Regulation of the mRNA-binding Protein AUF1 by Activation of the β-Adrenergic Receptor Signal Transduction Pathway*

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In both cell culture based model systems and in the failing human heart, β-adrenergic receptors (β-AR) undergo agonist-mediated down-regulation. This decrease correlates closely with down-regulation of its mRNA, an effect regulated in part by changes in mRNA stability. Regulation of mRNA stability has been associated with mRNA-binding proteins that recognize A + U-rich elements within the 3′-untranslated regions of many mRNAs encoding proto-oncogene and cytokine mRNAs. We demonstrate here that the mRNA-binding protein, AUF1, is present in both human heart and in hamster DDT1-MF2 smooth muscle cells and that its abundance is regulated by β-AR agonist stimulation. In human heart, AUF1 mRNA and protein was significantly increased in individuals with myocardial failure, a condition associated with increases in the β-adrenergic receptor agonist norepinephrine. In the same hearts, there was a significant decrease (−50%) in the abundance of β2-AR mRNA and protein. In DDT1-MF2 cells, where agonist-mediated destabilization of β2-AR mRNA was first described, exposure to β-AR agonist resulted in a significant increase in AUF1 mRNA and protein (−100%). Conversely, agonist exposure significantly decreased (−40%) β2-adrenergic receptor mRNA abundance. Last, we demonstrate that AUF1 can be immunoprecipitated from polysome-derived proteins following UV cross-linking to the 3′-untranslated region of the human β2-AR mRNA and that purified, recombinant p37AUF1 protein also binds to β2-AR 3′-untranslated region mRNA.

The condition of heart failure is associated with heightened activity of the adrenergic nervous system (1), the severity of failure correlating with increases in circulating and cardiac concentrations of the catecholamine, norepinephrine (2). As a consequence of this increased “adrenergic drive” the cardiac β-ARα1G-protein/adenyl cyclase pathway can become markedly desensitized. One major component of the desensitization is selective down-regulation of the dominant adrenergic receptor subtype within the human myocardium, the β2-AR (1, 3–5). Recently, we (6) and others (7) have demonstrated that the observed decrease in β2-adrenergic receptors in failing human heart is closely associated with a corresponding down-regulation of β2-AR mRNA. Therefore, it is of interest to better define potential mechanisms responsible for down-regulation of β2-AR mRNA.

Experiments performed using hamster DDT1-MF2 smooth muscle cells (8, 9) suggest that down-regulation of the endogenously expressed β2-AR mRNA does not appear to be caused by a decrease in the rate of transcription; rather, it appears that agonist exposure decreases the half-life of β2-AR mRNA from approximately 12 to 5 h (8). This regulatory mechanism has been demonstrated previously to be important for numerous mRNAs encoding proto-oncogenes, lymphokines, and cytokines (10–27). For these gene products regulation of mRNA stability has also been associated with the interaction of the mRNA with a family of cytosolic proteins (M, 30,000–40,000) that often bind to A + U-rich elements (AREs) commonly located within the 3′-untranslated region (3′-UTR) of the mRNA (28–34). This interaction induces mRNA degradation by mechanisms only partially understood. However, for some mRNAs including those containing AREs (35, 36), the degradation of mRNA may be associated with the process of translation. The cytosolic A + U-rich mRNA-binding proteins are in general considered to be distinct from other mRNA-binding proteins such as the heterogenous nuclear ribonucleoprotein particles (37, 38), however, the role of heterogenous nuclear ribonucleoprotein particles A1 and C proteins as cytoplasmic factors regulating mRNA stability is currently undergoing reassessment (39, 40).

From previous studies (41–43) using cytosolic extracts produced from DDT1-MF2 hamster smooth muscle cells, the properties of a β-AR mRNA-binding protein (β-ARB), which binds to hamster β2-AR and human β1-AR mRNAs, has undergone pre-

* The abbreviations used are: β-AR, β-adrenergic receptor; ARE, A + U-rich element; GM-CSF, granulocyte/macrophage colony-stimulating factor; 3′-UTR, 3′-untranslated region; β-ARB, β-AR mRNA-binding protein; RPA, ribonuclease protection assay; RSW, ribosomal salt wash; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

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liminary characterization. Binding of β-ARB to mRNA was determined to involve regions of the 3′-UTR of the hamster β2-AR mRNA containing an ARE (41, 42). In addition, agonist stimulation of the β-AR pathway or protein kinase A activation by a cAMP analogue resulted in significant up-regulation (3-4-fold) of β-AR protein as detected by UV cross-linking. Conversely, treatment of DDT-MF2 cells with dexamethasone, which up-regulates β2-AR mRNA, down-regulated β-AR by ~50%. Therefore, agents that regulate hamster β2-AR mRNA stability and abundance appear to affect reciprocally the abundance of β-AR protein. Among the family of G-protein-coupled receptors, the mRNAs of the hamster β2-AR, the human β1- and β2-AR, and the thrombin receptor have all been demonstrated to interact with β-AR (41–43). To date, the identity of β-AR has remained unresolved. However, β-AR does share characteristics in common with several described A + U-rich mRNA-binding proteins (41), including AUF1 (31).

The cytoplasmic RNA-binding protein, AUF1 (A + U-rich element RNA-binding/degradation factor), has recently been cloned and characterized (31). AUF1 binds to the 3′-UTRs of several highly regulated mRNAs including c-myc, GM-CSF, and c-fos. Further, there is evidence of “cause and effect” between AUF1 and regulation of mRNA stability in that partially purified AUF1 can selectively accelerate the degradation of c-myc mRNA in an in vitro mRNA decay system (34). Based on these findings, we endeavored to determine if AUF1 was expressed in human heart and in DDT-MF2 cells, and if so, if AUF1 abundance was regulated by stimulation of the β-AR pathway. Here we report that the mRNA encoding AUF1 protein is expressed in both human heart and DDT-MF2 cells. Furthermore, exposure of DDT-MF2 cells to β-AR agonist, or high adrenergic drive, as manifest in the failing human heart, results in up-regulation of the AUF1 gene product. In addition, we show that purified, recombinant p37UFT protein binds to an ARE within the 3′-UTR of the human β2-AR mRNA, and that cellular AUF1 can be immunoprecipitated from polysome-derived proteins following UV cross-linking to the 3′-UTR of the human β2-AR mRNA. These data link for the first time a specific mRNA-binding protein known to be associated with the regulation of mRNA stability, with the mRNA of a G-protein-coupled receptor. In addition, they demonstrate that the abundance of this mRNA-binding protein is up-regulated by adrenergic stimulation, an effect known to destabilize β-AR mRNA.

Tissue Procurement—Human ventricular myocardium was obtained from two categories of adult subjects. Failing hearts were obtained from patients undergoing heart transplantation for end-stage heart failure (n = 20) due exclusively to idiopathic dilated cardiomyopathy. These individuals had not received intravenous β-AR agonists, phosphodiesterase inhibitors, or β-blocking drugs prior to transplantation. Nonfailing hearts were obtained from adult organ donors whose hearts were unsuitable for cardiac transplantation due to blood type or size incompatibility (n = 14). Organ donors’ hearts had normal left ventricular function, as determined by echocardiography. Left ventricular aliquots were removed from the heart immediately upon explantation, and either immersed in liquid nitrogen for mRNA and protein quantification or placed in ice-cold, oxygenated Tyrodye’s solution for preservation of material for radioligand binding assays, as described previously (3).

Cell Culture—DDT-MF2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum (HyClone, Logan, UT), 100 U/ml penicillin (50 IU/ml streptomycin (60 μg/ml), and 1 μM (-)-isoproterenol, or vehicle (1 mM ascorbic acid) as described in each individual protocol.

AUF1 mRNA Measurement—A 233-base pair fragment of p37UFT cDNA (31) was cloned from human heart DNA by the use of reverse transcription-PCR. Primers utilized for this reaction spanned a segment of the human p37UFT coding region cDNA sequence from nucleotides 471 to 702 (31) and incorporated restriction enzyme recognition sites at the 5′ end (Smal for the forward primer and XbaI for the reverse primer). Primer sequences were 5′-CCCGAGAAGTTTGGAGAACATTAGGAGGCCC-3′ for the forward primer, and 5′-GACTCCTAGATGTTGGGCTTCTTTGAGAT-3′ for the reverse primer. Primer sequences were 5′-CCCCGGGAGCTTGGGAAAAGTGTATAGTGAGCG-3′ for the forward primer, and 5′-GACTCCTAGATGTTGGGCTTCTTTGAGAT-3′ for the reverse primer. The PCR product was subcloned into pbBluescript II KS (Stratagene, La Jolla, CA) and sequenced using the dideoxy method (Sequenase Version 2, U. S. Biochemical Corp.). Radiolabeled antisense riboprobe from the HindIII digested p37UFT fragment was used to probe a Northern blot of mRNA samples prepared from human heart tissue. The mRNA abundance of hamster β2-AR mRNA from DDT-MF2 cells was extracted by the method of Chomczynski and Sacchi (44) using RNA Stat-60 (Tel Test, Inc., Friendswood, TX), and RNA concentration was determined by absorbance at 260 nm (E3): 1 μg of RNA (RPA) of RNA were hybridized overnight with 10 000 cpm of radio-labeled AU1 riboprobe and a low specific activity 18 S rRNA riboprobe (Ambion, Inc.) using the RPA II kit (Ambion Inc.). Since 18 S rRNA abundance is in excess of mRNAs, 18 S probe was used at a low specific activity to assure molar excess of probe to target without producing a signal beyond the linear range when measured simultaneously with AUF1. The hybridization reaction was digested with RNase A and RNase T1. RNA-RNA hybrids were resolved by electrophoresis in an 8% polyacrylamide, 8 μm urea gel. Protected fragments corresponding to AUF1 and 18 S rRNA signals were quantified using a PhosphorImager (Bio-Rad).

β-AR mRNA Measurement—The abundance of hamster β2-AR mRNA from DDT-MF2 cells was determined by RPA using a specific riboprobe. All measurements were made as described above for AUF1 including normalization of the β2-AR mRNA signal to the signal for 18 S rRNA. The riboprobe (311 nucleotides) was generated from plasmid DNA encoding the hamster β2-AR using PCR primers corresponding to nucleotides 1201 to 1511 (45) and including restriction sites for XhoI and EcoRI. The forward primer was 5′-GATCCCGAGTTTGGGCTTCTTTGAGAT-3′ and the reverse primer was 5′-GATCGAATTCTTATGGCTTCCA-3′. The riboprobe was labeled using T7 RNA polymerase from riboprobe construct linearized with XhoI as described above.

β-AR mRNA Measurement—Human β2-AR mRNA abundance from human ventricular myocardium was measured by quantitative reverse transcription-PCR as described in detail previously (6). Briefly, poly(A)-enriched RNA was extracted from samples of human ventricular myocardium using oligo(dT)-cellulose (MicroFast Track™ mRNA Isolation Kit Version 1.2, Invitrogen Corp., San Diego, CA). mRNA was subjected to a reverse transcription reaction in the presence of a fixed amount of synthetic (84mer) RNA “internal standard” such that target mRNA (β2-AR) and internal standard were amplified co-linearly. PCR primers were end-labeled with 32P-ATP and the absolute amounts of β2-AR and internal standard PCR products were determined for each heart by linear modeling of at least 3 points on the linear portion of the amplification curves.

β-AR Quantification—β-AR density from human ventricular myocardium was determined in a crude membrane fraction as described previously (3). Briefly, the total population of β1- plus β2-AR was measured by the nonselective radiogand [3H]iodocyanopindolol with and without the use of 1 μM (-)-propranolol to determine total and nonspecific binding, respectively. Maximum binding (Bmax) and [125I]iodocyanopindolol dissociation constant (Kd) were determined by nonlinear least-squares computer modeling of the specific binding curve. β1- and β2-AR subtype proportions were determined using the β1-AR selective ligand CFP-20712A (3). Protein concentrations were determined by the Peterson method of Lowry (46).

Immunoblot Analysis of AUF1—Abundance of AUF1 polypeptides was determined in extracts of DDT-MF2 cells and in human heart tissues using a polyclonal antibody described previously (31). In human K562 cells, this antibody recognizes p37UFT/p40UFT, an apparent splice form of p37UFT/p40UFT, and 45-kDa protein, an immunologically related but in vitro translationally unmodulated protein (31). DDT-MF2 cells were treated with 10 μM (-)-isoproterenol for 24 or 48 h. Cells were harvested with ice-cold phosphate-buffered saline containing 1 mM EDTA, centrifuged for 5 min at 1000 × g, and resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 5 μM β-mercaptoethanol, 5 μg/ml aprotinin, and 5 μg/ml leupeptin). Cell lysates were subjected to eight rounds of freeze thaw (dry ice/ethanol for 1 min, 37 °C water bath for 1 min). The samples were centrifuged at 10 000 × g for 10 min, and the supernatant was collected. Total protein concentrations were measured using the BCA reagent kit (Pierce, Rockford, IL). Equal amounts
of protein were resuspended in Laemmli loading buffer (47), boiled for 5 min, and separated by SDS-PAGE (10% resolution phase and 5% stacking phase). Western blotting was performed as described previously (48). Proteins were transferred to 0.1-μm nitrocellulose membranes (Schleicher and Schuell) for 2 h at 40 V. Blots were blocked overnight in phosphate-buffered saline with 5% nonfat dry milk, washed 5× in 0.5% phosphatase-buffered saline, incubated with 1:1000 anti-AUF1 antibody (31), washed again in phosphate-buffered saline, and incubated with 1:1000 GAR (jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Signal was visualized using ECL detection using the manufacturer’s protocol (Amersham) and Kodak X-Omat AR film. Signal intensity was determined using an Alpha Innotech IS-1000 Digital Imaging System (San Leandro, CA). Linear range of the protein signal for AUF1 was determined by comparison of increasing amounts of protein (1–25 μg) on the immunoblot. All subsequent quantification was performed using 2.5 μg of total cellular protein, a concentration at the lower end of the linear range.

For human heart tissues, approximately 100 μg of tissue frozen in liquid N2 was placed in 200 μl of lysis buffer (20 mM Tris-HCl, 0.1% Triton X-100, 5 mM β-mercaptoethanol, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). The tissue was homogenized using 25 strokes of a Teflen pestle and a T-liner at 4°C. The homogenate was subjected to 8 rounds of freeze-thaw and centrifuged at 16,250 × g for 15 min. The supernatant was transferred to a fresh tube and the protein concentration determined as above. To perform Western analysis, 100 μg of total cellular protein was subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Marlborough MA) for 2.5 h at 40 V. Subsequent immunodetection methods were as outlined above.

Sequence of the cdNA Encoding the Human β3-Adrenergic Receptor 3'-UTR—The 2.4-kilobase cdNA encoding the human β3-AR (49) was subcloned into pBluescript KS at the EcoRI site and its orientation confirmed by cdNA sequencing. Nucleotide sequence was determined from purified, double stranded plasmid cdNA by the dyeode method (Sequenase Version 2). Sequencing primers corresponding to the published sequence of the β3-AR coding region and to the T3 primer were used initially. Internal primers were used once additional sequence had been obtained. A sequencing kit (U. S. Biochemical Corp.) was used to sequence the T-rich portion of the cdNA. Each cdNA strand was sequenced at least twice to insure accuracy. The cdNA sequence of the 3'-UTR of the human β3- AR has been submitted to GenBank (U29690).

In vitro Transcription of cdNA for UV Cross-linking—A 919-base pair cdNA fragment corresponding to the p37CR-3'-UTR was synthesized by PCR and subcloned into pcDNA3 (Invitrogen) utilizing the XbaI and XbaI restriction endonuclease sites. The resulting vector was linearized with XbaI, and in vitro transcription was performed as described previously (41). Briefly, radiolabeled cdNA was synthesized using T7 DNA-directed RNA polymerase and [3H]-UTP (800 Ci/mmol, DuPont New England Nuclear) to produce uniformly labeled, 5'-capped RNA. After transcription, the cdNA was mixed with 5 μg of yeast tRNA, 4 mM dithiothreitol, 5 μg heparin, and 65 units of RNasin in a total volume of 50 μl. After incubation for 10 min at 22°C, samples were placed in an ice slurry and exposed to short-wave (254 nm) UV radiation for 3 min in a Stratagene (model 1800) UV Stratalinker. The cross-linked cdNA was digested with RNase A (10.5 μg/ml) and RNase T1 (10 units/ml) at 37°C for 30 min. Samples were solubilized in 50 μl of Laemmli loading buffer for 10 min at 70°C, and proteins were resolved by SDS-PAGE. Gels were stained with Coomassie Blue R-250 (Sigma) followed by destaining and drying, and subjected to autoradiography for 1–5 days.

Immunoprecipitation of AUF1—Polysomes and RSW from DDT1-MF2 cells and radiolabeled human β3-AR 3'-UTR RNA were prepared as described above. Polysomes or RSW (2–106 cell equivalents) were mixed with 5 × 106 cell equivalents of β3-AR 3'-UTR RNA, UV cross-linked, and digested with RNase A and T1 as described above. Polysomes or RSW were preclaved with preimmune serum and Pansorbin cells (Calbiochem, La Jolla, CA) and immunoprecipitated as described by Zhang et al. (31). The pellet was resuspended in Laemmli buffer, boiled 5 min, and proteins resolved by SDS-PAGE (10%). Gels were dried, and radiolabeled proteins were visualized by autoradiography for 2 to 5 days.

RESULTS

Human Heart—In failing ventricular myocardium, β3-AR mRNA and receptor protein are significantly down-regulated to a similar extent (6, 7). Furthermore, as discussed below, sequencing of the cdNA for the 3'-UTR of the human β3-AR has revealed that there is at least one potential ARE. Based on the precedent of agonist-mediated destabilization of the hamster β2-AR mRNA (8) and the binding of β-ARB to this mRNA (41), we wished to determine if the gene encoding the mRNA-binding protein AUF1 was expressed in the human heart. Secondly, we wished to determine if AUF1 gene expression was affected by heart failure. Left ventricular myocardium was obtained from two groups: (i) individuals with idiopathic dilated cardiomyopathy (n = 20) undergoing orthotopic cardiac transplantation, and (ii) organ donors whose hearts were unsuitable for cardiac transplantation (n = 14). To measure AUF1 and human β3-AR mRNA, total cellular RNA was isolated from left ventricular myocardium. As determined by RPA, the mRNA encoding AUF1 was significantly up-regulated in failing heart (190% of control, p < 0.05) compared to nonfailing donor hearts (Fig. 1A). In each case the signal for AUF1 mRNA was normalized to that of 18 S RNA. Although 18 S RNA is a significantly more abundant RNA than AUF1, the signal for 18 S was often less intense than that of AUF1 due to the intentionally low specific activity of the 18 S probe. Therefore, the signal strength ratio of AUF1 to that of 18 S RNA was often >1. Heart failure had no effect on 18 S RNA expression (data not shown). In addition, immunoblot analysis of AUF1 proteins was performed on ventricular myocardium from nonfailing (n = 8) and failing (n = 8) human hearts. Fig. 1A is a representative immunoblot of eight of these hearts, four nonfailing and four failing. Compared to nonfailing hearts, the relative abundance of immunoreactive p45 and p40AUF1 protein were both significantly increased in failing hearts (Fig. 1B). p37AUF1 protein was distinctly present but variably detectable and at low abundance even when using as much as 100 μg of total cellular protein per lane. Therefore, quantitative analysis was not performed on the signal for p37AUF1. A more detailed analysis of AUF1 protein expression in response to β-agonist stimulation was performed in a cell culture model system, described below.

Although the identity of p45 is currently unknown, it is obviously immunologically related to p37AUF1 and to p40AUF1.
TABLE I

Expression of AUF1 mRNA and β1-AR mRNA and protein in nonfailing and failing human left ventricular myocardium

Tissue samples were obtained from patients undergoing heart transplantation for idiopathic dilated cardiomyopathy (Failing) or organ donors with normal contractile function (Nonfailing). Relative AUF1 mRNA abundance was measured by RPA and referenced to the signal for 18S RNA. The relative densitometric values for both AUF1 and 18S RNAs are arbitrary and dependent on the specific activity of each probe in each assay (a ratio of “2” does not imply twice as much AUF1 as 18S RNA). Absolute amounts of β1-adrenergic receptor mRNA were measured by quantitative reverse transcriptase (RT)-PCR from poly(A)-selected mRNA. β-AR density was measured in membrane preparations of human ventricular myocardium using multiple concentrations of the radioligand, [125I]iodocyanopindolol, to determine total adrenergic receptor binding. The density of the β1- and β2-AR subtypes was determined by competitive binding using the β1-selective antagonist CGP20712A.

| Group          | AUF1/18S mRNA ratio | β1-AR mRNA by RT-PCR | β1-AR density |
|----------------|---------------------|----------------------|---------------|
| Nonfailing (n = 14) | 10 ± 2              | 3.8 ± 0.6            | 80 ± 8        |
| Failing (n = 20)  | 19 ± 3              | 2.2 ± 0.3            | 31 ± 3        |

*a* 107 molecules/μg of poly(A) RNA.  
*b* X ± S.E.; p < 0.05, two-tailed unpaired t test.

**A**

**B**

![Image of AUF1 proteins in human left ventricular myocardium](http://www.jbc.org/)

**Fig. 1.** AUF1 proteins in human left ventricular myocardium. A, a representative immunoblot of AUF1 proteins from four nonfailing and four failing human hearts. Whole tissue lysates of human ventricular myocardium were prepared and assayed for AUF1 polypeptides using a polyclonal anti-AUF1 antibody (31). In each case, equivalent amounts of total cellular protein (100 μg) were analyzed for each subject. The data from these hearts and an additional eight hearts are summarized in B. B, the relative abundance of AUF1 immunoreactive proteins was investigated in nonfailing (n = 8) and failing (n = 8) human heart. Data are presented as X ± S.E. The relative abundance of p45 and p40 proteins is expressed in arbitrary units (A.U.). Statistical analysis of nonfailing compared to failing hearts was performed by use of a two-tailed, unpaired t test.

Furthermore, as demonstrated below (Fig. 6), p45 recognized and bound to A + U-rich RNAs thus appearing to be similar in this regard to AUF1 proteins. Lastly, like p40AUF1, p45 abundance is up-regulated in individuals with heart failure. The relationship of p45 to AUF1 or to other mRNA-binding proteins beyond these shared characteristics remains to be determined.

As determined by quantitative reverse transcriptase-PCR, β1-AR mRNA abundance was significantly decreased (~40%) in failing as compared to nonfailing, control hearts (Table I). These results are consistent with previous findings (6). β-AR density and subtype proportions also were determined in the same failing and nonfailing hearts. β1-AR density was also significantly reduced (~61%) in failing compared to nonfailing hearts (Table I). By contrast, β2-AR density was not different in failing compared to nonfailing hearts (23.2 ± 2.0 versus 27.4 ± 3.4 fmol/mg protein, respectively).

In summary, these data indicate that (i) AUF1 mRNA and protein are expressed in human ventricular myocardium; and (ii) in individuals with heart failure, AUF1 mRNA and protein are significantly up-regulated and, both β1-AR mRNA and protein are down-regulated. From these data we conclude that up-regulation of AUF1 in human heart may be involved in the regulation of β1-AR mRNA stability and thus may be at least partially responsible for the decline in β1-AR mRNA and subsequently protein abundance in the failing heart.

**DDT1-MF2 Cells—** The use of a cell culture model system and the cloning of p37AUF1 (31) has made it possible to explore in greater detail the role of β-agonist stimulation in regulating the expression of the mRNA-binding protein, AUF1. DDT1-MF2 cells were chosen because: (i) agonist-mediated destabilization of the endogenous hamster β2-AR mRNA was originally described in these cells (8), and (ii) β2-AR, which binds to the human β2-AR and hamster β2-AR mRNAs was also originally described in these cells (41). When DDT1-MF2 cells were treated with 10 μM (+)-isoproterenol for 24 h (n = 2) or for 48 h (n = 3), steady-state AUF1 mRNA abundance, as determined by RPA, was modestly increased to 129 ± 6% (p < 0.05, n = 5, pooled data) of untreated controls. AUF1 mRNA was increased to the same extent at both 24 and 48 h. In each case, mRNA abundance was normalized to signal for 18S RNA. Treatment with isoproterenol had no effect on 18S RNA expression (data not shown). Fig. 2A is a representative immunoblot of DDT1-MF2 whole cell lysates using polyclonal anti-AUF1 antibody. Three distinct bands are evident: p37AUF1, p40AUF1, and p45, an immunologically related polypeptide (31). Fig. 2B demonstrates the presence of AUF1 polypeptides in the polysome fraction of DDT1-MF2 cells under both basal and isoproterenol stimulated conditions. As is evident, AUF1 proteins are preferentially localized to the polysome fraction. This finding is consistent with localization determined previously for AUF1 in K562 cells (31) and for β2-AR in DDT1-MF2 cells (41).

As determined by immunoblot analysis, p37AUF1 protein was increased to 230 ± 50% of control (n = 5, pooled data) in cells treated with 10 μM (+)-isoproterenol for 24 h (n = 2) or 48 h (n = 3) compared to untreated controls (Table II). The relative abundance of p37AUF1 protein was increased to a similar extent by isoproterenol treatment at both the 24- and 48-h time points. In the same preparations, the relative amounts of p40AUF1 and p45 were also determined. In cells treated with isoproterenol, p40AUF1 increased to 160 ± 30% of control (n = 5, pooled data) and p45 increased to 180 ± 30% of control (n = 5, pooled data) (Table II). At each time point, the relative abundance of p40AUF1 and p45 were roughly similar, both being of considerably greater abundance than p37AUF1. Also of note is the finding that the relative abundance values of p37AUF1 and p40AUF1 are greater (in arbitrary units) at 48 h compared to 24 h. This may indicate an increased relative amount of AUF1 proteins as the cells continue to grow.

In the same cells, the relative abundance of the endogenous β2-AR mRNA was measured. Treatment of DDT1-MF2 cells with 10 μM (+)-isoproterenol for 24 h (n = 2) or 48 h (n = 3) produced a decrease in β2-AR mRNA to 63 ± 2% (p < 0.05, n = 5) of control. The degree of down-regulation was highly similar at 24 and 48 h (63% versus 62% of control). As with AUF1, the
protected signal for the hamster β2-AR mRNA was normalized to the invariant signal for 18 S rRNA (data not shown). The degree of down-regulation of β2-AR mRNA was in close agreement with a previous investigation (8) and correlates well with the degree of receptor down-regulation.

In summary, these results demonstrate that in DDT1-MF2 hamster smooth muscle cells: (i) stimulation of the β-AR pathway produces an increase in AUF1 mRNA and p37aufl protein. In addition, the abundance of p40aufl and p45 proteins are also increased; (ii) there is a reciprocal decrease in β2-AR mRNA abundance; and (iii) p37aufl protein is expressed and localized to a polysome fraction. We conclude that agonist-mediated up-regulation of AUF1 protein in DDT1-MF2 cells may contribute to agonist-mediated destabilization and down-regulation of the hamster β2-AR observed in these cells.

Human β2-AR 3'-UTR—Although previously cloned (49), the nucleotide sequence of the 3'-UTR of cDNA for the human β2-AR had not been determined. In order to determine if the β2-AR 3'-UTR contained potential mRNA stability regulatory domains such as an ARE and to facilitate mapping of mRNA-binding proteins, we sequenced this portion of the cDNA (Fig. 3). The β2-AR 3'-UTR contains a uniquely long poly(U) tract in its proximal region. This domain is similar to other AREs (20, 51). Several other A + U-rich regions are denoted including a putative mRNA destabilizing sequence “UUUAUUUAU” (52, 53). In addition, four potential poly(A) addition sites (AAUAAA or AUUAAA) are present. It is currently unknown which site or sites are used for poly(A) addition.

UV Cross-linking of Polyribosome-associated Proteins and Recombinant p37AUF1 Polypeptide to the β2-AR mRNA—To determine which mRNA-binding proteins bind to the human β2-AR mRNA, we performed UV cross-linking of radiolabeled RNA substrates to ribosome-associated proteins from isoprot-eronol (10 μM for 48 h) stimulated DDT1-MF2 cells. Proteins were solubilized from polyribosomes by extraction with 0.3 M KCl, termed a RSW. The rationale for using RSW rather than S100 cytosol or polysomes from DDT1-MF2 cells was analyzed for the presence of AUF1 polypeptides.

**Table II**

| Effect of β-agonist treatment on the relative abundance of AUF1 protein(s) in DDT1-MF2 cells | 24 h (n = 2) | 48 h (n = 3) |
|---|---|---|
| | p37 | p40 | p45 |
| CON | 125 ± 60 | 1750 ± 490 | 1850 ± 540 |
| ISO | 230 ± 10 | 2820 ± 110 | 2850 ± 50 |
| 48 h (n = 3) | p37 | p40 | p45 |
| CON | 540 ± 230 | 1940 ± 620 | 1500 ± 670 |
| ISO | 920 ± 270* | 2560 ± 420* | 2220 ± 510* |

* p < 0.02 CON versus ISO Student’s paired t test, two-tailed (data for 24 and 48 h combined, n = 5).
The 3'-UTR of the human β₁-AR was sequenced from the previously cloned cDNA (49) using the dideoxy method. The nucleotide sequence begins with the stop codon (UAG) at nucleotide 1432 and extends for an additional 932 nucleotides. A uniquely long U-rich region as well as several A + U-rich regions which are potential AREs are in bold and underlined including the putative mRNA destabilizing sequence, UUAUUUAU. Four canonical poly(A) addition sequences are shown in bold, underlined, and italics.

![Figure 3](image3.png)

**Fig. 3.** Nucleotide sequence of the 3'-untranslated region of the human β₁-AR. The 3'-UTR of the human β₁-AR was sequenced from the previously cloned cDNA (49) using the dideoxy method. The nucleotide sequence begins with the stop codon (UAG) at nucleotide 1432 and extends for an additional 932 nucleotides. A uniquely long U-rich region as well as several A + U-rich regions which are potential AREs are in bold and underlined including the putative mRNA destabilizing sequence, UUAUUUAU. Four canonical poly(A) addition sequences are shown in bold, underlined, and italics.

![Figure 4](image4.png)

**Fig. 4.** UV cross-linking of RSW proteins to multiple radiolabeled RNAs. Representative autoradiogram of RSW from DDT-MF2 cells treated with (-)isoproterenol (10 μM for 48 h) and UV cross-linked to capped, uniformly radiolabeled in vitro transcribed RNAs. Equal amounts of RSW (20 μl, 5 × 10⁶ cell equivalents/μl) and equal molar amounts of radiolabeled RNA were added to each reaction. Lane 1, non-UV cross-linked control; lane 2, β₁-AR 3'-UTR only; lane 3, β₁-AR coding region (CR) only; lane 4, c-myc 3'-UTR. A band at Mr 38,000, previously designated as β₁-AR (41–43), binds to the β₁-AR 3'-UTR, and to the c-myc 3'-UTR, but not to the β₁-AR CR.

![Figure 5](image5.png)

**Fig. 5.** Competitive displacement of β-ARB protein binding to β₁-AR 3'-UTR RNA. Radiolabeled RNA corresponding to the 3'-UTR of the human β₁-AR was UV cross-linked to RSW proteins in the presence of increasing amounts (0, 10-, and 50-fold molar excess) of unlabeled competitor RNAs encoding the human β₁-AR 3'-UTR (lanes 1–3), GM-CSF 3'-UTR (lanes 4–5) and ΔGM-CSF 3'-UTR (lanes 6 and 7). β₁-AR and GM-CSF but not ΔGM-CSF competed effectively for β-ARB binding.

- Proteins were UV cross-linked to the radiolabeled mRNA corresponding to the β₁-AR 3'-UTR. Following separation by SDS-PAGE, the gel was transferred to a polyvinylidene difluoride (Millipore) membrane and autoradiography and immunoblotting were performed to ensure that signals could be superimposed precisely. Here again, the proteins recognized by the anti-AUF1 antibody do not co-migrate with the M₉ 38,000 signal for β-ARB (Fig. 6C). By immunologic and migratory criteria, the results presented above in Fig. 6, A-C, all are strong against p38β-ARB being an AUF1 protein.

- Lastly, to determine directly if AUF1 could bind to the human β₁-AR 3'-UTR, radiolabeled RNA was incubated with purified, recombinant p37AUF1, subjected to UV irradiation, RNase A + T1 digestion, SDS-PAGE, and autoradiography. Recombinant p37AUF1 protein binds to the β₁-AR 3'-UTR and to the c-fos ARE but fails to bind to rabbit β-globin (Rβ) RNA (Fig. 7). Unlabeled β₁-AR 3'-UTR RNA effectively competes for AUF1 binding, while a 100-fold molar excess of β-globin does not (data not shown).

Together, the UV cross-linking and immunoprecipitation experiments indicate that: (v) a number of polysome-derived proteins between M₉ 38,000 and 45,000 bind to the 3'-UTR of the
human $\beta_1$-AR including a prominent polypeptide at $\sim M_r$ 38,000 as well as several polypeptides between $M_r$ 40,000 and 45,000 UV cross-link to the 3'-UTR but not the coding region of the human $\beta_1$-AR mRNA (a protein of the same apparent molecular weight binds to c-myc and GM-CSF mRNA; data not shown); (ii) anti-AUF1 antibody immunoprecipitates proteins between $M_r$ 40,000 to 45,000 when cross-linked to the human $\beta_1$-AR 3'-UTR, and (iii) purified recombinant p37$^{\text{AUF1}}$ binds to the 3'-UTR of the human $\beta_1$-AR mRNA. These results indicate that $M_r$ 38,000 ($\beta$-ARB) is not an AUF1-related protein. The results also demonstrate by multiple methods that AUF1 proteins bind to the mRNA encoding the $\beta_1$-AR 3'-UTR.

**DISCUSSION**

Agonist-mediated down-regulation of G-protein-coupled receptors is a well established regulatory paradigm. One of the ways in which the amount of receptor protein may be down-regulated is by an alteration in the steady-state abundance of its mRNA which, in turn, is controlled by transcription rate, or by mRNA stability, or both. Since the first report of agonist-mediated destabilization of an mRNA encoding a G-protein-coupled receptor by Hadcock et al. (8), the mRNA abundance of a number of other G-protein-coupled receptors have been reported to be regulated by changes in mRNA half-life (Table III). However, it should not be assumed that for each receptor of a particular subtype that it is regulated at the level of mRNA stability in all species or cell types. For example, the $\alpha_{1b}$-AR mRNA derived from rabbit aorta smooth muscle cells is regulated by an agonist-mediated response dependent on protein kinase C activity (54). By contrast, in DDT$_1$MF2 smooth muscle cells, the stability of hamster $\alpha_{1b}$-AR mRNA appears not to be regulated by agonist exposure (55). Furthermore, the hamster $\alpha_{1b}$-AR mRNA from DDT$_1$MF2 cells is not A + U-rich and does not interact with $\beta$-ARB (41). It is unknown whether or not the rabbit $\alpha_{1b}$-AR interacts with A + U-rich mRNA-binding proteins. As another example, the half-life of the mRNA encoding the m1-muscarinic receptor is decreased by its cognate agonist, carbachol, an effect that necessitates an intact 3'-UTR (56). While the 3'-UTR of the m1-muscarinic receptor does not contain A + U-rich regions, there are sequence motifs that may form hairpin loops that might act as binding sites for RNA-binding proteins. For example, 5'-AUU-3'/5'-UAA-3' motifs appear to be important for directing endonucleolytic cleavage in mRNAs containing stem-loop structures (62). These structures have been shown to interact with mRNA-binding proteins of $M_r$ 34,000 (63).

Several other mRNAs important to G-protein-coupled receptor signal transduction are also regulated at the level of mRNA.
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stability including protein kinase A1 (64), and the G-proteins \( \text{G}_{12}, \) and \( \text{G}_{13} \) (65). Most recently, we have demonstrated that the stability of the human \( \beta_2 \text{-AR} \) mRNA, the subject of the current investigation is regulated by \( \beta \)-agonist exposure in a cell culture-based model system, an effect that is dependent entirely on the presence of the 3'-UTR (61).

AUF1 shares several characteristics in common with the previously described \( \beta \)-AR mRNA-binding protein, \( \beta \)-ARB, which led us to hypothesize that they might be the same or related proteins. First, both proteins have similar electrophoretic mobilities. The apparent molecular weights of AUF1 are 37 and 40 kDa, whereas \( \beta \)-ARB was reported to be \( 
\text{Mr} = 35,000 \text{ by UV cross-linking of DDT1-MF2 S100 to the hamster } 
\beta_2 \text{-AR mRNA (41) and } -M, 38,000, \text{ by UV cross-linking of } 
polysomal-derived proteins from DDT1-MF2 cells to the human 
\beta_2 \text{-AR 3'-UTR (this article). Second, both proteins share the } 
same cellular fractionation profile, i.e. both are minimally repre-

sented in cytosolic fractions and are present at a much higher relative abundance in polysomes (Refs. 31 and 41; this article). Similarly, by immunoblotting, AUF1 is readily detectable in whole cell extracts (Fig. 2A), in the polysome fraction (Fig. 2B), and in RSW (data not shown). Third, in response to \( \beta \)-AR stimulation, both \( \beta \)-ARB and AUF1 are significantly up-regu-
lated at both 24 and 48 h (Ref. 41; this article). Fourth, \( \beta \)-ARB and AUF1 preferentially bind to the AREs of multiple mRNAs. \( \beta \)-ARB binds to the mRNAs encoding the human \( \beta_2 \text{-AR} \) and to 
the human and hamster \( \beta_2 \text{-ARs (41). More specifically, } \beta \)-ARB 
binds the proximal U-rich region of the 3'-UTR of human \( \beta_2 \text{-AR mRNA (data not shown). Similarly, } \beta \)-ARB also binds to 
the AREs of GM-CSF and the adenosine AII (42). More re-
cently, \( \beta \)-ARB has been demonstrated to bind to the throbmin 
receptor mRNA, another G-protein-coupled receptor regulated 
at the level of stability (43). Like the hamster \( \beta_2 \text{-AR, the half-life of the thrombin receptor mRNA is signifi-
cantly decreased by either agonist treatment or by stimulation of cells by } 
cAMP analogues (43). By contrast, \( \beta \)-ARB does not bind to 
the hamster \( \text{\alpha_2\text{-AR mRNA or to the } } \beta_2 \text{-globin mRNA (41) and } \text{weakly or not at all to the rat } \beta_2 \text{-, or human } \beta_2 \text{-AR mRNAs (43). } \beta \)-ARB, like purified recombinant p37\text{AUF1 protein, binds 
to the human } \beta_2 \text{-AR mRNA, as well as to the AREs for c-myc, 
c-fos, and GM-CSF (31), but not rabbit } \beta_2 \text{-globin mRNA. Fifth, both } 
\beta \)-ARB and p37\text{AUF1 protein increase with } \beta \)-agonist exposure.

However, the data from several different experimental approaches presented here indicate a lack of concordance for the molecular weights of \( \beta \)-ARB and AUF1 proteins and lead to the conclusion that \( \beta \)-ARB is not an AUF1 protein. These experiments include immunoprecipitation of AUF1 proteins from polysomes and immunoblotting of polysome-derived proteins to UV cross-linked \( \beta_2 \text{-AR mRNA. The biochemical and functional relativeness of human AUF1 to hamster } \beta \text{-ARB will necessitate the future purification and/or cloning of } \beta \text{-ARB.}

The observation that AUF1 is up-regulated in the failing human heart and in agonist-treated DDT1-MF2 cells is intriguing and leads to several important questions. The first and most obvious question: is AUF1 responsible for the observed down-regulation of \( \beta_2 \text{-AR mRNA in the failing human heart and/or the hamster } 
\beta_2 \text{-AR in DDT1-MF2 cells? The data presented here indicate that } \beta \text{-AR stimulation results in a recip-
rocal relationship: the up-regulation of AUF1 gene product(s) and the down-regulation of } \beta \text{-AR and the destabilization of } 
\beta_2 \text{-AR mRNA. This suggests that an increase in AUF1 protein(s) may be associated with increased mRNA turnover rates for the } 
\beta \text{-AR as well as for other mRNAs.}

It is of interest that AUF1 also binds to the human \( \beta_2 \text{-AR mRNA (data not shown). However, it is well established that the } 
mRNA \beta_2 \text{-AR is not down-regulated in failing human heart (1, 3–6). To date, there is no ready explanation for this finding beyond speculation. It is possible that human } \beta_2 \text{-AR mRNA undergoes agonist-mediated destabilization but that there is an offsetting increase in transcription rate such that steady-
state mRNA abundance does not appear to change. It is also possible that there may be differences in the affinity of AUF1 for the } 
\beta_2 \text{-AR and } \beta_2 \text{-AR mRNAs functionally affecting the role of } 
AUF1. Another possibility is that AUF1 and the target mRNA need to be appropriately co-localized physically and temporally for mRNA turnover to be accelerated. Lastly, multiple proteins co-immunoprecipitate with AUF1, however, these proteins have not yet been characterized (31). By virtue of its protein structure, which contains two consensus RNA recognition motifs (31), AUF1 unquestionably binds to the RNA directly and thus must be central to the actions of other pro-
teins associated with this complex. However, it is untested as to whether the same proteins always associate with AUF1 depend-
ing on which mRNA is bound. These points all address potential mechanisms conferring specificity of action for AUF1, a topic for future investigation.

In the failing human heart, changes in the expression of a number of other genes have also been documented including multiple components of several G-protein-coupled receptor signal transduction pathways, ion channels, and cytoskeletal/ structural proteins. Also well documented is the observation that c-myc and other mRNAs encoding proto-oncogenes are transiently up-regulated by stimuli associated with myocardial failure, e.g. high adrenergic drive (66–69). By contrast to the transient nature of c-myc and other proto-oncogene expression associated with adrenergic stimuli, increased expression of AUF1 appears to be persistent. In many cases, the individual hearts assayed for AUF1 mRNA and protein abundance had been “failing” for months if not years. Therefore, unlike the expression of proto-oncogenes and other immediate-early genes, AUF1 expression does not appear to accommodate to the chronic stimulus causing its up-regulation. An issue of impor-
tance to address in the future will be to determine the precise mRNA targets of AUF1 in the human heart. The role of AUF1 or other mRNA-binding proteins in regulating the expression of these mRNAs in the context of heart failure or in myocardial hypertrophy remains to be explored.

We conclude that AUF1 is present in both human heart and DDT1-MF2 cells and that it is up-regulated by \( \beta \)-agonist exposure in cells and by the process of heart failure. Furthermore, we have demonstrated that purified, recombinant AUF1 binds to the 3'-UTR of the human \( \beta_2 \text{-AR mRNA and can be immu-

nopedipitated from polysome-derived proteins UV cross-linked to 
human } \beta_2 \text{-AR 3'-UTR mRNA. Future experiments will at-
tempt to address more precisely the role of AUF1 in the desta-
blization of } \beta_2 \text{-AR mRNAs as well as exploring other potential 
target mRNAs for AUF1 binding.}

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| mRNA | Species | Ref. |
|------|---------|------|
| \( \beta_2 \text{-Adrenergic} \) | Hamster | 8 |
| \( \alpha_2 \text{-Adrenergic} \) | Rabbit | 54 |
| \( \alpha_1 \text{-Adrenergic} \) | Human | 57 |
| m1-muscarinic | Rat | 56 |
| AT1-Angiotensin II | Rat | 59 |
| \( \beta_2 \text{-Adrenergic} \) | Human | 43 |
| Thrombin | Human | 61 |

TABLE III
mRNAs encoding G-protein-coupled receptors regulated by mRNA stability

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Regulation of the mRNA-binding Protein AUFl by Activation of the -Adrenergic Receptor Signal Transduction Pathway
Aldo Pende, Kelli D. Tremmel, Christine T. DeMaria, Burns C. Blaxall, Wayne A. Minobe, Jonathan A. Sherman, John D. Bisognano, Michael R. Bristow, Gary Brewer and J. David Port

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