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Agonist-Mediated Down-Regulation of Rat β_1-Adrenergic Receptor Transcripts: Role of Potential Post-Transcriptional Degradation Factors

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ABSTRACT

The human β_1-adrenergic receptor (AR) and hamster β_2-AR transcripts can be post-transcriptionally regulated at the level of mRNA stability and undergo accelerated agonist-mediated degradation via interaction of their 3′ untranslated regions (UTR) with RNA binding proteins. Using RNase protection assays, we have determined that chronic isoproterenol exposure of rat C6 glioma cells results in the accelerated reduction of β_1-AR mRNAs. To determine the role of cellular environment on the agonist-independent and agonist-mediated degradation of β_1-AR mRNAs, we transfected rat β_1-AR expression recombinants into both hamster DDT$_1$MF2 cells and rat L6 cells. The rat β_1-AR mRNAs in the two transfectant cell pools retain longer agonist-independent half-lives than in the C6 environment and undergo accelerated degradation upon chronic agonist exposure. Using UV–cross-linking/immunoblot and immunoprecipitation analyses, we have determined that the rat β_1-AR 3′ UTR recognizes a predominant M_r 39,000 component, identified as the mammalian elav-like protein HuR, and several other minor components, including the heteronuclear protein hnRNP A1. HuR levels are more highly expressed in C6 cells than in DDT$_1$MF2 and L6 cells and are induced after chronic isoproterenol treatment. Furthermore, C6 transfectants containing an HuR expression recombinant exhibit reduced β_1-AR mRNA half-lives that were statistically comparable with half-lives identified in isoproterenol-treated C6 cells. These results imply that HuR plays a potential role in the agonist-independent and agonist-mediated down-regulation of β_1-AR mRNAs.

Post-transcriptional control mechanisms participate in the stability and degradation of various cellular RNAs, including those encoding G protein-coupled receptors (Nielsen and Shapiro, 1990; Schiavi et al., 1992; Jackson, 1993). AU-rich elements (AREs), identified in the 3′ untranslated regions (UTRs) of these mRNAs, initiate deadenylation as the first step in mRNA turnover (Greenberg et al., 1990; Brewer, 1991; Zubiaga et al., 1995; Xu et al., 1997). AREs may consist of AUUUA pentamers, AU-nonamers, and U-rich elements; specific combinations determine the degree of destabilization function. AREs have been divided into three classes (Xu et al., 1997): class I and II mRNAs contain multiple copies of AUUUA pentamers and display either processive or distributive deadenylation, respectively; class II mRNAs result in the formation of poly(A)$^-$ intermediates. Class III mRNAs do not contain AUUUA pentamers and undergo processive deadenylation. Clustering of multiple copies (5+) of the AUUUA pentamer seems to be important in the selection of processive versus distributive deadenylation (Xu et al., 1997). The optimal destabilization motif for c-fos mRNAs has been previously determined to be UUAUUUA(U/A)(U/A) (Zubiaga et al., 1995). However, reiteration of the AUUUA pentamer often results in the formation of overlapping nonamers; it is uncertain whether the multiple clustering of the core

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ABBREVIATIONS: ARE, AU-rich element; UTR, untranslated region; hnRNP, heterogeneous nuclear ribonuclear protein; AR, adrenergic receptor; βARB, β-adrenergic receptor mRNA-binding protein; bp, base pair(s); DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; RPA, RNase protection assay; ICER, inducible cAMP early repressor; CRE, cAMP response element.

pentamer or the presence of the overlapping nonamer represents the key determinant for initiation of mRNA degradation (Xu et al., 1997).

Among factors implicated in mRNA stability are several classes of RNA binding proteins (Derrigo et al., 2000). The AU-rich binding/degradation factor (or AUF1), also identified as the hnRNP D protein, is an important factor in the ARE-mediated decay pathway (Zhang et al., 1993; Loflin et al., 1999). AUF1 has been demonstrated to bind to AREs in transcripts encoding G protein-coupled receptors (Zhang et al., 1993, Pende et al., 1996) and undergoes high-affinity binding to AREs as a hexameric protein (DeMaria et al., 1997, Chkheidze et al., 1999). Other hnRNPs, including hnRNP A1 and hnRNP C, also bind to reiterated AUUUA sequences (Hamilton et al., 1993). The Hu proteins, primarily neuronal RNA binding proteins, are composed of four family members, HuA (or HuR), HuB, HuC, and HuD, that recognize AUUUA pentamers or derivative sequences (Ma et al., 1996; Akamatsu et al., 1999). HuR recognizes the UTRs of several transcripts, and has been implicated primarily in RNA stability (Levy et al., 1998; Ford et al., 1999) and most recently in RNA decay (Maurer et al., 1999).

Several G protein-coupled receptor mRNAs, including those encoding the α_1-adrenergic (Izzo et al., 1990), m_1-muscarinic (Lee et al., 1994), and β_2-ARs (Hadcock et al., 1989), undergo agonist-induced decreases in transcript stability. The β_2-AR mRNA contains an AU-rich 3′ UTR that can recognize several RNA binding proteins, including 1) the β-adrenergic receptor mRNA-binding protein (βARB), a cytosolic protein that requires both AUUUA pentamers and U-rich domains for RNA recognition and 2) the M_r 37,000 RNA degradation factor AUF1 (Port et al., 1992; Huang et al., 1993; Pende et al., 1996). In the presence of β-AR agonist, these RNA binding proteins seem to be up-regulated, concomitant with the down-regulation of β_2-AR mRNA. In humans undergoing cardiac failure, AUF1 and norepinephrine levels are increased, concomitant with significant decreases in β_1-AR mRNA and receptor (Pende et al., 1996). Specific AU-rich elements in the β_2-AR 3′ UTR, including a 20-bp core sequence, have been demonstrated to be essential for the agonist-mediated destabilization of β_2-AR mRNA (Tholanikunnel and Malbon, 1997; Danner et al., 1998; Tholanikunnel et al., 1999). Using chimeric receptor β-globin recombinants, the human β_1-AR mRNA 3′ UTR was determined to target β-globin mRNA for accelerated decay and was demonstrated to be necessary but not sufficient for agonist-mediated destabilization of transcripts (Mitchusson et al., 1998). Using mass spectrometry, βARB has recently been identified as a composite of HuR and hnRNP A1 (Blaxall et al., 2000). HuR seems to be the predominant RNA binding component of βARB; however, the precise component composition of βARB seems to be dependent on the 3′ UTR sequence of the target β-AR subtype mRNA (Blaxall et al., 2000).

In this report, we examined the agonist-independent and agonist-mediated regulation of the rat β_1-AR mRNA, endogenously expressed in C6 glioma cells and ectopically expressed in transfectant hamster DDT_1MF2 and rat L6 cells, and have identified factors that may potentially influence post-transcriptional β_1-AR mRNA decay. Our observations are consistent with the interpretation that the HuR proteins may play important roles in the agonist-mediated down-regulation of β_1-AR mRNAs and that expression of this factor may also influence agonist-independent β_1-AR mRNA degradation.

Materials and Methods

Cell Lines, Antibodies, Expression Recombinants, and Statistical Analyses. The rat C6 glioma (American Type Culture Collection, Manassas, VA), the hamster DDT_1MF2 (American Type Culture Collection), and the rat L6 skeletal muscle myoblast (American Type Culture Collection) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum, 1% penicillin/neomycin/streptomycin, and 1% L-glutamine. Low-glucose (1.5 g/l) DMEM was used to culture the C6 and L6 cell lines. High-glucose (4.5 g/l) DMEM was used to culture the DDT_1MF2 cell line. Antibodies were obtained that recognize HuR, the mammalian elav-like RNA binding proteins (Henry Furneaux, Cornell University School of Medicine, Ithaca, NY), and the heterogeneous nuclear ribonuclear proteins (hnRNP A1; provided by Gideon Dreyfuss, Howard Hughes Medical Institute, University of Pennsylvania School of Medicine, Phildelphia, PA). The monoclonal antibody recognizing the bacterial Flag antigen (anti-Flag M2 monoclonal) was obtained from Sigma (St. Louis, MO). The pSVZeo eukaryotic expression recombinants, containing the complete HuR coding sequence in sense or reverse orientations, were obtained from Andy Levy (Technion Faculty of Medicine, Haifa, Israel). Statistical analyses used to ascertain significance of β_1-AR mRNA half-lives were based on the two-tailed unpaired t test derived from the Microsoft Excel software program (Microsoft, Redmond, WA).

Construction of β_1-AR Recombinants Used for Transfection. We performed a two-step subcloning strategy to obtain the complete β_1-AR coding region and 3′ UTR. To obtain the β_1-AR region −23 to +1466, two primers were engineered, a 5′ primer containing an *Xba*I site and β_1-AR sequence from −23 and a 3′ primer containing a *Bam*HI site and β_1-AR sequence ending at +1466. Molecular clone pCoding plasmid DNA (50 ng) containing β_1-AR sequence −82 to +1573 was used as template for PCR amplification. To obtain the β_1-AR region +1467 to +2794, two additional primers were engineered, a 5′ primer containing a *Bam*HI site and β_1-AR sequence from +1467 and a 3′ primer containing an *Eco*RI site and β_1-AR sequence ending at +2794. Molecular clone pST3000–18 plasmid DNA (50 ng) containing β_1-AR sequence +1165 to +2967 was used as template for PCR amplification. PCRs were conducted using a core buffer consisting of 20 mM Tris-HCl, pH 8.75, 10 mM KCl, 10 mM NH_4SO_4, 2 mM $MgSO_4$, 0.1% Triton X-100, 100 μg/ml bovine serum albumin; 200 μM concentrations of each dNTP, 100 pmol of each primer, and 2.5 units of *Pyrococcus furiosus* DNA polymerase (Stratagene, La Jolla, CA). The reaction mixture was subjected to an initial denaturation at 95°C for 1 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, and 68°C for 2 min, with a final extension at 68°C for 20 min followed by a 4°C soak. Each PCR product was then subcloned into pGEM 3Zf(+) (Promega, Madison, WI), to develop large-scale quantities of the insert. Both recombinants were digested with *Bam*HI and *Eco*RI, and subjected to a three-way ligation into pGEM 3Zf(+). The 2.7-kilobase insert containing the complete β_1-AR coding sequence plus 3′ UTR was subcloned into the pcDNA 3.1(−) eukaryotic expression vector (Invitrogen, Carlsbad, CA). To confirm insert orientation and sequence, a majority of the recombinant was sequenced.

Cloning of the Hamster Cyclophilin Sequence by Reverse Transcription-Polymerase Chain Reaction. The hamster cyclophilin probe is composed of sequence extending from +63 to +273, relative to the translational start site, and was prepared by reverse transcription-PCR using DDT_1MF2 RNA as template. First strand synthesis was conducted (42°C, 30 min) using 5× first strand buffer (4 μl; 250 mM Tris-HCl, pH 8.3, 375 mM KCl, and 15 mM $MgCl_2$), 2.5 mM concentrations of each dNTP (2 μl), RNasin (2000 units), 1 mg/ml bovine serum albumin (2.5 μl), 0.1 M dithiothreitol (0.5 μl), reverse transcriptase (100 units; Moloney murine leukemia virus,

Invitrogen), DDT_1MF2 RNA (2 μg), and first strand primer (3′GACTCGAGGATCCA-TCGATTTTTTTTTTTTTTTTT5′). PCR was conducted using 10× PCR buffer (10 μl; 500 mM KCl, 100 mM Tris-HCl, pH 9.0, 1% Triton X-100; Bio Basic, Inc., Toronto, ON, Canada), 15 mM $MgCl_2$ (8 μl), 2.5 mM concentrations of each dNTP (8 μl), *Taq* polymerase (5 U), first-strand synthesis products (5 μl), and 100 pmol of upstream and downstream primer in a 100-μl total volume. The PCR mixture was subjected to an initial denaturation at 94°C for 1 min, followed by 30 cycles of 94°C for 1 min, 54°C for 45 s, and 72°C for 30 s. Upstream and downstream primers were derived from the Chinese hamster ovary (CHO) cyclophilin sequence available on GenBank (accession number X17105). The CHO and DDT_1MF2 hamster cyclophilin sequences differ in two bases within a common 211 bp sequence (position 93–303); there is a C-to-T conversion at position 235 and an A-to-G conversion at position 275 (in both cases, CHO-to-DDT_1MF2 sequence conversions). The DDT_1MF2 hamster cyclophilin sequence has been deposited in GenBank and has been assigned accession number AY004869.

Construction of the β_1-AR 3′ UTR Recombinants and Syntheses of cRNA Probes for RNA Mobility Shift Assays. PCR was used to generate probes encompassing the β_1-AR 3′ UTR subregions P1, P2, P3, and P4 (subregions 2187–2306, 2307–2454, 2455–2590, and 2591–2736, respectively), and the control β_1-AR upstream sequence containing subregion −408 to −323, relative to the translational initiation codon. Molecular clone DNA, containing the entire rat β_1-AR gene (50 ng), was used as PCR template. The PCR fragments encompassing the P1, P2, P3, and P4 subregions were individually subcloned by blunt-end ligation into pGEM3Z(+) (Promega). The resulting 3′ UTR recombinants, pGEM-P1, pGEM-P2, pGEM-P3, and pGEM-P4, and the upstream recombinant pβ_1-AR[−408,−323], were verified by sequence analyses and used to transcribe sense cRNA probes for RNA mobility shift assays. In vitro transcription of cRNA probes is described in Kirigiti and Machida (1999).

Transfection Procedures. pcDNA 3.1 recombinants containing the complete rat β_1-AR coding region and 3′ UTR (nucleotide position is from −23 to +2786) were electroporated into the DDT_1 MF2 or L6 cell lines (300 V, 900 μF capacitance; 3.0×10^6 cells in 0.3 ml); transfectants were then subjected to neomycin selection (500 μg/ml G418 [Geneticin; Invitrogen] for 10–14 days) to generate pooled stable clones expressing rat β_1-AR mRNAs. pSVZeo recombinants containing the HuR sequence in either sense or reverse orientation were electroporated into C6 cells using transfection parameters described above. Transfectants were then subjected to zeocin selection (600 μg/ml, product number 45–0430; Invitrogen) for 14 days to generate pooled stable clones; zeocin selection was continued for an additional 2 weeks or more before conducting agonist treatment experiments.

RNase Protection Assays. Total RNA was isolated using the QIA RNeasy Mini Kit, a product of QIAGEN, Inc. (Valencia, CA). Methods for RNA quantification, determination of sample integrity, and RNase protection assays (RPA) were conducted as described in Yang and Machida (1999). The probe pCS[−82, +273], which contains rat β_1-AR sequence extending from position −82 to +273 (relative to the translational start site), recognize all β_1-AR transcripts and was used to measure total β_1-AR mRNAs in RPA experiments. The hybridization probe used to discriminate the two polyadenylated forms of β_1-AR mRNA in C6 cells was p4Zβ1 probe [+2084, +2901], which contains β_1-AR sequence extending from +2084 to +2901. Antisense transcripts were generated with T7 RNA polymerase (20 units, 37°C, 1 h) using reaction components described in Yang and Machida (1999).

Total sample RNA (15 μg) was resuspended in hybridization solution with antisense transcripts (5×10^5 cpm for β_1-AR cRNAs and 1.5×10^3 cpm for cyclophilin cRNA), denatured and allowed to anneal following procedures described in Yang and Machida (1999). Hybridized RNAs were digested with RNase T1 following procedures described in Yang and Machida (1999). Ethanol-precipitated samples were suspended in formamide/dye solution and the products separated by electrophoresis on a 5% acrylamide, 7 M urea gel. RNase protection experiments were analyzed using standard autoradiography [autoradiographic bands were scanned and quantitated using the Adobe Photoshop 4.0 (Adobe Systems, Mountain View, CA) and the NIH Image software programs], or more recently with the Bio-Rad Molecular Imager FX system. In this system, radioactive signals from each protected band were detected and converted into digital quantitative values using the Quantity One software program (Bio-Rad). All intensity values for β_1-AR protected fragments were normalized by dividing these values with corresponding intensity values for the cyclophilin protected fragment obtained in the same lane. Normalization ratios (β_1-AR protected fragment intensity/cyclophilin protected fragment intensity) for each lane were divided by the normalization ratio determined for the lane corresponding to time 0. This second normalization resulted in the assignment of the time 0 lane with a numerical value of 1.0.

RNA Mobility Shift Assay and UV–Cross-Linking of RNA-Protein Complexes for SDS-Polyacrylamide Gel Electrophoresis. Preparation of cell extracts and binding reactions were conducted as described in Kirigiti and Machida (1999). RNA mobility shift assay experiments were electrophoresed in 4% nondenaturing polyacryamide gels and subjected to autoradiography. For use in protein size determinations, binding reactions were conducted using cellular extract (20 μg) and 1×10^6 cpm of ^{32}P-labeled cRNA probe for 20 min at room temperature (in a 50-μl final volume containing 4 mM dithiothreitol, 5 μg of yeast tRNA, 5 μg of heparin, and 50 units of Super RNasin). The reaction mixtures were then placed on ice and subjected to short wave (254 nm) ultraviolet irradiation for 5 min in a cross-linking oven (model FB-UVXL 100; Fisher Biotech, Belmont, WA). Noncovalently bound ribonucleotides were degraded by addition of RNase T1 (1 unit) and RNase A (1 μl of 0.5 μg/μl) for 30 min at 37°C. In some cases, as with the cross-linking associated with the hnRNP A1 analyses, RNase T1 digestion preceded cross-linking of radioactive probe with RNA binding proteins. The reaction mixtures were added to equal volumes of 2× loading buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 2% bromphenol blue, and 20% glycerol), boiled for 5 min, and subjected to electrophoresis in a 10% polyacrylamide gel (acrylamide/bis-acrylamide, 29:1; supplemented with SDS). Gels were transferred to nitrocellulose membranes before autoradiography (−85°C; 30–72 h) or analysis with the Bio-Rad Molecular Imager FX system.

Immunoprecipitation of UV–Cross-Linked RNA-Protein Complexes. In some cases, to enhance the autoradiographic signal of immunoprecipitated proteins, 5-fold additional probe and 3-fold additional protein were used in UV–cross-linking experiments. After UV–cross-linking of RNA-protein complexes and RNase A/T1 digestion, 1 to 2 μl of specific antibody was added to the reaction mix and allowed to incubate overnight at 4°C. Phosphate-buffered saline (PBS) was added to the mixture to bring the final volume to 200 μl. Protein A slurry (20–40 μl of a 1:1 mixture of PBS and protein A-Sepharose 4B beads; Sigma) was then added, and allowed to mix for 2 h at 4°C. The protein A beads were then washed three times by gentle centrifugation (2000*g*, 2 min) in PBS, and then resuspended in 6× loading buffer, before heating at 100°C for 2 min. RNA-protein complexes released from the protein A beads were then loaded into SDS-polyacrylamide gels. Electrophoresis and autoradiographic or molecular imaging analyses were conducted as described above.

Electrotransfer to Nitrocellulose Membranes and Immunoblotting Procedures. Proteins were electro-transferred onto nitrocellulose membranes, and blots were blocked and prepared for immunostaining using procedures described in Kirigiti et al. (2000) Primary antibodies include the mouse monoclonals to the mammalian elav-like protein HuR (1:5000 dilution) or to the heteroribonuclear protein hnRNP A1 (1:1000 dilution). The primary antibodies were then incubated for 2 to 5 h at room temperature with gentle agitation. The secondary antibodies for the mouse monoclonals were anti-mouse horseradish peroxidase (1:10,000 dilution used in both cases). Membranes were then washed four times with 1× Tris-

buffered saline/Tween 20, and then developed using the SuperSignal Western pico chemiluminescence substrate (Pierce Chemicals, Madison, WI).

Results

Short-Term Isoproterenol Agonist Treatment Induces Initial Up-Regulation Followed by Reduction of β_1-AR mRNA Levels in C6 Glioma Cells. Consistent with previous determinations (Hosoda et al., 1994; Rydelek-Fitzgerald et al., 1996), isoproterenol treatment (10 μM) of C6 glioma cells induces an initial short-term up-regulation of β_1-AR mRNA levels, followed by reduction of transcripts and return to basal levels (Fig. 1, A and B). Using RNase protection assays and the pCS[−82, +273] probe, which contains sequences present in all transcribed β_1-AR mRNAs, we observed a 1.5- to 2-fold elevation of β_1-AR mRNAs at 80 to 100 min after agonist addition. This was followed immediately by a diminution in β_1-AR mRNAs, reaching a 50% reduction from its peak levels within 20 to 40 min and steady-state residual levels within 180 min after agonist addition (Fig. 1, A and B). Multiple repeats of this experiment were conducted (n = 3), and all exhibited similar trends. In our experiments, agonist is removed and replaced with fresh culture medium following a short-term (60 min) isoproterenol treatment. In both Hosoda et al. (1994) and Rydelek-Fitzgerald et al. (1996), an initial up-regulation in β_1-AR mRNA levels was observed 1 h after isoproterenol treatment; this was followed by β_1-AR mRNA down-regulation to below basal levels during 4 h of continuous isoproterenol treatment. Thus, the conditions between our experiments and reports of Hosoda et al. (1994) and Rydelek-Fitzgerald et al. (1996) are not directly comparable. We believe that the additional isoproterenol treatment may trigger down-regulatory mechanisms, perhaps by transcriptional repression or by post-transcriptional mechanisms described in this study, that may account for the increased reduction of β_1-AR mRNA levels.

The biphasic effect in β_1-AR mRNA levels is also evident when using the p4Zβ1 probe (Fig. 1, C and D), which contains β_1-AR sequence extending from +2084 to +2901, relative to the translational start site. The rat β_1-AR mRNA has two functional polyadenylation signals at +2450 and +2732 (Searles et al., 1995). RNase protection assays using the

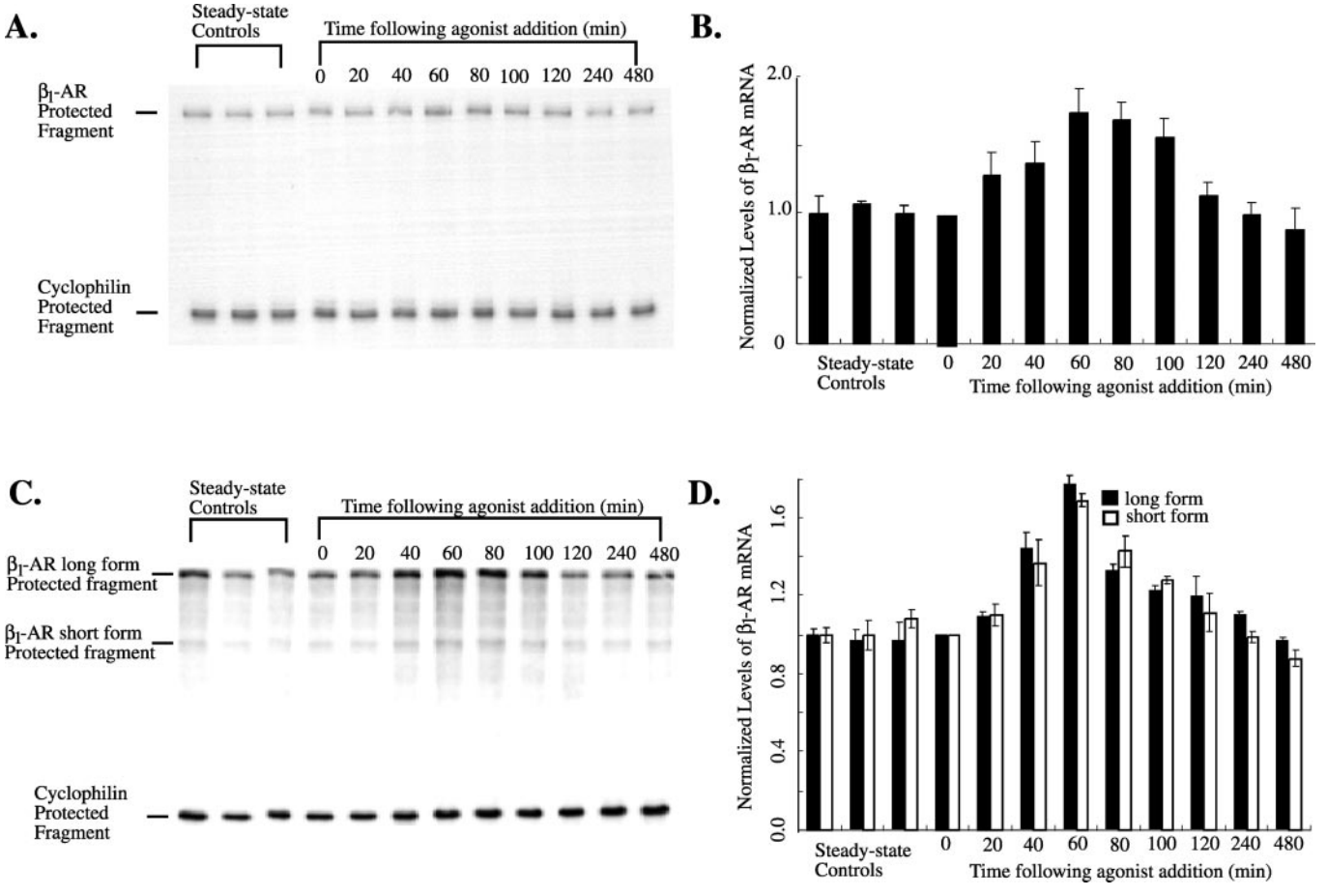


Fig. 1. β_1-AR transcripts obtained from isoproterenol-treated (60 min) C6 cells. Total RNA (15 μg) was hybridized to probe pCS[−82, +273] (contains rat β_1-AR sequence extending from −82 to +273) or the probe p4Zβ1 (contains rat β_1-AR sequence extending from +2084 to +2901) and pIB15.158 (the 158-bp coding fragment derived from the rat cyclophilin gene). A and C, RPA of representative experiments examining β_1-AR transcripts obtained from isoproterenol-treated (60 min) C6 cells, using probe pCS[−82, +273] and probe p4Zβ1, respectively. Steady-state β_1-AR mRNA levels at 240, 120, and 60 min (left to right in each panel) before agonist addition were also collected and analyzed by RPA. Samples collected before agonist treatment seem to be equivalent to the time 0 sample for the agonist-induction experiments. Note that β_1-AR mRNA levels may fall below pretreatment levels upon further extended sampling. B and D, statistical evaluation of multiple repeats of these experiments (n = 3 and n = 4 for A and C, respectively). B shows an evaluation of RPAs using probe pCS[−82, +273] (as in A) and D shows an evaluation of RPAs using probe p4Zβ1 (as in C). The β_1-AR mRNA levels are normalized with corresponding cyclophilin mRNA levels at each time point, followed by a series normalization of all ratios with the time 0 ratio being assigned the numerical value of 1.0.

p4Zβ1 probe can readily distinguish transcripts containing the proximal or distal polyadenylation signals; in prior studies (Searles et al., 1995), we have established that the distal polyadenylation site is highly preferred in C6 cells and in cardiac tissue. β_1-AR transcripts containing either the proximal (short-form transcript) or the distal (long-form transcript) polyadenylation signal seem to undergo simultaneous and equivalent induction after agonist treatment. In addition, although the level of β_1-AR transcripts containing the proximal polyadenylation site is very low during the reduction phase of the biphasic effect, it seems that the initiation time point for the reduction phase, and the degree of reduction of β_1-AR transcripts from peak levels, is equivalent for both polyadenylated transcripts (Fig. 1, C and D).

Actinomycin D Treatment Experiments Indicate That the Decline of β_1-AR Transcripts during the Reduction Phase after Short-Term Agonist-Induction Is Not Accelerated by Enhanced Transcript Degradation. Actinomycin D was applied at the peak of the up-regulatory phase after short-term agonist-induction (at 60 min), in an attempt to measure β_1-AR mRNA stability during the reduction phase of the biphasic curve (Fig. 2A). In control experiments, actinomycin D was applied to C6 cell cultures that were not treated with agonist. The transcript half-lives for β_1-AR mRNAs are not statistically different between the short-term agonist-treated or untreated C6 cell series ($t_{1/2}$ = 63 ± 2.3 min versus $t_{1/2}$ = 65 ± 4.3 min, respectively; p = 0.375; Table 1 and Fig. 2, B and C), indicating that the decline of β_1-AR mRNAs occurring during the reduction phase after short-term agonist treatment is not accelerated by enhanced transcript degradation. Figure 2B illustrates the semilogarithmic plot of β_1-AR mRNA levels versus time after initiation of actinomycin D treatment. We observed a rapid first-stage decay of β_1-AR mRNA levels during the initial 120 min after initiation of actinomycin D treatment, followed by a second-stage leveling of β_1-AR mRNA levels. Figure 2C illustrates a best-fit analysis of the first stage decline of β_1-AR mRNA levels, using a first-order decay equation; these equations were also used to determine β_1-AR mRNA half-lives under each experimental condition. In other short-term agonist/actinomycin D experiments using the p4Zβ1 RPA probe, the half-lives for β_1-AR transcripts containing either the distal or proximal polyadenylation sites were also not statistically different in C6 cells, compared with corresponding agonist-independent half-lives determined for both polyadenylated transcripts (Y. B. and C. A. M., unpublished observations)

Chronic Isoproterenol Treatment Results in Accelerated Reduction of β_1-AR Transcripts in C6 Cells. To address the possibility that chronic agonist treatment was required for induction of potential RNA degradation factors and for the resulting decrease in β_1-AR transcript half-life, we chronically treated C6 cells with isoproterenol (10 μM) for 24 h, before actinomycin D addtion and RPA analyses (Fig. 2D). In these experiments, β_1-AR transcripts retain half-lives ($t_{1/2}$) of 41 ± 5.9 min, statistically less (34.9% reduction) than the mRNA half-lives determined for either the untreated C6 cell series ($t_{1/2}$ = 63 ± 1.7 min) or during the reduction phase for the short-term isoproterenol treatment ($t_{1/2}$ = 63 ± 2.3 min; p = 0.013; Table 1 and Fig. 2, E and F). These results are consistent with the hypothesis that chronic agonist treatment of C6 cells results in the induction or increased induction of RNA degradation factors that can directly affect β_1-AR mRNA destabilization.

Rat β_1-AR Transcripts Expressed in Native C6 Cells or Hamster DDT$_1$MF2 and Rat L6 Cell Transfectants Retain Differential mRNA Half-Lives. DDT$_1$MF2 is a well-characterized hamster smooth muscle cell line that expresses only β_2- and not β_1-ARs; this cell line has been used extensively as a model to examine the agonist-mediated down-regulation of β_2-ARs transcripts and have been used as a source of extracts for protein interaction with the hamster β_2- and human β_1-AR UTRs (Port et al., 1992; Huang et al., 1993; Tholanikunnel and Malbon, 1997; Tholanikunnel et al., 1999). Danner and Lohse (1997) have previously examined the agonist-mediated down-regulation of β_2-AR mRNAs in the DDT$_1$MF2 and C6 cell lines. In these studies, the β_2-AR mRNA half-life in the DDT$_1$MF2 cell line ranged from 2 to 12 h (in suspension or monolayer cultures, respectively), that were reduced by 50% in the presence of agonist; the β_2-AR mRNA half-life in the C6 cell line was approximately 55 min and was not affected by agonist treatment. In these studies, the β_2-AR mRNA half-life was assessed after 30 min of agonist treatment, similar to the short-term isoproterenol treatment (60 min) and assessment of β_1-AR mRNA half-life conducted in our experiments above (Fig. 2A); the rat β_2-AR mRNA half-life results are similar to our rat β_1-AR mRNA half-life determinations ($t_{1/2}$ = 63 ± 2.3 min; Table 1 and Fig. 2, D and E). In addition, Hosoda et al. (1995) have determined the β_2-AR mRNA half-life in C6 cells to be approximately 60 min; in these studies, β_2-AR mRNA half-life was not influenced by short-term exposure to agonist. Danner and Lohse (1997) proposed that the agonist-mediated β_2-AR mRNA down-regulation in the DDT$_1$MF2 and C6 cell lines were moderated by distinct cell-specific mechanisms, potentially involving both transcriptional and post-transcriptional controls

We hypothesized that the DDT$_1$MF2 cell line provided a distinct cell environment that would differentially affect the agonist-independent rate of β_1-AR mRNA decay. Eukaryotic expression recombinants containing the rat β_1-AR coding region and 3′ UTR were electroporated into DDT$_1$MF2 cells; transfectants were selected for neomycin resistance and then pooled into a common cell population. This stable transfectant pool was subjected to chronic treatment with isoproterenol (10 μm) for 24 h, before actinomycin D addition and RPA analyses (Fig. 3A). Control experiments also included the use of actinomycin D without isoproterenol treatment. The rat β_1-AR mRNA expressed in DDT$_1$MF2 transfectants retained a much longer half-life ($t_{1/2}$ = 109 ± 4.0 min); chronic agonist treatment reduced (23.8% reduction) the rat β_1-AR mRNA half-life to 83 ± 5.1 min (p = 0.001; Table 1 and Fig. 3, B and C). These results are consistent with the interpretation that specific cell environment may provide distinct factors involved in both the agonist-independent and agonist-mediated, post-transcriptional destabilization of β_1-AR mRNAs.

To examine β_1-AR mRNA down-regulation in a broader range of cell lines, we also transfected the rat β_1-AR expression recombinant into the rat L6 skeletal muscle myoblast cell line. The L6 cell line endogeneously expresses β_2- but not β_1-ARs. The rat β_1-AR mRNA, expressed ectopically in L6 transfectants, retained basal half-lives that were statistically equivalent to those observed in the DDT$_1$MF2 transfectant cell line ($t_{1/2}$ = 109 ± 4.0 min and $t_{1/2}$ = 112 ± 2.5 min, for

DDT$_1$MF2 transfectants and L6 transfectants, respectively; Table 1 and Fig. 3, D–F). After chronic treatment with isoproterenol, the β_1-AR mRNA half-lives in the L6 transfectants were markedly reduced ($t_{1/2}$ = 97 ± 4.7 min; 13.4% reduction; Table 1 and Fig. 3, D–F). Thus, the rat β_1-AR mRNAs, expressed ectopically in the two transfectant cell pools, retain appreciably longer agonist-independent half-lives than that observed in the rat C6 environment, with all three transcript populations undergoing accelerated degradation upon chronic agonist exposure.

Multiple Cellular Proteins Recognize the β_1-AR 3′ UTR, Including Sequences Surrounding the Polyade-

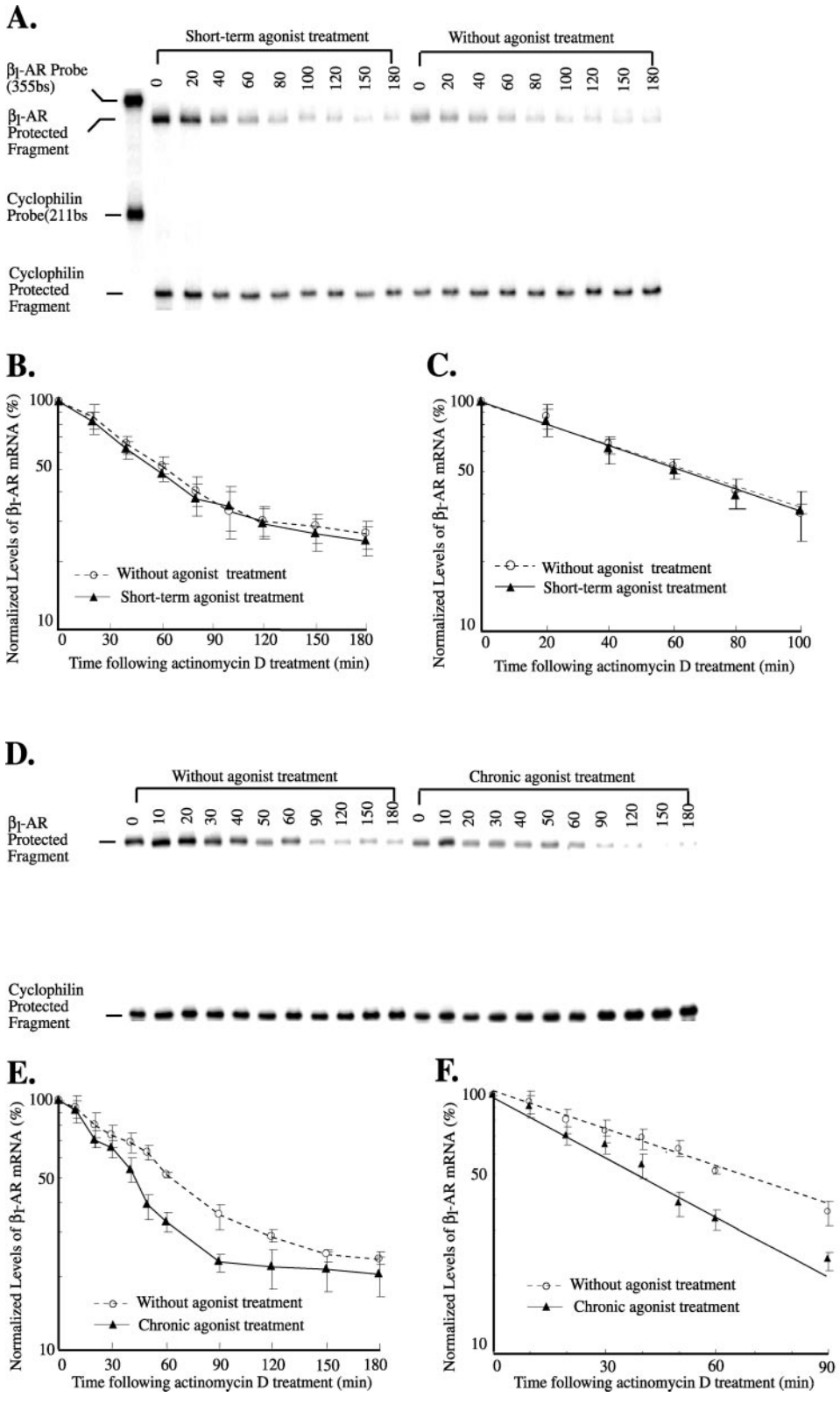


Fig. 2. A and D, β_1-AR mRNA half-lives in C6 cells undergoing short-term (60 min; A) or chronic (24 h; D) isoproterenol treatment. Illustrations depict representative RNase protection experiments. For all experiments, after either short-term or chronic isoproterenol treatment, actinomycin D was added to cells to inhibit de novo transcription. The hybridization probes used in these experiments were pCS[−82, +273] and pIB15.158. B and E, normalization analyses of β_1-AR mRNA levels. Normalization analyses for short-term agonist treatment in C6 cells (representative experiment shown in A) and chronic agonist treatment in C6 cells (representative experiment shown in D) are illustrated in B and E, respectively. Normalization calculations of β_1-AR mRNA levels against corresponding cyclophilin mRNA levels were conducted as described under *Materials and Methods*. Replicate experiments (n = 3) were conducted for all experimental series. Values were plotted on a semilogarithmic plot of normalized β_1-AR mRNA levels versus time after initiation of actinomycin D treatment. C and F, first-order decay of β_1-AR mRNAs. First-order decay analyses for short-term agonist treatment in C6 cells and chronic agonist treatment in C6 cells are illustrated in C and F, respectively. These panels illustrate statistical analyses (two-tailed unpaired t tests) of the first stage decline of β_1-AR mRNA levels described in B and E, by fitting data into a first-order decay equation. Data time points to develop first-order decay equations for the short-term agonist treatment experiment in C6 cells and chronic agonist treatment experiment in C6 cells were taken up to 100 and 90 min, respectively. First-order decay equations were derived and then used to determine β_1-AR mRNA half-lives under each agonist treatment condition.

TABLE 1

β_1-AR mRNA half-life determinations

Analyses were based on the two-tailed unpaired *t* test. Generalized equation to determine half-life: y = (intercept point on y-axis) × exp (mx) where y = percentage of β_1-AR mRNA remaining; m = slope of line; and x = time (in min). Statistical power of significance between A and B, $p = 0.375$; between C and D, $p = 0.013$; between E and F, $p = 0.001$; between G and H, $p = 0.028$; between I and J, $p = 0.23$; between K and L, $p = 0.049$; between E and G, $p = 0.35$; between C and I, $p = 0.009$. All half-life determinations are expressed in minutes; "iso" refers to isoproterenol. C6 cells were either untreated (A and C) or treated for 60 min (B) or for 24 h (D) with isoproterenol. DDT_1MF2 and L6 cells were stably transfected with the β_1-AR expression recombinant; transfectant cells were either untreated (E and G) or treated for 24 h with isoproterenol (F and H). C6 cells were stably transfected with HuR expression recombinants containing inserts in either sense (I and J) or reverse orientation (K and L); transfectant cells were either untreated (I and K) or treated for 24 h with isoproterenol (J and L). All cultures were treated with actinomycin D to inhibit de novo transcription before RNA extraction and RPA analyses.

	C6 (60 min iso)	C6 (24 h iso)	DDT_1MF2 transfectant (24 h iso)	L6 transfectant (24 h iso)	C6 HuR sense (24 h iso)	C6 HuR reverse (24 h iso)
			min			
Control	65 ± 4.3 (A)	63 ± 1.7 (C)	109 ± 4.0 (E)	112 ± 2.5 (G)	52 ± 2.7 (I)	60 ± 2.9 (K)
Iso	63 ± 2.3 (B)	41 ± 5.9 (D)	83 ± 5.1 (F)	97 ± 4.7 (H)	49 ± 2.5 (J)	50 ± 4.4 (L)

nylation Signals. With the use of RNA mobility shift assays and RNA probes recognizing discrete subregions of the β_1-AR 3′ UTR, we detected potential RNA binding protein(s) derived from extracts of C6 glioma cells (Fig. 4). Our initial strategy was to develop probes encompassing two basic subregions of the β_1-AR 3′ UTR (probe T1, position 2150–2441; probe T2, 2465–2736), delineating the 3′ ends of β_1-AR transcripts containing either the proximal or distal polyadenylation sites. However, probes of this size were too large for effective use in electrophoretic mobility shift assays (Kirigiti and Machida, 1999; also P. K. and C. A. M., unpublished observations). To generate more easily discernible mobility shifts, smaller probes encompassing the β_1-AR 3′ UTR were developed (probe P1, position 2187–2306; probe P2, position 2307–2454; probe P3, position 2455–2590; probe P4, position 2591–2736) (see map and sequence in Fig. 4, A and B). There seems to be multiple mobility shift complexes identified for the P2, P3, and P4 subregion probes (Fig. 4C), supporting the hypothesis that multiple protein interactions may occur for each subregion. We also conducted additional RNA mobility shift experiments using the P3 subregion probe and employing increasing dosages of cellular extract. In this experiment, as the amounts of cellular extract increases, the proportion of the observed RNA-protein complexes shifts to the higher molecular mass complex (Fig. 4D), indicating that there are at least two protein interaction sites on the P3 subregion.

UV–Cross-Linking and Denaturation of β_1-AR 3′ UTR-Protein Complexes Identifies a Major M_r 39,000 Binding Protein and Several Minor Binding Components. C6 cellular proteins were bound to β_1-AR probe pTail 1, which contains β_1-AR sequence extending from +2084 to +2901; probe-protein complexes were then subjected to UV–cross-linking, denaturation, and SDS-PAGE. This analysis identified a major M_r 39,000 binding protein and several minor components M_r 90,000, 82,000, 72,000, 63,000 and 48,000 (Fig. 5A). The presence of RNasin in our binding/UV–cross-linking reactions increases the electrophoretic resolution of the cross-linked components (Fig. 5A) but creates an experimental artifact (Fig. 5A; compare lanes containing probe with or without RNasin, and in the absence of protein extract, but subjected to UV–cross-linking). We conjecture that undetermined amounts of RNasin and the RNA probe have become UV–cross-linked and that subsequent RNase digestion of cross-linked components results in the radioactive tagging of small amounts of RNasin molecule. Hence, we are unable to rule out the possibility that the M_r 63,000 protein band may represent RNasin protein artifact. When the β_1-AR probe pTail 1 was bound to cellular extracts from the C6, DDT_1MF2, and L6 cell lines, binding protein patterns were very similar (Fig. 5B). Notable differences in binding pattern included variations in the intensities of the M_r 39,000 band, highest in the C6 cell line and faint in the DDT_1MF2 and L6 cell lines, and the absence of the M_r 48,000 band and the appearance of a new M_r 56,000 band in the DDT_1MF2 cell line (Fig. 5, A and B).

UV–Cross-Linking/Immunoblot Analyses Indicate That the Major M_r 39,000 Molecule Is the Mammalian Elav-Like Protein HuR. When cross-linked proteins were electro-transferred onto nitrocellulose membranes and the blot was immunostained with HuR antibodies, we observed coincident immunostaining with the M_r 39,000 protein (Fig. 5C). This molecular mass determination is in good agreement with the calculated M_r 36,069 for HuR.

Immunoreactive bands, equivalent to the position of the free RNA probe, were also visualized in all samples, including the RNA probe alone lane (Fig. 5C). Consistent with observations in Fig. 5A, we believe that the RNasin protein contained in all samples to inhibit RNase activity is tagged to small amounts of RNA probe by UV–cross-linking (see RNA probe alone lane) and is nonspecifically reactive to either the primary or secondary antibody. To verify this possibility, we conducted additional UV–cross-linking/immunoblot experiments where RNasin was omitted from the binding reactions, with subsequent blotting and immunostain reactions; in these experiments, we observed no immunostaining of bands equivalent to the electrophoretic position of the free RNA probe (P. K. and C. A. M., unpublished observations).

UV–Cross-Linking/Immunoblot Analyses Indicate That the Rat β_1-AR 3′ UTR Probe Also Weakly Recognizes hnRNP A1. Parallel UV–cross-linking and immunoblot experiments were also conducted with heteroribonuclear protein A1 antibodies (Fig. 5D); immunoblot experiments demonstrate two immunoreactive bands positioned immediately above and below the M_r 39,000 protein, consistent in size with the 43- and 36- kDa hnRNP A1 proteins characterized in human lymphocytes (Hamilton et al., 1993). Careful laser scanning and alignment of the radioactive bands in this autoradiogram with the immunostained bands in the immunoblot revealed that neither of these two hnRNP A1 bands were coincident with the electrophoretic position of the M_r 39,000 protein (Fig. 5C; see black lines denoting alignment positions of the hnRNP A1 proteins).

In a different experiment, using extended electrophoresis and conducting only partial RNase digestion during the UV–cross-linking procedure, we visualized cross-linked products of M_r 44,000 and a protein doublet of M_r 37,000 to 39,000

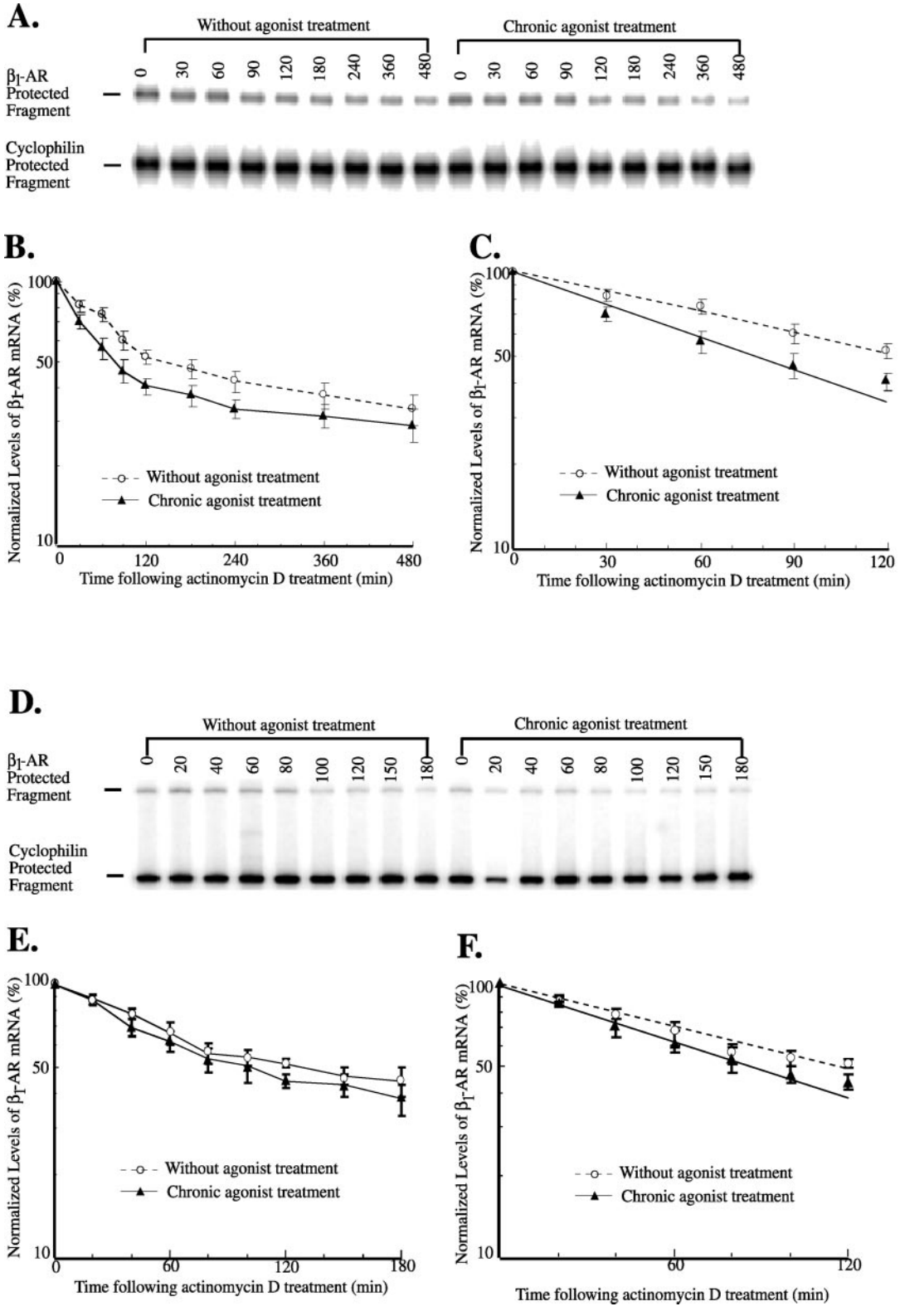
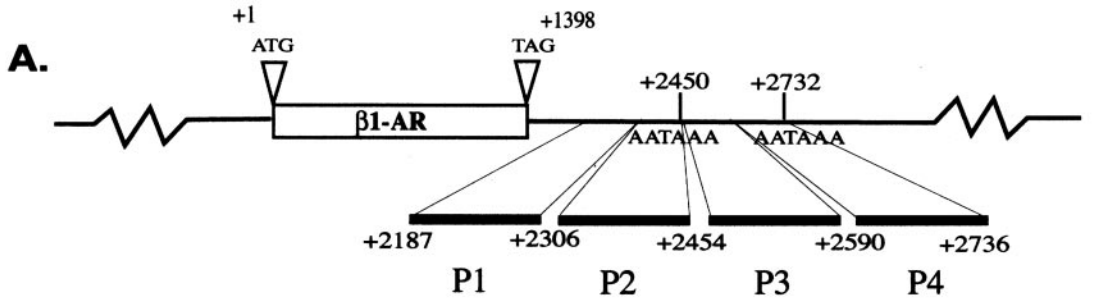


Fig. 3. A and D, rat β_1-AR mRNA half lives in DDT_1MF2 (A) and L6 (D) transfectants undergoing chronic (24 h) isoproterenol treatment. Illustration depicts representative RNase protection experiment. The hybridization probes used in this experiment series were pCS[−82, +273] and the 211-bp hamster cyclophilin probe (A) or pIB15.158 (D). B and E, normalization analyses of β_1-AR mRNA levels. Normalization calculations of β_1-AR mRNA levels against corresponding cyclophilin mRNA levels were conducted as described under *Materials and Methods*. Replicate experiments (n = 3 or 4 for B and E, respectively) were conducted. Values were plotted on a semilogarithmic plot of normalized β_1-AR mRNA levels versus time after initiation of actinomycin D treatment. C and F, first-order decay of β_1-AR mRNAs. These panels illustrate statistical analyses (two-tailed unpaired t tests) of the first-stage decline of β_1-AR mRNA levels described in B and E, respectively, by fitting data into first-order decay equations. Data time points to develop first-order decay equations for the chronic agonist treatment experiment in DDT_1MF2 and L6 transfectants were taken up to 120 min. First-order decay equations were derived and then used to determine β_1-AR mRNA half-lives.



B.

P1 RNA Sequence

AUAUUAGUUGGAAGGACCAGGCGCUGAGUCUCUCUCUGUGACAUGUGAUUCUGUCAACUAAAGAUA
GGACAGAACAAAAGGAAACAGUUGGGAUUAUUGCACAUGUGGCUAAAAACAAAG

P2 RNA Sequence

AUGACAAAAAAAAAAAGGCAGUGGUUGAAAGGCCAUUUUGCACAGUGUUAGACAUUGUAAAAUCCAUA
GAAGUUGUUAACUUGCACAAAAAAUUAAAUAUUUUUAAUGGGACGGGGAGGUGGGCAGAUCUUUAA
AAUGCUAAAAAAUAA

P3 RNA Sequence

AAUUCAAACUCAGUUUCUCUUGUCUAUUAUGUUAUUGAGUUGAUGAUUUAUUGGGAAAUCACUUUU
UAUACUCUUAUCAUGGUACUGUAACUGUAUCCAUAUUAUAAAUAUUAUUAUCUUACGUAUUUUUAUUAUA

P4 RNA Sequence

AUUUUUUAAUGUCCAAGUGCCCACGUGAAUCUGCUGGUAAAAUUUAGCAUUCGUGUGUAUAUUCAA
UCUCUUCUUGUGUGUGUGACCAAGUAUUUAUACUCUGGUGCGACUAAGUACCAUGUGGGGAAUUGG
UCCAUGUGCAAUAA

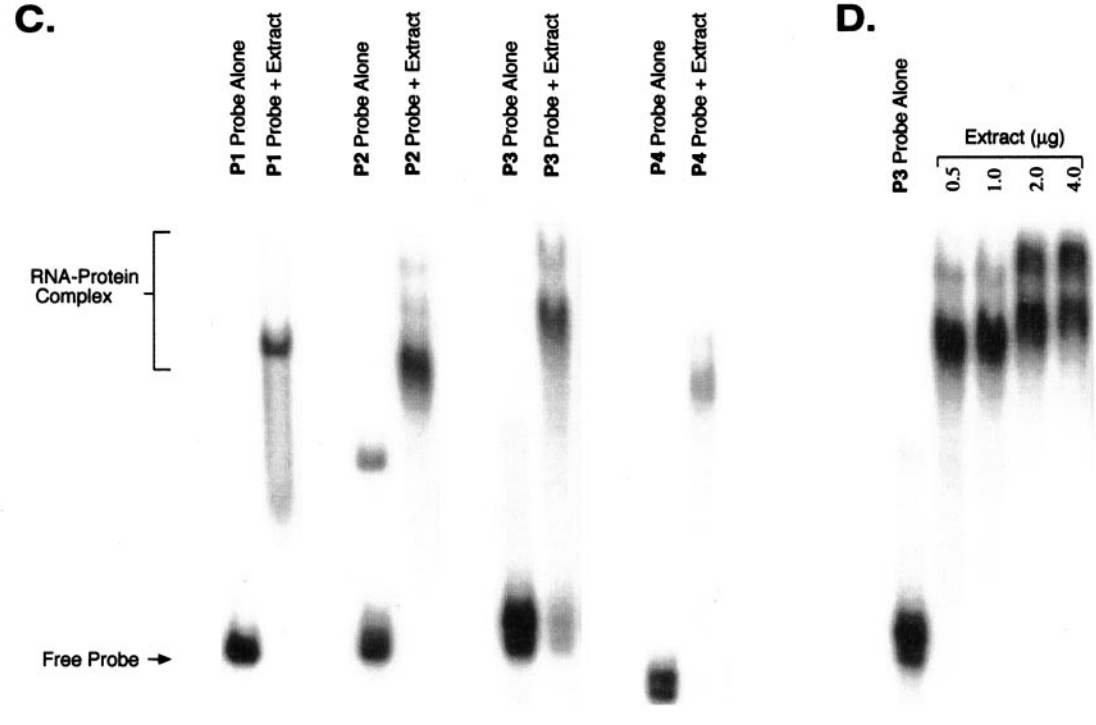


Fig. 4. A, description of β_1-AR cRNA probes used in RNA mobility shift experiments. B, nucleotide sequence of the β_1-AR 3′ UTR subregion probes P1, P2, P3, and P4. C, RNA mobility shift experiments using the subregion probes P1, P2, P3, and P4. For each subregion analyses, probe was bound to C6 cellular extract (1.0 μg) and loaded into polyacrylamide gels. Electrophoresis was conducted in 5% polyacrylamide gels in Tris-glycine EDTA at 150 V (25 mA) for 3 h. D, RNA mobility shift experiment using the subregion probe P3 with increasing amounts of cellular extract. In this experiment, varying amounts of cellular extract (0.5, 1.0, 2.0, and 4.0 μg) obtained from C6 glioma cells were used in independent binding reactions. Binding reactions were conducted using 2.5×10^4 cpm P3 probe for 15 min at room temperature.

(Fig. 5E). The extended electrophoresis resolved a doublet band; we believe that the larger component is the M_r 39,000 protein identified in Fig. 5A. We conjecture that incomplete RNase digestion allowed weaker or less-tightly cross-linked protein-probe interactions to remain undigested and permitted visualization of the faint M_r 44,000 and 37,000 bands (Fig. 5E). This reduced RNase digestion is also evident by the appearance of a larger proportion of undigested probe (electrophoretic position equivalent to the M_r 63,000 protein) (Fig. 5A). Immunoblot analyses of these cross-linked proteins indicate immunoreactivity of the hnRNP A1 antibody to the M_r 44,000 and M_r 37,000 bands (Fig. 5E), consistent with the 43- and 36-kDa hnRNP A1 proteins identified in human lymphocytes.

When β_1-AR 3′ UTR subregion P3 is used as a probe for UV–cross-linking analyses, we observed that this specific subregion recognizes proteins electrophoretically equivalent to those observed when using the complete β_1-AR 3′ UTR probe (pTail 1); subsequent immunoblot analyses of UV–cross-linked proteins indicates that the M_r 39,000 protein is also HuR (Fig. 5F). As a nonrelevant control probe, we used the rat β_1-AR upstream region, extending from position −408 to −323 (relative to the translational initiation codon) and were unable to visualize the major M_r 39,000 protein and several other minor cross-linked components (Fig. 5G).

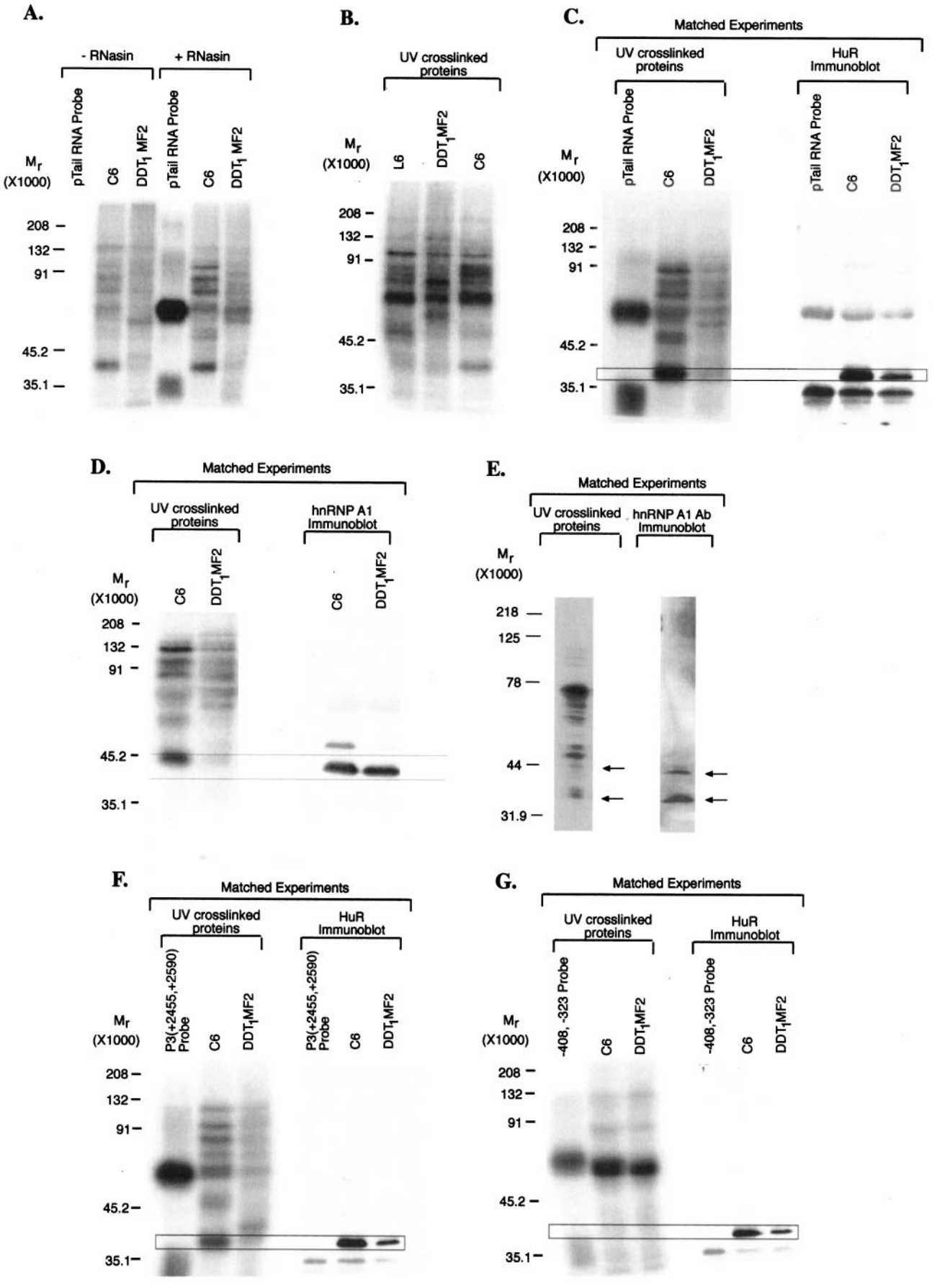
UV–Cross-Linking/Immunoprecipitation Analyses Verify Interaction of the β_1-AR 3′ UTR Probe to HuR and hnRNP A1. To augment the UV–cross-linking/immunoblot analyses, we conducted additional experiments using direct immunoprecipitation of UV–cross-linked proteins obtained from C6 cells and antibodies recognizing HuR and hnRNP A1. The UV–cross-linking/immunoblot analyses provided an initial determination in the recognition of the ARE-binding proteins to the rat β_1-AR 3′ UTR; however, this assay could not distinguish ARE-binding proteins that were not bound directly to the probe, but present as an unbound single or cross-linked multimeric protein present in the original binding reaction. Using this immunoprecipitation approach, we verified direct interaction of the β_1-AR 3′ UTR probe with HuR and hnRNP A1 obtained from C6 cell extracts (Fig. 6). The immunoprecipitated HuR protein seems to be electrophoretically coincident with the M_r 39,000 protein identified by UV–cross-linking (compare lanes in Fig. 6) and with the HuR molecule identified in cellular extracts by Western blot analyses (Fig. 7). In addition, both the M_r 44,000 and 37,000 hnRNP A1 molecules were immunoprecipitated from UV–cross-linked proteins obtained from C6 cells (Fig. 6), consistent with the UV–cross-linking/immunoblot results using the hnRNP A1 antibody (Fig. 5E) and with the sizes of the two molecular forms identified by direct Western blot analyses of C6 cellular extracts (Fig. 7). Negative control experiments, which do not demonstrate presence of immunoprecipitated proteins, include the use of nonrelevant antibody, such as the monoclonal antibody recognizing the bacterial Flag antigen, or the systematic removal of individual components in the immunoprecipitation procedure (Fig. 6). UV–cross-linking/immunoprecipitation experiments using cell extracts from the DDT_1MF2 line also resulted in the recovery of the HuR protein and the two molecular forms of hnRNP A1 (X. L. and C. A. M., unpublished observations).

Immunoblot Analyses Indicate That HuR Is Constitutively Expressed in the C6 Cell Line and Undergoes Further Induction after Isoproterenol Treatment. We systematically examined constitutive (or agonist-independent) and isoproterenol-induced levels of the RNA binding proteins HuR and hnRNP A1 in the rat C6 and hamster DDT_1MF2 cell lines. The M_r 39,000 HuR becomes elevated upon chronic isoproterenol treatment in both C6 and DDT_1MF2 cells (Fig. 7, A and C; also Table 2). These observations are consistent with the interpretation that the induction of this factor after isoproterenol treatment may lead to increased agonist-mediated β_1-AR transcript degradation. In addition, using immunoblot analyses conducted on the same blot, we have determined that the constitutive levels of HuR in C6 cells are statistically higher (1.28×) than corresponding levels in DDT_1MF2 and L6 cells (Fig. 7D).

The M_r 44,000 hnRNP A1 protein in C6 cells seems to be constitutively expressed at high levels even in the absence of agonist (compare 7-fold difference in band intensities between the M_r 44,000 hnRNP A1 molecules in C6 versus DDT_1MF2 cells; Fig. 7B). In addition, the M_r 37,000 hnRNP A1 protein in C6 cells is significantly induced after chronic isoproterenol treatment (compare values in Table 2: 3573 ± 424 versus 2367 ± 311 for isoproterenol and uninduced conditions, respectively; 1.5-fold induction; $p = 0.0019$).

C6 Stable Transfectants Containing HuR Expression Recombinants Exhibit Reduction in β_1-AR mRNA Half-Lives. To provide additional information concerning the role of HuR in β_1-AR mRNA degradation, we transfected expression recombinants containing the complete HuR coding sequence into the C6 cell line, and isolated stable transfectants. As a transfection control, we also conducted transfections using HuR expression recombinants containing inserts in reverse orientation. C6 cells transfected with the sense HuR expression recombinant exhibit a reduced β_1-AR mRNA half-life ($t_{1/2}$ = 52 ± 2.7 min; 13.3 to 17.5% reduction; $p = 0.009$) compared with either nontransfected C6 cells ($t_{1/2}$ = 63 ± 1.7 min) or C6 cells transfected with the control HuR recombinant ($t_{1/2}$ = 60 ± 2.9 min) (Fig. 8 and Table 1). Western blot analyses indicate that HuR levels are elevated approximately 10.8 to 19.9% in the sense HuR transfectants compared with nontransfected C6 cells (Y. B. and C. A. M., unpublished observations). In stable transfectants containing the control HuR recombinant, after chronic isoproterenol treatment for 24 h, the β_1-AR mRNA half-lives were significantly reduced ($t_{1/2}$ = 60 ± 2.9 min versus $t_{1/2}$ = 50 ± 4.4 min; absence and presence of chronic isoproterenol treatment, respectively; 16.7% reduction), verifying the capacity of these cells to undergo agonist-mediated β_1-AR mRNA down-regulation (Table 1). The agonist-mediated reduction in β_1-AR mRNA half-lives in the stable transfectants containing the sense HuR expression recombinant seems to be statistically equivalent with the β_1-AR mRNA half-life measured in the absence of isoproterenol (Table 1).

Constitutive β_1-AR mRNA Levels in HuR Stable Transfectants Are Reduced to Levels below Those Observed in Isoproterenol-Treated C6 cells. We conducted RNase protection analyses comparing basal and isoproterenol-induced levels of β_1-AR mRNAs in C6 cells, the DDT_1MF2 and L6 transfectants ectopically expressing β_1-AR mRNAs and the C6 transfectants containing HuR expression recombinants (Table 3). Analyses for all cell lines were conducted on the same gel to obtain relative and comparable values of β_1-AR mRNA levels. This experiment was con-



ducted multiple times ($n = 4$), with the value obtained for basal β_1-AR mRNA levels in the C6 cell line normalized as the unit measure 1. In these experiments, cell cultures were subjected to 24 h of isoproterenol treatment and then harvested immediately. This experiment measures the composite β_1-AR mRNA level and is a reflection of transcription activation/repression and post-transcriptional degradation. As expected, after chronic agonist treatment, the levels of β_1-AR mRNAs in C6 cells retained a reduced value of 0.84 ± 0.03 compared with basal uninduced levels (ascribed a unit value of 1). Interestingly, when C6 cells are transfected with the sense HuR expression recombinant, basal β_1-AR mRNAs levels in these transfectant cells retained a value of 0.63 ± 0.02, statistically reduced from the isoproterenol-induced β_1-AR mRNA levels observed in nontransfectant C6 cells (0.84 ± 0.03). When the sense HuR transfectants were treated chronically with isoproterenol, β_1-AR mRNA levels remained at 0.60 ± 0.02, indicating that the effect of HuR expression in the uninduced condition was potentially near saturation. In stable transfectants containing the control HuR recombinant in reverse orientation, basal β_1-AR mRNA levels were 1.15 ± 0.01, statistically higher than the basal level observed in uninduced C6 cells (normalized unit value of 1). Although untested, this may indicate the potential effect of antisense RNA hybridization occurring with endogeneous HuR transcripts, resulting in the overall diminution of HuR expression. Overall, these experiments support the hypothesis that HuR plays a role in the agonist-independent and agonist-mediated down-regulation of β_1-AR mRNAs.

In a parallel experiment, we also determined relative levels of β_1-AR mRNAs in the isoproterenol-induced and uninduced DDT_1MF2 and L6 transfectants. As anticipated from expression from a strong promoter, the ectopic expression of the rat β_1-AR mRNA in DDT_1MF2 transfectants was higher (3.0 ± 0.34) than the endogenously expressed level identified in C6 cells (normalized unit value of 1; Table 3). We also simultaneously compared the ectopically expressed levels of rat β_1-AR mRNAs in L6 transfectant cells. The L6 transfectants expressed β_1-AR mRNAs at only 15% (0.15 ± 0.05) of the level observed in C6 cells (Table 3). Thus, in two transfectant cell pools, where the ectopic agonist-independent expression of β_1-AR mRNAs bracket above and below the levels observed in C6 cells, the constitutive half-lives of the β_1-AR mRNAs were statistically equivalent ($t_{1/2}$ = 109 ± 4.0 min and $t_{1/2}$ = 112 ± 2.5 min, for DDT_1MF2 transfectants and L6 transfectants, respectively; $p = 0.35$). This seems to diminish the possibility that ectopic overexpression of β_1-AR mRNAs results in saturation of degradation components. However, we cannot rule out the possibility that differences in the 5′ end of the ectopic and endogenous β_1-AR mRNAs, or potential differences in the intracellular localization of the ectopically-expressed transcripts, may play a role in differences in degradation rate.

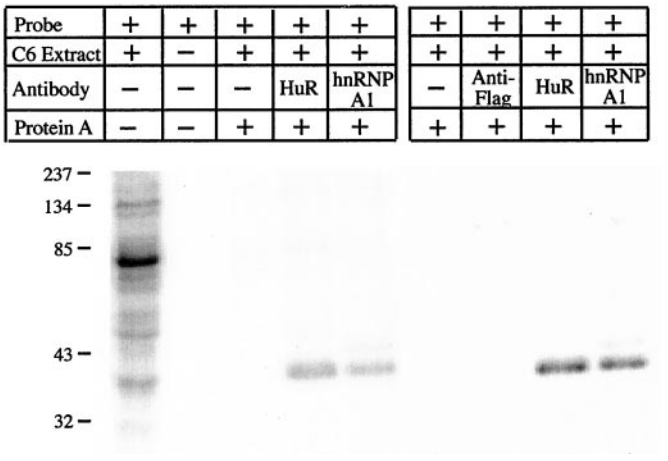


Fig. 6. UV–cross-linking and immunoprecipitation of RNA-protein complexes. RNA complexes using the β_1-AR probe pTail 1, which contains β_1-AR sequence extending from +2084 to +2901 and C6 cellular extracts (25 μg) were prepared as described under *Materials and Methods*. Complexes were irradiated with UV light for 5 min on ice using a UV–cross-linking oven and treated with RNase A and T1. Specific antibody (1–2 μl of anti-HuR and anti-hnRNP A1) was added to the reaction mix and allowed to incubate for 1 h at 4°C. PBS was added to the mixture to bring the final volume to 200 μl. Protein A slurry (20–40 μl) was then added and allowed to mix overnight at 4°C. The protein A beads were then washed twice by gentle centrifugation and then resuspended in 1× loading buffer before heating at 100°C for 5 min. RNA-protein complexes released from the protein A beads were then loaded into SDS-polyacrylamide gels. Negative control experiments include the use of nonrelevant antibody, such as the monoclonal antibody recognizing the bacterial Flag antigen, or the systematic removal of individual components in the immunoprecipitation procedure. Note that all control experiments, including probe lane, have undergone UV–cross-linking and RNase digestion. Molecular mass markers (in kilodaltons) are displayed in the left margin.

Discussion

We have used an array of experimental tools, including RNase protection assays, RNA mobility shifts, immunoblotting and immunoprecipitation experiments, and UV–cross-linking/denaturation analyses, to develop a basic understanding of the molecular determinants underlying

Fig. 5. UV–cross-linking and denaturation of RNA-protein complexes for polyacrylamide gel electrophoresis and electro-transfer and staining of nitrocellulose membranes with HuR and hnRNP A1 antibodies. RNA complexes using the β_1-AR probe pTail 1, which contains β_1-AR sequence extending from +2084 to +2901 and C6 cellular extracts (25 μg) were prepared as described under *Materials and Methods*. Complexes were irradiated with UV light for 5 min on ice using a UV–cross-linking oven and treated with RNase A and T1. Samples were then denatured and subjected to SDS-PAGE. Proteins from the polyacrylamide gels were electro-transferred onto nitrocellulose membranes; blots were subsequent immunostained with primary and secondary antibodies as described under *Materials and Methods*. For all panels, the sizes of the relative molecular mass markers (in kilodaltons) are illustrated on the left side of each panel. In A, parallel binding/UV–cross-linking experiments were conducted in the presence or absence of RNasin. Note presence of experimental artifact in RNA probe lane (in the presence of RNasin). In B, binding/UV–cross-linking experiments, all conducted in the presence of RNasin were conducted using extracts from C6, DDT_1MF2, and L6 cells. Duplicate UV–cross-linking reactions for C and D were electrophoresed in the same gel. Cross-linked proteins were transferred to a single nitrocellulose membrane and membranes were subsequently cut to conduct parallel HuR and hnRNPA1 immunostaining reactions. Immunostained blots using primary antibodies recognizing HuR (C) and hnRNPA1 (D) are displayed. Note boxes which denotes coincident localization of the immunoreactive HuR band with the M_r 39,000 cross-linked protein (C and F). Lines in D denote the electrophoretic positions of the hnRNPA1 immunoreactive proteins on the original autoradiogram displaying the cross-linked proteins. For experiment described in E, formation, UV–cross-linking and denaturation of RNA-protein complexes, SDS-PAGE, and electrotransfer of proteins are as described under *Materials and Methods*, with modifications concerning the addition of RNase T1 before conducting the cross-linking reactions. The blot in E was immunostained with antibodies recognizing hnRNPA1. The M_r 44,000 and 37,000 cross-linked proteins are perfectly aligned with corresponding immunoreactive hnRNPA1 molecules (see arrows in E). F and G contain binding/UV–cross-linking experiments using the P3 subregion probe (containing rat β_1-AR 3′ UTR sequence +2455 to +2590; F) and a nonrelevant control RNA probe (containing β_1-AR upstream sequences −408 to −323; G).

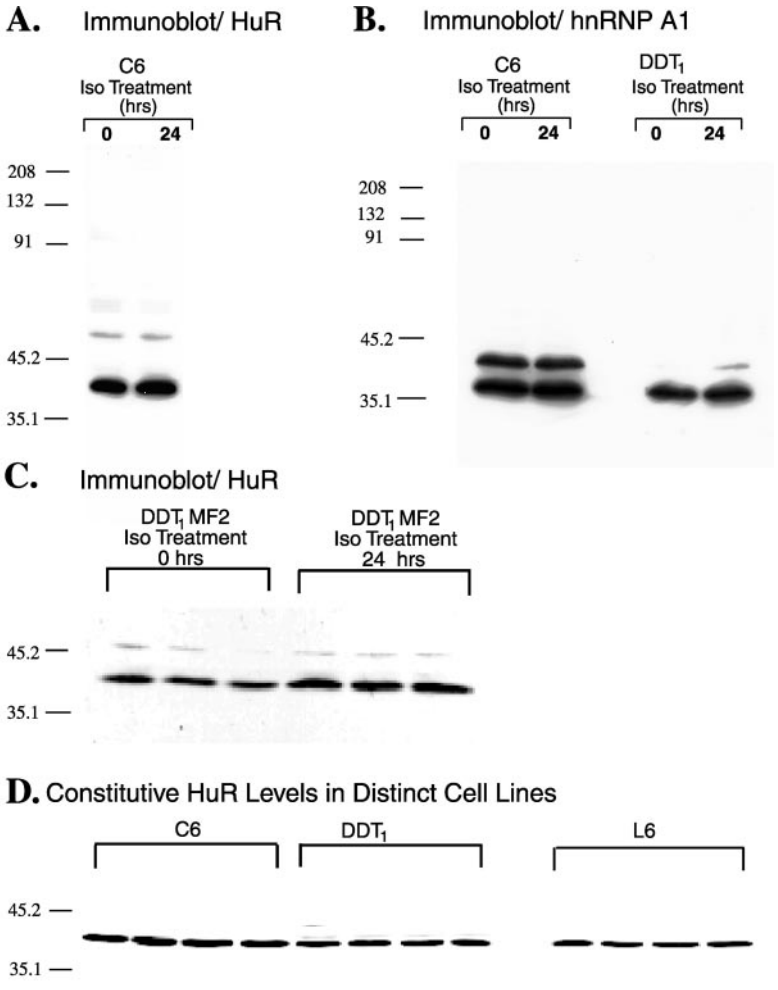


Fig. 7. Immunoblot analyses of constitutive and isoproterenol-induced HuR (A and C) and hnRNPA1 (B) expression in C6 and DDT_1MF2 cell lines. C6 and DDT_1MF2 cells were treated with isoproterenol for 24 h. Cellular extract (25 μg) were subjected to polyacrylamide gel electrophoresis, electrotransfer to nitrocellulose membranes, and immunoblot analyses using antibodies as described under *Materials and Methods*. Untreated controls (equivalent to zero hour sample) and isoproterenol-treated samples are displayed. Note high constitutive expression of the M_r 44,000 hnRNPA1 protein in C6 cells versus DDT_1MF2 cells. Experiments were conducted in replicate ($n = 4$ for agonist-mediated induction of HuR in C6 cells, A; $n = 3$ for agonist-mediated induction of HuR in DDT_1MF2 cells, C) and are quantified in Table 2. D, Immunoblot analyses comparing constitutive levels of HuR in C6, DDT_1MF2, and L6 cell extracts. Preparation of cell extracts, SDS-PAGE and immunoblot analyses using the HuR antibody were conducted as described under *Materials and Methods*. Band intensities were examined using the Bio-Rad Molecular Imager FX system. The average intensities (and standard deviations) for constitutive HuR expression are: C6, 3376 ± 176; DDT_1MF2, 2634 ± 61.9; and L6, 2374 ± 93.2.

agonist-mediated degradation of β_1-AR mRNAs. We have accumulated evidence that the mammalian elav-like protein HuR and the heteroribonuclear protein hnRNP A1 interact with the rat β_1-AR 3′ UTR and that the induction of HuR by isoproterenol may play a role in the agonist-mediated modulation of β_1-AR mRNA half-life.

We were originally uncertain why the rat β_1-AR mRNA half-life in C6 cells ($t_{1/2}$, 63 - 65 min; Fig. 2) was much shorter than the reported half-lives of the hamster β_2-AR mRNA in DDT_1MF2 cells (Danner and Lohse, 1997). Furthermore, after chronic isoproterenol treatment of DDT_1MF2 cells, the reported half-life of the hamster β_2-AR mRNA underwent a significant reduction, to either 50 min in the case of suspension cultures or 6.5 h in the case of monolayer cultures (Danner and Lohse, 1997). In this same study, isoproterenol had no effect on β_2-AR mRNA stability in C6 cells, although agonist treatment reduced transcript levels by approximately 50%.

In our studies, the agonist-independent half-life for the rat β_1-AR mRNA was perceptibly longer ($t_{1/2}$ = 63 ± 1.7 min) than the half-life determined after chronic agonist treatment ($t_{1/2}$ = 41 ± 5.9 min), indicating that chronic isoproterenol induction can trigger a significant decrease in β_1-AR mRNA stability in C6 cells. Our experimental results for the effects of isoproterenol treatment on β_1-AR mRNA levels in C6 cells are similar to determinations reported previously for β_2-AR mRNA in the same cell line. We hypothesized that cellular environment, dictating differences in expression of key RNA degradation factors in the C6 and DDT_1MF2 cell lines could account for the agonist-mediated observed differences in both the agonist-independent and -mediated β-AR mRNA stability. We tested this hypothesis by transfecting an expression vector encoding the rat β_1-AR into hamster DDT_1MF2 cells and creating a stable transfectant pool. In these experiments, the agonist-independent half-life of the rat β_1-AR transcript ($t_{1/2}$ = 109 ± 4.0 min; Fig. 3 and Table 1) in transfectant DDT_1MF2 cells was much longer than in its endogenous environment in C6 cells ($t_{1/2}$ = 63 ± 1.7 min) and seemed to be similar to the half-life determined for the hamster β_2-AR mRNA in the DDT_1MF2 cell line. In addition, in the DDT_1MF2 cell transfectants, chronic isoproterenol treatment resulted in decreases in rat β_1-AR mRNA half-life ($t_{1/2}$ = 83 ± 5.1 min). Similar results were also obtained using the rat L6 cell transfectants that ectopically express β_1-AR mRNAs. Using the L6 transfectants, the β_1-AR mRNA half-lives, determined both in the absence and presence of chronic isoproterenol treatment, were comparable with half-lives observed in DDT_1MF2 transfectants (Fig. 3 and Table 1). These observations provide evidence that functional β_1-AR mRNAs are expressed in the DDT_1MF2 and L6 transfectant cell lines, and that the rat β_1-AR mRNAs undergo differential agonist-mediated degradation rates dependent on cell environment.

TABLE 2

HuR and hnRNP A1 levels in C6 cells and DDT_1MF2 cells

Multiple dishes of C6 and DDT_1MF2 cells (n = 4 dishes for each experimental condition and each cell type) were uninduced or treated with isoproterenol for 24 h. Proteins were extracted, electrophoresed on SDS-PAGE, and electrotransfered to membranes for Western Analyses using antibodies recognizing HuR and hnRNP A1. Band intensities were measured using the Bio-Rad Molecular Imager FX system. Each cell line (with both experimental conditions) for each antibody was analyzed on individual blots. Thus, band intensity values between isoproterenol-treated and uninduced conditions for each cell line are on the same intensity scale, but not values for band intensities between cell lines or between antibodies. Displayed values are mean intensity values ± S.D.

	C6 Cells		DDT_1MF2 Cells	
	+ Isoproterenol	Uninduced	+ Isoproterenol	Uninduced
HuR	8580 ± 681[a]	6696 ± 929[a]	656 ± 59[a]	472 ± 45[a]
hnRNP A1				
M_r 44,000	2219 ± 358	1750 ± 281	665 ± 22	684 ± 27
M_r 37,000	3573 ± 424[b]	2367 ± 311[b]	4293 ± 105	4422 ± 101

[a] Difference between average band intensities for HuR in C6 cells under uninduced and isoproterenol-treated conditions is statistically significant (n = 4; p = 0.024). Difference between average band intensities for HuR in DDT_1MF2 cells under uninduced and isoproterenol-treated conditions is statistically significant (n = 3; p = 0.012).
[b] Difference between band intensities of M_r 37,000 hnRNP A1 in C6 cells under uninduced and isoproterenol-treated conditions is statistically significant (p = 0.0019). All other observed differences in the M_r 44,000 hnRNP A1 molecule in C6 cells and the M_r 37,000 hnRNP A1 molecule in C6 and DDT_1MF2 cells between uninduced and isoproterenol-treated conditions were not statistically significant.

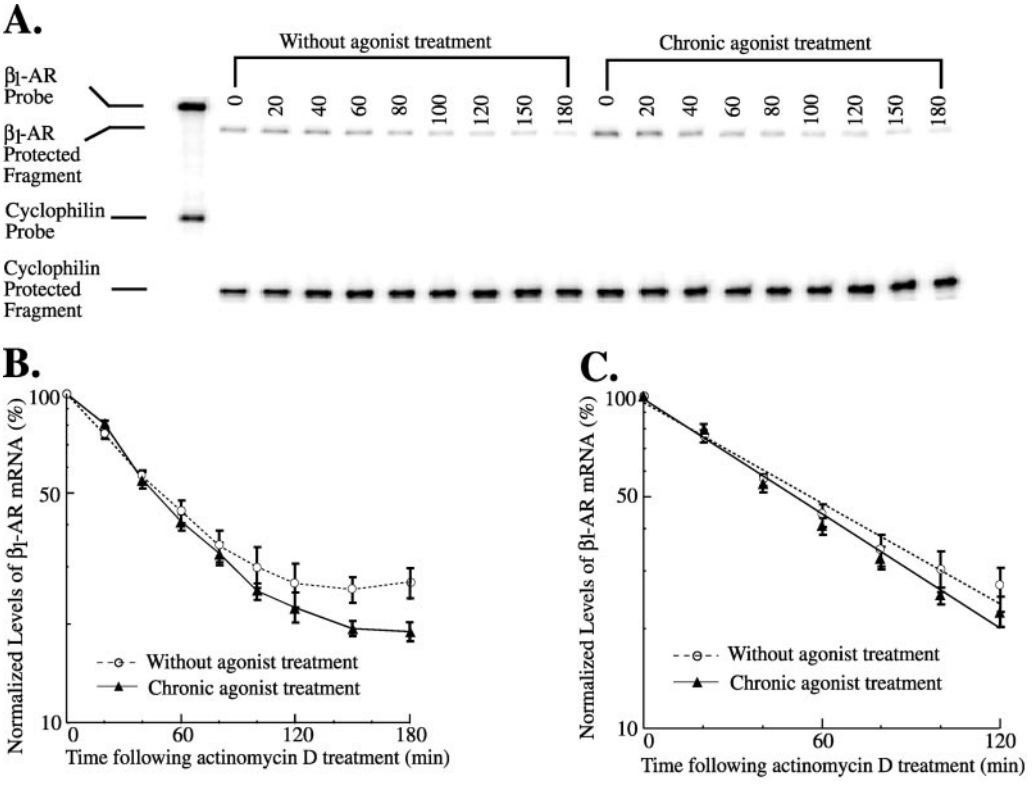


Fig. 8. A, rat β_1-AR mRNA half lives in C6 transfectants containing HuR expression recombinants and subjected to chronic (24 h) isoproterenol treatment. Illustration depicts representative RNase protection experiment. The hybridization probes used in this experimental series were pCS[−82, +273] and pIB15.158. Eukaryotic expression recombinants containing HuR inserts in sense or reverse orientation were transfected into C6 cells. B, normalization analyses of β_1-AR mRNA levels. Normalization calculations of β_1-AR mRNA levels against corresponding cyclophilin mRNA levels were conducted as described under *Materials and Methods*. Replicate experiments (n = 4) were conducted. Values were plotted on a semilogarithmic plot of normalized β_1-AR mRNA levels versus time after initiation of actinomycin D treatment. C, first-order decay of β_1-AR mRNAs. This panel illustrates statistical analyses (two-tailed unpaired t tests) of the first stage decline of β_1-AR mRNA levels described in B, by fitting data into first-order decay equations. Data time points to develop first-order decay equations for the chronic agonist treatment experiment in C6 transfectants were taken up to 120 min. First-order decay equations were derived and then used to determine β_1-AR mRNA half-lives.

TABLE 3

β_1-AR mRNA levels in C6 cells and transfectants

Stable transfectants were generated using expression recombinants containing the complete rat β_1-AR cDNA, including 3′ UTR and HuR in either sense or reverse orientation. Chronic isoproterenol treatment was 10 μM for 24 h. RNA was harvested immediately after isoproterenol treatment. RNA measurements are a reflection of composite transcriptional and post-transcriptional activity. β_1-ARmRNA levels were determined for all cell types and treatment conditions by RNase protection analyses. Analyses for each experiment were conducted on a single gel to obtain comparative values. This experiment was repeated multiple times (n = 4). Intensity values were quantified using the system described under *Materials and Methods*. Values for each cell type and condition were averaged and then normalized using the intensity value identified in the uninduced C6 cell line, which was ascribed a unit measure of 1.00. Standard deviations were calculated using Microsoft Excel.

Cell type	Transfecting Plasmid	Chronic Isoproterenol	β_1-AR mRNA Levels	Standard Deviation
C6	None	−	1.00	N/A
C6	None	+	0.84	0.03
C6	HuR sense	−	0.63	0.02
C6	HuR sense	+	0.60	0.02
C6	HuR reverse	−	1.15	0.01
C6	HuR reverse	+	0.95	0.03
DDT_1MF2	Rat β_1-AR	−	3.01	0.34
DDT_1MF2	Rat β_1-AR	+	1.69	0.05
L6	Rat β_1-AR	−	0.15	0.05
L6	Rat β_1-AR	+	0.08	0.03

The rat β_1-AR mRNA has two functional polyadenylation signals at +2450 and +2732 (Searles et al., 1995). In Fig. 1, β_1-AR transcripts containing either the proximal (short-form transcript) or the distal (long-form transcript) polyadenylation signal seem to undergo simultaneous and equivalent induction after agonist treatment, consistent with the common transcription and promoter/enhancer apparatus affecting these two polyadenylated mRNAs.

We recognize that the reduced levels of the shorter polyadenylated β_1-AR transcript is at the extreme lower limits of measurement for the RPA procedure; with this caveat and by using repeated evaluations ($n = 4$), we can detect no appreciable difference in stability of the two polyadenylated β_1-AR mRNAs (Y. B. and C. A. M., unpublished observations). Other investigators have confirmed the presence of two human β_1-AR transcripts, using consensus polyadenylation signals at +2469 and +2751 (Ellis and Frielle, 1998). In these studies, using ventricular heart RNA isolated from patients with idiopathic dilated cardiomyopathy, both polyadenylated transcripts underwent substantial decreases compared with nonfailing controls, with the larger transcript undergoing significantly greater decreases (2.1- and 2.7-fold decreases for the shorter and longer transcripts, respectively). These investigators conjectured that the differences in transcript levels were caused by differences in mRNA stability mediated by unique AREs present only in the longer transcript, but did not experimentally rule out potential transcriptional regulators. We cannot eliminate the potential role of sequence differences between the rat and human β_1-AR 3′ UTR and the differences in cell environment (C6 cells versus cells in the left ventricular heart) for creating these distinctly different observations.

Because of the common AU-rich characteristics of the β_1- and β_2-AR 3′ UTR sequences, including the presence of several AUUUA pentamers, and from evidence that RNA binding proteins may influence the post-transcriptional regulation of β_2-AR mRNAs, we hypothesized that the agonist-induced down-regulation of β_1-AR mRNAs is mediated, at least in part, by post-transcriptional destabilization of transcripts via interaction with RNA degradation molecules. However, transcriptional repression of β_1-AR mRNAs may also occur, by interaction of the inducible cAMP early repressor (ICER) with the cyclic AMP response element (CRE site) in the β_1-AR promoter region. Our studies with collaborators have demonstrated that ICER interacts with the CRE at position −1314 to −1307 in the rat β_1-AR gene and that cotransfection experiments using an ICER expression vector in C6 cells represses transcription of the rat β_1-AR promoter-luciferase recombinant containing the CRE (Rydelek-Fitzgerald et al., 1996). We believe that the transcriptional repression results identified in Rydelek-Fitzgerald et al. (1996) is consistent with our current findings, where agonist-mediated post-transcriptional control mechanisms occur after long-term (24 h) but not short-term (60 min) agonist treatment. As shown in Fig. 1, we determined that enhanced post-transcriptional degradation of β_1-AR mRNAs does not occur after short-term isoproterenol treatment. Thus, we believe that our current findings are complementary with Rydelek-Fitzgerald et al. (1996) concerning transcriptional repression. In addition, differences in the basal levels of the polyadenylated rat or human β_1-AR mRNAs may be caused by differences in the translational efficiency of the two polyadenylated transcripts. Two subtypes of the α_2-ARs (the $\alpha_{2a/d}$-ARs and α_{2c}-ARs) retain dramatically different translational efficiencies; this difference is caused by a 288-bp sequence in the 3′ UTR of the α_{2c}-AR mRNA (Yang et al., 1997).

UV–cross-linking and denaturation analysis of the pTail 1-C6 protein complexes identified a major M_r 39,000 binding protein and several minor components M_r 90,000, 82,000, 72,000, 63,000, and 48,000 (Fig. 5A). When the β_1-AR probe pTail 1 was bound to cellular extracts from the C6, DDT_1MF2, and L6 cells, binding protein patterns were very similar (Fig. 5A). The notable differences were variations in the intensities of the M_r 39,000 band, highest in the C6 cell line and faint in the DDT_1MF2 and L6 cell lines; the absence of the M_r 48,000 band; and the appearance of a new M_r 56,000 band in the DDT_1MF2 cell line.

Immunoblot and immunoprecipitation analyses of the UV–cross-linked proteins indicate that the predominant M_r 39,000 protein is HuR (Fig. 5C and Fig. 6). In addition, when partial RNase digestion is used for the UV–cross-linking/hnRNP A1 immunoblot analyses (Fig. 5, A and B), allowing conservation of weaker probe-protein cross-linking products to occur, we observe additional minor protein components of M_r 44,000 and 37,000; these molecules are immunoreactive to antibodies recognizing hnRNP A1. Our immunoblot analyses indicate that HuR levels in the C6 cells are statistically higher (1.28 X) than corresponding levels observed in DDT_1MF2 and L6 cells (Fig. 7D; also Table 2). Thus, differences in the intensity of the M_r 39,000 binding protein observed in the UV–cross-linking of the β_1-AR 3′ UTR probe to distinct cell extracts (as shown in Fig. 5B) can be partially attributed to abundance differences in HuR. We hypothesize that these intensity differences may also be caused by variations in binding avidity of the HuR molecule, perhaps conferred by post-translational structural modifications (e.g., phosphorylation) or by the requirement of additional cofactors. Four HuR ligands, known as SETα and SETβ, pp32, and APRIL (acidic protein rich in leucine), have been recently identified and seem to influence the ability of HuR to recognize ARE-containing mRNAs (Brennan et al., 2000); thus, the existence of other cofactors or binding molecules that can influence the avidity of HuR to recognize β_1-AR mRNAs is a distinct possibility. These experiments infer that HuR abundance and/or HuR recognition to the rat β_1-AR mRNA 3′ UTR seem to correlate with differences in basal transcript stability. These observations also support the interpretation that other RNA binding molecules, including hnRNP A1, can recognize elements within the rat β_1-AR 3′ UTR.

Our observations are consistent with the report by others (Blaxall et al., 2000), who find that both HuR and hnRNP A1 can undergo induction by isoproterenol and bind to distinct β-AR 3′ UTRs with differential affinities. In the βARB complex, HuR seems to be the predominant RNA binding component; the precise ratio of the HuR and hnRNP A1 binding components are dependent on the 3′ UTR sequence of the target β-AR subtype mRNA (Blaxall et al., 2000). In these studies, HuR and hnRNP A1 bind to human β_1-AR 3′ UTR sequences, whereas only HuR is found to bind to hamster β_2-AR 3′ UTR sequences. In our experiments, both HuR and two molecular forms of hnRNP A1 are identified directly in cross-linking/immunoblot and immunoprecipitation analyses using cellular extracts and the rat β_1-AR 3′ UTR probe (Fig.

5, C and E; Fig. 6). Our evidence indicates that the two molecular forms of hnRNP A1 recognize the rat β_1-AR 3′ UTR, but with perhaps weaker avidity compared with HuR. HuR seems to be induced in isoproterenol-treated C6 and DDT$_1$MF2 cells (Fig. 7, A and C; Table 2) and is constitutively expressed at higher levels in the C6 cell line (Fig. 7D and Table 2). In addition, the M_r 40,000 hnRNP A1 molecule is constitutively expressed at high levels in C6 cells, and undergoes agonist-mediated induction (Fig. 7B; also Table 2).

To test the hypothesis that HuR played a role in β_1-AR mRNA down-regulation, we transfected eukaryotic expression recombinants containing HuR inserts in sense or reverse orientation into C6 cells. C6 stable transfectants containing the sense HuR recombinant expressed elevated levels of HuR, and exhibited a shorter agonist-independent β_1-AR mRNA half-life and lower basal β_1-AR mRNA levels compared with the corresponding mRNA degradation rate and levels observed in nontransfected C6 cells (Fig. 8 and Tables 1 and 3). Furthermore, after chronic isoproterenol treatment, the β_1-AR mRNA half-life and levels did not seem to be statistically altered (Fig. 8; Tables 1 and 3), indicating that constitutive HuR expression in the stable transfectants were potentially saturating and that isoproterenol induction had no additional effect. The constitutive β_1-AR mRNA decay rate in the HuR sense transfectant was not maximally accelerated to the rate observed in isoproterenol-treated nontransfected C6 cells, but the β_1-AR mRNA levels in the HuR sense transfectant seem to be depressed to a much-reduced basal level. C6 stable transfectants containing the control HuR recombinant in reverse orientation exhibited an agonist-independent β_1-AR mRNA half-life that was statistically equivalent to the degradation rate observed in nontransfected C6 cells. Observations described in this manuscript are consistent with the interpretation that the rat β_1-AR 3′ UTR retains high avidity to HuR, and that HuR may both play a role in the agonist-independent and agonist-mediated post-transcriptional degradation of the β_1-AR mRNAs. Thus, work conducted in this study and by Rydelek-Fitzgerald et al. (1996) implicates the existence of at least three regulatory mechanisms influencing β_1-AR mRNA levels in C6 cells: 1) an initial, acute up-regulation induced by transcriptional activation, 2) an intermediate down-regulation moderated by transcriptional repression, potentially by ICER, and 3) a chronic down-regulation mediated by post-transcriptional degradation mechanisms, potentially influenced by HuR, a component of βARB, and/or by other RNA binding proteins.

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References

Akamatsu W, Okano HJ, Osumi N, Inoue T, Nakamura S, Sakakibara A-I, Miuram M, Matsui N, Darnell RB, and Okano H (1999) Mammalian elav-like neuronal RNA-binding proteins HuB and HuC promote neuronal development in both central and peripheral nervous systems. *Proc Natl Acad Sci USA* **96:**9885–9890.

Blaxall BC, Pellett AC, Wu SC, Pende A, and Port JD (2000) Purification and characterization of beta-adrenergic receptor mRNA-binding proteins. *J Biol Chem* **274:**4290–4297.

Brennan CM, Gallouzi I-E, and Steitz JA (2000) Protein ligands to HuR modulate its interaction with target mRNAs in vivo. *J Cell Biology* **151:**1–13.

Brewer G (1991) An A + U rich element RNA-binding factor regulates c-myc mRNA stability in vitro. *Mol Cell Biol* **11:**2460–2466.

Chkheidze AN, Lyakhov DL, Makeyev AV, Morales J, Kong J, and Liebhaber SA (1999) Assembly of the α-globin mRNA stability complex reflects binary interaction between the pyrimidine-rich 3′ untranslated region determinant and poly (C) binding protein αCP. *Mol Cell Biol* **19:**4572–4581.

Danner S, Frank M, and Lohse MJ (1998) Agonist regulation of human β_2-adrenergic receptor mRNA stability occurs via a specific AU-rich element. *J Biol Chem* **273:**3223–3229.

Danner S and Lohse MJ (1997) Cell type-specific regulation of β_2-adrenoceptor mRNA by agonist. *Eur J Pharmacol* **331:**73–78.

DeMaria CT, Sun Y, Long L, Wagner BJ, and Brewer G (1997) Structural determinants in AUF1 required for high affinity binding to A+U-rich elements. *J Biol Chem* **272:**27635–27643.

Derrigo M, Cestelli A, Savettieri G, and Di Liegro I (2000) RNA-protein interactions in the control of stability and localization of messenger RNA. *Int J Mol Med* **5:**111–123.

Ellis CE and Frielle T (1999) Characterization of two human β_1-adrenergic receptor transcripts: cloning and alterations in failing heart. *Biochem Biophys Res Commun* **258:**552–558.

Ford LP, Watson J, Keene JD, and Wilusz J (1999) Elav proteins stabilize deadenylated intermediates in a novel in vitro mRNA deadenylation/degradation system. *Genes Dev* **13:**188–201.

Greenberg ME, Shyu A-B, and Belasco JG (1990) Deadenylation: a mechanism controlling c-fos mRNA decay. *Enzyme* **44:**181–192.

Hadcock JR, Wang H-Y, and Malbon CC (1989) Agonist-induced destabilization of β-adrenergic receptor mRNAs. *J Biol Chem* **264:**19928–19933.

Hamilton BJ, Nagy E, Malter JS, Arrick BA, and Rigby WFC (1993) Association of heterogeneous nuclear ribonuclear protein A1 and C proteins with reiterated AUUUA sequences. *J Biol Chem* **268:**8881–8887.

Hosoda K, Feussner GK, Rydelek-Fitzgerald L, Fishman PH, and Duman RS (1994) Agonist and cyclic AMP-mediated regulation of beta 1-adrenergic receptor mRNA and gene transcription in rat C6 glioma cells. *J Neurochem* **63:**1635–1645.

Hosoda K, Fitzgerald LR, Vaidya VA, Feussner GK, Fishman PH, and Duman RS (1995) Regulation of β_2-adrenergic receptor mRNA and gene transcription in rat C6 glioma cells: effects of agonist, forskolin, and protein synthesis inhibition. *Mol Pharmacol* **48:**206–211.

Huang L-Y, Tholanikunnel BG, Vakalopoulou E, and Malbon CC (1993) The M_r 35,000 β-adrenergic receptor mRNA-binding protein induced by agonists requires both an AUUUA pentamer and U-rich domains for RNA recognition. *J Biol Chem* **268:**25769–25775.

Izzo NJ, Seidman CE, Collins S, and Colucci WS (1990) Alpha 1-adrenergic receptor mRNA level is regulated by norepinephrine in rabbit smooth muscle cells. *Proc Nat Acad Sci USA* **87:**6268–6271.

Jackson RJ (1993) Cytoplasmic regulation of mRNA function: the importance of the 3′ untranslated region. *Cell* **74:**9–14.

Kirigiti P and Machida CA (1999) Electrophoretic mobility shift assay for detection of DNA binding proteins recognizing beta-adrenergic receptor gene sequences, in *Adrenergic Receptor Protocols* (Machida CA ed) pp 431–451, Humana Press, Totowa, New Jersey.

Kirigiti P, Yang YF, Li X, Li B, Midson CN, and Machida CA (2000). Rat beta 1-adrenergic receptor regulatory region containing consensus AP-2 elements recognizes novel transactivator proteins. *Mol Cell Biol Res Commun* **3:**181–192.

Lee NH, Earle-Hughes J, and Fraser CM (1994) Agonist-mediated destabilization of m1 muscarinic acetylcholine receptor mRNA: elements involved in mRNA stability are localized in the 3′ untranslated region. *J Biol Chem* **269:**4291–4298.

Levy NS, Chung S, Furneaux H, and Levy AP (1998) Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. *J Biol Chem* **273:**6417–6423.

Loflin P, Chen C-YA, and Shyu A-B (1999) Unraveling a cytoplasmic role for hnRNP D in the in vivo mRNA destabilization directed by the AU-rich element. *Genes Dev* **13:**1884–1897.

Ma W-J, Cheng S, Campbell C, Wright A, and Furneaux H (1996) Cloning and characterization of HuR, a ubiquitously expressed elav-like protein. *J Biol Chem* **271:**8144–8151.

Maurer F, Tierney M, and Medcalf RL (1999) An AU-rich sequence in the 3′ UTR of plasminogen activator inhibitor type 2 (PAI-2) mRNA promotes PAI-2 mRNA decay and provides a binding site for nuclear HuR. *Nucleic Acids Res* **27:**1664–1673.

Mitchusson KD, Blaxall BC, Pende A, and Port JD (1998) Agonist-mediated destabilization of human beta 1-adrenergic receptor mRNA: role of the 3′ untranslated region. *Biochem Biophys Res Commun* **252:**357–362.

Nielsen DA and Shapiro DJ (1990) Insights into hormonal control of messenger RNA stability. *Mol Endocrinol* **4:**953–957.

Pende A, Tremmel KD, DeMaria CT, Blaxall BC, Minobe WA, Sherman JA, Bisognano JD, Bristow MR, Brewer G, and Port JD (1996) Regulation of the mRNA-binding protein AUF1 by activation of the β-adrenergic receptor signal transduction pathway. *J Biol Chem* **271:**8493–8501.

Port JD, Huang L-Y, and Malbon CC (1992) β-adrenergic agonists that down-

regulate receptor mRNA up-regulate a M_r 35,000 protein(s) that selectively binds to β-adrenergic receptor mRNAs. *J Biol Chem* **267:**24103–24108.
Rydelek-Fitzgerald L, Li Z, Machida CA, Fishman PH, and Duman RS (1996) Adrenergic regulation of ICER (inducible cAMP early repressor) and β_1-adrenergic receptor gene expression in C6 glioma cells. *J Neurochem* **67:**490–497.
Schiavi SC, Belasco JG, and Greenberg ME (1992) Regulation of proto-oncogene mRNA stability. *Biochem Biophys Acta* **1114:**95–106.
Searles RP, Midson CN, Nipper V, and Machida CA (1995) Transcription of the rat β_1-adrenergic receptor gene: characterization of the transcript and identification of important sequence elements. *J Biol Chem* **270:**157–162.
Tholanikunnel BG and Malbon CC (1997) A 20-nucleotide (A+U)-rich element of β_2-adrenergic receptor (β_2-AR) mRNA mediates binding to the β_2-AR-binding protein and is obligate for agonist-induced destabilization of receptor mRNA. *J Biol Chem* **272:**11471–11478.
Tholanikunnel BG, Raymond JR, and Malbon CC (1999) Analysis of the AU-rich elements in the 3′ untranslated region of the β_2-adrenergic receptor mRNA by mutagenesis and identification of the homologous AU-rich region from different species. *Biochem* **38:**15564–15572.
Xu N, Chen C-YA, and Shyu A-B (1997) Modulation of the fate of cytoplasmic mRNA by AU-rich elements: Key sequence features controlling mRNA deadenylation and decay. *Mol Cell Biol* **17:**4611–4621.
Yang Q, McDermott PJ, Duzic E, Pleij CWA, Sherlock JD, and Lanier SM (1997) The 3′ UTR of the α_{2c}-AR mRNA impedes translation of receptor message. *J Biol Chem* **272:**15466–15473.
Yang Y-F and Machida CA (1999) Ribonuclease protection assay for the detection of β_1-adrenergc receptor RNA, in *Adrenergic Receptor Protocols* (Machida CA ed) pp 139–153, Humana Press, Totowa, New Jersey.
Zhang W, Wagner BJ, Ehrenman K, Schaefer AW, DeMaria CT, Crater D, DeHaven K, Long L, and Brewer G (1993) Purification, characterization, and cDNA cloning of an AU-rich element RNA-binding protein, AUF1. *Mol Cell Biol* **13:**7652–7665.
Zubiaga AM, Belasco JG, and Greenberg ME (1995) The nonamer UUAUUUAUU is the key AU-rich sequence motif that mediates mRNA degradation. *Mol Cell Biol* **15:**2219–2230.

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