Analysis of the AU-Rich Elements in the 3′-Untranslated Region of β_2-Adrenergic Receptor mRNA by Mutagenesis and Identification of the Homologous AU-Rich Region from Different Species†

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ABSTRACT: The 35000-M_r β-adrenergic receptor mRNA binding protein (βARB) is induced by β-adrenergic agonists and binds to G-protein-linked receptor mRNAs that exhibit agonist-induced destabilization. Recently, we identified a 20-nucleotide, AU-rich region in the 3′-untranslated region of the hamster β_2-adrenergic receptor mRNA consisting of an AUUUUA hexamer flanked by U-rich regions, which constitutes the binding domain for βARB. U to G substitution in the hexamer region attenuates the binding of βARB, whereas U to G substitution of hexamer and flanking U-rich domains abolishes binding of βARB and stabilizes β_2-adrenergic receptor mRNA levels in transfectant clones challenged with either isoproterenol or cyclic AMP. In the study presented here, we mutated the 20-nucleotide ARE region to establish the minimal AU-rich sequence required for βARB binding. U to G substitutions of flanking poly(U) regions and of the hexamer established the nature of the binding properties. Using various mutants, we demonstrated also that binding of βARB correlates with the extent of destabilization of β_2-adrenergic receptor mRNA in response to agonist stimulation. High-affinity binding of hamster, rat, mouse, porcine, and human ARE sequences to βARB was revealed by SDS−polyacrylamide gel electrophoresis following UV-catalyzed cross-linking and by gel mobility shift assays. Further, βARB was shown to bind more avidly to the 20-nucleotide ARE region than to well-established mRNA destablization sequences of tandem repeats of five pentamers. Thus, for β_2-adrenergic receptor, mRNA destabilization likely occurs via conserved AU-rich elements present in the 3′-untranslated regions of receptor mRNAs.

Agonist-induced downregulation of G-protein-linked receptors, typified by the β_2-adrenergic receptor, provides a major explanation for long-term adaptation to chronic stimuli characteristically observed for members of this receptor family (*1*−*5*). Levels of the β_2-adrenergic receptor and its mRNA decline following a challenge with agonist (*2*, *3*), reflecting a post-transcriptional destabilization of receptor mRNAs (*6*). We have previously identified a 35000-M_r β_2-AR mRNA-binding protein (βARB)[1] that might mediate this destabilization (*7*). This protein exhibits several properties (*8*, *9*) similar to those of AU-rich element (ARE) binding RNA destabilizing proteins reported by others (*10*−*13*). βARB recognizes a 20-nucleotide ARE in the 3′-untranslated region (UTR) of hamster β_2-AR mRNA constituted by an AUUUUA hexamer flanked by U-rich regions (*14*). β_2-AR mRNA appears to be similar to other highly regulated mRNAs [e.g., mRNAs of granulocyte/macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), and the oncogenes *c-myc* and *c-fos*] that are markedly influenced by alterations of their rates of degradation (*15*−*19*). In addition to the ARE of β_2-AR mRNA, cognate sequences of 3′-untranslated regions (UTR) of mRNA, such as the tandem repeats of AUUUA pentamers (*10*−*13*, *20*−*22*) and nonamers, such as UUAUUUA(U/A) (U/A) (*23*) and UUAUUUAUU (*24*), are important elements in determining RNA stability.

RNA-binding proteins implicated in regulating mRNA stability and turnover include the following: heterogeneous, nuclear ribonucleoprotein particles (hnRNPs; *25*−*27*); small nuclear RNA-binding proteins that participate in the splicing and further processing of pre-mRNAs possessing introns, a 5′-cap, and a 3′-poly(A)+ tract (*28*, *29*); cytosolic mRNA binding protein such as the 72000-M_r poly(A)-binding protein which binds to long stretches (∼25 nucleotides per protein) of poly(A)+ and stabilizes the RNA to 3′- to 5′-nuclease activity (*30*); and a subset of smaller (30000−40000-M_r) cytosolic mRNA-binding proteins that recognize the AREs

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[1] Abbreviations: ARE, AU-rich element; ORF, open reading frame; UTR, untranslated region; β_2-AR, β_2-adrenergic receptor; βARB, β-AR mRNA-binding protein.

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in the 3′-UTR (*7*, *9*−*12*, *31*, *32*). In addition to βARB (*7*−*9*), two GPLR mRNA-binding proteins have been identified. Those are an 85000-M_r factor identified in rat hepatocytes, which binds to β_2-AR transcripts (*33*), and a 37000-M_r AU-rich element RNA-binding/degradation factor (AUF1), which has been shown to bind β_1-AR mRNAs (*34*). The 35000-M_r βARB, in contrast, binds selectively to β_1- and β_2-adrenergic and thrombin receptor mRNAs (examples of G-protein-linked receptors with mRNAs exhibiting AU-rich domains), and fails to bind both rat and human β_3-AR mRNA (*9*). βARB is induced by agonist treatment, and its levels vary inversely with the level of receptor mRNA (*7*). Although AUF1 has been shown to bind to β_2-AR mRNA via the 3′-UTR, its nonidentity with βARB was established by immunoprecipitation and immunoblotting analysis of AUF1 polypeptides of UV cross-linking products (*34*).

We previously identified the cognate β_2-adrenergic receptor mRNA sequence necessary for binding of βARB by site-directed mutagenesis in tandem with stable expression in Chinese hamster ovary cells of wild-type receptors harboring mutated 3′-UTRs (*14*). A 20-nucleotide, AU-rich sequence consisting of an AUUUUA hexamer flanked by U-rich regions was shown to be obligate for binding of βARB and regulation of β_2-adrenergic receptor mRNA stability in vivo. In the work presented here, we identified the sequence determinants of the 20-nucleotide region by introducing three different U to G mutations into each of the flanking poly(U) regions, as well as into the core hexamer. Stable expression of receptor mRNA with and without mutations in the 3′-UTR region in Chinese hamster ovary cells demonstrates that the level of binding of βARB correlates with the extent of destabilization of the β_2-adrenergic receptor mRNA in response to agonist stimulation. We also tested highly homologous AREs of β_2-AR from rats, porcine, mice, and humans for binding properties to βARB.

EXPERIMENTAL PROCEDURES

Cell Culture. DDT_1-MF2 vas deferens smooth muscle cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (Hyclone), penicillin (60 μg/mL), and streptomycin (100 μg/mL) as described by Scarpace et al. (*35*). CHO cells were grown in F-12 medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone). Cells were treated with agonist (10 μM isoproterenol) for 24 h and used for the preparation of the S100 fraction.

Preparation of Cytosolic (S100) Extracts. Following drug treatment, cells were washed twice with phosphate-buffered saline (PBS), and removed from the plate with 1.0 mM EDTA in PBS. Approximately 5×10^7 cells were collected gently by low-speed (1000*g*) centrifugation, resuspended in PBS, transferred to a sterile polypropylene ultracentrifuge tube, and collected again gently by centrifugation. The PBS was aspirated from the cell pellet, and 5 μL aliquots of each of the protease inhibitors (10 mg/mL), aprotinin and leupeptin, were added to the cell pellet. The cells were then subjected to ultracentrifugation (100000*g*) for 90 min at 4 °C. The resulting supernatant fraction was transferred to Eppendorf tubes and maintained in an ice bath for immediate use. This cytosolic fraction is called the S100 fraction throughout the paper. The protein concentration was determined by method of Lowry et al. (*36*).

Mutagenesis and Plasmid Construction. Mutagenesis of β_2-adrenergic receptor cDNA in pSP70 was performed by overlap extension polymerase chain reaction as described previously (*14*). The mutated cDNAs were verified for the appropriate base substitution by direct sequencing and then cloned to expression vector pcDNA3.1 for stable transfection in CHO cells. Plasmids containing the 20-nucleotide AUUUUA hexamer flanked by poly(U) regions (nucleotides 1592−1611; *37*) as well as those with U to G substitutions M1, M2, M3, and DEC (decamer) and tandem copies of five AUUUA pentamers were constructed by using complementary synthetic oligonucleotides flanked by restriction sequences for *Hin*dIII at the 5′-end and *Cla*I at the 3′-end. Complementary oligonucleotides were annealed and cloned into pSP70. The resultant plasmids were linearized immediately 3′ to the AU-rich region and employed as templates for in vitro transcription. Similarly, AU-rich regions identified from rat (*38*), mouse (*39*), porcine (*40*), and human (*41*) β_2-AR cDNA sequences, which are homologous to the hamster sequence, were cloned as described for the hamster sequence. All constructs were sequenced to verify the orientation and sequence of the insert.

In Vitro Transcription. All of the constructs were made in the pSP70 plasmid vector using *Hin*dIII at the 5′-end and *Cla*I at the 3′-end, which were linearized immediately 3′ to the AU-rich region and employed as templates for in vitro transcription. Transcription was performed in vitro using SP6 DNA-directed RNA polymerase to produce uniformly labeled RNA, using the technique of Melton et al. (*42*). Briefly, mRNAs were transcribed in the presence of RNasin (Promega), and radiolabeled [α-^{32}P]UTP (800 Ci/mmol, Dupont NEN), with nucleotides and buffer conditions as described in detail by Promega. After the RNA was transcribed, RNase-free DNase was added to the mixture to remove template DNA. The labeled transcript was extracted with phenol after addition of 5 μg of yeast tRNA, and then with chloroform, and precipitated finally with 2.5 volumes of ice-cold ethanol and 0.1 volume of 3 M sodium acetate. The labeled transcript was reconstituted in RNase-free water, maintained at −80 °C, and used within 24 h of synthesis. The size and integrity of the transcripts were verified immediately prior to use by electrophoresis on a 5% acrylamide, 7 M urea gel.

UV Cross-Linking and Label Transfer. An aliquot of radiolabeled mRNA ($1-4 \times 10^6$ cpm), 5 μg of yeast tRNA, and competing unlabeled RNA transcripts (at the indicated molar excess over probe) were each added to a mixture containing the S100 cytosolic fraction (30−50 μg of total protein), 4 mM dithiothreitol, 5 μg of heparin, and 65 units of RNasin in a total volume of 50 μL. Aliquots of the mixture of the S100 cytosolic fraction and radiolabeled RNA were distributed in wells of a 96-well microtiter plate and allowed to incubate for 10 min at 22 °C. Samples were exposed to short-wave (254 nm) UV irradiation at a distance of 7 cm for 30 min. The RNA not cross-linked to protein was digested with RNase A (0.5 mg/mL) and RNase T1 (10 units/mL) at 37 °C for 30 min.

SDS−Polyacrylamide Gel Electrophoresis of RNA Protein Adducts. Samples were solubilized in 50 μL (1:1) of Laemmli loading buffer (*43*) for 10 min at 67 °C. The samples were then loaded onto a SDS−polyacrylamide gel (10% acrylamide separating gel with 5% acrylamide stack) and subjected to electrophoresis. Resolved proteins were stained with

Coomassie Blue R, and the gels were destained, dried, and subjected to autoradiography for 12−24 h. The relative intensities of radiolabeled species on the gel were quantified by direct analysis of radioactivity using a PhosphorImager (Molecular Dynamics).

RNA Gel Retardation Assay. Five to twenty-five milligrams of cytoplasmic lysates was incubated with different radiolabeled RNA probes ($1-4 \times 10^5$ cpm) in 15 mM HEPES (pH 7.9), 15 mM KCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM spermine, 1 mg/mL yeast tRNA, 5 mg/mL heparin, and 20 units of RNasin in a total volume of 20 mL for 10 min at 25 °C. Reaction mixtures were incubated on ice for 20 min, and complexes were resolved by electrophoresis on a 5% native polyacrylamide gel in 45 mM TBE (tris-borate-EDTA) as the running buffer. The gel was dried and visualized on a PhosphorImager and subjected to autoradiography. For competition experiments, different amounts of unlabeled RNAs and radiolabeled RNAs were mixed and incubated on ice for 10 min before addition of the cytosolic extract.

Transfection of CHO Cells. CHO wild-type cells were transfected with pcDNA3 harboring mutant or wild-type receptor cDNAs or empty vector plasmids, using Lipofectin (Life Technologies, Inc.). Stable transfectant clones were selected for neomycin resistance in F-12 medium containing 10% fetal bovine serum and G418 (400 μg/mL). The level of expression of β_2-adrenergic receptor was determined for CHO clones stably expressing receptors with wild-type and mutant 3′-UTRs by measuring receptor mRNA levels. Clones expressing similar levels of β_2-AR mRNA were used for the RNA stability study.

Determination of the β_2-AR mRNA Half-Life. CHO cells were pretreated with isoproterenol (10 μM) for 24 h, and actinomycin D (5 μg/mL) was added to arrest transcription at specific times. Total RNA was extracted from individual culture dishes at the indicated time, and the amount of receptor mRNA was quantified by using an RNase protection assay, as described previously (*9*). Radiolabeled, antisense riboprobes corresponding to a region 285 (1201−1486) nucleotides from the coding region of β_2-AR mRNA were employed for the RNase protection assay (*14*).

RESULTS

To explore the individual roles for 5′- and 3′-localized poly(U) regions and the central hexamer AUUUUA separately with respect to binding of βARB, we engineered three different mutations within the 20-nucleotide ARE region (Figure 1). U to G substitutions in the 5′- and the 3′-flanking poly(U) regions were termed mutants 1 and 3, respectively. Mutant 2 was constructed by U to G substitutions in the AUUUUA hexamer. The ability of the uniformly radiolabeled RNAs transcribed from these plasmids to bind βARB of S100 cytosolic extracts in vitro was determined using a UV-catalyzed cross-linking, label transfer assay, as well as using the ability of unlabeled wild-type and mutant RNA transcripts to compete with ^{32}P-labeled transcripts for binding to βARB. The cross-linked, radiolabeled RNA-binding protein adducts were subjected to SDS−polyacrylamide gel electrophoresis, made visible by autoradiography (Figure 2, left panel), and quantified from at least four independent experiments by phosphorimaging (Figure 2, right panel).

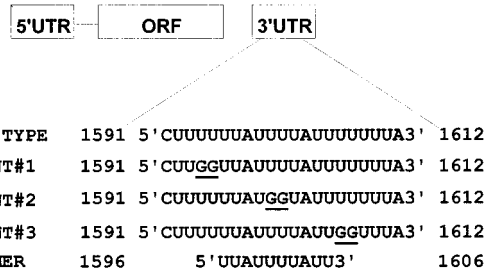

FIGURE 1: Mutations of the 3′-untranslated ARE region of the hamster β_2-AR cDNA designed to disrupt the hexamer AUUUUA and 5′- and 3′-flanking poly(U) regions. The 20-nucleotide ARE region from hamster β_2-AR cDNA harboring the AUUUUA hexamer (wild-type) was employed for mutagenesis. U to G substitutions were created in the 5′-flanking (mutant 1), 3′-flanking (mutant 3), poly(U) regions and in the core hexamer AUUUUA (mutant 2) so their roles in the binding by βARB could be studied. The sequence for the decamer (DEC) consists of an AUUUUA hexamer flanked by two U on either side. The various AREs were constructed using sequences from residue 1592 to 1611 of hamster β_2-AR cDNA, as described in Experimental Procedures.

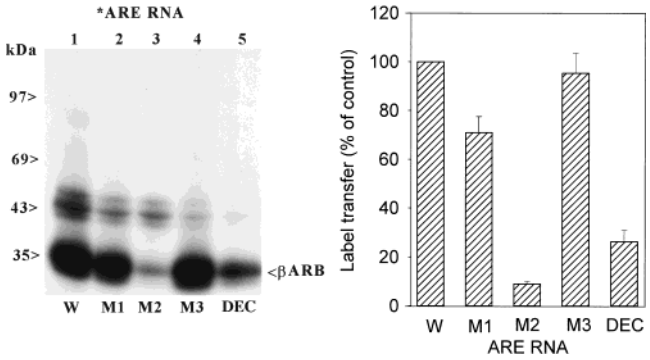

FIGURE 2: Mutational analysis of the 20-nucleotide 3′-UTR ARE region from hamster β-adrenergic receptor mRNAs. Effects on binding to βARB. (Left) Representative autoradiogram of products from UV-catalyzed cross-linking of S100 cytosolic fractions from DDT_1-MF2 cells with uniformly radiolabeled, in vitro-transcribed RNAs corresponding to the 20-nucleotide wild-type ARE region (lane 1, W), mutant 1 (lane 2, M1), mutant 2 (lane 3, M2), mutant 3 (lane 4, M3), or the decamer (lane 5, DEC). Equal amounts of S100 cytosolic protein and equimolar concentrations for each radiolabeled ARE RNA were employed for these studies. A prominently labeled species with an M_r of 35 000 in lanes 1, 2, and 4 is the βARB. (Right) Quantification of the ^{32}P-label transferred from RNAs with either the wild-type ARE or mutant ARE region to the 35000-M_r βARB. The values are displayed as "percent label transfer", setting the value of label transfer from the RNA harboring the wild-type ARE as 100%. Each value represents the mean ± the standard deviation of at least three separate experiments.

βARB (35000-M_r) was labeled prominently by the wild-type (W) 20-nucleotide ARE of the hamster receptor mRNA, while a few other, slower migrating proteins, less prominently labeled, were also visible. Mutation of the 3′-poly(U) region flanking the AUUUUA (M3) did not alter the ability of the transcript to bind βARB, whereas a similar mutation to the 5′-flanking region reduced the level of binding to βARB by only ∼25%. In contrast, interruption of the core TTTT of the hexamer (M2) nearly abolished the binding to βARB.

Next, we tested the role of the poly(U) tracts flanking the AUUUUA hexamer in the binding to βARB (Figure 2). Evidence has been presented indicating that the nonamer UUAUUUAUU is the minimal AU-rich sequence motif that triggers rapid deadenylation and decay of mRNA (*23*, *24*). Loss of one uridine residue from either end of the nonamer

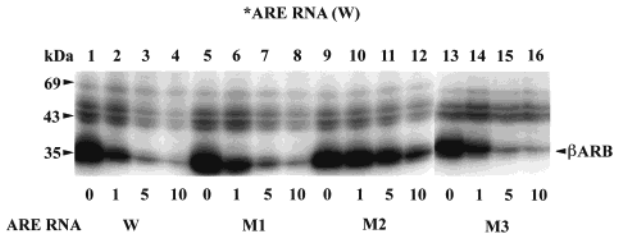

FIGURE 3: AREs harboring mutations in the central hexamer ARE region, but not the 3′-flanking poly(U) region, fail to compete with binding of wild-type ARE of β_2-AR mRNA to the βARB. Autoradiogram of UV-catalyzed cross-linking of S100 cytosolic fractions prepared from DDT_1-MF2 cells with capped, uniformly radiolabeled, in vitro-transcribed RNA corresponding to the wild-type ARE region from hamster β_2-adrenergic receptor. ^{32}P-labeled wild-type ARE RNA binding to βARB was subjected to competition by adding increasing amounts (5-, 10-, and 20-fold molar excesses) of unlabeled RNAs transcribed from the hamster ARE region with either wild-type ARE (lanes 2−4, W), mutant 1 (lanes 6−8, M1), mutant 2 (lanes 10−12, M2), or mutant 3 (lanes 14−16, M3) AREs harboring disruptions of the 5′-flanking poly(U), hexamer AUUUUA core, or 3′-flanking poly(U) region. Unlabeled RNAs and ^{32}P-radiolabeled RNA were added simultaneously to the mixtures containing the S100 cytosolic extracts. The mixture was incubated for 10 min, prior to UV-catalyzed cross-linking.

provokes a decrease in the destabilizing activity of the nonamer (*24*). To test the ability of a sequence like the nonamer to bind to βARB, we created a plasmid expressing a 10-nucleotide ARE consisting of TT on either end of a hexamer AUUUUA (Figure 1). Because mutation of the hexamer to a pentamer has been shown to reduce transcript binding of βARB by 50% (*14*), we decided to test the hexamer core flanked by UU on each end. The extent of label transfer of the radiolabeled 10-nucleotide decamer (DEC) to βARB was less than 25% of that observed with the 20-nucleotide ARE of the β_2-AR mRNA (Figure 2).

The binding of each mutant to βARB was tested further via competition studies. Unlabeled transcripts of the wild-type 20-nucleotide ARE of the β_2-AR mRNA and of the mutants with U to G substitutions in the 5′- and 3′-flanking, as well as a mutant of U to G in the hexamer region, were employed as competitors for the binding of the radiolabeled wild-type ARE RNA to βARB (Figure 3). The unlabeled M1 and M3 transcripts exhibited some capacity [less than that of the wild-type (W) unlabeled transcripts] to compete with ^{32}P-labeled wild-type ARE RNA for label transfer to βARB. The M2 competed poorly with ^{32}P-labeled wild-type ARE RNA for label transfer to βARB, even at a 10-fold molar excess. The results from multiple competition studies revealed a rank order of competition as follows: wild-type ARE (greatest) $\approx$ M3 > M1 $\gg$ M2 (least), confirming the results from the label transfer studies (Figure 2).

Agonist-mediated changes in receptor mRNA levels can be studied in cell lines stably transfected and expressing G-protein-linked receptors (*14*, *44*). Wild-type CHO cells express few endogenous β_2-AR and provide an ideal cell type for the study of agonist-induced regulation of β_2-AR, once stably transfected with expression vectors (*14*). Direct label transfer studies performed with S100 fractions from the CHO cells confirmed the presence of βARB in these cells (*14*). To address the influence of βARB binding on agonist-induced destabilization of β_2-AR mRNA, we transfected CHO cells with vectors expressing wild-type (TTTTTATTTTATTTTTTA) β_2-AR mRNA as well as two receptor mRNAs with mutantions M4 (TTGGTTATGGTATTTTTTTA) and M5 (TTTTTTATTTTATTGGTTTA) introduced into the ARE region. Each mutant was selected for the study of mRNA stability on the basis of their βARB binding properties. U to G substitutions in the AUUUUA hexamer and 5′-poly(U) region of the ARE (M4) abolished binding of βARB to β_2-AR mRNA (Figure 2 and ref *14*). Mutations in the 3′-poly(U) region flanking the AUUUUA hexamer (M5) did not alter the ability of the transcript to bind βARB (Figure 2). CHO clones stably transfected and expressing similar levels of β_2-AR mRNA were created for the wild-type and mutated 3′-untranslated regions. The stability of the receptor transcript was assessed in cells challenged with β-adrenergic agonist. These clones were treated with isoproterenol (10 μM) for 24 h, and receptor mRNA levels were quantified at the indicated time by using an RNase protection assay (Figure 4A) following the addition of actinomycin D to arrest transcription.

In the CHO clones expressing the wild-type β_2-AR mRNA, the half-life of the receptor mRNA was found to be greater than 12 h as reported previously (*14*). The half-life for β_2-AR mRNA in DDT1-MF2 cells expressing endogenous receptor was established to be 12−15 h (*6*). In the clones expressing the wild-type β_2-AR mRNA, the half-life was reduced to $\sim$7 h by challenge with agonist (left panels of parts A and B of Figure 4). CHO clones expressing M4 were tested for agonist-induced downregulation of β_2-AR mRNA. Disruption of the AUUUUA hexamer and the 5′-poly(U) region not only abolished the binding of the transcript to βARB (Figure 2) but also attenuated sharply the destabilization of the β_2-AR mRNA in response to agonist treatment (middle panels of parts A and B of Figure 4). In clones expressing β_2-AR with mutation of the 3′-poly(U) region alone (M5), agonist-induced downregulation was similar to that of clones expressing wild-type β_2-AR mRNA (right panels of parts A and B of Figure 4). Taken together, these results demonstrate that binding of the βARB is critical in the agonist-induced destabilization of β_2-AR mRNA.

The AUUUA pentameric sequence is highly conserved and often repeated in tandem in the 3′-untranslated region of RNAs that encode short-lived cytokines and protooncogenes. The presence of the multiple copies of this pentamer appears to alter the stability of the mRNAs in which they reside (*13*). Tandem repeats of more than three copies of AUUUA pentamers are thought to increase the effectiveness of the ARE region in protein binding and mRNA destabilization (*45*). On the basis of these observations, we compared the ability of the 20-nucleotide ARE region of the β_2-AR mRNA to that of tandem repeats of an AUUUA pentamer to bind βARB. A 21-nucleotide ARE region consisting of five tandem repeats of the AUUUA pentamer was transcribed from the same plasmid vector and restriction sites that were used to generate the hamster ARE sequence. In the direct label transfer assay, the uniformly labeled 21-nucleotide ARE consisting of five AUUUA pentamers (Figure 5, upper panel) exhibited <50% of the level of binding to βARB of that of the 20-nucleotide wild-type ARE of β_2-AR mRNA (Figure 5, upper panel). Binding of hamster ARE versus tandem repeats of AUUUA pentamer was investigated further through competition studies in which we assessed the ability of the unlabeled, 20-nucleotide ARE from hamster and tandem copies of pentamers to compete with radiolabeled

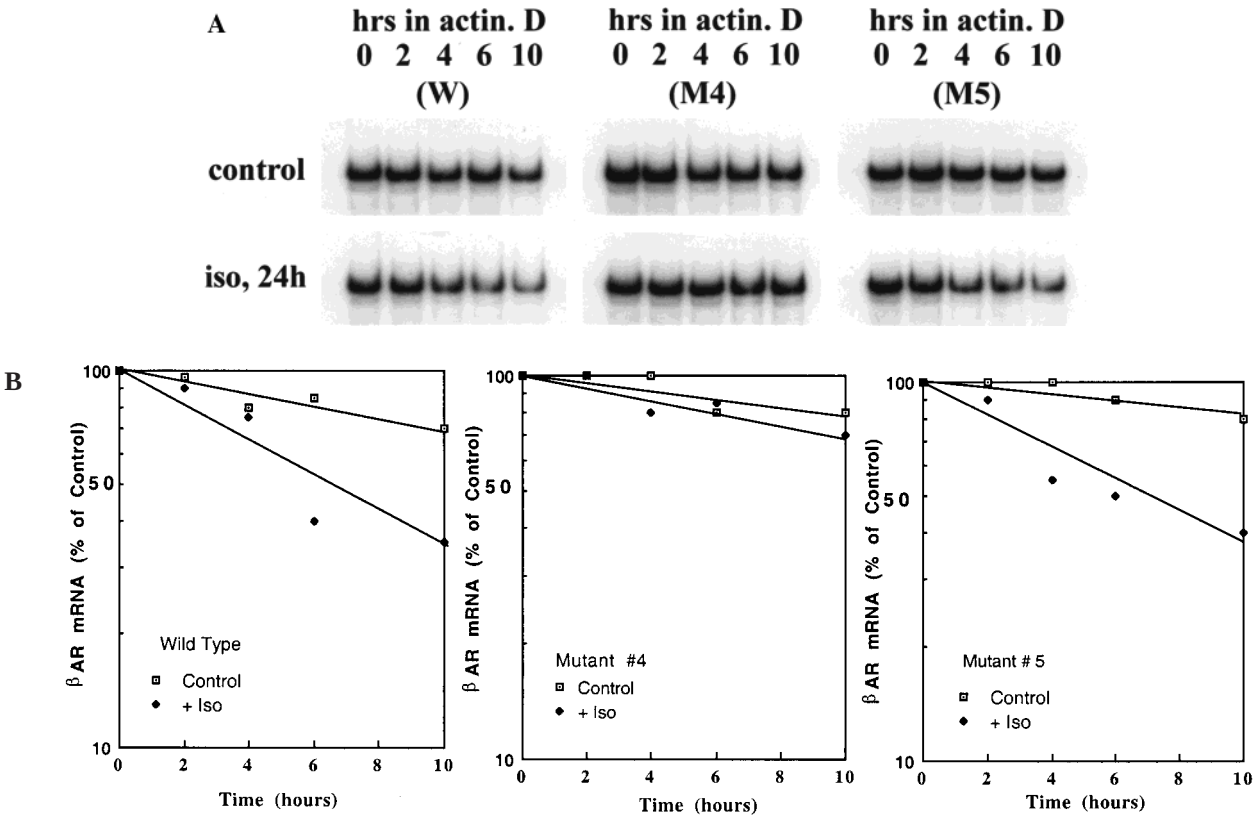

FIGURE 4: Disrupting the βARB binding domain of the ARE region in the β_2-AR mRNA abolishes agonist-induced destabilization of the receptor mRNA in vivo. (A) Autoradiograms obtained from RNase protection analysis of β_2-AR mRNA isolated from CHO cells stably transfected with β_2-AR harboring wild-type 3′-UTR (left panel), a mutation interrupting the AUUUUA hexamer and 5′-flanking poly(U) regions (M4, middle panel), or a mutation interrupting the 3′-flanking poly(U) regions (M5, right panel). Cells were challenged with either no agent (control) or β-adrenergic agonist (isoproterenol, iso), and the half-life of β_2-AR mRNA was determined, as described in Experimental Procedures. (B) Quantification of autoradiograms obtained from RNase protection analysis of β_2-AR mRNA isolated from CHO cells transfected with the wild type and mutants 4 and 5. The RNase-resistant bands were quantified by phosphorimaging analysis of each band. The autoradiograms are representive of at least three replicate experiments. Quantitative data are the mean values ± the standard deviation of three replicate experiments for each, β_2-AR mRNA with wild-type 3′-UTR or 3′-UTR with mutation 4 or 5.

hamster ARE for binding to βARB (Figure 5, upper panel). The unlabeled tandem pentamer ARE RNA competed poorly with the radiolabeled hamster ARE for binding to βARB. Quantification of data from replicate, independent label transfer studies in which both labeled AREs were prepared and used simultaneously revealed that the hamster (hexamer-containing) ARE is the preferred binding species for βARB (Figure 5, lower panel).

The binding of wild-type, 20-nucleotide hamster ARE and of the 21-nucleotide ARE containing tandem repeats of five pentamers $(AUUU)_5$ was analyzed independently by mobility shift assays on nondenaturing gels (Figure 6). Radiolabeled RNA corresponding to the 20-nucleotide hamster ARE and tandem repeats of five AUUUA pentamers were prepared under identical conditions. Equal amounts of radiolabeled RNA transcripts were incubated with increasing amounts of the cytosolic fraction from hamster vas deferens DDT_1-MF2 cells. The reaction products were then resolved by polyacrylamide gel electrophoresis under native, nondenaturing conditions. Formation of protein−RNA complexes of both labeled RNA species with the cytosolic extract was evident in the autoradiograms of the native gels following electrophoresis. Increasing the amount of cytosolic extract led to a mobility shift for the labeled RNA probes that was much more prominent for the complexes with the 20-nucleotide wild-type ARE. With higher amounts of cytosolic protein, the 20-nucleotide wild-type ARE, but not the 21-nucleotide RNA harboring five pentameric AUUUAs, was shifted quantitatively to a slower-migrating complex (Figure 6). Thus, the ARE region of β_2-AR mRNA has a higher affinity for βARB than do multiple pentamers.

To further characterize the βARB binding motif, we identified highly homologous ARE regions present in the 3′-UTR of β_2-AR mRNAs of rat, mouse, porcine, and human cDNA, and employed them as naturally occurring mutant forms of the 20-nucleotide hamster ARE (Figure 7A). These potential β_2-AR mRNA AREs represent those in all mammals for which sequence information on the 3′-untranslated regions is available. We engineered pSP70 plasmids expressing the putative AREs (identified solely on the basis of sequence homology) in the 3′-untranslated regions from rat, mouse, porcine, and human cDNA. Uniformly radiolabeled, capped RNAs corresponding to each of these probes were prepared under identical conditions, and their binding to βARB was tested by direct label transfer. The radiolabeled ARE regions from mouse, rat, porcine, and human species were shown to bind to the βARB with a magnitude similar to that of the 20-nucleotide hamster ARE (Figure 7B). Quantification of the data from replicate label transfer experiments revealed essentially equivalent amounts of binding of each of the species-specific AREs to βARB. Next, competition experiments in which unlabeled RNA made from

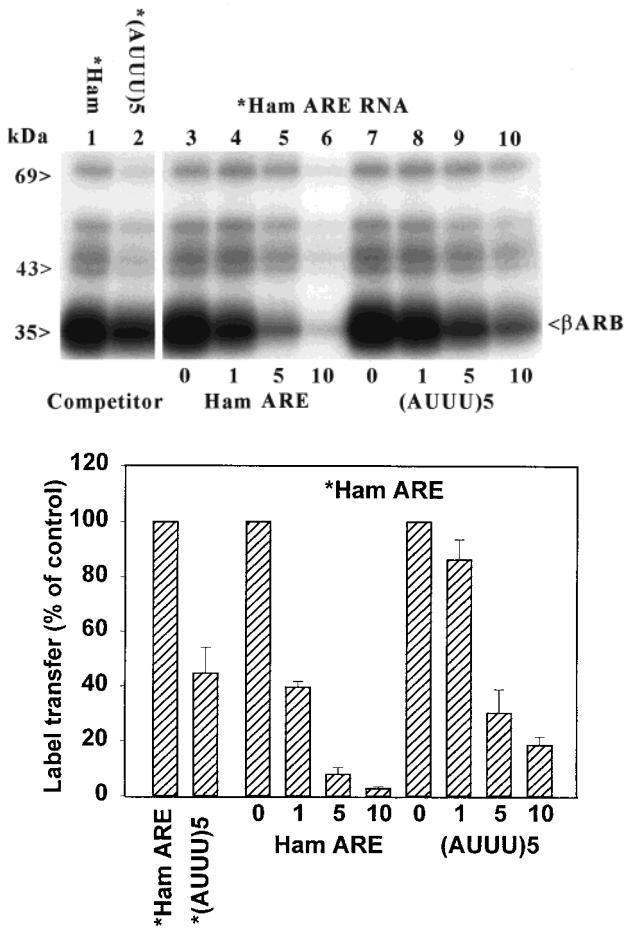

FIGURE 5: βARB binding of hamster 20-nucleotide ARE as compared to that of an ARE harboring tandem repeats of five AUUUA pentamers. (Top) Representive autoradiogram of products from UV-catalyzed cross-linking of S100 cytosolic fractions from DDT_1-MF2 cells using uniformly radiolabeled, in vitro-transcribed RNAs corresponding to either the 20-nucleotide hamster β_2-AR mRNA ARE (lane 1, *Ham) or the 21-nucleotide tandem pentamer AUUUA-containing ARE [lane 2, *(AUUU)5]. Competition experiments for βARB binding by molar excesses of unlabeled, 20-nucleotide hexamer-containing ARE (Ham ARE, lanes 4−6) and 21-nucleotide tandem repeats of five pentamers [(AUUU)5, lanes 8−10] vs radiolabeled 20-nucleotide hexamer-containing ARE were performed. Note that the hexamer-containing ARE is preferred to the ARE with tandem repeats of five pentamer AUUUAs. (Bottom)-Quantification of the ^{32}P-label transferred from hamster ARE and ARE with tandem repeats of five pentamers as well as the competition studies. The values are displayed as "percent label transfer", setting the value of label transfer from the hamster ARE as 100%. Each value represents the mean ± the standard deviation of at least three separate experiments.

hamster, mouse, rat, porcine, and human ARE regions were employed to compete with the binding of the radiolabeled hamster ARE RNA to βARB were performed (Figure 8). The autoradiograms revealed that unlabeled ARE RNA from mice, rats, pigs, and humans competed as well as the hamster ARE for binding of labeled hamster ARE to βARB. Quantification of data from replicate competition experiments confirmed the results obtained by direct label transfer studies (data not shown).

We employed a mobility shift assay in a manner complementary to the UV-catalyzed cross-linking and direct label transfer assay to test further the binding of the species-specific ARE regions to βARB. When cytosolic extracts from DDT_1-MF2 cells were incubated with radiolabeled, in vitro-transcribed RNA corresponding to the 20-nucleotide ARE region from hamster β_2-AR cDNA and subjected to electrophoresis, we observed a shift of the free probe to a slower-

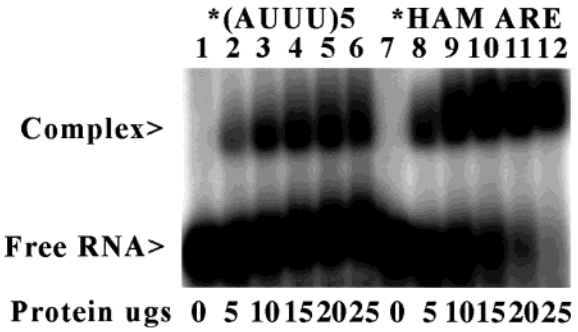

FIGURE 6: Gel mobility shift assay for binding to cytosolic extracts and hamster 20-nucleotide ARE as compared to that of an ARE harboring tandem repeats of AUUU motifs. The binding of a radiolabeled ARE containing tandem repeats of five AUUUA motifs as compared to that of the hamster 20-nucleotide ARE of β_2-AR mRNA was analyzed by electrophoretic mobility shift assays. Increasing amounts of cytosolic extracts of DDT_1-MF2 smooth muscle cells were incubated with ^{32}P-labeled ARE RNA probes and analyzed by electrophoresis on nondenaturing, native gels, as described in Experimental Procedures.

A

HAMSTER	127	5'CUUUUUUAUUUUAUUUUUUUA3'
RAT	129	5'CUUUUUUAUUUUAGGUUUUUA3'
MOUSE	130	5'CUUUUUUUAAAUUUUAUUUUAUUUUUUA3'
PORCINE	124	5'CUUUUUAUUUUUUUAUUUUUUUA3'
HUMAN	139	5'CUUUUUAUUUUUUUA3'

B

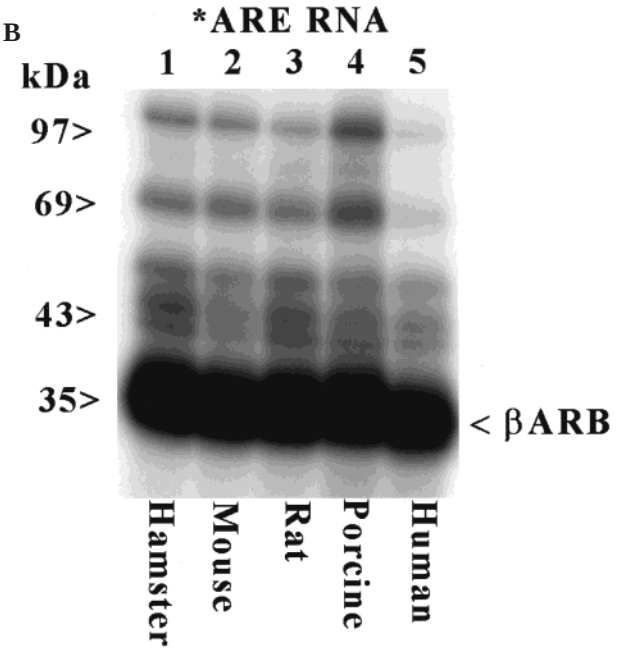

FIGURE 7: Homologous AREs of rat, mouse, porcine, and human β_2-AR mRNA bind to βARB. (A) Identification of the homologous βARB-binding region from rat, mouse, porcine, and human β_2-AR cDNA. The numbers indicate the position of the ARE region starting from the first nucleotide in the 3′-UTR. (B) Representive autoradiogram of UV-catalyzed cross-linking experiments of S100 cytosolic fractions from DDT_1-MF2 cells with uniformly radiolabeled, in vitro-transcribed RNA probes corresponding to the AREs of several mammalian species. Equal amounts of S100 cytosolic protein and equimolar concentrations for each radiolabeled ARE RNA probes were employed for these studies (lane 1, hamster; lane 2, mouse; lane 3, rat; lane 4, porcine; and lane 5, human).

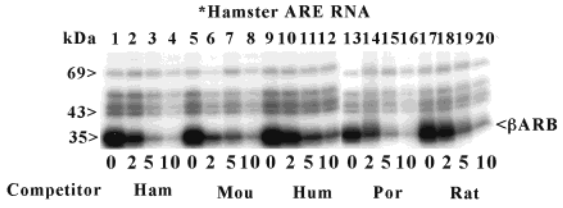

FIGURE 8: Molar excesses of unlabeled ARE RNA corresponding to the hamster, mouse, rat, porcine, and human β_2-AR mRNA compete with binding of the radiolabeled hamster 20-nucleotide ARE for binding to βARB. Autoradiogram of UV-catalyzed cross-linking of S100 cytosolic fractions prepared from DDT_1-MF2 cells with capped, uniformly radiolabeled, in vitro-transcribed RNA corresponding to the hamster ARE region. The binding of the ^{32}P-labeled hamster ARE RNA probe to βARB was subjected to competition by adding increasing amounts (2-, 5-, and 10-fold molar excesses) of unlabeled RNAs transcribed from AREs of hamster (lanes 2−4), mouse (lanes 6−8), human (lanes 10−12), porcine (lanes 14−16), and rat (lanes 18−20) β_2-adrenergic receptor mRNAs. Unlabeled RNAs and ^{32}P-radiolabeled RNA were added simultaneously to the mixture containing the S100 cytosolic extracts. The mixture was incubated for 10 min, prior to UV-catalyzed cross-linking.

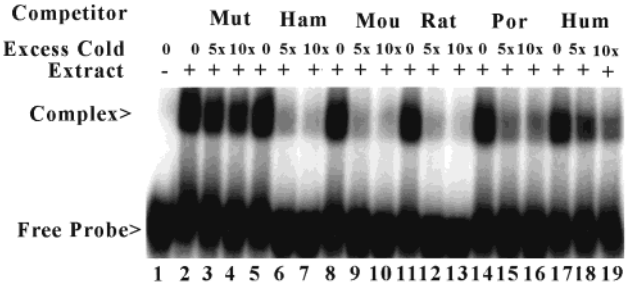

FIGURE 9: RNA gel mobility shift assays. Specificity of ARE RNA−protein complex formation. The autoradiogram shows the results of RNA gel mobility shift analysis performed using ^{32}P-labeled hamster ARE RNA incubated without (lane 1) and with 15 μg of cytosolic extracts from DDT_1-MF2 smooth muscle cell (lanes 2−19). In competition studies, unlabeled ARE RNA corresponding to mutant 2 (lanes 2−4), hamster (lanes 5−7), mouse (lanes 8−10), rat (lanes 11−13), porcine (lanes 14−16), and human (lanes 17−19) ARE RNA was mixed with radiolabeled hamster ARE RNA (~3 pmol) before adding cytosolic extract. Cytoplasmic lysates were added, and the mixture was incubated for 10 min at 25 °C. The complexes were resolved on 5% native polyacrylamide gels, dried, and subjected to autoradiography.

migrating RNA−protein complex (Figure 9). To evaluate possible nonspecific interactions between charged proteins and RNA molecules, we used unlabeled RNA corresponding to the hamster M2 mutant (devoid of the intact hexamer core) as a control for the specificity of the formation of RNA−protein complexes. Unlabeled ARE RNA of the M2 mutant competed poorly with labeled wild-type ARE RNA in the mobility shift assay, even when the unlabeled M2 RNA was employed at a 10-fold molar excess. Unlabeled ARE RNA corresponding to unmutated hamster, mouse, and rat sequences, in contrast, competed effectively with radiolabeled hamster ARE RNA for complex formation at a 5-fold molar excess. The unlabeled porcine and human ARE sequences also competed effectively with radiolabeled hamster ARE RNA for complex formation (Figure 9).

DISCUSSION

G-Protein-linked receptors exhibit agonist-mediated down-regulation of receptor expression. For one prominent member of the G-protein-linked receptor superfamily, the β_2-AR, agonist-induced downregulation of receptor reflects destabilization of pre-existing receptor mRNA (*6*). Other members of the G-protein-linked receptor superfamily shown to be regulated at the level of mRNA stability include the rat m1-muscarinic (*44*), AT1-angiotensin II (*46*), rat serotonin 5-HT_{2A} (*47*), and human thrombin receptors (*9*). The mRNAs of these receptors possess well-known destabilizing elements, i.e., AREs (*9*). AREs may mediate RNA destabilization through interaction with proteins. The 35000-M_r βARB exhibits specificity for binding to mRNA of β_2-AR and other receptors which manifest agonist-induced destabilization of mRNA. It fails to recognize the mRNA of homologous β-ARs, such as rat and human β_3-ARs (*9*), which do not display agonist-induced alterations in the $t_{1/2}$ of the receptor mRNA (*48*). We previously performed site-directed mutagenesis of the 3′-untranslated region of β_2-AR and identified a 20-nucleotide AU-rich element (ARE) consisting of an AUUUUA hexamer flanked on 5′- and 3′-reaches by U-rich regions as a βARB binding region (*14*). Agonist stimulation of β_2-ARs provokes a significant "upregulation" of βARB, as established by UV-catalyzed cross-linking (*7*). Treatment of DDT_1-MF2 smooth muscle cells with the glucocorticoid dexamethasone, in contrast, increases the level of β_2-AR expression, increases β_2-AR mRNA levels, and simultaneously "downregulates" the level of βARB (*7*). On the basis of the pattern of induction and the direction of the change in β_2-AR mRNA levels, the 35000-M_r βARB appears to destabilize β_2-AR mRNA.

ARE-containing mRNAs can be divided into classes based on the number and distribution of AU-rich elements in the 3′-UTR. AUUUA pentamer-containing mRNAs are usually found in oncogenes and cytokines and can be of two different types (*49*). Oncogene mRNAs have either one to three AUUUA sequences spaced throughout the 3′-UTR region or multiple clusters of pentamers as found in cytokine mRNAs. Although both ARE regions cause rapid decay of mRNA, typically the poly(A) tail of oncogene mRNAs is degraded synchronously whereas the poly(A) tail of cytokine mRNAs shows a progessive mechanism of poly(A) tail decay (*49*). The ARE regions identified in β_2-AR mRNA are classified into a third group where AUUUA pentamers are not a requirement. To emphasize this distinction, the level of binding of βARB actually is reduced when the hexamer core of the β_2-AR mRNA ARE is replaced with a pentamer (*14*). A fourth motif consisting of a nonconsensus AU-rich nonamer also has been reported in the human β_2-AR 3′-UTR to influence agonist-induced destabilization of the receptor mRNA (*50*).

Our analysis of the 20-nucleotide ARE of the hamster β_2-AR mRNA took advantage of specific mutations to evaluate the roles of the hexamer AUUUUA core and its two flanking, poly(U)-rich regions as well as of the naturally occurring AREs in other mammalian species to probe the cognate nucleotide binding requirements of βARB. By comparing the sequences and label transfer data, we gained several key insights (Figure 10). The poly(U) region that is located 3′ to the hexamer AUUUUA core is not obligate for the interaction with βARB, since mutant 3 and the rat ARE lack this feature but bind well to βARB. In contrast, the integrity of the poly-(U) track within the hexamer AUUUUA is essential for binding to βARB. Interrupting the track abolishes binding

SOURCE	SEQUENCE	LABEL TRANSFER (Percent)
HAMSTER	5'CUUUUUUAUUUUAUUUUUUUUA3'	100
HAMSTER MUT#1	5'CUUGGUUAUUUUAUUUUUUUA3'	70
HAMSTER MUT#2	5'CUUUUUUAUGGUAUUUUUUUA3'	10
HAMSTER MUT#3	5'CUUUUUUAUUUUAUUGGUUUA3'	100
HAMSTER MUT#4	5'CUUUUUUAUUUAUUGGUUUA3'	50
DECAMER	5'UUAUUUUAUU3'	25
RAT	5'CUUUUUUAUUUUAGGUUUUUA3'	100
MOUSE	5'CUUUUUUUUAAAUUUUAUUUUAUUUUUUA3'	100
PORCINE	5'CUUUUUAUUUUUUUUAUUUUUUUUA3'	100
HUMAN	5'CUUUUUAUUUUUUUUA3'	100

FIGURE 10: Alignment of wild-type and mutant forms of the hamster ARE of the β_2-adrenergic receptor with putative AREs of mRNA from β_2-adrenergic receptors of several mammalian species.The preferred ARE sequence for βARB binding in β_2-AR mRNA is underlined (see the text for details and discussion).

to βARB both in the label transfer and in the mobility shift assays. βARB recognizes a pentamer AUUUA if it is embedded within the proper flanking regions, but does so to a lesser extent than when a hexamer AUUUUA is present (*9*, *14*). Although the hamster β_2-AR mRNA contains both a pentamer ARE which is not flanked by poly(U) regions and a hexamer ARE flanked by poly(U) region, mutagenesis and studies of the ability of the mRNA to be regulated in vivo demonstrate that it is the hexamer containing ARE, rather than the pentamer, that is essential for agonist-induced downregulation of receptor mRNA (*14*). The study presented here further supports a role for βARB in agonist-induced destablization of β_2-AR mRNA through its interaction with the ARE region present in the 3′-UTR region. Analysis of the human and porcine ARE demonstrates the ability of an expanded nonamer AUUUUUUUA core to bind to βARB. One could speculate that the 3′ A-nucleotide bracketing the hexamer motif may well be unnecessary. Studies of the β_3-AR mRNA (which harbors UUUAUUUUU tracks) show that this 3′-UTR fails to bind βARB (*9*). Unlike the poly(U)-rich domain that is 3′ to the hexamer AUUUUA core which is nonessential, the poly(U) on the 5′-flanking side of the hexamer appears to be critical to the ability of the RNA to bind the βARB. Taken together, these studies reveal a preferred sequence for binding to the βARB as a central hexamer AUUUUA, which can tolerate loss of one or gain of several U nucleotides, flanked by a poly(U)-rich sequence that is 5′ to the hexamer AUUUUA core.

Although humans and rodents diverged approximately 75 million years ago, the similarities of the β_2-AR 5′- and 3′-flanking regions are calculated to be 73 and 79%, respectively (*51*). On the basis of these values, it has been suggested that the 5′- and 3′-flanking regions must contain genetic elements which are required for appropriate regulation of receptor expression (*51*). Much of the previous work has focused on regulatory elements identified in the 5′-UTR of β_2-AR cDNA such as glucocorticoid response elements, cAMP response elements, and phorbol ester response elements (*1*, *2*, *51*). The work presented here identifies an ARE region located in the 3′-UTR region of β_2-AR mRNA which is present in all the mammalian species. The extent of homology for the receptor mRNA AREs suggests that these elements are obligate in receptor regulation and have therefore been subject to selective pressure to preserve their sequences. What is new about the current work is that we have defined the minimal sequence of a unique RNA destabilizing region in the 3′-UTR of β_2-AR. This sequence requires a core hexamer AUUUUA and a U-rich region located 5′ to the core AUUUUA for efficient binding to βARB, a putative RNA-destabilizing protein, to occur.

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