Translational Control of \( \beta_2 \)-Adrenergic Receptor mRNA by T-cell-restricted Intracellular Antigen-related Protein*

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Cellular expression of the \( \beta_2 \)-adrenergic receptor (\( \beta_2 \)-AR) is suppressed at the translational level by 3'-untranslated region (UTR) sequences. To test the possible role of 3'-UTR-binding proteins in translational suppression of \( \beta_2 \)-AR mRNA, we expressed the full-length 3'-UTR or the adenylate/uridylate-rich (\( A+U \)-rich element (ARE)) RNA from the 3'-UTR sequences of \( \beta_2 \)-AR in cell lines that endogenously express this receptor. Reversal of \( \beta_2 \)-adrenergic receptor translational repression by retroviral expression of 3'-UTR sequences suggested that ARE RNA-binding proteins are involved in translational suppression of \( \beta_2 \)-adrenergic receptor expression. Using a 20-nucleotide ARE RNA from the receptor 3'-UTR as an affinity ligand, we purified the proteins that bind to these sequences. T-cell-restricted intracellular antigen-related protein (TIAR) was one of the strongly bound proteins identified by this method. UV-catalyzed cross-linking experiments using in vitro transcribed 3'-UTR RNA and glutathione S-transferase-TIAR demonstrated multiple binding sites for this protein on \( \beta_2 \)-AR 3'-UTR sequences. The distal 340-nucleotide region of the 3'-UTR was identified as a target RNA motif for TIAR binding by both RNA gel shift analysis and immunoprecipitation experiments. Overexpression of TIAR resulted in suppression of receptor protein synthesis and a significant shift in endogenously expressed \( \beta_2 \)-AR mRNA toward low molecular weight fractions in sucrose gradient polysome fractionation. Taken together, our results provide the first evidence for translational control of \( \beta_2 \)-AR mRNA by TIAR.

\( \beta_2 \)-Adrenergic receptors (\( \beta_2 \)-AR)\(^1\) are low abundance membrane-integrated G-protein-coupled receptors that are activated by catecholamines at the cell surface. \( \beta_2 \)-AR mRNA is transcribed from a single intronless gene (1–3), and both transcriptional (4) and post-transcriptional mechanisms (5–10) are known to regulate receptor expression. Post-transcriptional mechanisms include regulation at the level of receptor mRNA translation (5–7) as well as regulation of mRNA stability (8, 9). A highly conserved 5'-leader cistron present in the 5'-untranslated region (UTR) of this receptor inhibits the translation of receptor mRNA (5, 6). \( \beta_2 \)-Adrenergic receptors from several mammalian species have a highly conserved 3'-untranslated region (10) with multiple adenine/uridine-rich (ARE) regions (11–13). The 3'-untranslated region of the \( \beta_2 \)-adrenergic receptor contains regulatory elements that can alter stability of the receptor transcript in response to agonist challenge (11–13). Recently, we demonstrated that the 3'-UTR sequences negatively regulate the translation of the receptor mRNA (7).

The modulation of translational efficiency is an important post-transcriptional control mechanism for eukaryotic gene expression. In most cases, translational control results from interaction of RNA-binding proteins with the 5'- and/or 3'-UTR sequences (14–28). Many sequence-specific RNA-binding proteins that bind to 3'-UTR of mRNAs have also been identified (29–33). These interacting proteins could bind to linear sequences on the RNA and regulate its translation both positively and negatively by affecting localization and circularization of mRNA molecules (27, 28). A + U-rich RNA found in the 3'-UTRs of many mRNAs represents one of the well studied translational and stability determinants in eukaryotic mRNAs (34, 35). Several ARE-binding proteins have been identified that can stabilize, destabilize, or otherwise regulate the translational efficiency of transcripts to which these proteins bind (29–32, 36–38). T-cell-restricted intracellular antigen-1 (TIA-1) and its closely related homologue TIA-1-related protein (TIAR) are RNA-binding proteins (39) that have been shown to act as translational silencers by specific associations with 3'-UTR sequences of tumor necrosis factor \( \alpha \) (29, 30) and matrix metalloproteinase-13 (31). Both proteins possess three RNA recognition motifs that confer high affinity binding to U-rich and A + U-rich RNA (40). The TIAR gene encodes two isoforms, TIAR-a and TIAR-b, both of which arise from alternative splicing of a common precursor transcript (41).

Recently, we used deletion constructs and reporter constructs to demonstrate that the 3'-UTR of \( \beta_2 \)-AR mRNA inhibits the translation of the receptor transcript (7). In the current work, we demonstrate that \( \beta_2 \)-AR expression is transcriptionally repressed in two different cell lines, both of which endogenously express this receptor. Ectopic expression of \( \beta_2 \)-AR 3'-untranslated region sequences or the A + U-rich region present within the receptor 3'-UTR sequence was capable of altering receptor expression at the post-transcriptional level, suggesting that translational repression is caused by binding of a soluble factor or factors to sequences within the 3'-UTR. RNA affinity purification and immunoprecipitation experiments demonstrated high affinity binding of TIAR and TIA-1 to re-
receptor 3′-UTR RNA. In support of the ability of TIAR to regulate β2-AR expression, ectopic expression of TIAR resulted in suppression of receptor expression and sequestration of receptor mRNA into nonpolysonal fractions in sucrose density gradient fractionation. Taken together, our results suggested that TIAR, by binding to 3′-UTR sequences, regulates translation of β2-AR mRNA.

**Experimental Procedures**

**Cell Culture**—DDT1-MF2 and A431 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 5% heat-inactivated feline bovine serum (Invitrogen), penicillin (60 μg/ml), and streptomycin (100 μg/ml) as described before (11). A431 cells were grown in the same medium with 10% heat-inactivated feline bovine serum. CHO cells were grown in F-12 medium (Invitrogen) supplemented with 10% heat-inactivated feline bovine serum.

**Retroviral Expression of Receptor 3′-UTR and A+U-Rich RNA in DDT1-MF2 and A431 Cells**—The complete 3′-UTR region, a 21 nt A+U-rich element (nucleotides 1591–1611; 5′-CTTTTTTTTTTTTTT-A-3′) present within the 3′-UTR of hamster β2-AR cDNA (13) and a 15 nt A+U-rich element (nucleotides 1383–1401; 5′-CTTTTTTTTTTTTTTTT-TTA-3′) of human β2-AR DNA 3′-UTR sequences (13) were cloned into the unique sites of the retroviral vector pLNCX2 (BioScience Clontech). A vector having the same number of nucleotides with randomly introduced mutations (5′-CGTTGTATCTGTATCTTTT-CAT-3′) served as a control. Plasmids containing the A+U-rich elements and the 21 nt scrambled sequences were constructed by using complementary synthetic oligonucleotides flanked by restriction sequences for HindIII at the 5′-end and ClaI at the 3′-end. Complementary oligonucleotides were annealed and cloned into the pLNCX2 vector. Plasmid DNA concentrations were determined by UV spectrophotometry and confirmed by agarose gel electrophoresis of linearized plasmid DNA.

DDT1-MF2 cells and A431 cells were transfected with pLNCX2 containing the full-length 3′-UTR, the A+U-rich sequences, or the control vector, using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. Stable transfectant clones were selected using 650 μg/ml G418 for DDT1-MF2 cells and 0.1 mg/ml G418 for A431 cells. The levels of expression of β2-AR were determined in DDT1-MF2 and A431 cells by radioligand binding assay (42) of whole cells and crude cytosolic extracts made from both control and ARE cells by radioligand assay (43) and were determined in DDT1-MF2 and A431 cells using RNA STAT-60 reagent (Tel-Test B, Inc., Tyler, TX) as described previously (43). Antisense riboprobes corresponding to 445 nt downstream of the CAT start codon (nt 696–1140) from the coding region were obtained by RT-PCR amplification using total RNA from DDT1-MF2 and A431 cells using the forward primer 5′-ATGGAGAATGAGATGCCCAA-3′ and reverse primer 5′-GACCAGTGATGGAGGATGTAAACTTCCT-3′. The PCR-amplified fragments were sequenced and cloned into pSP70 (Promega) for in vitro transcription and into pGEM3Z (Promega) for in vivo transcription. The RNA concentrations were determined using standard methods. TIAR, TIA-1, HuR, AU1, or TTP antibodies were used in Western blot analysis. All of the antibodies were used at a dilution of 1:1,000 in phosphate-buffered saline containing 3% bovine serum albumin. After incubation with alkaline phosphatase-linked secondary antibodies, immunoreactive bands were visualized by a chemiluminescent method (CVP star; New England Biolabs).

**RT-PCR Isolation of TIAR and TIA-1**—The cDNAs for both TIAR and TIA-1 were obtained by RT-PCR amplification using primers described previously (31). For TIAR, the forward primer was 5′-ATGGAGAAGACGGCAGGGCCA-3′, and reverse primer was 5′-TACATGTGTGTTGTAACCTTG-3′. The cDNAs for both short and long isoforms of TIAR were obtained using these primers. Similarly, TIA-1 was PCR-amplified by using the forward primer 5′-ATGGAGAAGACGGCAGGGCCA-3′ and reverse primer 5′-TACATGATTGTTGTAACCTTG-3′. The PCR-amplified fragments were sequenced on both sense and antisense strands and cloned into the expression vector pcDNA3 (Invitrogen).

**GST Fusion Protein Constructs Using Full-length TIAR and TIA-1**—The cDNAs for TIAR and TIA-1 were obtained by RT-PCR from A431 cells were cloned into the pET-22a (+) vector (Novagen). The sequences of the inserts were verified by DNA sequencing and grown in the presence of kanamycin at 37 °C. After the OD reached 0.6, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM, and chloroform and precipitated with 2.5 volumes of ice-cold ethanol and 0.1 volume of 3 ml of sodium acetate, washed twice in 70% ethanol, and then reconstituted in RNase-free water. About 800 μg of ARE RNA were made by in vitro transcription of ARE RNA using T7 polymerase (Promega). ARE RNA was made as described above by biotinylated using PHOTOPROBE (long arm) kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s recommendations. Briefly, ARE RNA and phototope riboprobe were mixed and exposed to light (365 nm), which covalently couples bis RNA to RNA. Unincorporated RNA was removed by extracting the mixture twice with 2-butanol, and the biotinylated RNA was precipitated with ammonium acetate, washed with 70% ethanol, and dissolved in 1.0 ml of RNase-free TE (10 mM Tris, pH 8.0, 1 mM EDTA).

**Purification of β2-AR ARE RNA-binding Proteins—**Cytosolic extracts (about 125 μg of protein) were subjected to pre-clearance by incubating on a rotator with 3 ml of VECTREX Avidin D (Vector Laboratories, Burlingame, CA) for 2 h at 4 °C on a rotator. After pre-clearance, the pellets were washed twice with 2 ml of the same buffer and centrifuged at 10,000 × 105 cpm in 15 mM HEPES (pH 7.9), 15 mM KCl, 5 mM MgCl2, 2 mM DTT, 0.1 mM spermine, 1 mg/ml yeast tRNA, and 20 units of RNase in a total volume of 20 μl at 40 °C for 30 minutes. The reaction mixtures were incubated on ice for 20 min, and complexes were resolved by electrophoresis on 5% native polyacrylamide gels in 45 mM TBE (Tris borate–EDTA) for the running buffer. For supershift experiments, binding mixtures were incubated with 1.0 μg each of the affinity-purified antibodies for hRNP-A1, TIAR, TIA-1, HuR, AU1, or TTP antibodies, and then incubated with the T7 RNA polymerase and/or TTP (rabbit polyclonal), and HuR (mouse monoclonal) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-AUF-1 (rabbit polyclonal) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Nonimmune serum was used as a control. All of the samples were incubated for an additional 15 min, and loading buffer containing 1% SDS was added. For in vitro transcription, a 2× SDS-PAGE buffer was added to the PCR amplicons and to be used in Western blot analysis. All of the antibodies were used at a dilution of 1:1,000 in phosphate-buffered saline containing 3% bovine serum albumin. After incubation with alkaline phosphatase-linked secondary antibodies, immunoreactive bands were visualized by a chemiluminescent method (CVP star; New England Biolabs).

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**GST Fusion Protein Constructs Using Full-length TIAR and TIA-1**—The cDNAs for TIAR and TIA-1 were obtained by RT-PCR from A431 cells were cloned into the pET-22a (+) vector (Novagen). The sequences of the inserts were verified by DNA sequencing and grown in the presence of kanamycin at 37 °C. After the OD reached 0.6, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM, and
growth was continued for 3 h to induce expression of the GST fusion proteins. The bacteria were chilled on ice and spun at 5000 x g for 5 min at 4 °C. The pellet was washed with cold 20 mM Tris-HCl, pH 8.0, and stored frozen at −70 °C. The bacteria were lysed in the presence of lysosome, proteasome inhibitors, and benzonase (25 units) and incubated at room temperature for 20 min. The lysates were centrifuged at 16,000 x g for 20 min at 4 °C, and the supernatants were loaded onto a GST affinity column with a flow rate of 10 ml/h. Columns were washed with 10 volumes of wash buffer, and the fusion proteins were eluted using 3 volumes of elution buffer containing glutathione. Hamster β2-AR Deletion Constructs—The pcDNA vector (Invitrogen) was used to make the various deletion constructs of β2-AR cDNA as described previously (7). The entire 5'-UTR and the coding region from hamster β2-AR cDNA including the stop codon was PCR-amplified using Ffu polymerase and cloned into pcDNA3. Similarly, the proximal 120- and 190-nt and distal 340-nt sequences from the 3'-UTR were PCR-amplified and inserted into pcDNA3. All of the constructs were sequenced to verify the orientation and sequence of the PCR-amplified region and used for in vitro transcription after linearization with a restriction enzyme that cleaves the plasmid immediately 3' to the inserted receptor sequences.

In Vitro Transcription and UV-cross-linked Label Transfer Assay—Transcription was performed in vitro using T7 DNA-directed RNA polymerase to produce uniformly labeled RNA corresponding to various deletion constructs as described previously (11, 13). Briefly, RNAs were transcribed in the presence of [32P]UTP (800 Ci/mmol; PerkinElmer Life Sciences), nucleotide, and buffer conditions as detailed by Promega. After the RNA was transcribed, RNase-free DNase was added to the mixtures to remove template DNA. The labeled transcripts were extracted with phenol and then with phenol and precipitated finally with 2.5 volumes of ice-cold ethanol and 0.1 volumes of 3 M sodium acetate. The labeled transcripts were reconstituted in RNase-free water. The size and integrity of the transcripts were verified, and the label transfer assay was performed as described previously (11). An aliquot of radiolabeled mRNA (4 x 10⁶ cpm), 5 µg of yeast tRNA, and competing unlabeled RNA transcripts when used (at the molar excess over probe indicated) were each added to mixtures containing the GST-TIAR or GST-TIA-1, 4 mM dithiothreitol, 5 µg of heparin, and 65 units of RNasin in total volumes of 50 µl. Mixtures were incubated for 10 min at 22 °C and exposed to short wave (254-nm) UV irradiation at a distance of 7 cm for 30 min. The mRNA not cross-linked to protein was digested with RNase A (0.5 mg/ml) and RNase T1 (10 units/ml) at 37 °C for 30 min. Samples were subjected to SDS-PAGE analysis.

SDS-Polyacrylamide Gel Electrophoresis of RNA Protein Mixtures—50 µl of 2× Laemmli loading buffer was added to the digested protein RNA mixture and heated for 10 min at 89 °C. The samples were loaded onto SDS-polyacrylamide gels (10% acrylamide separating gel with 5% acrylamide stack) and subjected to gel electrophoresis. Resolved proteins were stained with Coomassie Blue R, and the gels were destained, dried, and subjected to autoradiography.

Determination of GST-TIAR Binding Affinity to Different Regions of β2-AR 3'-UTR RNA by Gel Shift Assay—The affinity of purified GST-TIAR for RNA substrate was determined by RNA gel mobility shift assay as described by DeMaria and Brewer (45). Two fmol of [32P]-labeled, in vitro transcribed RNA (constant) were incubated with increasing concentrations of GST-TIAR protein. Reaction mixtures were incubated on ice for 20 min, and complexes were resolved by electrophoresis on a 5% native polyacrylamide gel in 45 mM TBE (Tris borate-EDTA) as the running buffer. The gel was dried and visualized on a PhosphorImager and then subjected to autoradiography. The protein concentration required to shift 50% of the free substrate RNA above the baseline is defined as the Kd value.

TIAR Immunoprecipitation (IP) and Identification of Target 3'-UTR Transcripts—IP experiments were performed following the method of Tenenbaum et al. (46). In vitro transcribed radiolabeled receptor 3'-UTR transcripts were incubated and UV-cross-linked with cytosolic extracts from DDT1-MF2 cells and then subjected to IP with TIAR- and HuR-specific antibodies. IP using nonimmune serum served as a control in these experiments. Immunoprecipitated complexes were subjected to SDS-PAGE analysis followed by autoradiography as described above for the UV-cross-linked label transfer assay. Quantification of the [32P] label transferred from full-length 3'-UTR and proximal 190-nt and distal 340-nt regions to TIAR protein was done by direct analysis of radioactivity using a PhosphorImager (Amersham Biosciences).

Polyosite Profile Analyses of β2-AR mRNA from A431 Cells—A431 cells were transiently transfected with TIAR cDNA or empty vector, and after 48 h of transfection, cytoplasmic extracts were made, and polysome profile analyses were carried out as described in detail previously (7). After ultracentrifugation, the tubes were punctured at the bottom, and the fractions were displaced upwards using Fluorimeter FC-40 (Sirma). Absorbance was monitored continuously at 254 nm using ISCO-UA5 (model 640) density gradient fraction collector. A total of 14 fractions were collected, and total RNA was extracted from each fraction. To obtain enough receptor RNA for RNase protection assays, two successive fractions were pooled, making a total of seven fractions. For Western blots of TIAR in sucrose gradient fractions, aliquots of individual fractions were dialyzed to remove sucrose and concentrated using Centricron centrifugal filter devices (Millipore Corp.). Equal volumes from each fraction were used for Western blot analyses with polyclonal antibodies raised against TIAR.

RESULTS

Expression of 3'-UTR Sequences Leads to Reversal of β2-AR Adrenergic Receptor Translational Repression in DDT1-MF2 and A431 Cells—Our previous work demonstrated that 3'-UTR sequences of the β2-AR mRNA suppress the translation of receptor mRNA (7). Such transcript-specific translational inhibition usually involves the binding of proteins to cis-acting elements present in the 3'-UTR sequences of RNA. Thus, β2-AR translational control by 3'-UTR sequences could possibly be achieved by factors that bind to 3'-UTR in a sequence-specific manner. We tested this possibility by using retroviral transcripts that contain receptor 3'-UTR sequences as decoys to relieve the translational inhibition of receptor mRNA in cell lines that endogenously express β2-AR. For this purpose, DDT1-MF2 cells were transfected with a retroviral expression vector carrying the full-length receptor 3'-UTR sequences or a control vector. To assess the effect of receptor 3'-UTR expression, G418-resistant clones expressing receptor 3'-UTR RNA and control vector-transfected cells were assayed for β2-AR expression by radioligand binding assay. The results from these experiments demonstrated an increased expression of receptor protein in DDT1-MF2 cells expressing receptor 3'-UTR RNA as compared with vector-transfected cells (Fig. 1A), suggesting that trans-acting proteins are involved in negatively regulating the receptor expression in these cells.

In earlier studies, we demonstrated that several cytosolic proteins bind to a 21-nt conserved A+U-rich region present within the 3'-UTR of β2-AR mRNA (11, 13). To localize the region within the 3'-UTR RNA that causes increased receptor expression, we expressed the conserved A+U-rich RNA or scrambled RNA in DDT1-MF2 cells. Those experiments demonstrated a 2-fold increase in receptor expression in cells expressing A+U-rich RNA sequences as compared with cells expressing scrambled RNA (Fig. 1A), consistent with a role for A+U-RNA-binding proteins in regulating receptor expression in DDT1-MF2 cells.

To confirm the above results in another cell line, we expressed A+U-rich or scrambled RNA sequences in A431 (human epidermal carcinoma) cells using the retroviral expression vector. A431 cells are commonly used to study the regulation of endogenously expressed β2-AR. Radioligand binding assays using membranes and whole cells demonstrated 80–100% increases in receptor expression in A431 cells expressing A+U-rich RNA sequences as compared with vector-transfected cells (Fig. 1B). Expression of both hamster (ARE RNA-1) and human β2-AR A+U-rich RNA (ARE RNA-2) resulted in increased receptor expression in A431 cells. There was no change in receptor expression in cells that expressed scrambled RNA as compared with untransfected cells. These results further support the possibility that A+U-rich sequence-binding proteins are involved in suppressing the receptor expression in both cell lines.

Increased Receptor Expression Is Not Accompanied by Receptor mRNA Stabilization—It is possible that sequestration of A+U-rich RNA sequences from the 3'-UTR results in increased receptor expression. Hence, we
quantified receptor mRNA levels by RNase protection assays in DDT1-MF2 and A431 cells. A, DDT1-MF2 cells were transfected with a retroviral expression vector (pLNCX2) carrying the full-length 3’-UTR (530 3’-UTR) sequences of hamster β2-AR cDNA or the conserved ARE region (ARE RNA 1) from the 3’-UTR of hamster β2-AR mRNA cDNA (see “Experimental Procedures” for details of the sequences). G418-resistant clonal transfectants were used to measure receptor expression by radioligand binding assays using a saturating concentration (400 pM) of the β2-AR ligand [125I]CYP. Assays were performed in triplicate in whole cells, and receptor levels were calculated as fmol/10^5 cells. The results were confirmed by a radioligand binding assay using crude membrane preparations of the transfected cells. G418-resistant clones were pooled (more than 100 clones from each transfection) and used for ligand binding assays. Data represent mean ± S.D. of four separate transfections. Cells expressing retroviral transcripts containing scrambled RNA served as controls (control RNA). B, A431 cells were transfected with a retroviral expression vector carrying the A+U-rich region (see “Experimental Procedures” for details of the sequences) present in the 3’-UTR of hamster or human β2-AR cDNA (13). G418-resistant clones were selected and assayed for receptor expression as described above for DDT1-MF2 cells. Cells transfected with the same retroviral expression vector carrying a scrambled 20-nt region served as controls. Data represent mean ± S.D. of five separate transfections with retroviral vectors expressing scrambled RNA (Control RNA), hamster ARE RNA (11), or human ARE RNA (2) β2-AR 3’-UTR RNA. C, steady state levels of β2-AR mRNA in DDT1-MF2 cells expressing ARE or scrambled (SCR) RNA. Total RNA was extracted from G418-resistant clones of DDT1-MF2 cells, and 25 μg of total RNA was used in RNase protection assays as described before (50). Antisense riboprobes specific for DDT1-MF2 cells were obtained by RT-PCR from total RNA isolated from these cells using primers as specified under “Experimental Procedures.” Shown are data from three independent transfection experiments with ARE and scrambled RNA. D, equal quantities of total RNA isolated from DDT1-MF2 cells were run on agarose gel and stained with ethidium bromide to check for equal loading. E, steady state levels of β2-AR mRNA in A431 cells expressing ARE or scrambled RNA. Total RNA was extracted from G418-resistant clones of A431 cells, and experiments were performed as described for C, except that antisense riboprobes specific for A431 cells were obtained by RT-PCR from total RNA isolated from these cells using primers as specified under “Experimental Procedures.”

Fig. 1. Expression of full-length 3’-untranslated region sequences or the A+U-rich elements leads to increased receptor expression in DDT1-MF2 and A431 cells. A, DDT1-MF2 cells were transfected with a retroviral expression vector (pLNCX2) carrying the full-length 3’-UTR (530 3’-UTR) sequences of hamster β2-AR cDNA or the conserved ARE region (ARE RNA 1) from the 3’-UTR of hamster β2-AR mRNA cDNA (see “Experimental Procedures” for details of the sequences). G418-resistant clonal transfectants were used to measure receptor expression by radioligand binding assays using a saturating concentration (400 pM) of the β2-AR ligand [125I]CYP. Assays were performed in triplicate in whole cells, and receptor levels were calculated as fmol/10^5 cells. The results were confirmed by a radioligand binding assay using crude membrane preparations of the transfected cells. G418-resistant clones were pooled (more than 100 clones from each transfection) and used for ligand binding assays. Data represent mean ± S.D. of four separate transfections. Cells expressing retroviral transcripts containing scrambled RNA served as controls (control RNA). B, A431 cells were transfected with a retroviral expression vector carrying the A+U-rich region (see “Experimental Procedures” for details of the sequences) present in the 3’-UTR of hamster or human β2-AR cDNA (13). G418-resistant clones were selected and assayed for receptor expression as described above for DDT1-MF2 cells. Cells transfected with the same retroviral expression vector carrying a scrambled 20-nt region served as controls. Data represent mean ± S.D. of five separate transfections with retroviral vectors expressing scrambled RNA (Control RNA), hamster ARE RNA (11), or human ARE RNA (2) β2-AR 3’-UTR RNA. C, steady state levels of β2-AR mRNA in DDT1-MF2 cells expressing ARE or scrambled (SCR) RNA. Total RNA was extracted from G418-resistant clones of DDT1-MF2 cells, and 25 μg of total RNA was used in RNase protection assays as described before (50). Antisense riboprobes specific for DDT1-MF2 cells were obtained by RT-PCR from total RNA isolated from these cells using primers as specified under “Experimental Procedures.” Shown are data from three independent transfection experiments with ARE and scrambled RNA. D, equal quantities of total RNA isolated from DDT1-MF2 cells were run on agarose gel and stained with ethidium bromide to check for equal loading. E, steady state levels of β2-AR mRNA in A431 cells expressing ARE or scrambled RNA. Total RNA was extracted from G418-resistant clones of A431 cells, and experiments were performed as described for C, except that antisense riboprobes specific for A431 cells were obtained by RT-PCR from total RNA isolated from these cells using primers as specified under “Experimental Procedures.”
Expressed ARE Sequences Bind Cytosolic Proteins—To confirm that ARE RNA is expressed in cells and that the observed changes in receptor levels are mediated by protein binding to ARE RNA molecules, we performed in vivo label transfer experiments in transfected cells. A431 and DDT₁-MF2 cells selected with G418 and expressing ARE RNA and control cells expressing scrambled RNA sequences were incubated with \(^{[3H]}\)uridine, and cell monolayers were exposed to shortwave (254 nm) UV light after washing and removing unincorporated \(^{[3H]}\)uridine. Equal quantities of cytosolic proteins from A⁺/U-rich RNA-expressing and scrambled RNA-expressing cells were subjected to SDS-PAGE and autoradiography. The autoradiogram (Fig. 2) shows several protein bands between the apparent molecular masses of 30 and 50 kDa only in cells expressing ARE RNA. Similar results were obtained in both A431 (Fig. 2A) and DDT₁-MF2 (Fig. 2B) cells. These results suggest that cytosolic proteins are bound to ARE RNA in cells expressing retroviral transcripts containing \(\beta_\text{2-AR} \) ARE RNA and also support the possibility that sequestration of ARE RNA-binding proteins may be responsible for the observed up-regulation of \(\beta_\text{2-AR} \) expression.

Purification of Receptor ARE-binding Proteins—To explore further the possibility that A⁺/U-rich region-binding proteins regulate \(\beta_\text{2-AR} \) mRNA at the level of translation, we set out to purify the receptor mRNA 3'-UTR sequence-binding proteins. We employed a \(\beta_\text{2-AR} \)-specific ARE RNA sequence for affinity purification of cytosolic proteins that are specifically bound to receptor ARE RNA. To that end, we followed the method of Grosset et al. (47) with modifications as stated under “Experimental Procedures.” Cytosolic proteins bound to biotinylated ARE RNA were isolated using VECTREX avidin. Both DDT₁-MF2 and A431 cells express \(\beta_\text{2-AR} \) endogenously, and cytosolic extracts from both cell lines exhibited more or less identical patterns of protein binding to ARE RNA (Fig. 3A). We proceeded with purification of ARE-binding proteins using extracts of A431 cells. To reduce nonspecific protein binding to avidin, cytosolic extracts were precleared of avidin binding factors and used for RNA affinity chromatography. Fig. 3B is a silver-stained gel showing the protein pattern of fractions eluted from the RNA affinity column. Six well separated proteins in the molecular weight range of 30–50 kDa (Fig. 3B, lane 6) were eluted with 2 M KCl. A more concentrated aliquot of the purified fraction was run separately (Fig. 3B, lane 7) to demonstrate the presence of a protein band at \(~30\) kDa in the purified fraction. In addition to these six proteins, three high molecular weight proteins were also visible in silver-stained gels (Fig. 3B, lanes 6 and 7).

A UV-catalyzed label transfer assay was performed using radiolabeled ARE RNA and aliquots of samples from the silver stain experiments, revealing almost identical patterns of cross-linked proteins using crude cytosolic extract and purified proteins (Fig. 3C, lane 1 and 5). This suggests that all of the cytosolic proteins that bind to ARE RNA were isolated using this purification method. The high molecular weight proteins eluted by 2 M KCl did not show any label transfer (Fig. 3C, lane 5) using labeled ARE RNA, suggesting that these proteins do not bind directly to ARE RNA and hence were not included in further analysis.

Identification of TIAR and TIA-1 as \(\beta_\text{2-AR} \) mRNA-binding Proteins—Using the affinity approach, the amount of proteins available for peptide analysis was limited. Therefore, gel shift and Western blot analyses were used to identify the purified receptor ARE-binding proteins. We reasoned that formation of high molecular weight RNA-protein complexes (supershift) and/or disruption of the RNA-protein complexes in the presence of antibodies to known ARE-RNA binding proteins would be an indication of the presence of specific protein in the purified fraction. Nonimmune serum served as control in such experiments. Purified proteins formed two complexes in control experiments (Fig. 4A, lanes 2). The use of antibodies against AUF-1 and hnRNP-A1 did not result in significant changes in RNA-protein complex formation (Fig. 4A, lanes 3 and 4). HuR, TIAR, and TIA-1 antibodies, when included in the mixture, produced supershifts that were accompanied by reductions in RNA-protein complex formation (Fig. 4A, lanes 5–7). The use of anti-TTP and nonimmune serum also did not result in any changes in RNA-protein complex formation (lanes 8 and 9). These results suggested that TIAR, TIA-1, and HuR (but not TTP or AUF1) are present in the purified fractions. Also, it is clear from Fig. 4A that the use of HuR antibodies shifted the lower RNA-protein complex (complex-1), whereas TIAR and
TIA-1 antibodies shifted the upper RNA-protein complex (complex-2). The addition of excess cold ARE RNA completely abolished the complex formation with RNA (Fig. 4A, last two lanes), demonstrating the specificity of the interactions.

To confirm these results, we performed Western blot analyses of the purified proteins. Blots demonstrated the presence of all five of the ARE-binding proteins tested in the unpurified cytoplasmic extracts from A431 cells (Fig. 4B). However, purified fractions showed that TIAR, TIA-1, and HuR were the major proteins. Minor quantities of two isoforms of AUF1 proteins were also detected in purified fractions by Western blot analyses. TTP, although detected in the cytosolic extracts, did not bind to \( \beta_2 \)-AR ARE RNA. The \(-50 \text{kDa}\) protein band (protein band marked 1 in Fig. 3B, lane 7) was identified as actin by peptide sequence analyses. The combined results from gel shift and Western blot analyses suggested that TIAR, TIA-1, and HuR are the major proteins that bind to \( \beta_2 \)-AR ARE RNA.

**TIAR and TIA-1 Bind to Receptor mRNA through the 3′-UTR Sequence**—TIAR and TIA-1 are translational silencer proteins (30–32) that sediment exclusively into monosomal fractions in

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**FIG. 3. Purification of receptor ARE RNA-binding proteins by RNA affinity method.** The details of the purification are given under “Experimental Procedures.” The purification of ARE-binding proteins was reproducible, since the two times this was repeated using cytosolic extracts gave similar results. A, cytosolic extracts of DDT1-MF2 and A431 cells show a similar pattern of protein binding to \( \beta_2 \)-AR ARE RNA. The autoradiogram shows UV cross-linking and label transfer using cytosolic extracts from DDT1-MF2 and A431 cells with radiolabeled in vitro transcribed RNA corresponding to the 21-nt ARE RNA. B, SDS-PAGE and silver stain analysis of proteins from each step of the ARE RNA affinity purification. Aliquots of A431 cell cytosolic extracts (starting material in lane 2), pre cleared cytosolic extracts (lane 3), effluent from the RNA avidin column (lane 4), last wash with 0.3 M KCl (lane 5), and proteins eluted with 2 M KCl (lane 6) were subjected to SDS-PAGE and silver stain analysis. A more concentrated aliquot of the sample eluted with 2 M KCl (lane 6) was separated to SDS-PAGE analysis (lane 7) followed by silver stain. Lane 1 shows a protein ladder (Invitrogen). C, autoradiogram of UV-catalyzed cross-linking of proteins from each step in the purification with radiolabeled ARE RNA. \( ^{32} \text{P} \)-Labeled ARE RNA probe was incubated with aliquots of various fractions obtained by RNA affinity purification as described above (Fig. 3B) and subjected to UV-catalyzed cross-linking followed by digestion with RNase prior to SDS-PAGE analysis and autoradiography. Lane 1, starting material; lane 2, pre cleared cytosolic extracts; lane 3, effluent from the RNA affinity column; lane 4, last wash with 0.3 M KCl; lane 5, proteins eluted with 2 M KCl.
sucrose density gradient analysis of cytosolic extracts (48). We previously demonstrated that significant quantities of $\beta_2$-AR mRNA also sediment to monosomal fractions in a 3'-UTR-dependent manner (7). In contrast, HuR (49), a member of the embryonic lethal abnormal vision family of RNA-binding proteins (50), has been reported to sediment into polysomal fractions (47, 51). Because actin did not bind to ARE RNA, we focused on TIAR and TIA-1. Therefore, we further investigated roles for TIAR and TIA-1 by constructing GST fusion proteins.

The binding of GST fusion proteins of TIAR or TIA-1 to receptor RNA was assessed by UV-catalyzed cross-linking and label transfer using radiolabeled $\beta_2$-AR mRNA corresponding to the full-length 5'-UTR with the receptor coding region or the 3'-UTR alone (Fig. 5A). Since use of radiolabeled $\beta_2$-AR RNA and GST did not result in any label transfer in UV-catalyzed cross-linking experiments (data not shown), all of the RNA binding studies were done using GST fusion proteins of TIAR and TIA-1. Such direct label transfer studies using equal quantities of TIAR and TIA-1 and equimolar concentrations of radiolabeled RNAs demonstrated that both TIAR and TIA-1 bind to full-length $\beta_2$-AR mRNA, albeit to TIAR with much higher affinity (Fig. 5B). In contrast, deletion of the 3'-UTR sequences, while retaining the 5'-UTR and coding region, completely abolished the binding of both of the proteins to the transcripts. This suggests that both TIAR and TIA-1 bind to $\beta_2$-AR mRNA through the 3'-UTR sequences. To confirm this further, we examined the ability of the unlabeled receptor mRNA corresponding to full-length and 3'-UTR deletion constructs to compete with labeled full-length receptor transcripts. In those experiments, full-length receptor mRNA competed effectively for the binding of labeled full-length receptor mRNA to TIAR. Unlike the wild-type full-length transcripts, the unlabeled transcripts for the coding region and 5'-UTR sequences (3'-UTR) failed to compete with 32P-labeled transcripts for binding to TIAR (Fig. 5C).

TIAR Has Multiple Binding sites on $\beta_2$-AR 3'-UTR Sequences—To more fully characterize the TIAR binding regions within the 3'-UTR sequences, we used uniformly labeled in vitro transcribed RNA corresponding to various deletions within the 3'-UTR sequences (Fig. 6A). These experiments demonstrated that there are multiple binding sites for TIAR protein in the 3'-UTR of $\beta_2$-AR mRNA (Fig. 6B). The proximal 120-nt region showed minimal label transfer, indicating that this region may not bind efficiently to TIAR. The addition of the next 70-nt region (proximal 190 nt) to labeled RNA resulted in significant label transfer with GST-TIAR. There is a 20-nt A+U-rich region present within this 70-nt region that probably contributes to TIAR binding. Radiolabeled RNA corresponding

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to the distal 345-nt region of the 3'-UTR sequences also demonstrated significant label transfer to GST-TIAR (Fig. 6B). There are A+U-rich and poly(U) regions within the distal 340-nt RNA of the receptor 3'-UTR also (1). Using equimolar concentrations of uniformly radiolabeled probes and equal quantities of GST-TIAR, the distal 340-nt 3'-UTR RNA displayed more prominent binding to TIAR, as compared with the proximal 190-nt 3'-UTR region (Fig. 6B).

**GST-TIAR Binding to Proximal 190-nt Versus Distal 340-nt 3'-UTR RNA**—In our earlier studies, we showed that the distal 340-nt region from the 3'-UTR sequences is necessary for translational inhibition of receptor mRNA (7). Thus, it is important to identify the binding affinities of different regions of the 3'-UTR RNA to TIAR. To address more directly the binding affinities of TIAR to proximal and distal 3'-UTR RNA, non-denaturing gel shift experiments were performed using GST-TIAR and equimolar concentrations of in vitro transcribed, radiolabeled RNA corresponding to distal and proximal 3'-UTRs. The results of non-denaturing gel shift analysis (Fig. 6C) demonstrated that TIAR binds with higher affinity to the distal 340-nt RNA (−10 nm) as compared with the proximal 190-nt RNA region (−20 nm). Thus, the results of gel shift analyses are consistent with the UV-cross-linked label transfer assays.

**Identification of TIAR Binding Site on β2-AR 3'-UTR RNA by Immunoprecipitation (IP)**—In the above experiments, we used GST fusion proteins to identify the number of binding sites and binding affinities of different regions of β2-AR 3'-UTR RNA. Additional evidence for TIAR binding to 3'-UTR sequences of β2-AR RNA was obtained by UV-cross-linking of cytosolic lysates from DDT1-MF2 cells with radiolabeled receptor 3'-UTR RNA, followed by IP of the resulting complexes in either the presence or absence of specific anti-TIAR and anti-HuR antibodies. Radiolabeled RNA corresponding to the full-length 530-nt 3'-UTR and proximal 190-nt and distal 340-nt regions of the β2-AR were separately assessed to identify the region(s) of the 3'-UTR RNA that preferentially associate with TIAR. Aliquots of UV-cross-linked material that were not subjected to IP showed that all three 3'-UTR RNA sequences were associated with several proteins (Fig. 7A, lanes 1, 3, and 8). IP of the cross-linked material without a specific antibody (nonimmune serum) produced no radiolabeled bands (Fig. 7A, lane 4). In contrast, use of a specific anti-TIAR antibody showed that both radiolabeled 530-nt and distal 340-nt transcripts were strongly associated with TIAR (Fig. 7A, lanes 1, 2, and 6) as compared with proximal 190-nt (lane 9) transcripts. The IP efficiency was calculated to be ~90% for TIAR and ~70% for HuR in our experiments (data not shown). Thus, although the binding affinities of different proteins cannot be compared in such experiments, the binding affinities of the same protein to different transcripts can be compared. Using equimolar concentrations of proximal 190-nt and distal 340-nt transcripts and equal quantities of cytosolic extracts, 2.5–3.5-fold more incorporation of radioactivity was observed when the distal 340-nt region was used as the probe (mean ± S.D. of three separate experiments) (Fig. 7B). Thus, the results of IP experiments using total cytosolic extracts support the results obtained by using GST-TIAR in direct label transfer (Fig. 6B) and gel mobility shift assays (Fig. 6C). IP experiments also revealed that both isoforms of TIAR bind to β2-AR 3'-UTR RNA sequences (Fig. 7A, lanes 2 and 6).

**Co-transfection of TIAR and β2-AR cDNA in CHO Cells**—We reasoned that if the 3'-UTR of the β2-AR is necessary for...
binding of TIAR to receptor mRNA, then co-transfection of CHO cells with receptor cDNA constructs with and without 3'-UTR sequences along with TIAR should reflect a 3'-UTR-mediated translational suppression of receptor synthesis. To that end, CHO cells were co-transfected with equal quantities (5 μg) of β2-AR cDNA and TIAR cDNAs. For the corresponding controls, CHO cells were co-transfected with receptor cDNA having 3'-UTR deletions along with TIAR cDNAs (5 μg each). Because 3'-UTR deletions result in increased receptor expression in transfected cells (7), CHO cells were also transfected separately with 5 μg each of β2-AR cDNA with and without 3'-UTR sequences along with (5 μg) empty vector. Receptor expression was measured in each group at 48, 72, and 96 h after transfections. Although the co-transfections decreased the expression of β2-AR in both groups, there was a clear difference between the two groups. Cells co-transfected with TIAR and full-length receptor cDNAs showed ~45–50% greater decreases in receptor expression after 48 h of co-transfection (Fig. 8A, compare lanes 2 and 6) when compared with cells co-transfected with 3'-UTR deletions of β2-AR and TIAR. Receptor measurement after 72- and 96-h transfection showed rapid increases in receptor expression only in cells that were co-transfected with full-length β2-AR cDNA and TIAR (Fig. 8A, compare lanes 3 and 4 with lanes 7 and 8).

Increased expression of TIAR was confirmed in CHO cells that were co-transfected with β2-AR and TIAR by Western blot analysis using the cytosolic extracts (Fig. 8B). As shown in Fig. 8B, TIAR expression was high at 24 and 48 h after transfection and returned to control values after 72 h. Receptor expression increased significantly after 72 and 96 h of transfection only in cells that were co-transfected with wild type β2-AR and TIAR and not in CHO cells that are co-transfected with 3'-UTR deletion constructs of β2-AR and TIAR. These results further suggest that overexpression of TIAR suppresses the translation of β2-AR mRNA and that the increase in receptor expression after 72 h of transfection correlates with the loss of TIAR overexpression.

To confirm that the decreased receptor expression in CHO cells that are co-transfected with β2-AR and TIAR cDNAs is due to translational suppression of receptor mRNA, we compared the steady-state levels of receptor mRNA in these transfected cells. Receptor mRNA levels were comparable in both control and experimental groups (Fig. 8C), suggesting that the decreased receptor expression is caused by translational suppression of receptor mRNA by TIAR. We also tested the short and long isoforms of TIAR in co-transfections with β2-AR cDNAs to check for specificity. A similar inhibitory pattern was obtained using both of the isoforms (data not shown).

Polysome Profile Analysis of β2-AR mRNA in Cells Overexpressing TIAR—To examine more directly the role of TIAR in
**FIG. 7.** Identification of preferential binding sites for TIAR on β₂-AR 3′-UTR RNA using cytosolic extracts from DDT1-MF2 cells. A, representative autoradiogram showing label transfer to TIAR and HuR from uniformly labeled in vitro transcribed 3′-UTR transcripts of β₂-AR mRNA. Equimolar quantities of radiolabeled β₂-AR 3′-UTR transcripts corresponding to the full-length (530-nt) 3′-UTR and the proximal 190-nt and distal 340-nt regions were synthesized and incubated with equal quantities of cytosolic lysates from DDT1-MF2 cells (50 μg) and then subjected to UV-cross-linked label transfer. The resulting complexes were treated with RNase A + T1 and subjected to IP using anti-TIAR and anti-HuR antibodies. IP with nonimmune serum served as a control. The immunoprecipitated materials and controls were subjected to SDS-PAGE analysis as described under “Experimental Procedures.” Lanes 1, 5, and 8, UV-cross-linked material that was not subjected to IP; lanes 2, 6, and 9, IP with anti-TIAR antibody; lanes 3, 7, and 10, IP with anti-HuR antibody. Lane 4, no labeled protein is pulled in the IP using nonimmune serum, thus demonstrating the specificity of the IP experiments. B, quantification of the 32P label transferred from uniformly labeled distal 340-nt RNA and proximal 190-nt RNA to TIAR. The values are displayed as percentages of label transfer obtained by setting the values obtained for the 530-nt RNA to 100%. The data represent the mean ± S.D. of three separate experiments.

**FIG. 8.** The 3′-UTR of the β₂-AR decreased receptor expression in CHO cells when co-transfected with the TIAR. A, CHO cells were co-transfected with equal quantities of full-length (lanes 1–4) or 3′-UTR deletion constructs (lanes 5–8) of β₂-AR cDNA (5 μg each) with TIAR in pcDNA3 or empty vector. Receptor expression levels were measured by radioligand binding assay in both groups of cells 48 h (lanes 2 and 6), 72 h (lanes 3 and 7), and 96 h (lanes 4 and 8) following transfections. The values displayed are percentages of receptor expression taking the values obtained for receptor expression at the appropriate time points for cells that were co-transfected with β₂-AR cDNA with empty vector as 100% (lanes 1 and 5). Each value represents the mean ± S.D. of at least three separate experiments. W-type, wild type. B, Western blot analysis was carried out using cytoplasmic extracts of CHO cells to compare the expression levels of TIAR in untransfected cells with β₂-AR- and TIAR-co-transfected cells. Equal quantities of cytosolic extracts from CHO cells at 0, 24, 48, 72, 96, and 120 h after transfections were used for Western blot analysis using polyclonal antibody to TIAR. C, representative autoradiogram showing steady-state levels of β₂-AR mRNA in CHO cells transfected with β₂-AR and TIAR. Total RNA was extracted after 48 h of transfection, and 25 μg of total RNA was used in RNase protection assays using β₂-AR-specific antisense probe as described previously (7).

β₂-AR mRNA localization in sucrose density gradient fractions, we compared the distribution of endogenously expressed β₂-AR mRNA in A431 cells with and without TIAR overexpression. Comparison of cytoplasmic levels of TIAR protein in DDT1-MF2 and A431 cells showed lower levels of TIAR in A431 cells (data not shown). Hence, we chose this cell line for these studies. Polyosome fractionation and RNA extraction were performed on cytosolic extracts using sucrose gradients as described previously (7). Monosomes and polysomes were identified by absorbance at 254 nm (Fig. 9A). Fourteen continuous fractions, from lightest to heaviest, were collected, and total RNA was extracted from each fraction as described previously (7). RNA from successive fractions was pooled to obtain a total of seven fractions, and the distribution of β₂-AR mRNA was determined in these fractions by RNase protection assay. The results demonstrated the presence of significant quantities of β₂-AR mRNA in monosomes (Fig. 9B, upper panel), suggesting that β₂-AR mRNA is distributed in both monosomes and polysomes. When cytosolic extracts from A431 cells overexpressing TIAR were subjected to sucrose density gradient centrifugation, there was a significant shift in receptor mRNA distribution from heavy polysomes to low molecular weight fractions containing monoribosomes (Fig. 9B, lower panel), suggesting that TIAR overexpression can cause redistribution of
TIAR Regulates β2-AR mRNA Translation

**Fig. 9.** Polysome profile analysis of β2-AR mRNA in cells overexpressing TIAR. These experiments using cytosolic lysates of vector-transfected and TIAR-overexpressing A431 cells were done three times, showing similar results. A, representative UV absorption profile (254 nm) of the cytosolic extracts of A431 cells across sucrose density gradient analysis. Cytosolic lysates (1.0 ml) were prepared and layered over a 10–50% sucrose gradient and centrifuged at 38,000 rpm for 3 h in an SW40Ti rotor. The gradient profile was monitored continuously by measuring the absorbance at 254 nm with an ISCO UA-5 detector. The gradients were collected from top to the bottom by displacing them upwards, and a total of 14 fractions were collected. B, RNase protection assay showing the distribution of β2-AR mRNA in sucrose density gradient fractions. The upper panel shows distribution of β2-AR mRNA using cytosolic extracts of vector-transfected A431 cells. Total RNA was extracted from all 14 fractions, successive fractions were pooled to obtain a total of seven fractions (since β2-AR mRNA has low abundance in cells, it was necessary to pool two successive fractions to obtain sufficient quantities of receptor mRNA for the RNase protection assay), and β2-AR mRNA levels were quantitated by an RNase protection assay. The lower panel shows distribution of β2-AR mRNA using cytosolic extracts of A431 cells overexpressing TIAR. Experimental details are same as in the upper panel. C, quantification of β2-AR transcripts in individual fractions of A431 cells with (○) and without TIAR (□) overexpression was done by densitometric analysis. Distribution of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA did not change with TIAR overexpression. Data represent the means ± S.D. of three separate transfections and gradient analysis. D, Western blot analysis showing distribution of TIAR in sucrose density gradient fractions of A431 cells. Fractions obtained from sucrose gradients were processed as described under "Experimental Procedures" and subjected to SDS-PAGE analysis followed by Western blot analyses using polyclonal anti-TIAR antibodies.

**DISCUSSION**

In our earlier studies, we reported that 3′-UTR sequences of β2-AR mRNA participate in the post-transcriptional regulation of receptor mRNA, including translational suppression and agonist-mediated mRNA destabilization (7, 11, 13). We have also demonstrated that the distal 340-nt region from the 3′-UTR is necessary for translational suppression of receptor mRNA (7). In the present study, we have provided new information related to the mechanisms underlying the translational control of β2-AR mRNA by its 3′-UTR sequences. The following are the major findings from this work: 1) translational inhibition by 3′-UTR binding proteins is a normal control mechanism in both DDT1-MF2 and A431 cells; 2) identification of TIAR and TIA-1 as proteins that bind to β2-AR 3′-UTR RNA; 3) identification of multiple binding sites for TIAR on β2-AR 3′-UTR RNA and high affinity binding of TIAR to distal 340-nt β2-AR 3′-UTR RNA; and 4) overexpression of TIAR resulted in suppression of receptor protein expression and a significant shift in endogenously expressed β2-AR mRNA toward nonpolysomal fractions in sucrose density gradient analyses. Increased expression of β2-AR in DDT1-MF2 and A431 cells expressing retroviral transcripts that contain receptor 3′-UTR sequences as compared with vector-transfected cells suggests that 3′-UTR sequences could relieve translational inhibition of endogenous receptor mRNAs by competing for proteins that bind to receptor 3′-UTR sequences. In addition, the decoy experiments also suggested that β2-AR mRNA is translationally repressed by 3′-UTR sequence-binding proteins in cell lines that endogenously express this receptor, supporting our earlier
results in transfected CHO cells (7). Thus, an excess of the exogenous A+U-rich RNA could titrate out receptor A+U-rich region-binding proteins, resulting in reversal of β2-AR translational repression by 3′-UTR-binding proteins. In our experiments, using ARE RNA as decoys, it is not possible to assess quantitatively the extent to which A+U-rich binding proteins are sequestered. However, in vivo UV-catalyzed label transfer experiments using live cells demonstrated that A+U-rich RNA is expressed in cells and that cytosolic proteins are bound to such RNA molecules. A comparison of in vivo (Fig. 2, A and B) and in vitro label transfer experiments (Fig. 3, A and C) clearly demonstrates that multiple proteins in the molecular weight range of 30–50 kDa can bind to a relatively short region of A+U-rich RNA. These results were further confirmed by affinity purification of ARE-binding proteins from cytoplasmic extracts. The finding that multiple proteins bind to a relatively short region of ARE RNA is not unprecedented in that a multimeric protein complex has been reported to bind the ARE of cyclooxygenase-2 mRNA (36).

Expression of highly conserved regions from creatine kinase (16) and vimentin (52) 3′-UTR sequences enhanced the expression of both of these gene products without significant changes in their mRNA levels, suggesting that conserved RNA regions from 3′-UTR can alter gene expression posttranscriptionally at the level of mRNA translation. Expression of 3′-UTR sequences of α-tropomyosin in neoplastic NMu2 cells (a mutant myogenic cell line) has been reported to suppress anchorage-independent growth and tumor formation in NMu2 cells (53, 54). These examples clearly demonstrate that 3′-UTR-binding proteins play a significant role in post-transcriptional regulation of several genes.

The finding that TIAR and TIA-1 can form a complex with β2-AR mRNA provides the first evidence that these proteins might specifically regulate the expression of β2-AR. Both of these proteins have been shown to function as translational silencer proteins by binding to tumor necrosis factor α (29, 30) and cyclooxygenase-2 mRNA (32). In addition, a species-dependent translational silencing of human matrix metalloproteinases-13 is reported by an alternatively spliced form of TIA-1 (31). TIA-1 and TIAR are structurally and functionally related RNA-binding proteins that interact with short stretches of uridylate and A+U-rich RNA (40). The 3′-UTRs of β2-AR mRNA from different mammalian species (1, 2) contain multiple A+U-rich and poly(U) regions, and such sequences are present in their proximal and distal regions.

The current study also identifies TIAR and TIA-1 as β2-AR mRNA-binding proteins by multiple approaches. Using receptor-specific ARE RNA as an affinity ligand, we isolated both TIAR and TIA-1 from cytoplasmic extracts of A431 cells. The use of GST fusion proteins for TIAR demonstrated multiple binding sites for TIAR and differential binding affinities for different regions of β2-AR 3′-UTR RNA. This observation was further confirmed by immunoprecipitation experiments using DDT1-MF2 cytosolic extracts and TIAR-specific antibodies. Thus, although we used an ARE RNA from the proximal 3′-UTR of β2-AR RNA for the purification and identification of TIAR, this region of the receptor 3′-UTR RNA did not inhibit the translation of β2-AR mRNA (7). However, identification of multiple binding sites for TIAR on β2-AR mRNA and preferential binding of both of the isoforms of TIAR to the distal 340-nucleotide 3′-UTR RNA (Fig. 7A) support our earlier finding that this region of the 3′-UTR RNA is necessary for translational control (7).

Sucrose density gradient analysis with cytoplasmic extracts of A431 cells overexpressing TIAR demonstrated that TIAR can complex with β2-AR under in vivo conditions and sequester receptor mRNA into nontranslating monosomes. These results are in good agreement with our previous results on receptor mRNA distribution in transfected CHO cells (7). We also demonstrated that in the absence of 3′-UTR, the receptor mRNA is shifted completely to the polysomal fractions in sucrose density gradient centrifugation (7). Since TIAR has no binding site in the 5′-UTR and coding regions of the receptor mRNA, we speculate that receptor mRNA bound to TIAR by its 3′-UTR sequences are sequestered into untranslatable mRNA-protein complexes. Also, sequestration of TIAR and other related proteins by excess ARE RNA expressed by retroviral transcripts may be responsible for releasing the receptor mRNA binding to ribonucleoproteins for subsequent translational activation.

The increased production of receptor protein in ARE RNA-expressing cells could not have resulted from increased receptor mRNA stability, because the receptor mRNA levels decreased whereas the receptor protein levels increased in both DDT1-MF2 and A431 cells. These seemingly contradictory observations suggest that 3′-UTR-binding proteins not only translationally suppress but also stabilize the receptor mRNA. We also identified HuR, another ARE-binding protein, by the receptor ARE RNA affinity purification method. HuR has been reported to selectively stabilize ARE-containing mRNAs (55, 56). Although HuR has been reported to bind β1 and β2-AR mRNAs (57, 58), its role in receptor mRNA regulation is not understood. From the present data, it is not possible to conclude whether the translational suppression and stabilization is achieved by the same protein or by different proteins that bind to different regions in the 3′-UTR of the receptor mRNA. Specific mRNA “knock down” by RNA interference of HuR and TIAR will be able to identify which of the RNA-binding proteins is necessary for stabilization of the receptor mRNA.

Although translational control by 3′-UTR has been reported for G-protein-coupled receptor mRNAs (7, 59), the current report is the first to identify a particular RNA-binding protein and the regulatory sequences that recruit that protein, in this case TIAR. From the current work, it can be concluded that translational suppression of β2-AR mRNA by 3′-UTR-binding proteins is a normal control mechanism that works in at least two different cell lines of human and hamster origin. The high extent of homology of the β2-AR 3′-UTR sequences (10, 13) between different mammalian species suggests that translational control by 3′-UTR sequences is an integral part of the post-transcriptional regulation of β2-AR expression. This study also identified high affinity binding of TIAR to the distal 340-nt region of receptor mRNA that is obligate for the translational suppression of β2-AR mRNA (7). The significant shift in β2-AR mRNA from polysomes to monosomes in sucrose density gradient fractions of A431 cells overexpressing TIAR suggests that TIAR, by binding to the receptor 3′-UTR RNA, enhances receptor mRNA association with monosomes.

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Translational Control of β2-Adrenergic Receptor mRNA by T-cell-restricted Intracellular Antigen-related Protein
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