreal, PQ); phorbol-12-myristate-13-acetate (PMA) and diethylpyrocarbonate (DEPC) were from Sigma (St. Louis, MO); actinomycin C_1 and hygromycin B were purchased from Boehringer Mannheim Canada (Laval, PQ); tissue culture media and fetal bovine serum were from Gibco/BRL (Burlington, ON); iron-supplemented calf serum was from HyClone (Logan, UT); and restriction enzymes were from Gibco/BRL, Promega (Madison, WI), and New England Biolabs (Mississauga, ON). T4 DNA ligase and the Klenow fragment of *E. coli* DNA polymerase I were from Gibco/BRL. All other chemicals were obtained from BDH (Toronto, ON).

Plasmid constructions

Subcloning procedures were performed as described (24) unless otherwise noted. The fidelity of all plasmid constructs was verified by restriction mapping and dideoxy sequencing with Sequenase v2.0 (US Biochemical, Cleveland, OH). The full length LDL receptor 3′UTR fusion clone pC7βG-LDLR3′UTR was constructed by subcloning a 2.7 kbp *Xho*I fragment from pLDLR3 (American Type Culture Collection : ATCC, Rockville, MD) containing the 3′ 39 nucleotides of the receptor coding sequence and entire 3′UTR into the *Xho*I site of pC7βG (23). Similarly, Δ4230 was constructed by subcloning the 1.6 kbp *Xho*I + *Pvu*II fragment of pLDLR3 into the polylinker of pC7βG which had been digested with *Asc*I, filled in with Klenow, and digested with *Xho*I. All other LDL receptor cDNA fragments subcloned in fusion with β-globin were generated by polymerase chain reaction (PCR) amplification of pLDLR3 from various oligonucleotide primer sets (**Fig. 1** and **Fig. 2**) using *Pfu* polymerase and Perfect Match® polymerase enhancer (Stratagene, La Jolla, CA). Amplified fragments were subcloned into pC7βG after digestion with appropriate restriction enzymes, listed in Fig. 2.

Cell culture and transfections

The hepatoblastoma cell line HepG2 (ATCC) was maintained in minimum essential medium (MEM) supplemented with 10% iron-supplemented calf serum. Cells were maintained in MEM with 10% fetal calf serum during transfections. HepG2 cells were transfected by calcium phosphate co-precipitation (24), and stably transfected cells selected in 300 μg/mL hygromycin B as described previously (23).

Preparation of RNA and Northern analyses

Total RNA was isolated from cultured cells using TRIzol reagent (Gibco/BRL), according to the manufacturer's instructions. The poly(A)$^+$ RNA fraction was isolated using the polyATtract system (Promega). Total or poly(A)$^+$ RNA was fractionated on 1.2% formaldehyde agarose gels and transferred onto nylon membranes as described previously (22).

RNA blots were probed with cDNA fragments that had been radiolabeled by random priming with [^{32}P-α]dATP (25). The *Kpn*I + *Nhe*I coding region fragment of β-globin cDNA from pC7βG or the *Pac*I + *Hpa*II fragment upstream of the SV40 late polyadenylation signal from the same plasmid were used to probe for β-globin and β-globin-fusion mRNAs expressed in HepG2 cells. Blots were also probed with an *Xba*I + *Pst*I fragment of human GAPDH cDNA from pHcGAP (ATCC) as a loading control. Blot hybridization and washing conditions were identical to those described previously (22).

Assays of mRNA decay

Actinomycin time course assays were used to monitor the decay of β-globin or β-globin-fusion mRNAs in stably transfected cell populations essentially as described (22). Cells were maintained in MEM + 10% FCS without hygromycin B for 24 h prior to actinomycin treatment. First-order decay constants and resulting mRNA half-life values were calculated as described (22). Assessment of significance between mRNA decay constants was performed using the paired Student's *t* test at *n*-2 degrees of freedom (26). Significant differences were considered to be those with $P < 0.05$.

Analyses of cytoskeletal-mRNA association

Fractionation of polysomes from HepG2 cells into soluble and cytoskeletal-associated pools was performed by a modification of the protocol of Zambetti et al. (27). Briefly, cell monolayers were washed in 1 × PBS containing 0.5 mm EDTA. Cells were scraped from plates in lysis buffer (10 mm PIPES [pH 6.8], 100 mm KCl, 2.5 mm $MgCl_2$, 0.5% Triton X-100: 5 mL/10^7 cells) containing 0.3 m sucrose. DEPC (0.25%) was included to inhibit endogenous ribonuclease activity. Lysates were incubated on ice for 3 min and centrifuged at 500/*g* for 3 min. The cytoskeletal-associated polysome fraction (pellet) was resuspended directly in TRIzol

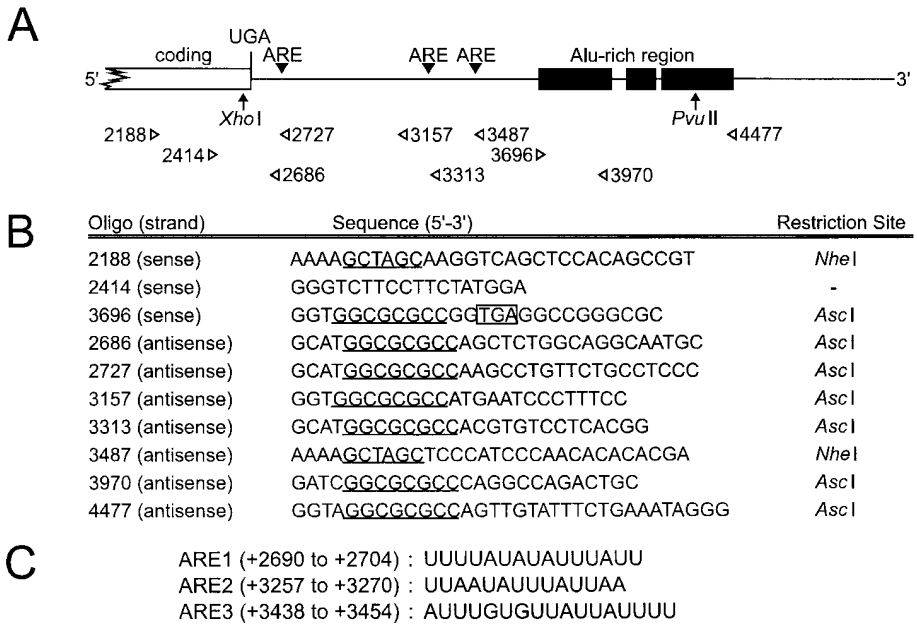

Fig. 1. Oligonucleotides used in construction of pC7βG-LDL receptor fusion clones. (A) A schematic of the 3′ end of the coding sequence and 3′ untranslated region of LDL receptor mRNA. Shown are the location of three putative AU-rich elements (AREs : solid triangles) in the proximal 3′UTR as well as a cluster of three *Alu*-like repetitive sequences (solid boxes) in the more distal 3′UTR. The location of *Xho*I (+2544) and *Pvu*II (+4230) restriction sites used in the construction of several deletion clones are indicated by arrows. The location and orientation of each oligonucleotide primer used for PCR-amplification of receptor cDNA sequences is shown by the open triangles. All numbering is relative to the A residue of the translational initiation codon. (B) The sequence of each oligonucleotide is shown, and incorporated restriction sites are underlined. The translational termination codon included in oligo 3696 is boxed. (C) The sequence of each putative ARE in the proximal 3′UTR of LDL receptor mRNA is shown. Candidate AREs were selected based on homology to the nonameric sequence UUAUUUAUU, identified as the minimal element contributing to rapid turnover of several mRNAs encoding immediate early genes and cytokines (29).

reagent. Soluble polysomes (supernatant) were recovered by precipitation with ethanol and resuspended in TRIzol. Resuspension of each fraction in TRIzol required repeated passage through a 23-gauge needle. Total RNA was then isolated from each TRIzol lysate according to the manufacturer's instructions. For detection of low abundance mRNAs, poly(A)$^+$ RNA fractions were isolated as described above. Specific mRNAs were quantified from equal cellular equivalents of each RNA fraction by Northern blotting and quantitative densitometry.

RESULTS

Localization of determinants of constitutive stability of LDL receptor mRNA and regions required for PMA-induced stabilization

In HepG2 cells, endogenous LDL receptor mRNA was shown to decay with a half-life of approximately 45 min, and was stabilized 2-fold after treatment with 160 nm PMA for 1.5 h (22). To determine whether elements in the 3′ untranslated region of receptor mRNA contributed to the regulation of its stability, the 3′UTR of human LDL receptor cDNA was subcloned into the polylinker of pC7βG. The expression cassette of this vector, termed pC7βG-LDLR3′UTR, encoded a fusion mRNA containing a short 5′UTR and the complete coding sequence from β-globin mRNA linked to the 3′ 39 nucleotides of coding sequence and the entire 3′UTR of LDL receptor mRNA (**Fig. 3A**). Transcription of the cassette was initiated from the human cytomegalovirus immediate early promoter, and terminated by the late polyadenylation/termination signal from SV40. Maintenance of the open reading frame of β-globin ensured that translation of the βG-LDLR3′UTR fusion mRNA would be terminated at the endogenous STOP codon of LDL receptor mRNA.

HepG2 cells stably transfected with pC7βG-LDLR3′ UTR expressed a 3.3 kb β-globin-LDL receptor fusion mRNA. Actinomycin C_1 time course analyses demonstrated that this mRNA (βG-LDLR3′UTR) decayed with a constitutive half-life of 26 min (**Fig. 3B**, **C**). Treatment of these cells for 1.5 h with 160 nm PMA stabilized the fusion mRNA 2- to 2.5-fold ($P < 0.01$) resulting in an mRNA half-life of 59 min. Previously, the half-life of β-globin mRNA expressed from pC7βG in stably transfected HepG2 cells had been measured at approximately 10 h (23). These data indicate that elements within the 3′UTR of receptor mRNA contribute to both the short constitutive half-life and stabilization in response to PMA treatment associated with endogenous LDL receptor mRNA in HepG2 cells.

To identify discrete elements in the receptor 3′UTR that contribute to the regulation of its turnover, a series of βG-LDLR3′UTR fusion constructs were made in which various regions of the 3′UTR of LDL receptor mRNA had been deleted (Fig. 2). Episomes encoding these fusion

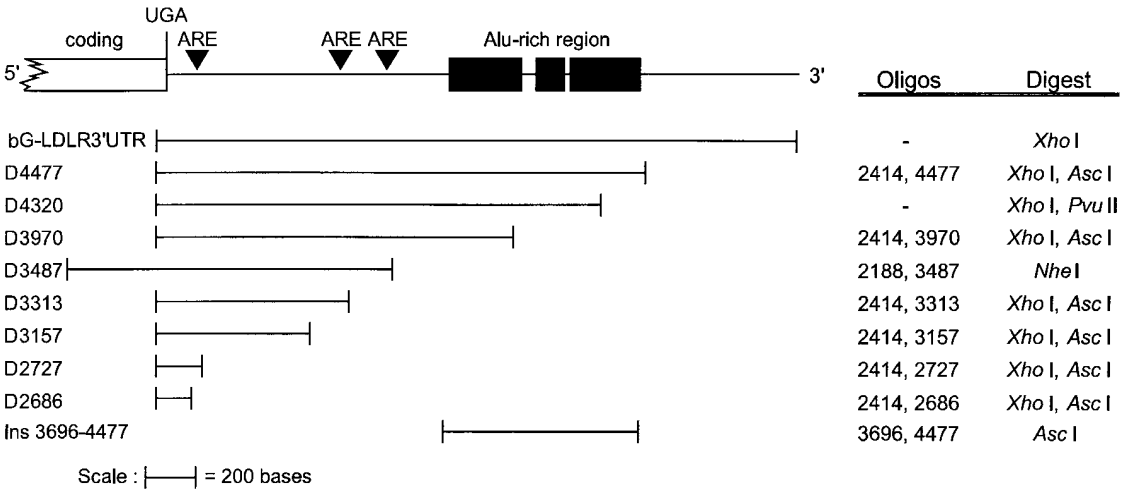

Fig. 2. Deletion clones of pC7βG-LDLR3′UTR. This figure shows the fragments of LDL receptor cDNA subcloned in fusion with the coding region of β-globin cDNA in the transcription cassette of pC7βG. All fragments were PCR-amplified from pLDLR3 using the oligonucleotide primer sets shown at right, with the exceptions of βG-LDLR3′UTR and Δ4230 (detailed in Materials and Methods). Amplified fragments were then digested with the restriction enzymes listed at far right and subcloned into the appropriate restriction sites in the pC7βG polylinker.

mRNAs were stably transfected into HepG2 cells, and mRNA half-lives were determined in the presence or absence of PMA.

Fusion of 3′UTR sequences upstream of the first putative AU-rich element (ARE) had little effect on constitutive mRNA stability (cf. Δ2686 and β-globin) (**Fig. 4A**). However, addition of the following 40 nucleotides, encompassing this ARE, resulted in a greater than 3-fold in-

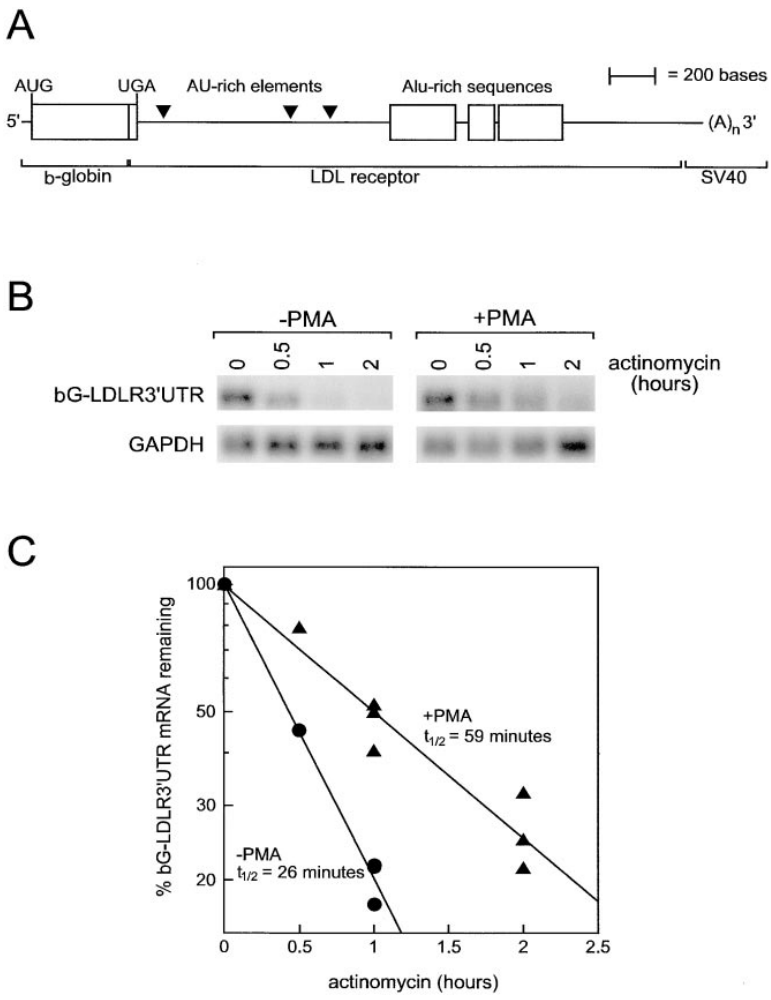

Fig. 3. Stability of βG-LDLR3′UTR mRNA in HepG2 cells. (A) Schematic of the mRNA encoded by the expression cassette of pC7βG-LDLR3′UTR. The 5′UTR and coding sequence from β-globin were fused in-frame to the 3′ 39 nucleotides of the LDL receptor coding region and entire 3′UTR of receptor mRNA. The late polyadenylation signal from SV40 terminated transcription of the construct. (B) pC7βG-LDLR3′UTR was stably transfected into HepG2 cells as described in Materials and Methods. Actinomycin C_1 time course assays were used to monitor the turnover of βG-LDLR3′UTR-fusion mRNA in these cells after treatment for 1.5 h with 0.02% DMSO (−PMA) or 160 nm PMA (+PMA). At indicated actinomycin time points, poly(A)$^+$ RNA was isolated, and βG-LDLR3′UTR and GAPDH mRNAs were quantified by Northern analysis of poly(A)$^+$ RNA. A representative blot is shown in this panel. A fragment immediately upstream of the SV40 polyadenylation signal was used to probe for βG-LDLR3′UTR mRNA to ensure that the fusion transcript was not terminated from the upstream LDL receptor poly(A) signal. (C) Scanning densitometry was used to quantify the relative levels of βG-LDLR3′UTR and GAPDH mRNAs at each actinomycin time point. Data were plotted as the percentage of βG-LDLR3′UTR mRNA remaining normalized to GAPDH mRNA levels either in the absence (−PMA: circles, solid line) or presence (+PMA: triangles, dashed line) of 160 nm PMA. mRNA half-life values were calculated as described in Materials and Methods.

crease in the rate of fusion mRNA turnover (cf. Δ2727 and Δ2686). Similar AU-rich sequences have been extensively implicated in the rapid turnover of many cytokine and proto-oncogene mRNAs (28, 29). Inclusion of downstream AREs was also shown to accelerate fusion mRNA decay, to the extent that Δ3487, containing all three AREs, decayed with a half-life of 48 min, similar to the 45 min constitutive half-life of endogenous LDL receptor mRNA in HepG2 cells (22). The constitutive mRNA destabilizing activity contributed by the 800 nucleotides of receptor 3′UTR containing these three AREs was thus greater than 10-fold (cf. Δ2686 and β-globin with Δ3487).

Stabilization of β-globin-fusion mRNAs in response to PMA was observed for the three longest constructs, Δ4230, Δ4477, and βG-LDLR3′UTR (Fig. 4B). The Δ3970 fusion mRNA, containing the first *Alu* repeat, demonstrated no stabilization in response to PMA treatment. Δ4230 mRNA contained the two 5′ *Alu*-like repeats and the proximal half of the 3′ repeat, while all three *Alu*-like repeats are contained within Δ4477 mRNA.

Localization of elements in the LDL receptor 3′UTR involved in cytoskeletal association of polysomes

Previously, we have demonstrated that LDL receptor polysomes in HepG2 cells are largely cytoskeletal-associated, suggesting that LDL receptor polysomes are associated with both the endoplasmic reticulum and the cytoskeleton (22). To test whether determinants of cytoskeletal-association might be located in the 3′UTR of LDL receptor mRNA, polysomes from several populations of HepG2 cells stably transfected with β-globin-LDL receptor fusion clones were separated into free and cytoskeletal-associated fractions based on differential solubility in a non-ionic detergent (27). It was anticipated that β-globin mRNA should not localize to the membranes of the endoplasmic reticulum because it lacks the appropriate signal se-

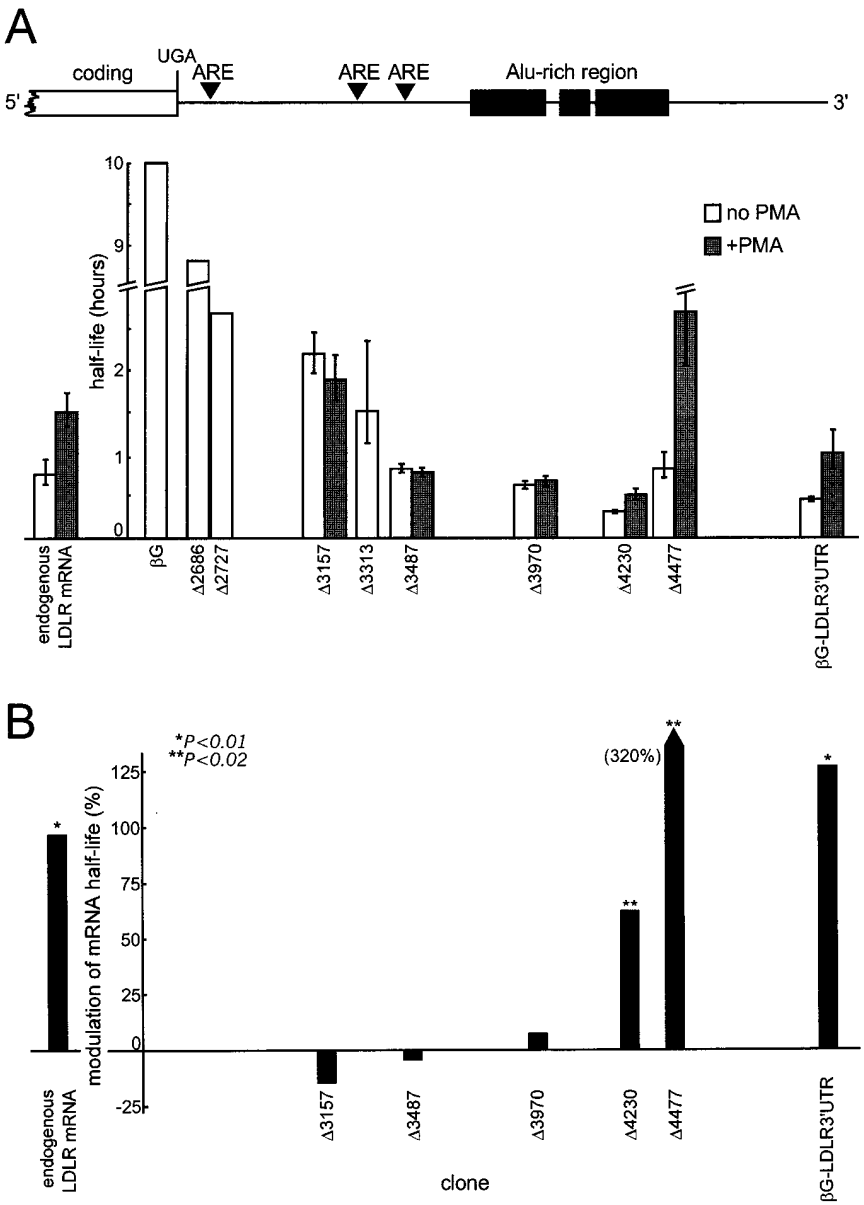

Fig. 4. Decay kinetics of β-globin-LDL receptor fusion mRNAs in HepG2 cells. The half-lives of β-globin-fusion mRNAs were determined by actinomycin C_1 time course assays as described in Materials and Methods. (A) This histogram illustrates the half-lives of fusion mRNAs in stably transfected HepG2 cells after treatment with 0.02% DMSO (−PMA: open bars) or 160 nM PMA (+PMA: shaded bars) for 1.5 h. The horizontal position of each data set indicates the most 3′ nucleotide of LDL receptor mRNA sequence in that fusion mRNA as compared with the schematic above. The height of each bar illustrates the half-life of each fusion mRNA ± SE. As the first-order rate constant, *k*, is calculated based on regression slopes of actinomycin time course data, s_e becomes large relative to *k* when the decay of stable ($t_{1/2} > 2.5$ h) mRNAs is examined. Accordingly, rigorous quantitative comparisons of many longer-lived mRNA species were not possible. At far left, the half-life of endogenous LDL receptor mRNA in HepG2 cells ± PMA is shown for comparison (22). (B) In this panel, the relative change in half-life after PMA treatment is shown for several β-globin-LDL receptor fusion mRNAs. Again, the stabilization of endogenous LDL receptor mRNA after PMA treatment of HepG2 cells is indicated at left (22). Significant differences in mRNA decay kinetics were determined by paired Student's *t* test as described in Materials and Methods and are highlighted by asterisks.

quences for membrane targeting. When HepG2 cells stably expressing β-globin mRNA were lysed in 0.5% Triton X-100, essentially all β-globin polysomes were recovered in the Triton-soluble fraction, indicating that β-globin polysomes did not associate with components of the cytoskeleton in these cells (**Fig. 5**). In contrast, when HepG2 cells stably expressing βG-LDLR3′UTR mRNA were fractionated by this technique, greater than 70% of βG-LDLR3′UTR polysomes were recovered in the Triton-insoluble fraction, strongly suggesting that elements within the 3′UTR of LDL receptor mRNA were capable of binding components of the cytoskeleton. Pre-treatment of these cells with 160 nm PMA for 1.5 h did not affect the subcellular distribution of LDL receptor polysomes (data not shown). Similar analyses using HepG2 cells expressing β-globin-LDL receptor deletion mRNAs demonstrated that sequences between +2727 and +3157 of the receptor 3′UTR could confer some ability to associate with the cytoskeleton (≈25% of Δ3157 polysomes recovered in the insoluble fraction), but additional 3′ sequences were required for maximal localization (cf. 43% of Δ3313 polysomes recovered in the insoluble fraction; 62% of Δ3970 polysomes). The observation that a fusion mRNA lacking sequences between +2727 and +3157 was also partially recovered in the Triton-insoluble fraction (Ins 3696–4477) indicated that at least two distinct regions of the LDL receptor 3′UTR were capable of associating with cytoskeletal components (Fig. 5). Based on the increased recovery of the longer fusion mRNAs in cytoskeletal-associated polysome fractions (βG-LDLR3′UTR and Δ3970 versus Δ3157 and Ins 3696–4477), it seems likely that cytoskeletal-binding from these domains is additive.

The data shown in Fig. 5 initially suggested that the lengths of transcripts recovered in the soluble and cytoskeletal-associated polysome fractions differed. However, several additional control experiments revealed that the difference in electrophoretic mobility was attributable to sample composition rather than mRNA size. First, Northern analyses of RNA extracted from whole cells did not reveal two size populations of any of the β-globin-fusion transcripts tested (Fig. 3B and data not shown). Second, agarose gels of Triton-fractionated polysomal RNAs showed similar differences between the mobilities of 18S and 28S ribosomal RNAs in the Triton-soluble and -insoluble fractions(data not shown). Finally, mixing Triton-soluble and -insoluble mRNAs containing Δ3313 mRNA prior to electrophoresis and Northern analysis revealed migration of this chimeric mRNA in a single size population (data not shown).

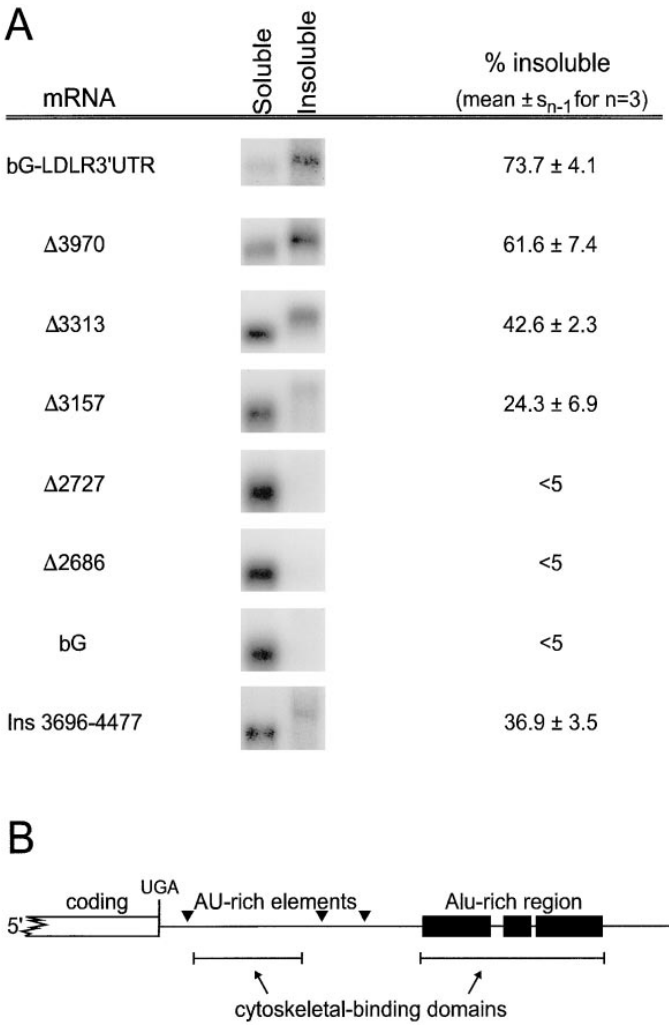

Fig. 5. Cytoskeletal-association of β-globin-LDL receptor fusion mRNAs in HepG2 cells. (A) Polysomes from HepG2 cells stably transfected with each of the listed β-globin-LDL receptor fusion clones were separated into Triton-soluble and -insoluble fractions as described in Materials and Methods. Equal cellular equivalents of total or poly(A)$^+$ RNA from each fraction were loaded in adjacent lanes of 1.2% formaldehyde agarose gels and Northern blotted. Blots were probed for β-globin-fusion mRNAs and signals on the resulting autoradiographs were quantified by scanning densitometry. An example of the distribution of polysomes containing each β-globin-fusion mRNA is shown in this panel, while the mean distributions ± standard deviations of triplicate experiments are listed at right. β-globin, Δ2686, and Δ2727 mRNAs were virtually undetectable in RNA extracted from Triton-insoluble fractions. An upper limit of 5% insoluble has been quoted in each of these cases, as this represents the range of linearity of the densitometer used. (B) This panel illustrates the location of two putative cytoskeletal-binding domains within the LDL receptor 3′UTR, based on the polysome distributions shown in (A).

DISCUSSION

Fusion of the 3′UTR of LDL receptor mRNA to the coding region of β-globin mRNA was shown to decrease the constitutive stability of the fusion transcript approximately 20-fold relative to β-globin mRNA alone. PMA treatment of HepG2 cells stably expressing this fusion mRNA also resulted in a 2- to 2.5-fold increase in fusion mRNA half-life, which is similar to the phorbol ester-induced stabilization of endogenous LDL receptor mRNA (22). These results indicate that sequences in the receptor 3′UTR function as *cis*-acting determinants of both constitutive mRNA stability and the increase in stability induced by phorbol ester treatment.

The majority of RNA sequences implicated in the determination of mRNA decay rates have been localized to 3′UTRs (30). In particular, AU-rich elements (AREs) have been identified in the 3′UTRs of many short-lived mRNA species and are believed to exert their destabilizing effects through interactions with specific ARE-binding proteins (28). In the LDL receptor 3′UTR, three putative AREs were identified in the proximal 3′UTR based on their similarity to the nonameric motif UUAUUUAUU (Fig. 1). Insertion of a single copy of this element into a heterologous fusion mRNA has been shown to significantly accelerate fusion mRNA turnover, while multiple overlapping copies function as even more potent destabilizers (29). The binding affinity of one ARE-binding protein, termed AUF1, has been shown to correlate closely with the destabilizing potential of its target RNA sequence (31). Inclusion of the most 5′ ARE (ARE1) of the LDL receptor 3′UTR into a β-globin-fusion mRNA (Δ2727) resulted in a 3-fold increase in its turnover rate. ARE1 includes two overlapping copies of the UUAUUUAUU motif, with a single U→A substitution. Addition of receptor 3′UTR sequences containing all three AREs to the coding region of β-globin mRNA resulted in further destabilization of the β-globin-fusion transcripts. Based on our half-life studies of βG-LDLR3′UTR deletion clones, we have demonstrated that sequences in the proximal 3′UTR of LDL receptor mRNA, including these ARE-like sequences, significantly contribute to the short constitutive half-life exhibited by the endogenous receptor mRNA.

While sequences in the proximal 3′UTR of LDL receptor mRNA dramatically increased the constitutive turnover rate of β-globin-fusion mRNAs in HepG2 cells, stabilization of these mRNAs was not observed following PMA treatment. Fusion mRNAs containing more distal 3′UTR sequences, however, were stabilized in cells treated with the phorbol ester. In particular, inclusion of sequences containing the second and third *Alu*-like repeats conferred PMA-induced stabilization, suggesting that an inducible *cis*-acting mRNA stability determinant is present in this region.

mRNA stabilization in response to phorbol ester treatment has been intensively studied in the regulation of ribonucleotide reductase expression. In murine fibroblasts, mRNAs encoding both the R1 and R2 subunits of ribonucleotide reductase are stabilized after PMA treatment by a mechanism involving short *cis*-acting sequences in their 3′UTRs (32, 33). Furthermore, binding of specific cytoplasmic proteins to these elements has been shown to correlate with rapid turnover of these mRNAs. Phorbol ester treatment of murine fibroblasts results in a decrease in binding of these *trans*-acting factors and concomitant stabilization of the ribonucleotide reductase mRNAs (33, 34). It is unclear, however, whether a similar mechanism applies to the regulation of LDL receptor mRNA stability, as the distal 3′UTR of receptor mRNA contains no sequences similar to those identified in ribonucleotide reductase mRNAs.

Having defined the region responsible for PMA-induced stabilization to the distal 3′UTR of LDL receptor mRNA, we investigated whether sequences responsible for cytoskeletal-association of this mRNA might be similarly located. We found that association between LDL receptor polysomes and components of the cytoskeleton occurs through at least two distinct elements in the receptor mRNA 3′UTR (Fig. 5B). In HepG2 cells, β-globin-fusion mRNAs containing LDL receptor 3′UTR sequences between +2727 and +3157 (Δ3157) as well as +3696 to +4477 (Ins 3696–4477) were partially recovered in detergent-insoluble cellular fractions. For fusion mRNAs containing sequences from both of these regions (Δ3970, βG-LDL R3′UTR), larger proportions (up to 65% of the fusion transcript) were found associated with the cytoskeletal fraction, suggesting that multiple determinants of subcellular localization may operate in an additive or cooperative fashion in these mRNAs.

In chicken fibroblasts, a 68 kDa protein has recently been purified and cloned that binds the cytoskeletal-association domain of β-actin mRNA (35). In this case, two adjacent ACACCC sequences have been shown to be critical for high affinity binding. Mutations in these sequences that inhibit protein binding also disrupt cytoskeletal-targeting of β-actin polysomes. However, similar sequences are not present in the cytoskeletal-association domains of the receptor 3′UTR, suggesting that distinct binding mechanisms may be involved.

We have shown previously that disruption of the cytoskeleton with cytochalasin results in stabilization of LDL receptor mRNA to the same extent as treatment with PMA (22). Furthermore, in the absence of an intact cytoskeleton, PMA has no additional effect on mRNA stability. A possible explanation for these observations is that the attachment of the mRNA to the cytoskeleton via sequences in the 3′UTR may destabilize the mRNA by increasing the proximity between some components of the degradative machinery and their targets which are also present in the 3′UTR. If so, PMA could increase stability by causing either dissociation or inactivation of components of the degradative machinery from the cytoskeleton or by protecting labile sites in the distal 3′UTR. A corollary of this model is that cytoskeletal attachment should be a prerequisite for PMA-induced stabilization. For the β-globin-LDL receptor fusion mRNAs examined to date, this relationship has been maintained.

Association of mRNAs with cytoskeletal structures has

been extensively studied as a mechanism of targeting nascent polypeptide products (36). However, our studies suggest that cytoskeletal-association of a subset of polysomal RNAs including LDL receptor mRNA may be involved in the regulation of mRNA turnover. Such a mechanism could conceivably induce major posttranscriptional changes in gene expression patterns during periods of cytoskeletal stress or reorganization which may occur at certain stages of development or cellular differentiation.

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