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# The 3′-Untranslated Region of the $\alpha_{2C}$-Adrenergic Receptor mRNA Impedes Translation of the Receptor Message*

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**Qing Yang‡, Paul J. McDermott§, Emir Duzic¶, Cornelius W. A. Pleij‖, John D. Sherlock, and Stephen M. Lanier****

*From the Department of Pharmacology, §Division of Cardiology, Department of Medicine, Medical University of South Carolina, Charleston, South Carolina 29425 and ‖Leiden Institute of Chemistry, Leiden University, 2300 RA Leiden, The Netherlands*

**We report that two subtypes of $\alpha_2$-adrenergic receptors ($\alpha_{2A/D}$- and $\alpha_{2C}$-AR) are ectopically expressed with dramatically different efficiencies and that this difference is due to a 288-nucleotide (nt) segment in the 3′-untranslated region (3′-UTR) of the $\alpha_{2C}$-AR mRNA that impairs translational processing. NIH-3T3 fibroblasts were transfected with receptor constructs (coding region plus 552 nt, $\alpha_{2C}$-AR; coding region plus 1140 nt, $\alpha_{2A/D}$-AR) and a vector conferring G418 resistance. Transcription was driven by the murine sarcoma virus promoter element, and the receptor gene segment was upstream of an SV40 polyadenylation cassette. Drug-resistant transfectants were evaluated for expression of receptor mRNA and protein. 90% of the NIH-3T3 $\alpha_{2C}$-AR transfectants expressed receptor mRNA, but only 14% of the clonal cell lines expressed receptor protein. In contrast, 90% of the NIH-3T3 $\alpha_{2A/D}$-AR transfectants expressed receptor protein (200–5000 fmol/mg). Similar results were obtained following transfection of $DDT_1MF$-2 cells with the two receptor constructs. The role of the 3′-UTR of the $\alpha_{2C}$-AR in mRNA processing was determined by generating new constructs in which the 3′-UTR was progressively truncated from 552 to 470, 182, 143, or 74 nt 3′ to the stop codon. Truncation of the 3′-UTR resulted in the expression of receptor protein in the G418-resistant transfectants (nt 74, 100%; nt 143, 80%; nt 182, 50%). The level of mRNA in the transfectants expressing the receptor protein was not greater than that in nonexpressing clones, and the differences in protein expression did not reflect altered mRNA stability in the truncated construct. The $\alpha_{2C}$-AR mRNA with the longer 3′-UTR underwent translational initiation as it was found in the polysome fraction, indicating that the lack of receptor protein was due to impaired translational elongation or termination. These data suggest that translational efficiency is a key mechanism for regulating $\alpha_{2C}$-AR expression and associated signaling events.**

The response of the cell to hormones/neurotransmitters is an integrated process that involves varying numbers of molecules. Several factors interact to engineer a specific cell response to a particular hormone. The cell-specific and developmentally regulated expression of entities involved in the signaling process is a key component in this process, allowing different cells to respond to the same hormone but with dramatically different results depending on the receptor subtype expressed and/or the cell phenotype. To maintain signaling specificity and diversity in higher organisms, the system has evolved such that the components of the signaling pathway are expressed as isoforms or closely related molecules subserving similar but distinct functions. The preceding thought is particularly evident for cell-signaling events initiated through heptahelical membrane receptors coupled to heterotrimeric guanine nucleotide-binding proteins. For example, the adrenergic signaling system includes two agonists (norepinephrine and epinephrine) that interact to varying degrees with nine different receptors. Signaling by this system is tightly regulated by mechanisms involving the expression and turnover of members of the adrenergic receptor family. Regulatory mechanisms influence receptor gene transcription, receptor mRNA stability, receptor trafficking, and posttranslational events such as receptor phosphorylation.

The $\alpha_2$ subfamily of adrenergic receptors consists of three distinct proteins that differ in their ligand recognition properties, tissue distribution, signaling efficiency, and regulation (1, 2). Heterologous expression of the three $\alpha_2$-AR[1] subtypes in various cells indicates that the three subtypes also exhibit different trafficking patterns within the cell (3, 4) and are selectively phosphorylated by receptor kinases (5). The $\alpha_{2A/D}$-AR subtype is widely distributed in both peripheral tissues and within the central nervous system, whereas in the rat the $\alpha_{2B}$-AR is found primarily in the kidney, liver, and neonatal lung. The rat $\alpha_{2C}$-AR is primarily expressed in the central nervous system, and recently we identified cis elements in the 5′ upstream region of the rat $\alpha_{2C}$-AR important for cell type-specific transcription of the receptor gene (6). In contrast to the $\alpha_{2A/D}$-AR, there is an apparent dissociation between $\alpha_{2C}$-AR protein expression and receptor mRNA observed in discrete areas of the central nervous system and the NG108-15 neuroblastoma × glioma cell hybrid (7–10), suggesting that translation of the $\alpha_{2C}$-AR mRNA is a regulated event. To address this possibility, we compared the relationship between mRNA and protein expression following ectopic expression of the $\alpha_{2C}$-AR and $\alpha_{2A/D}$-AR in two cell lines. We report that the 3′-untranslated region of the $\alpha_{2C}$-AR impedes translational processing of the receptor mRNA.

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‡ Visiting scientist from the Institute of Cardiovascular Basic Research, Beijing Medical University, Beijing, People's Republic of China.

¶ Present address: Cadus Pharmaceuticals, Tarrytown, NY 10591.

** To whom correspondence should be addressed: Dept. of Pharmacology, Medical University of South Carolina, 171 Ashley Ave., Charleston, SC 29425. Tel.: 803-792-2574; Fax: 803-792-2475; E-mail: laniersm@musc.edu.

[1] The abbreviations used are: AR, adrenergic receptor; 3′-UTR, 3′-untranslated region; nt, nucleotide(s); MSV, murine sarcoma virus.

## EXPERIMENTAL PROCEDURES

*Materials*—[$^3$H]RX821002 (52 Ci/mmol) and [$^3$H]rauwolscine (87 Ci/mmol) were purchased from Amersham Corp. [$^{32}$P]dCTP (3000 Ci/mmol) and $^{35}$S-dATP (1320 Ci/mmol) were purchased from DuPont NEN. The multiprime DNA labeling system was obtained from Amersham Corp. Sequenase version 2.0 DNA sequencing kit was from U. S. Biochemical Corp. Restriction enzymes and DNA sizing markers were obtained from New England Biolabs Inc. (Beverly, MA). Tissue culture supplies were obtained from JRH Biosciences (Lenexa, KS). Rauwolscine was obtained from Atomergic Chemetals Corp. (Farmingdale, NY). RNA isolation kits were obtained from Stratagene (La Jolla, CA).

*Cell Culture, Membrane Preparations, and Radioligand Binding*—NIH-3T3 fibroblasts were maintained in a monolayer culture in Dulbecco's modified Eagle's medium at 37 °C under 95% atmosphere and 5% $CO_2$ supplemented with 10% bovine calf serum and containing penicillin (100 units/ml), streptomycin (100 $\mu$g/ml), and Fungizone (0.25 $\mu$g/ml). Cell membranes were prepared, and radioligand binding assays were performed as described previously (11).

*Generation of Receptor Expression Constructs and Cell Transfection*—The $\alpha_{2C}$-AR (*RG10*) gene (1929 nt) or $\alpha_{2A/D}$-AR (*RG20*) gene fragments (2,493 nt) were inserted into the expression vector 3′ to the MSV long terminal repeat and upstream of the SV40 polyadenylation signal as described previously (12). These gene segments began at the translational start AUG within the context of a Kozak consensus sequence for translational initiation and contained varying lengths of sequence 3′ to the translational stop codon. To generate $\alpha_{2C}$-AR constructs with a truncated 3′-UTR, the 1,929-nt gene segment was subcloned into the *Eco*RI-*Not*I restriction sites of pSK. pSK.$\alpha_{2C}$-AR was linearized at the 3′ end of the gene fragment and digested with exonuclease III using the Erase-A-Base system (Promega, Madison, WI) to generate clones containing 74-, 143-, 182-, and 470-nt sequences 3′ to the translational termination codon. The $\alpha_{2C}$-AR constructs were restricted with *Eco*RI-*Not*I, blunt-ended, and modified with *Hin*dIII linkers for ligation into the expression vector pMSV. We also generated constructs in a separate vector that contained both the MSV long terminal repeat and the neomycin drug resistance cassette. The p$\alpha_{2A/D}$-AR/$\alpha_{2C}$-AR-3′-UTR construct was generated by a three-component ligation using (I) pGEM7 containing the $\alpha_{2A/D}$-AR (nt 1–782 of the protein coding region), (II) a fragment of $\alpha_{2A/D}$-AR (nt 782–1353 of the protein coding region plus 64 nt 3′ to the translational stop codon), and (III) the $\alpha_{2C}$-AR 3′-UTR from nt 113 to 552 following the translational stop codon. pGEM7.$\alpha_{2A/D}$-AR (receptor gene inserted 5′ at *Eco*RI and 3′ at *Bam*HI in the polylinker) was digested with *Kpn*I and *Hin*dIII to remove the last 568 nt of the coding region and the 3′-UTR to generate component I. To generate component II, pGEM7.$\alpha_{2A/D}$-AR was digested with *Acc*I (restriction sites at nt 644 and at nt 64 3′ to the translational stop codon), and the 708-nt fragment was purified, blunt-ended, and digested with *Kpn*I, yielding component II. To generate component III, we took advantage of an *Rsa*I restriction site at nt 113, 3′ to the translational stop codon. pGEM7.$\alpha_{2C}$-AR was restricted with *Rsa*I, and the 2237-nt fragment containing the 3′-UTR (nt 113–552) and a portion of the plasmid was purified and restricted with *Hin*dIII, yielding component III. The three purified components were then ligated to generate the $\alpha_{2A/D}$-AR/$\alpha_{2C}$-AR-3′-UTR construct in pGEM7, and its sequence was confirmed by restriction mapping and DNA sequencing. The $\alpha_{2A/D}$-AR/$\alpha_{2C}$-AR-3′-UTR insert was then inserted into the *Hin*dIII cloning site of the pMSV.neo vector as described above. Segments of the various constructs were sequenced by the dideoxy chain termination method.

NIH-3T3 fibroblasts were transfected with a calcium phosphate precipitate containing 16 $\mu$g of expression vector and 4 $\mu$g of a plasmid encoding neomycin resistance or with 20 $\mu$g of plasmid when the vector containing both the receptor construct and the neomycin resistance cassette was used. Transfected cells were selected for their resistance to the antibiotic G418 (0.5 mg/ml). Selection was begun 3 days after transfection and continued for 3–4 weeks. G418-resistant clones were screened for expression of the receptor subtypes by RNA blot analysis and by their ability to bind the $\alpha_2$-selective antagonists [$^3$H]rauwolscine or [$^3$H]RX821002. The expected sizes of receptor mRNA were calculated from the expression vector construct and served as indicators of appropriate gene insertion. Selected transfectants were characterized by determining the ligand recognition properties of the receptor subtype proteins and their apparent molecular weight as described previously (13, 14).

*Preparation of RNA and RNA Blot Analysis*—Total cellular RNA was isolated as described previously (15). For preparation of cytoplasmic RNA, NIH-3T3 cells were washed with ice-cold phosphate-buffered saline (137 mM NaCl, 2.6 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$) and harvested by gentle scraping of the plate. Cells were pelleted and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl, and 0.5% Nonidet P-40). The homogenate was centrifuged at 10,000 × *g*, and the supernatant was used to prepare cytoplasmic RNA. Cytoplasmic and total cellular RNA was isolated using the Stratagene RNA isolation kit according to the manufacturer's instructions. Isolated RNA was subjected to electrophoresis on 1% agarose, 3% formaldehyde gels followed by transfer to a nylon filter (Hybond-N) by pressure blotting. The filter was then baked for 2 h at 80 °C in a vacuum oven and prehybridized in phosphate buffer containing 0.5 M $Na_2HPO_4$, pH 7.2, 1% bovine serum albumin, 7% SDS, 1 mM EDTA at 65 °C for 1 h before the addition of probe as described previously (6, 15). Radiolabeled probes were generated by random priming using the receptor gene segment as a template. The stability of receptor mRNA was determined by harvesting cells at different times after the blockade of transcription with actinomycin D (5 $\mu$g/ml) (15). RNA blots were then hybridized with the appropriate probe as described above. mRNA degradation rate was calculated after densitometric scanning of the autoradiographs.

*Analysis of Ribosomal Distribution of Receptor mRNA*—Polysomes were isolated as described previously (16, 17). Monolayer cultures of NIH-3T3 transfectants were washed with Hanks' balanced salt solution at 4 °C (5.4 mM KCl, 0.3 mM $Na_2HPO_4$, 0.4 mM $KH_2PO_4$, 4.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM $MgSO_4$, 137 mM NaCl, 5.6 mM D-glucose, 0.02% phenol red) containing 0.01% cycloheximide and harvested by gentle scraping of the plate. Cells were pelleted and then resuspended in 1 ml of lysis buffer (16 mM Tris-HCl, pH 7.5, 250 mM KCl, 10 mM $MgCl_2$, 0.5% Triton X-100, 2 mM dithiothreitol, 0.1 mg/ml cycloheximide, and 2 $\mu$l/ml RNasin. 150 $\mu$l of 10% Tween 80, 5% deoxycholate was added to the homogenate, the intact nuclei and mitochondria were removed by centrifugation, and the supernatant was loaded onto 15–50% linear sucrose gradients. The gradients were spun at 4 °C at 35,000 rpm in an SW 41 rotor for 2 h and then displaced upward through a modified 0.5-cm flow cell in an ISCO fractionator set to continuously monitor absorbance at 254 nm. Each fraction was extracted with phenol:chloroform, and the RNA was precipitated. The RNA pellet was dissolved in water and denatured by 50% formamide, 6% formaldehyde, 1 × SSC (150 mM NaCl, 15 mM $NaC_6H_5N_3O_7$) at 68 °C for 15 min, and ~5 $\mu$g of RNA was blotted directly onto a nylon membrane in a slot-blot apparatus. The blot was hybridized as described above. Following the removal of bound receptor subtype probe, the blot was hybridized with a nick-translated probe derived from rat 28 S RNA to provide controls for sample loading.

*Secondary Structure of RNA*—The secondary structure of the $\alpha_{2C}$-AR 3′-UTR was determined by the use of a genetic algorithm, which is able to simulate RNA folding pathways. The essential features of the algorithm involve mutations and crossovers in the population of solutions, with subsequent processing of the fittest solutions to generate new solutions as described previously (18, 19). At every algorithm iteration, the population of structures was expanded via the mutation/crossover process and subsequently diminished to that of the original population by fitness criteria. A particular analysis was considered completed when the free energy was not improved after a chosen number of repetitions. The program MFOLD in the University of Wisconsin GCG sequence analysis package was used for energy-minimum calculations. The analysis was achieved using the APL programming language in the program STAR.

## RESULTS

*Receptor Expression in NIH-3T3 Fibroblasts and DDT$_1$MF-2 Cells*—A stable transfection system was used to evaluate the role of the 3′-UTR in regulating expression of the $\alpha_{2C}$-AR. A fragment of the rat $\alpha_{2C}$-AR gene consisting of the 1374-nt coding region and a 552-nt segment of the gene sequence 3′ to the translational stop codon was inserted into an expression vector downstream of the pMSV promoter and upstream of an SV40 polyadenylation cassette (11) (Fig. 1*A*). The $\alpha_{2C}$-AR gene construct was introduced into NIH-3T3 fibroblasts by calcium phosphate coprecipitation, and G418-resistant clones were evaluated for receptor expression by radioligand binding assays and RNA blot analysis. Only ~14% of the drug-resistant clones expressed $\alpha_{2C}$-AR protein, whereas 90% of the clones expressed receptor mRNA (Fig. 1, *B* and *C*). Similar results were obtained in three different transfections in which a total of >100 individual clones were screened for receptor expres-

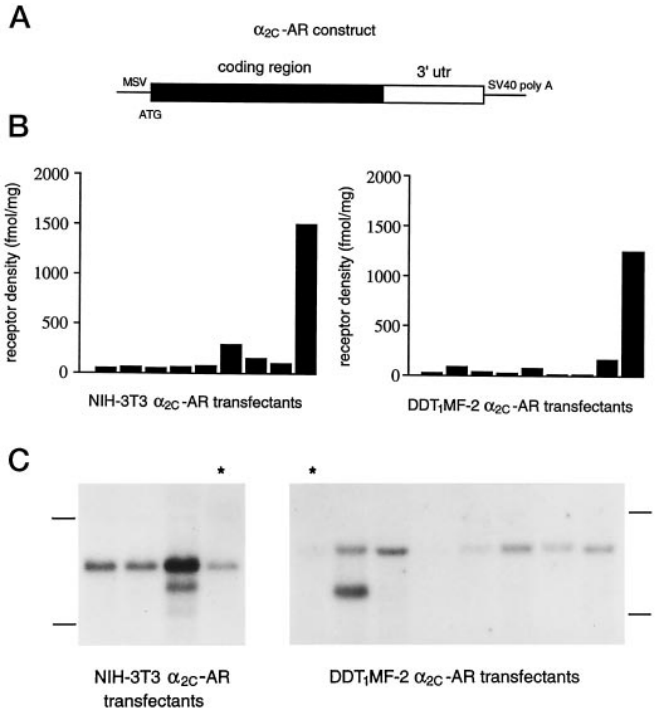


FIG. 1. **Expression construct and transfection efficiency for the $\alpha_{2C}$-AR in NIH-3T3 fibroblasts and DDT$_1$MF-2 cells.** NIH-3T3 fibroblasts and DDT$_1$MF-2 cells were stably transfected with the receptor subtype gene fragment (*A*) as described under "Experimental Procedures." G418-resistant clonal transfectants were screened for receptor subtype expression by radioligand binding assays using the $\alpha_2$-AR-selective ligand [$^3$H]RX821002 at saturating concentrations (~20 nM), and the results are expressed as fmol of receptor/mg of membrane protein (*B*). $\alpha_{2C}$-AR transfectants were further evaluated for gene expression by RNA blot analysis (*C*). Total RNA was prepared from selected transfectants and processed as described under "Experimental Procedures." The RNA blot was hybridized with a random-primed probe generated from the gene insert contained in the expression vector. The *asterisks above* the *lanes* in *C* indicate that the RNA was prepared from a transfectant expressing $\alpha_{2C}$-AR protein as determined in radioligand binding assays. The *lines* to the *right* of each RNA blot indicate the migration of 28 S and 18 S rRNA. The results are representative of the data obtained from similar studies in which 100 individual clones were screened for receptor expression in each cell type.

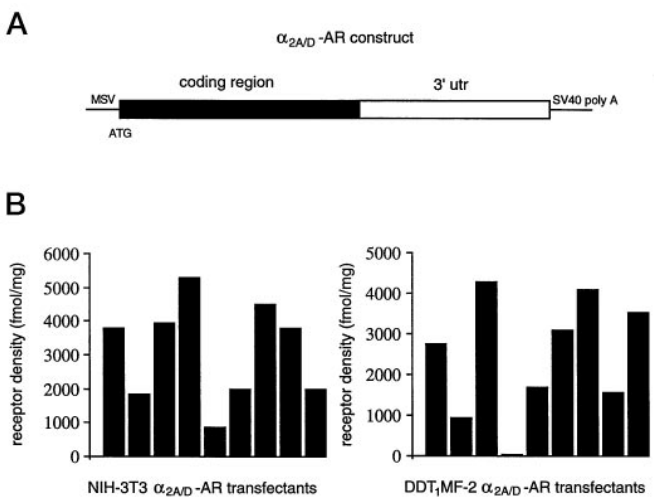


FIG. 2. **Expression construct and transfection efficiency for the $\alpha_{2A/D}$-AR in NIH-3T3 fibroblasts and DDT$_1$MF-2 cells.** Cells were stably transfected with the $\alpha_{2A/D}$-AR as described under "Experimental Procedures." G418-resistant clonal transfectants were screened for receptor subtype expression by radioligand binding assays using the $\alpha_2$-AR-selective ligand [$^3$H]RX821002 (~20 nM, 5–25 $\mu$g of membrane protein/tube). Data in *B* are representative of three separate transfections involving the analysis of a total of 20–30 individual clones for each cell type.

sion, and results from a subset of such clones are shown in Fig. 1. In the few clones that expressed receptor protein, the level of $\alpha_{2C}$-AR mRNA was not greater than that in clones lacking receptor protein (Fig. 1*C*). Similar results were obtained when NIH-3T3 fibroblasts were transfected with a receptor construct in which the drug resistance cassette was inserted into the receptor expression vector.[2] The dissociation between $\alpha_{2C}$-AR protein and mRNA was also observed following stable transfection of DDT$_1$MF-2 cells derived from hamster smooth muscle, indicating that the failure to process the receptor mRNA is not restricted to a fibroblast cell line (Fig. 1, *B* and *C*).

The dissociation between mRNA and expressed protein for the $\alpha_{2C}$-AR was not observed in similar experiments using constructs encoding the $\alpha_{2A/D}$-AR subtype. A fragment of the $\alpha_{2A/D}$-AR gene consisting of the 1350-nt coding region and a 1140-nt segment of the gene sequence 3′ to the translational stop codon was inserted into the pMSV expression vector and introduced into NIH-3T3 fibroblasts and DDT$_1$MF-2 cells as described above (Fig. 2*A*). Approximately 95% of the $\alpha_{2A/D}$-AR transfectants expressed receptor protein, suggesting that the $\alpha_{2C}$-AR and $\alpha_{2A/D}$-AR mRNAs are processed with different efficiencies by the two cell lines (Fig. 2*B*). The two receptor constructs contained identical 5′ upstream regions derived from the vector and exhibited 64% nucleotide sequence identity in the protein coding region. The two receptor constructs encoded proteins that exhibited 55% overall homology. A major difference between the two constructs was the gene segment 3′ to the translational stop codon, and subsequent studies focused on the influence of this region on $\alpha_{2C}$-AR expression.

*$\alpha_{2C}$-Adrenergic Receptor Expression Using Truncated Constructs*—Sequence analysis of the 3′-UTR of the $\alpha_{2C}$-AR identified a polyadenylation signal AAUAAA at nt 469 (Fig. 3). The sequence of the genomic clone in this region was identical to that of a rat $\alpha_{2C}$-AR cDNA (20). The $\alpha_{2C}$-AR 3′-UTR sequence from nt 1 to 221 is rich in GC nt (67%), whereas the GC content decreases to 40% from nt 222 to 481. To determine if the 3′-UTR of the $\alpha_{2C}$-AR influenced receptor expression, NIH-3T3 cells were transfected with $\alpha_{2C}$-AR gene constructs in which the 3′-UTR was progressively truncated to 470, 182, 143, and 74 nt 3′ to the translational stop codon (Fig. 4*A*). In contrast to the limited expression of the original receptor construct, ~50 (nt 182), 80 (nt 143), and 100% (nt 74) of the cells transfected with $\alpha_{2C}$-AR constructs in which the 3′-UTR was truncated expressed receptor protein (Fig. 4*B*). In terms of the number of clones expressing receptor protein, the p$\alpha_{2C}$-AR.3′-UTR-182 transfectants were intermediate relative to the p$\alpha_{2C}$-AR.3′-UTR-143 and the p$\alpha_{2C}$-AR.3′-UTR-470 transfectants. Photoaffinity labeling of the expressed $\alpha_{2C}$-AR with the $\alpha_2$-AR photoprobe $^{125}$I-AzRAU and radioligand binding studies indicated that the receptor exhibited the ligand recognition properties and $M_r$ expected of an $\alpha_{2C}$-AR (13, 14).[2] The expression of receptor in the 3′-truncated constructs but not in the p$\alpha_{2C}$-AR.3′-UTR-470 or the p$\alpha_{2C}$-AR.3′-UTR-552 was independent of the relative levels of receptor mRNA (Fig. 4*C*). Truncation of the 3′-UTR to nt 470 removed the polyadenylation signal in the receptor gene sequence, and p$\alpha_{2C}$-AR.3′-UTR-470 transfectants were similar to p$\alpha_{2C}$-AR.3′-UTR-552 transfectants in that ~90% of the clones expressed receptor message but not receptor protein (Fig. 4*B*). These data indicated that the presence of a polyadenylation site in addition to that in the expression vector did not account for the observed lack of mRNA processing and also suggest that there is no long range inter-

[2] J. D. Sherlock and S. M. Lanier, unpublished observations.

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5′ .....c cgtaggagga gaaggggctt caggcagtga

1   CCCTCTGGCT GCCTGGACTT GGCCCCACTG ACCTCCTGGA CAGCTCCGAA
51  CTCGGGTAGA TAGGGGGACC AACCAGTGTG GCTTCTCCAG AGTTCCAGGG
101 CGGACTTCAG GGTACAGTGT GGAATGTGGC CAGCAGGAAC TGGGAGAGAG
151 ACAACTGGGC CTCTGGGAGT GGGGAGGAGA AAGGGAGACC CTTTGCCTTC
201 CCATCTCAGC GAGGGGCTGT GGCTAGATCC AGTTCCTAAG AAGGCTTCTG
251 TGGAGTGTGG CTGTGAACTT AGTTAGGGTT TTAGAGCTCA GACAACTGCA
301 CCAGAAAGGA GACCCCCCCC CCCAACTCTC GGTCTTCCCT GTGAGCAAGG
351 GCTGACTTCC TATGACCTGA AAAAGGTATC TGTCTGGGGG AGGAGAGATA
401 GCACAGGCAA TCTCTGGTTA CTGAGAGTGT TTGCCAAATA CAATGACAGC
451 CAAACAAAAC CAAACTTTTT TTTTAAAATA AACCTTTGTA ATCTAAGTGT
501 TAGGTGCATT GGTCAGTCCT TAAGCCAGGG TGTGAGAGAA CTCTCCAGAT
551 CC  3′
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FIG. 3. **Nucleotide sequence of the $\alpha_{2C}$-AR gene 3′ to the protein coding region.** The *lowercase letters* correspond to the 3′ end of the protein coding region. The *underlined* nucleotides indicate a consensus signal for polyadenylation. The gene segment was sequenced in both sense and antisense directions using the automated DNA sequencing facility at the Medical University of South Carolina.

action of this region with the 5′ region of the transcript. These data indicated that a segment of the $\alpha_{2C}$-AR 3′-UTR from nt 183 to 470 (3′ to the translational stop codon) regulated the processing of the receptor transcript.

Computer simulation of RNA folding in the 3′-UTR generates a relatively stable secondary structure (Fig. 5). The most stable structural elements of the $\alpha_{2C}$-AR 3′-UTR are the hairpins from nt 13–90, 135–237, and the long branched hairpin between nt 260 and 450. The 3′-UTR also contains a motif (nt 469–476, UUUUUUAA) similar to sequences UUAUUUAU associated with message instability. Relative to the results of receptor expression in Fig. 4, the hairpin from nt 13–90 is apparently not involved in the inhibition of translational processing of the $\alpha_{2C}$-AR mRNA, as receptor expression was observed with the p$\alpha_{2C}$-AR.3′-UTR-143 construct. As the most efficient processing of the receptor mRNA occurs with the p$\alpha_{2C}$-AR.3′-UTR-74 and the p$\alpha_{2C}$-AR.3′-UTR-143 constructs, the stable hairpin from nt 135–237 may contribute to the observed results. However, the p$\alpha_{2C}$-AR.3′-UTR-182 construct also expressed receptor protein in ~50% of the transfectants, suggesting that the large branched hairpin between nt 260–450 also played a role in the translational processing of the receptor message.

*Cellular Localization and Stability of $\alpha_{2C}$-AR Transcripts*—The processing of transcripts involves several steps including capping, polyadenylation, splicing, transport out of the nucleus, and movement through various populations of ribosomes in the cytoplasm. The role of the 3′-UTR in these events was addressed by determining the distribution of full-length and truncated mRNA species within the cell and the relative stability of the different $\alpha_{2C}$-AR transcripts. The apparently poor processing of the full-length *versus* truncated mRNA may reflect a failure of the full-length transcript to move out of the nucleus and associate with a translationally active population of ribosomes in the cytoplasm. This issue was addressed by comparing the relative amounts of $\alpha_{2C}$-AR mRNA in the cytosol in the transfectants that expressed the receptor protein with those that did not. Analysis of cytosolic *versus* total cellular $\alpha_{2C}$-AR mRNA indicated that a portion of the mRNA species generated from the p$\alpha_{2C}$-AR.3′-UTR-74 and p$\alpha_{2C}$-AR.3′-UTR-552 constructs were both found in the cytosol (Fig. 6). Thus, the failure of the p$\alpha_{2C}$-AR.3′-UTR-552 to be processed into receptor protein was not due to the lack of potential mRNA access to the

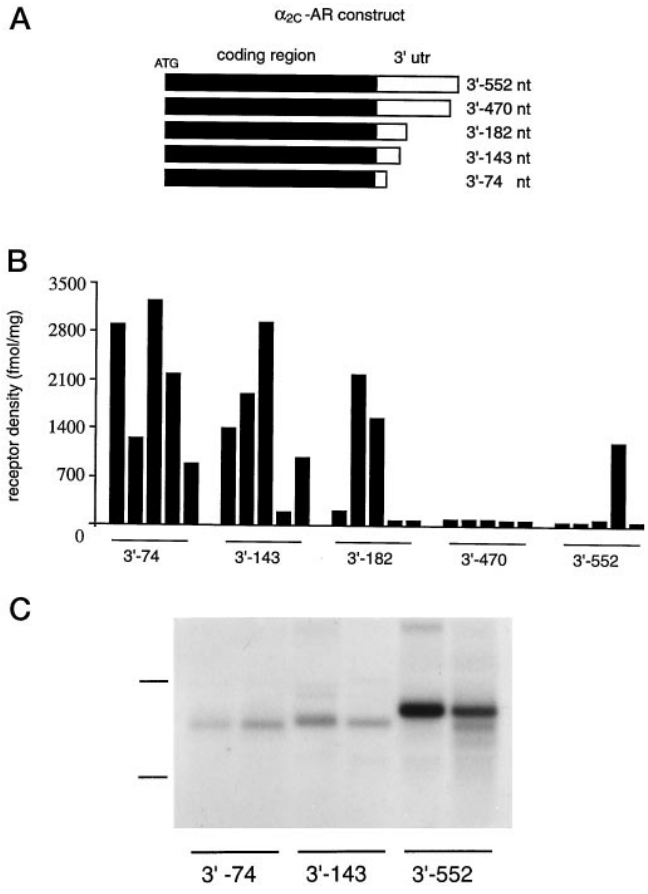


FIG. 4. **Expression of $\alpha_{2C}$-AR in NIH-3T3 fibroblasts following truncation of the 3′-UTR.** Expression constructs containing progressively shorter segments of the 3′-untranslated region of the receptor gene were generated by digestion with exonuclease III, and NIH-3T3 fibroblasts were stably transfected with the receptor subtype gene fragment (*A*) as described under "Experimental Procedures." Transfectants were screened for receptor protein by radioligand binding (*B*) and for gene transcription by RNA blot analysis (*C*) as described in the legend to Fig. 1. Analysis of an additional five clones isolated from the $\alpha_{2C}$-AR.3′-UTR-182 transfection indicated that 50% of the drug-resistant clones expressed receptor protein.

translational machinery. Fig. 6 also indicates that the lack of receptor protein in the p$\alpha_{2C}$-AR.3′-UTR-552 transfectants was not due to lower amounts of receptor mRNA relative to those observed in p$\alpha_{2C}$-AR.3′-UTR-74 transfectants.

Once in the cytosol, the p$\alpha_{2C}$-AR.3′-UTR-552 mRNA underwent translational initiation as indicated by the presence of the mRNA in the polysome complex of translationally active ribosomes (Fig. 7). These data suggest that the presence of the 3′-UTR segment in p$\alpha_{2C}$-AR.3′-UTR-552 impedes the movement of the ribosome along the mRNA and that removal of the 3′-UTR segment between nt 74 and 552 removes this constraint. The failure to complete the translational processing of the mRNA was not associated with any differences in the relative stabilities of the full-length and truncated mRNAs (Fig. 8). The stability of the $\alpha_{2C}$-AR mRNA species was determined following the transcription block with actinomycin D and compared with that of $\beta$-actin as an internal control for RNA loading. Analysis of the degradation rate of receptor mRNA in p$\alpha_{2C}$-AR.3′-UTR-74 and p$\alpha_{2C}$-AR.3′-UTR-552 transfectants revealed a similar $t_{1/2}$ (~6 h) for both species, indicating that the 3′-UTR segment from nt 74 to 552 did not influence mRNA stability (Fig. 8*C*). These data indicated that the $\alpha_{2C}$-AR 3′-UTR was interfering with translational processing of the $\alpha_{2C}$-AR mRNA. To determine if the 3′-UTR of the $\alpha_{2C}$-AR could regulate translation of a heterologous mRNA, we generated a

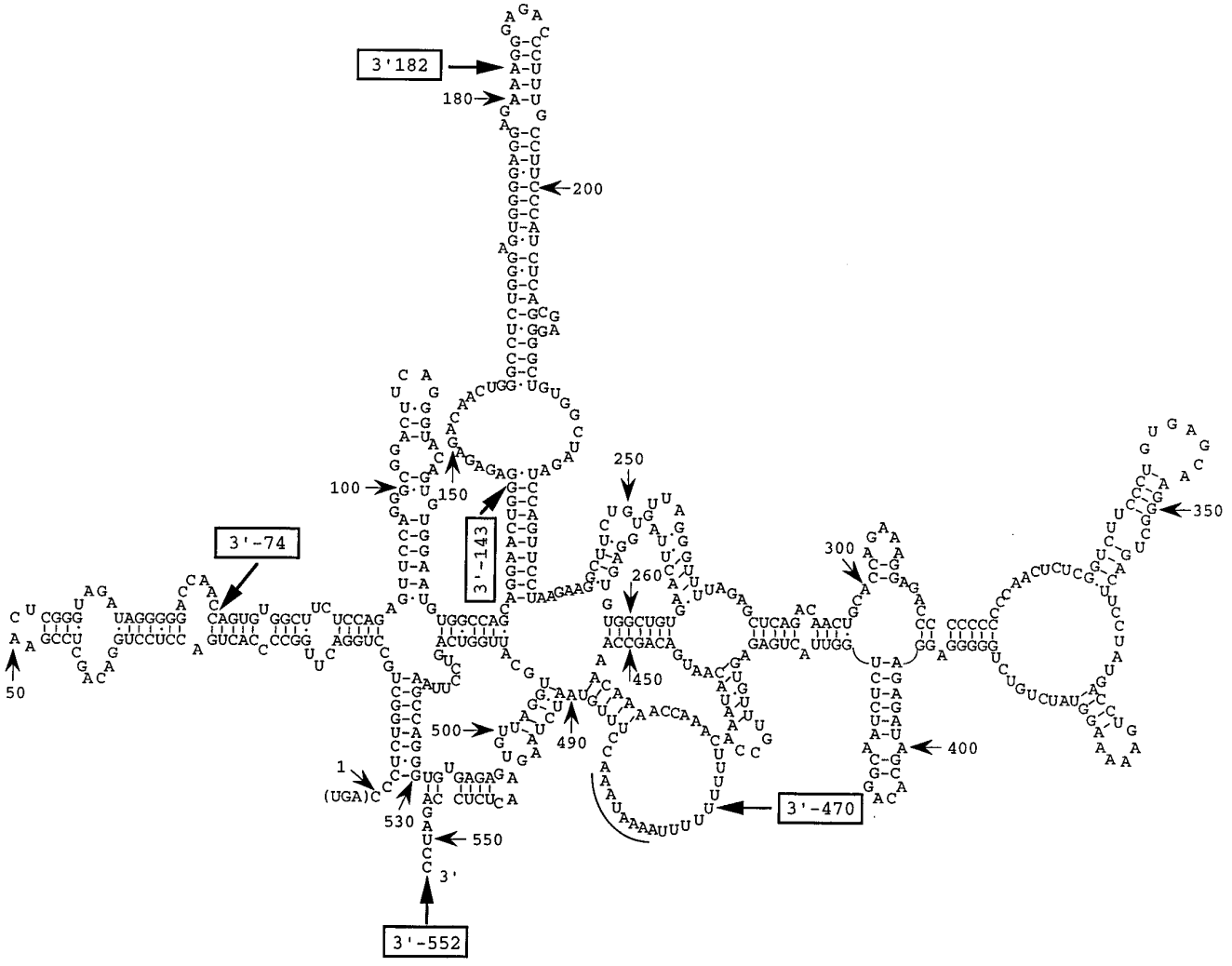


FIG. 5. **Predicted structure of the 3′-UTR of the $\alpha_{2C}$-AR mRNA.** mRNA folding pathways were simulated using a genetic algorithm to generate the displayed secondary structure as described under "Experimental Procedures." The sites of truncated constructs described in Fig. 4 are indicated by the *boxed numbers*. The polyadenylation signal is *underlined*.

construct in which the 3′-UTR of the $\alpha_{2C}$-AR was substituted for the 3′-UTR of the $\alpha_{2A/D}$-AR (Fig. 9). The segment of the $\alpha_{2C}$-AR 3′-UTR appended to the $\alpha_{2A/D}$-AR contained the portion that apparently impeded translation of the $\alpha_{2C}$-AR mRNA (Figs. 4 and 9*A*). NIH-3T3 cells were transfected with the $\alpha_{2A/D}$-AR/$\alpha_{2C}$-AR-3′-UTR construct, and receptor expression was compared with that obtained in parallel transfections with the wild-type $\alpha_{2A/D}$-AR construct indicated in Fig. 2. The p$\alpha_{2A/D}$-AR/$\alpha_{2C}$-AR-3′-UTR and p$\alpha_{2A/D}$-AR transfectants behaved similarly in terms of receptor expression (Fig. 9). All of the six clonal cell lines examined from each transfection expressed receptor protein as determined in radioligand binding assays (Fig. 9). RNA blot analysis indicated that similar amounts of $\alpha_{2A/D}$-AR/$\alpha_{2C}$-AR-3′-UTR and $\alpha_{2A/D}$-AR mRNA were expressed in the two series of transfections.[2]

## DISCUSSION

The processing of mRNA to the mature protein is subject to regulation at several steps including translational initiation, elongation, and termination (21–23). These events are influenced by several factors and often involve cis elements in the 5′- and 3′-untranslated regions of the mRNA that are recognized by specific RNA-binding proteins and participate in mRNA masking, message stabilization, and/or movement of messages among different ribosome populations within the cell (24–33). The regulation of protein expression at a translational level has evolved to play significant roles in various aspects of cell-signaling events. One of the first points of regulation in the translation process is translational initiation and the association of the mRNA with polysomes via the 43 S complex. This step is rate-limiting for the translation of most mRNA molecules, primarily due to stoichiometric issues concerning the factors required to form the translational initiation complex. One of the best understood examples of translational regulation involves the iron-responsive elements present in the 5′-UTR of ferritin mRNA and their influence on translational initiation (30). Translational initiation is also influenced by the 3′-UTR as indicated by the masking of maternal mRNAs. The masking of maternal mRNAs in *Xenopus* involves the binding of proteins to specific sequences in the 3′-UTR of the mRNA, resulting in a translational block. At appropriate stages of development, the mRNA species are unmasked with subsequent expression of the protein. These and other observations related to posttranscriptional editing of the poly(A) tail and its influence on translation indicate that there is a possibility of physical interplay between the 5′- and 3′-untranslated regions of mRNAs. Such an interaction of these two domains may be an important component of translational regulation. The 3′-UTR is also an important determinant of stability for several mRNA species (*e.g.* β-tubulin, $\beta_2$-AR) and plays a key role in the segregation of specific mRNAs within the cell (21, 31, 32, 34).

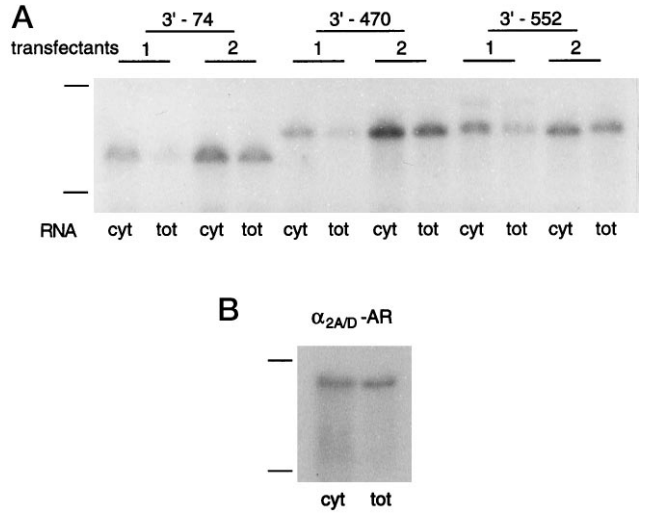


FIG. 6. **Cellular distribution of $\alpha_{2C}$-AR mRNA in $\alpha_{2C}$-AR.3′-UTR-74, $\alpha_{2C}$-AR.3′-UTR-470, and $\alpha_{2C}$-AR.3′-UTR-552 NIH-3T3 transfectants.** Total RNA (*tot*) or cytosolic RNA (*cyt*) was isolated from NIH-3T3 transfectants as described under "Experimental Procedures." Cells were transfected with $\alpha_{2C}$-AR constructs containing various segments of the 3′-UTR (*A*) or the $\alpha_{2A/D}$-AR expression construct (*B*) illustrated in Figs. 1, 2, and 4. Blots were hybridized with random-primed radiolabeled probes generated using the coding region of the two receptor subtypes. The blot is representative of two experiments using different RNA preparations.

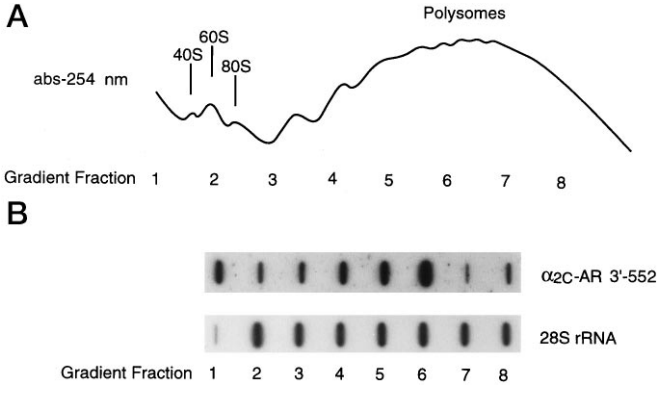


FIG. 7. **Distribution of $\alpha_{2C}$-AR.3′-UTR-552 mRNA in polysome preparations.** *A*, cells were lysed, and polysomes were isolated by sucrose density gradient centrifugation as described under "Experimental Procedures." Fractions that eluted from the gradient were monitored by optical density, and aliquots were blotted onto nylon membranes (*B*) and screened for the distribution of receptor mRNA. The probe was then stripped from the blot, and the blot was rescreened with the 28 S rRNA probe to verify RNA loading. This experiment was repeated twice with similar results.

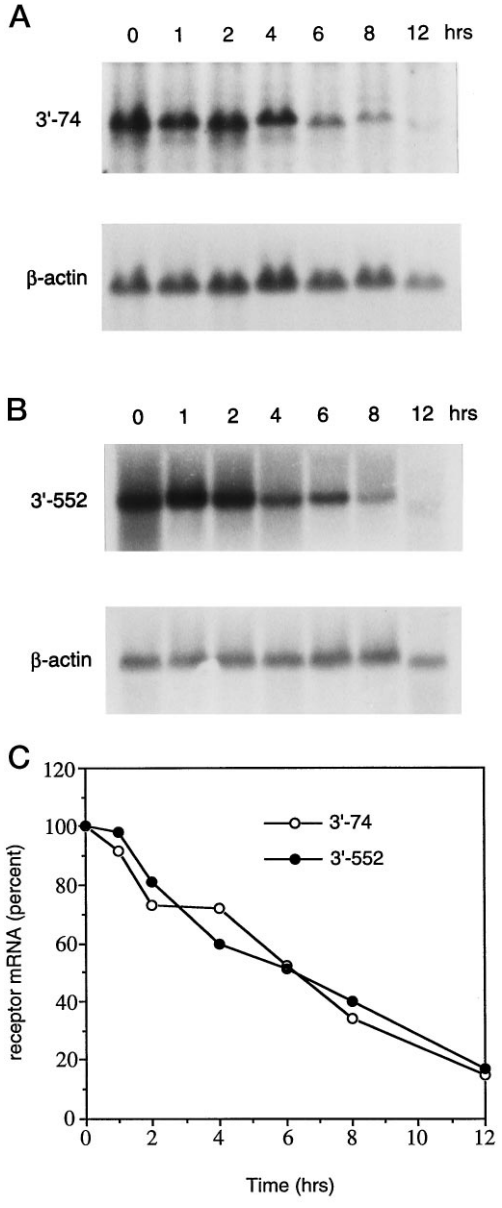


FIG. 8. **Stability of $\alpha_{2C}$-AR mRNA in $\alpha_{2C}$-AR.3′-UTR-74 and $\alpha_{2C}$-AR.3′-UTR-552 NIH-3T3 transfectants.** Total RNA was prepared from the $\alpha_{2C}$-AR.3′-UTR-74 and $\alpha_{2C}$-AR.3′-UTR-552 NIH-3T3 transfectants before and at 2, 4, 6, 8, and 12 h after the addition of actinomycin D as described under "Experimental Procedures." RNA samples were processed, and nylon blots were hybridized with random-primed radiolabeled probes derived from the coding region of the $\alpha_{2C}$-AR gene or $\beta$-actin cDNA (*A* and *B*). The autoradiographs were scanned for signal intensity, and the signal generated by the $\alpha_{2C}$-AR mRNA species was normalized to the $\beta$-actin signal to provide an internal control for RNA loading (*C*). Data in *C* are plotted as a percent of the signal at zero time. The results are representative of three similar experiments using different RNA preparations.

A second point of regulation occurs as the translational machinery searches for the start codon positioned in the most favorable context for initiation of protein synthesis. Once translation is initiated, elongation proceeds at varying rates for different messages, eventually terminating at the stop codon through the action of the release factor and the subsequent dissociation of the peptide from the ribosome. The elongation process is engineered through the action of elongation factors, and it is fairly rapid, incorporating 4–6 amino acids per s. The rate of the elongation process is also potentially subject to regulation, although the mechanisms involved in such regulation are poorly understood. A decrease in elongation rate (*i.e.* translational stalling) may result in an increased amount of mRNA associated with the polysome fraction in the cytosol, as it is not efficiently processed through the translation process. In contrast, mRNAs that are elongated at normal rates would spend less time in the polysome complex. Thus, there are several points during translation at which the processing of a particular mRNA can be specifically regulated. Depending upon the type of translational regulation, a situation could exist where there is detectable mRNA but the corresponding protein is absent. Such is the situation for the $\alpha_{2C}$-AR mRNA.

The translational processing of $\alpha_{2C}$-AR mRNA appears to be a regulated event, and this regulation involves a 278-nt segment in the 3′-UTR of the $\alpha_{2C}$-AR. The importance of this segment in the processing of the $\alpha_{2C}$-AR mRNA is indicated by the expression of the protein following removal of this domain. As the protein coding region is identical in the truncated construct, it is not possible to explain the observed data based on

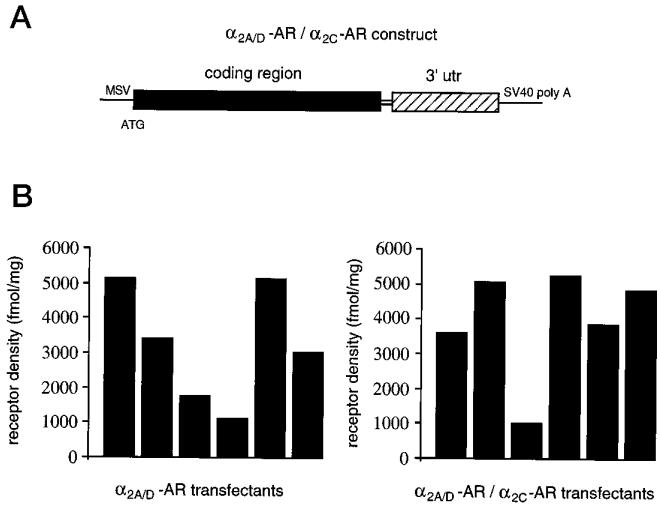


FIG. 9. **Influence of the $\alpha_{2C}$-AR 3′-UTR on the transfection efficiency of the $\alpha_{2A/D}$-AR.** The $\alpha_{2A/D}$-AR/$\alpha_{2C}$-AR-3′-UTR construct in *A* was generated as described under "Experimental Procedures" and consists of 1350 nt of the $\alpha_{2A/D}$-AR coding region, 67 nt of the $\alpha_{2A/D}$-AR 3′-UTR, and 439 nt of the $\alpha_{2C}$-AR 3′-UTR from nt 113 to 552 following the translational stop codon. NIH-3T3 fibroblasts were transfected with pMSV.neo.$\alpha_{2A/D}$-AR/$\alpha_{2C}$-AR-3′-UTR113–552 (*B*, *right panel*) or pMSV.neo.$\alpha_{2A/D}$-AR (*B*, *left panel*) (see construct in Fig. 2*A*). Six G418-resistant clonal transfectants were propagated and evaluated for receptor mRNA and protein expression. Although not shown in this figure, both series of transfectants expressed similar levels of receptor mRNA. Receptor protein expression was determined in radioligand binding assays using the $\alpha_2$-AR selective ligand [$^3$H]RX821002 (~20 nM, 5–25 $\mu$g of membrane protein/tube). The results were generated from duplicate determinations following a single transfection.

differences in protein turnover. In terms of known mechanisms by which the 3′-UTR can influence mRNA processing (*i.e.* translational initiation and mRNA stabilization), the translation of $\alpha_{2C}$-AR mRNA is of particular interest. Neither message stability nor transport of the $\alpha_{2C}$-AR mRNA to the cytosol is influenced by the 3′-UTR. In addition, the p$\alpha_{2C}$-AR.3′-UTR-552 mRNA species was found in the polysome complex, indicating that it undergoes translational initiation. Thus the differences in the generation of the protein product must be due to a decreased rate of elongation of the initiated products and/or failure to properly terminate protein synthesis in the p$\alpha_{2C}$-AR.3′-UTR-552 transfectants. A similar mechanism is proposed to participate in the translational control of expression of HSP70 in chicken reticulocytes (35) and of $\alpha$-myosin in cardiac myocytes (36). The inability of the polysome complex to process the p$\alpha_{2C}$-AR.3′-UTR-552 mRNA species is likely dependent on the secondary structure generated by the 3′-UTR and/or RNA-binding proteins, both of which might impede elongation/termination. The inability of the $\alpha_{2C}$-AR 3′-UTR to influence mRNA processing in a heterologous fashion suggests that there are additional important interactions of the 3′-UTR with other domains of the $\alpha_{2C}$-AR mRNA.

Among the three $\alpha_2$-AR subtypes, the $\alpha_{2A/D}$-AR has the widest tissue distribution in the rat as determined by both analysis of mRNA and radioligand binding studies. $\alpha_{2A/D}$-AR mRNA is found in kidney, liver, pancreas, adipocytes, vascular smooth muscle cells, and RIN-5AH pancreatic beta cells. Each of these tissues also expresses the receptor protein. In peripheral tissues and within the central nervous system, the distribution of $\alpha_{2A/D}$-AR mRNA correlates with receptor expression as determined by *in situ* hybridization, immunoblotting, and radioligand binding (37–39). In contrast to the $\alpha_{2A/D}$-AR, the expression of the rat $\alpha_{2B}$-AR and $\alpha_{2C}$-AR is more restricted. $\alpha_{2C}$-AR mRNA and protein are primarily found in the central nervous system, although low levels are detected by *in situ* hybridization in the kidney (7, 38, 39). The distribution of $\alpha_{2C}$-AR and $\alpha_{2A/D}$-AR mRNA and immunoreactivity in the central nervous system is discussed by Rosin *et al.* (7) and Talley *et al.* (37). The distribution of $\alpha_{2C}$-AR mRNA within the central nervous system is not entirely consistent with the receptor distribution defined by immunohistochemistry. In contrast to the close relationship between mRNA and detectable protein for the $\alpha_{2A/D}$-AR, there are selected sites within the central nervous system (islands of Calleja, nucleus accumbens, superior and inferior colliculus, caudate putamen) in which there is a dissociation between $\alpha_{2C}$-AR mRNA and detectable protein as determined by either immunohistochemistry or radioligand binding. A dissociation between $\alpha_{2C}$-AR mRNA and detectable protein is also observed in the neuroblastoma × glioma cell line NG108-15. Although transcripts encoding the $\alpha_{2B}$-AR and $\alpha_{2C}$-AR are identified in NG108-15 cells (6–10), receptor purification and radioligand binding studies indicate expression of the $\alpha_{2B}$-AR but not the $\alpha_{2C}$-AR protein (10, 40). The presence of $\alpha_{2C}$-AR mRNA, but the absence of receptor protein, is exactly the situation observed in the present studies. Precise interpretation of receptor mRNA *versus* protein distribution in the rat central nervous system can be complicated by potential neuronal transport of mRNA and/or proteins. However, the dissociation between the $\alpha_{2C}$-AR mRNA and receptor protein contrasts with the close relationship between mRNA and protein for other membrane receptors in the central nervous system and suggests that translation may be an important point of regulation for the expression of the $\alpha_{2C}$-AR. The data presented in the present manuscript are consistent with this possibility and suggest that the 3′-UTR of the $\alpha_{2C}$-AR mRNA exerts a strong influence on translational processing of the receptor message.

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