Functional role for the angiotensin II receptor (AT_{1A}) 3′-untranslated region in determining cellular responses to agonist: evidence for recognition by RNA binding proteins

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We demonstrate a functional role for the 3′-untranslated region (3′-UTR) of the angiotensin II (Ang II) receptor subtype AT_{1A} mRNA in Chinese hamster ovary (CHO-K1) cells by stably transfecting the coding region of the receptor gene with or without the 845 bp 3′-UTR. Two cell lines expressing similar levels of cell-surface receptors (with 3′-UTR, $B_{max} = 571$ fmol/mg protein; without 3′-UTR, $B_{max} = 663$ fmol/mg protein) were used in the present study. Both cell lines expressed high-affinity receptors (with 3′-UTR, $K_d = 0.83$ nM; without 3′-UTR, $K_d = 0.82$ nM), and binding studies with ^{125}I-labelled Ang II in the presence of GTP[S] demonstrated that both coupled to heterotrimeric G-proteins. Despite these similarities, significant differences were observed for receptor-mediated cell signalling pathways. In cells without the 3′-UTR, Ang II stimulated an increase in cAMP accumulation (11-fold above control) and in cells with the 3′-UTR no stimulation was observed, which was consistent with previous observations in most endogenous Ang II receptor (AT_1)-expressing cells. Activation of cAMP by Ang II in cells without the 3′-UTR correlated with an inhibition of DNA synthesis, determined by [^{3}H]thymidine incorporation. Ang II-mediated responses were blocked by EXP3174, a selective non-peptide receptor antagonist. We also observed differences in the transient profiles of intracellular calcium between cells with and without the 3′-UTR in response to Ang II. In cells with the 3′-UTR, a sustained level of intracellular calcium was observed after Ang II stimulation, whereas cells without the 3′-UTR displayed a full return to basal level within 50 s of Ang II treatment. Even though the expressed exogenous gene is under the control of a constitutively expressing promoter (cytomegalovirus promoter), Northern-blot analysis revealed a considerably greater accumulation of AT_{1A} mRNA in cells without the 3′-UTR compared with cells with the 3′-UTR. Analysis of the decay rate of the AT_{1A} mRNA in cells with and without the 3′-UTR revealed that the normally unstable AT_{1A} receptor mRNA became highly stable by removing its 3′-UTR, identifying a role for the 3′-UTR in mRNA destabilization. Interestingly, both cells express similar levels of receptors at the cell surface, suggesting that the 3′-UTR is also involved in the efficient translation and/or translocation of the receptor protein to the plasma membrane. We hypothesized that these 3′-UTR-mediated functions of the receptor are regulated by RNA-binding proteins. To identify possible RNA-binding proteins for the AT_{1A} 3′-UTR, cellular extracts were prepared from parental CHO-K1 cells and 3′-UTR-binding assays, electrophoretic mobility-shift assays and UV crosslinking studies were performed. A major cellular protein of 55 kDa was identified, which specifically interacted with the 3′-UTR. Our data suggest that the 3′-UTR of the AT_{1A} can control specific receptor functions, perhaps via selective recognition of the 3′-UTR by RNA-binding proteins.

INTRODUCTION

Messenger RNA consists of a 5′-untranslated region, a coding sequence which is translated into protein, a downstream 3′-untranslated sequence (3′-UTR) and a poly(A) tail. A role for the 3′-UTR in mRNA stability and degradation, particularly for early response and structural genes, is well established [1], and this mRNA stability appears to be dictated by sequence motifs within 3′-UTRs, that are recognized by specific RNA-binding proteins. The destabilization sequences have been mapped to AU-rich elements within the 3′-UTR of mRNAs that display rapid turnover [2,3].

Recent studies have established a broader role for the 3′-UTR, which extends beyond its capacity to influence mRNA stability and one of the important developments in this area has been the identification of a role for the 3′-UTR in cell growth and differentiation [4,5]. Genetic complementation studies in differentiation-defective myoblast mutant cells have revealed that the 3′-UTR of troponin I, tropomyosin and α-cardiac actin mRNA were able to inhibit cell proliferation and to promote cell differentiation [5]. Moreover, expression of these 3′-UTRs in non-myogenic 10T1/2 fibroblasts suppressed cellular proliferation, suggesting that the effects are not restricted to a particular phenotype. Another role recently attributed to the 3′-UTR is the intracellular localization of specific mRNAs [4,6,7]. For example, in the *Drosophila* embryo, the proteins oskar, bicoid and nano, which control the overall morphology of the adult fly, are regulated by their respective 3′-UTRs, which localize the mRNAs to specific regions of the developing embryo [6]. Similarly, the localization of the mRNAs for the structural genes α- and *β-actin* is also directed by their 3′-UTR [7]. Such localization requires the transport and retention of the mRNAs at specific locations

Abbreviations used: Ang II, angiotensin II; 3′-UTR, 3′ untranslated region; AT_{1A}, AT_{1B}, AT_{1C}, Ang II AT_1-receptor subtypes; +3′-UTR, AT_{1A} with 848 bp of its 3′-UTR; −3′-UTR, AT_{1A} with only 56 bp of its 3′-UTR; CHO-K1, Chinese hamster ovary cells; CMV, cytomegalovirus; EXP3174, a non-peptide agonist specific for AT_1; α-MEM, α-minimal essential medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GTP[S], guanosine (5′ → O^3)-1-thiotriphosphate; G418, geneticin; HBSS, Hanks balanced salt solution; CMV, cytomegalovirus.

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within the cell, presumably through recognition by specific nuclear and cytoplasmic RNA binding proteins [8]. Other studies have also shown that the 3′-UTR can influence the efficiency of protein translation independently of polyadenylation. For example, a specific mRNA binding protein of 48 kDa has been identified that binds to the 3′-UTR of the 15-lipoxygenase mRNA, to inhibit its translation, suggesting that the 3′-UTR regulates translation [9]. Thus, control of cell function by the 3′-UTR appears to occur at multiple levels and recent interest has focused on identifying sequences and motifs within the 3′-UTR, as well as in the binding proteins that recognize them.

Angiotensin II (Ang II) is the active component of the renin–angiotensin system which has a well established role in blood pressure regulation and fluid and salt homoeostasis [10]. Ang II also appears to participate in mediating numerous other actions, including fetal development, cell growth (normal and pathophysiological), neuromodulation and cytokine-like effects [11]. These actions of Ang II are mediated by two major types of Ang II receptors, AT_1 and AT_2, which are both putative members of the seven transmembrane G-protein coupled receptor superfamily [11,12]. Most Ang II effects reported to date are mediated by the activation of AT_1. Interestingly, cDNA cloning has identified multiple subtypes of AT_1 (e.g. AT_{1A}, AT_{1B}, AT_{1C}), however, the AT_{1A} receptor appears predominant with respect to tissue distribution and biological function [13–15]. The mRNA species for AT_{1A} is transcribed primarily as a 2.4 kb transcript of which 848 bp are the 3′-UTR. Although no function has been attributed to the 3′-UTR of Ang II receptors it is interesting to note that, despite highly similar coding regions for AT_1 subtypes, the 3′-UTRs show little sequence similarity [16], suggesting a possible function in cellular discrimination of these subtypes.

In this study, we determined the contribution of the 3′-UTR of the AT_{1A} receptor by stably expressing the receptor with and without its 3′-UTR in Chinese hamster ovary (CHO-K1) cells, and determining cellular responses to Ang II. We report that cells expressing similar levels of AT_{1A} receptors, with and without 3′-UTR, displayed similar affinities for Ang II and coupling to heterotrimeric G-proteins, but markedly different AT_{1A} mRNA content and rate of degradation, consistent with a role of the 3′-UTR in mRNA stability. Remarkably, these receptors differed both in their capacity to couple to intracellular signalling pathways (cAMP accumulation and calcium transients) and to cell growth in response to Ang II stimulation. Using an mRNA mobility-shift assay, we show that at least one cellular protein (55 kDa) specifically binds the 3′-UTR of the AT_{1A} receptor. Our data suggest that the 3′-UTR of the AT_{1A} receptor plays an important role in the cellular responses to Ang II, perhaps through identification of the 3′-UTR by specific RNA-binding proteins, in a manner analogous to that described for some early response and structural genes.

EXPERIMENTAL

Materials

EXP3174 was provided by DuPont–Merck Pharmaceutical Co. (Wilmington, DE, U.S.A.). α-Minimal essential medium (α-MEM), Geneticin (G418), fetal-calf serum (FCS), penicillin, streptomycin and trypsin–EDTA were from Life Technologies (Gaithersburg, MD, U.S.A.). ^{125}I-Labelled Ang II, [α-^{32}P]dCTP, [γ-^{32}P]ATP and [α-^{35}S]dATP were from Dupont–New England Nuclear (Boston, MA, U.S.A.). Restriction enzymes were from Promega Co. (Madison, WI, U.S.A.), Ang II and sequencing kits were from United States Biochemical Co. (Cleveland, OH, U.S.A.) and Taq polymerase was from Perkin-Elmer Co. (Norwalk, CT, U.S.A.). Electrophoresis reagents and all other chemicals were from Bio-Rad (Richmond, CA, U.S.A.) and Sigma Chemicals (St. Louis, MO, U.S.A.).

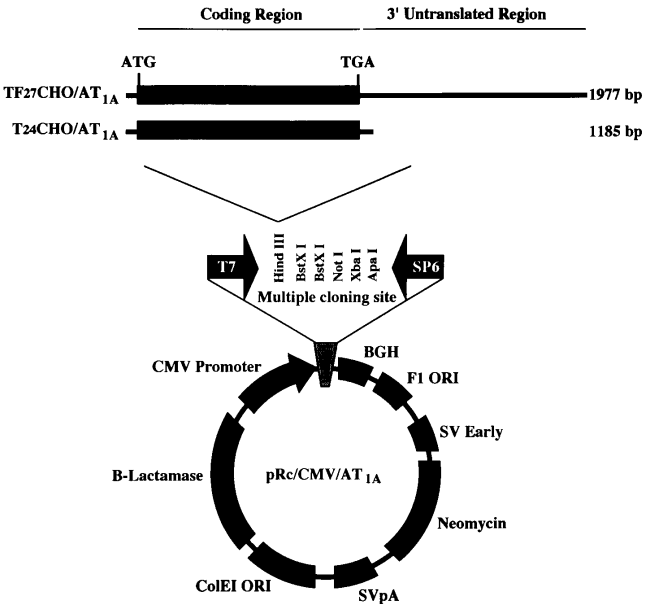

Figure 1 Expression vector pRc/CMV containing the Ang II (AT_{1A}) receptor with and without 3′-UTR, derived from the rat genomic clone λ12 [17]

The +3′-UTR AT_{1A} receptor with 848 bp of untranslated 3′ sequence and the −3′-UTR AT_{1A} receptor with 52 bp of untranslated 5′ sequence and only 56 bp of untranslated 3′ sequence were subcloned separately into the *Hin*dIII site of the expression vector. These constructs are under the constitutive control of a CMV promoter and enhancer and contain the neomycin resistance gene for selection. TF27CHO/AT_{1A} and T24CHO/AT_{1A} refer to CHO-K1 cells stably transfected with the +3′-UTR and −3′-UTR construct respectively.

Stable expression of the AT_{1A} receptor in CHO-K1 cells

The isolation of a genomic clone (λ12) for the rat AT_{1A} receptor has been described previously [17]. A cDNA fragment coding for the AT_{1A} receptor with 848 bp of its 3′-UTR (1977 bp; +3′-UTR) was amplified by PCR from the λ12 genomic clone, with primers (sense 5′-GTAAAGCTTAAGTGGATTTCG-3′ and antisense 5′-TACATTACAATAAAATTACTTTATTTAGAGG-3′) based on the published sequence [13], and subcloned into the *Hin*dIII site of pRc/cytomegalovirus (CMV), a eukaryotic expression vector (Figure 1). The pRc/CMV vector contains the human CMV promoter and enhancer for high-level constitutive expression and the neomycin-resistance gene for selection. The authenticity of the insert was confirmed by sequencing. PCR amplification of the AT_{1A} receptor with only 56 bp of its 3′-UTR (−3′-UTR) and subcloning into pRc/CMV has been described previously [17]. CHO-K1 cells were grown in α-MEM containing 10% (v/v) FCS, and 60% confluent cultures were transfected with pRc/CMV AT_{1A}, +3′-UTR or −3′-UTR by the polybrene method [18]. Individual colonies expressing transfected receptors were selected for the ability to grow in the presence of G418 (600 μg/ml) and isolated G418-resistant clones were screened for ^{125}I-labelled Ang II binding as described previously [17]. Numerous clones expressing variable levels of AT_{1A} receptor, +3′-UTR and −3′-UTR, were obtained. The experiments described in the present work were performed on two cell lines (TF27CHO/AT_{1A}, +3′-UTR and T24CHO/AT_{1A}, −3′-UTR), chosen because they possess similar levels of receptor expression.

Receptor-binding studies

Cells were plated in 35-mm diam. multiwell plates and radioligand binding studies were performed as described previously [17]. Competition binding studies were performed at 22 °C in the presence of 36 pM ^{125}I-labelled Ang II and increasing concentrations (1 pM–1 μM) of unlabelled Ang II, in a buffer containing 50 mM Tris/HCl (pH 7.5)/120 mM NaCl/5 mM $MgCl_2$ containing 10 μg/ml of bacitracin and 2 mg/ml of D-glucose. Data were analysed and non-linear regression curves were obtained using the computer software Prism (GraphPAD software, San Diego, CA, U.S.A.); K_d and B_{max} were calculated as described previously [19]. High-affinity agonist binding requires association of the receptor and the heterotrimeric G-protein with the α-subunit in the GDP-bound form [11,16]. The ability of plasma membrane AT_{1A} receptors to couple to G-proteins was determined using GTP[S], a non-hydrolysable GTP analogue which lowers the affinity of the receptor for Ang II. Plasma membranes were prepared from confluent cultures of transfected cells as described previously [17] and binding of ^{125}I-labelled Ang II (50 pM) was performed at 22 °C for 60 min using membranes (200 μg of protein/ml) and increasing concentrations (10 nM to 100 μM) of GTP[S].

RNA isolation and Northern-blot analysis

Total RNA from untransfected CHO-K1 cells, TF27CHO/AT_{1A} and T24CHO/AT_{1A} cells was isolated as described previously [20] and separated by 1.5% agarose-gel electrophoresis in the presence of 6.5% (v/v) formaldehyde [21]. RNA was transferred to a GeneScreen membrane (NEN Research Products, Boston, MA, U.S.A.), UV cross-linked and prehybridized at 60 °C for 2 h in 0.5 M sodium phosphate (pH 7.5)/1 mM EDTA/1% (w/v) bovine serum albumin/7% (w/v) SDS. A ^{32}P-labelled nick-translated probe, corresponding to a 550 bp fragment of the coding region of the AT_{1A} receptor, was used to probe the membrane overnight at 60 °C. The membrane was washed twice with 1 × SSC (0.15 M NaCl/0.015 mM sodium citrate)/0.1% (w/v) SDS at room temperature and twice at 60 °C. After two washes with 0.1 × SSC/0.1% (w/v) SDS at 65 °C, the blot was air dried and exposed to X-ray film for the indicated times and temperatures.

Determination of mRNA half-life

To determine the half-life of the +3′-UTR and −3′-UTR AT_{1A} mRNAs, cells were grown to 80% confluency in α-MEM containing 10% (v/v) FCS. To inhibit transcription, cells were immersed in fresh media containing 5 μg/ml actinomycin D and incubated at 37 °C. At the indicated times, cells were harvested and total cellular RNA was isolated from individual dishes as described previously [20]. Slot blotting on to nylon membranes was performed with 12 μg of RNA and this was immobilized by UV cross-linking. The cDNAs for the rat AT_{1A} receptor and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were radioactively labelled with [^{32}P]dCTP by the nick-translation method [21]. The membrane was hybridized with radioactively labelled AT_{1A} cDNA probe at 60 °C for at least 16 h. The membrane was washed twice at room temperature in 1 × SSC/0.1% (w/v) SDS and twice at 65 °C in 0.1 × SSC/0.1% (w/v) SDS. The membrane was autoradiographed with an intensifying screen at −70 °C. The AT_{1A} receptor probe was removed and the membrane was reprobed with radioactively labelled GAPDH, which served as an internal control. The AT_{1A} receptor mRNA levels were quantified by densitometry and standardized to GAPDH. The decay rate (mean ± S.E.M.) was calculated from three independent experiments.

Measurement of cAMP

Cells were plated in 6-well plates at 300000 cells/well. On the following day, cells were depleted of serum for 12 h and were washed twice with Hepes-buffered MEM and exposed to various compounds (Ang II, Ang II + Exp3174 or forskolin) for 7 min in the same medium, in the presence of the phosphodiesterase inhibitor Ro 20-1724 (100 μM). The medium was removed and the cells were treated with 0.6 ml of 0.1 M HCl at 4 °C for 10 min. The HCl solution was removed and cAMP was measured in the acid extract using an automated radioimmunoassay system as described previously [22]. Total protein was estimated by the Bradford method [23].

Calcium measurements

Intracellular calcium measurements were made using cell suspensions as described previously [17]. In brief, transfected cells were incubated at 37 °C for 1 h with 50 μg Fura-2/AM (penta-acetoxymethyl ester) in Hanks balanced salt solution (HBSS) containing 20 mM Hepes, 1mg/ml bovine serum albumin and 2.2 mg/ml glucose. After being washed with HBSS, cells were treated with trypsin and transferred to 4 ml HBSS. The cells were centrifuged and resuspended to a density of 10^6 cells/ml in HBSS. Agonist-mediated emission signals from 2 ml of cell suspension were measured in a thermostatically controlled DM3000-F spectrophotometer (Spex Industries, Edison, NJ, U.S.A.), as described previously [17].

[^{3}H]Thymidine incorporation

Measurement of [^{3}H]thymidine incorporation, as an indicator of DNA synthesis, was performed as described previously [24]. Briefly, the cells were plated in 6-well plates in α-MEM containing 10% (v/v) FCS at a density of 300000 cells/well. The cells were depleted of serum for 48 h and Ang II (1 μM) was added for 18 h. [^{3}H]thymidine was added during the final 2 h of incubation. The radioactivity in trichloroacetic acid precipitable material was measured in a γ-radiation counter (LS 3801; Beckman).

Plasmid construct and RNA transcription *in vitro*

The 3′-UTR of the rat AT_{1A} receptor (848 bp) was amplified by PCR from pRc/CMV + 3′-UTR, inserted into the *Eco*RV site of pBluescript II-KS$^+$ and sequenced to confirm its authenticity. The DNA was restriction digested with *Hin*dIII to generate a linear plasmid and the reaction was digested with Proteinase K. After extraction into phenol/chloroform (1:1, by vol.) and precipitation with ethanol, the DNA was resuspended in RNase-free 10 mM Tris/EDTA buffer (pH 7.9). Approx. 1 μg of the linear plasmid was transcribed *in vitro* into sense RNA in the presence or absence of [α-^{32}P]UTP using bacteriophage T7 RNA polymerase [21]. The reaction was incubated with 10 units of RNase-free DNase 1 for 30 min at 37 °C. The transcript was extracted into phenol/chloroform (pH 4.7) (5:1, by vol.), precipitated with ethanol and resuspended in 50 μl of RNase-free 10 mM Tris/EDTA buffer (pH 7.9).

RNA–protein binding reaction and mobility-shift assay

High salt cellular extracts were prepared as described [25] with minor modifications. CHO-K1 cells were grown in α-MEM in the presence of 10 % (v/v) FCS until subconfluent. Cultures were washed twice with HBSS, suspended in 1ml of ice-cold HBSS, sedimented by centrifugation at 1000 ***g*** for 10 min and the cell pellets were resuspended in 300 μl of lysis buffer [10 mM Hepes (pH 7.9)/10 mM KCl/1.5 mM $MgCl_2$/300 mM NaCl/0.5 mM dithiothreitol/0.5 mM PMSF/10 μM leupeptin/10 μM aprotein containing 1 unit/μl of RNasin]. The cells were homogenized in a glass homogenizer with 15 strokes of a loose fitting pestle, incubated on ice for 30 min and sedimented at 1000 ***g*** for 10 min. The resulting supernatant was used as the high salt cellular extract. High salt cellular extract protein (15 μg) and 5×10^5 c.p.m. of 3′-UTR mRNAs were incubated in 10 mM Hepes (pH 7.9)/10 mM KCl/1.5 mM $MgCl_2$/0.5 mM dithiothreitol containing 200 ng/μl yeast tRNA in a total volume of 20 μl at 30 °C for 15 min. The reaction mixture was then incubated at 37 °C for 30 min with 100 units of RNase T1, to digest unprotected riboprobe, followed by incubation on ice for 10 min with 5 μg/μl of heparin to reduce non-specific binding. The RNA–protein complex was separated by PAGE on a 4 % (w/v) acrylamide non-denaturing gel with 0.5×44.5 mM Tris/borate/10 mM EDTA buffer (pH 8.0). The gel was dried and exposed to X-ray film (Fuji RX).

UV crosslinking and SDS/PAGE electrophoresis

RNA–protein binding reactions were performed as described above, using 15 μg of cellular extracts and 5×10^5 c.p.m. of radioactively labelled RNA per reaction. After treatment with heparin the binding-reaction samples were irradiated with UV light on autocrosslinking setting (Stratalinker 2400; Stratagene, La Jolla, CA, U.S.A.). The samples were boiled for 10 min in Laemmli sample buffer and separated by SDS/PAGE (8 % acrylamide gel) [21]. The gel was dried and the radioactive bands were exposed by autoradiography.

RESULTS

Stable expression and agonist-binding studies of the AT_{1A} receptor in CHO-K1 cells

cDNAs corresponding to the full length AT_{1A} receptor mRNA (+3′-UTR) and truncated receptor mRNA (−3′-UTR) were subcloned into the pRC/CMV vector and transfected into CHO-K1 cells. Individual neomycin resistant colonies were isolated and screened for ^{125}I-labelled Ang II binding. Numerous clones of various receptor density were identified and propagated. Clones expressing the AT_{1A} receptor with the 3′-UTR had less than 600 fmol of cell surface receptors/mg of protein, whereas receptor transfection without the 3′-UTR produced clones expressing more than 500 fmol of cell surface receptors/mg of protein (results not shown). Two clonal lines (TF27CHO/AT_{1A} and T24CHO/AT_{1A}) were selected for the present study, based on similar levels of Ang II receptor binding. The results obtained for these clones, as detailed below, were confirmed in multiple clonal cell lines expressing different levels of +3′-UTR and −3′-UTR AT_{1A} receptor (+3′-UTR cell lines: TF22CHO/AT_{1A}, B_{max} = 397 fmol/mg of protein and TF32CHO/AT_{1A}, B_{max} = 306 fmol/mg of protein; −3′-UTR cell lines: T5CHO/AT_{1A}, B_{max} = 1207 fmol/mg of protein and T11CHO/AT_{1A}, B_{max} = 593 fmol/mg of protein). Figure 2 (upper panel) shows competition binding studies in TF27CHO/AT_{1A} (+3′-UTR) and T24CHO/AT_{1A} (−3′-UTR) cells using radioactively labelled Ang II in the presence of increasing concentrations of unlabelled

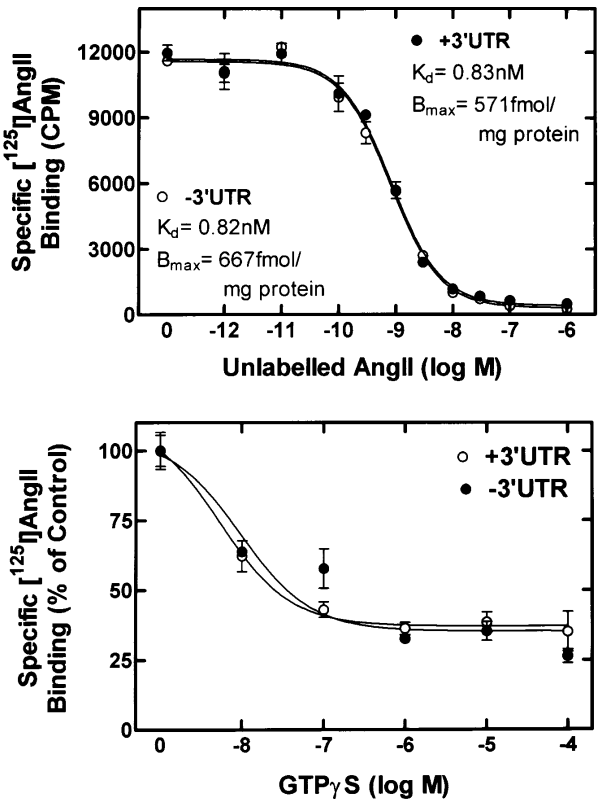

Figure 2 Receptor characterization of CHO-K1 cells expressing the AT_{1A} +3′-UTR and −3′-UTR

Upper panel, displacement of ^{125}I-labelled Ang II specific binding to TF27CHO/AT_{1A} (+3′-UTR) and T24CHO/AT_{1A} (−3′-UTR) cells. Confluent cells were assayed for ^{125}I-labelled Ang II binding at 22 °C for 1 h in the presence 1 pM–1 μM of unlabelled Ang II as a competitor (mean ± S.E.M., $n = 3$). Lower panel, effect of GTP[S] on the dissociation of bound ^{125}I-labelled Ang II from membranes of TF27CHO/AT_{1A} and T24CHO/AT_{1A} cells. Membrane fractions were prepared as described in the Experimental section. Equilibrium binding of ^{125}I-labelled Ang II to the membranes was performed for 1 h at 22 °C in the presence of 10 nM–100 μM of GTP[S]. The data shown are the means of triplicate determinations from a representative experiment.

Ang II. Both cell lines expressed similar levels (+3′-UTR, B_{max} = 571 fmol/mg of protein and −3′-UTR, B_{max} = 667 fmol/mg of protein) of high affinity (+3′-UTR, K_d = 0.83 nM and −3′-UTR, K_d = 0.82 nM) cell surface receptors. Since AT_{1A} receptors are normally G-protein coupled, we determined whether these receptors coupled to G-proteins by examining the inhibitory effect of GTP[S], a non-hydrolysable GTP analogue, on Ang II binding. GTP[S] inhibited the binding of ^{125}I-labelled Ang II to the receptor in a concentration-dependent manner by reducing the receptors to a low-affinity state (Figure 2, lower panel), showing that both +3′-UTR and −3′-UTR receptors are similarly affected by GTP[S].

Angiotensin II AT_{1A} receptor mRNA stability in transfected CHO-K1 cells.

Since the 3′-UTR has been reported to play an important role in mRNA stability and degradation, we determined the steady-state level and the decay rate of AT_{1A} mRNA expressed by TF27CHO/AT_{1A} (+3′-UTR) and T24CHO/AT_{1A} (−3′-UTR) cells. In these cells, receptor expression is under the control of the constitutively expressing human CMV promoter, and therefore we presume a similar level of transcription. Northern-blot

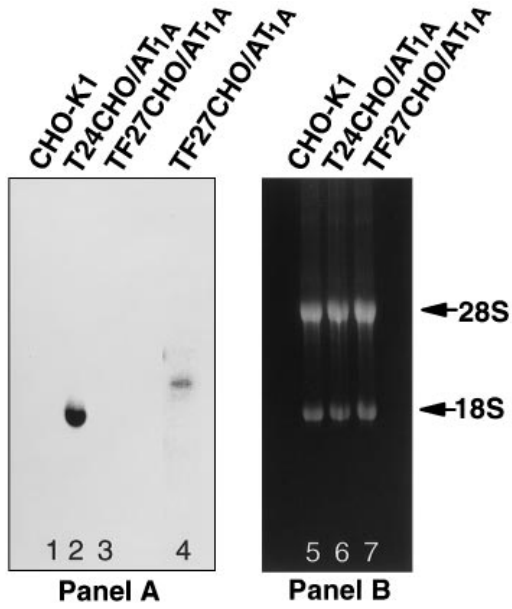

Figure 3 Northern-blot analysis of TF27CHO/AT_{1A} (+3′-UTR), T24CHO/AT_{1A} (−3′-UTR) and CHO-K1 cells

Total RNA (5 μg) from each cell line was separated by gel electrophoresis, blotted and hybridized with a nick-translated AT_{1A}-receptor cDNA probe as described in the Experimental section. (**A**) Lanes 1–3, 6 h exposure at room temperature. While a strong signal was readily observed in RNA extracted from T24CHO/AT_{1A} (−3′-UTR), no signal was seen in RNA from TF27CHO/AT_{1A} (+3′-UTR) cells. Lane 4, 60 h exposure of lane 3 at 70 °C with intensifying screens. A weak signal was detected at an higher molecular mass corresponding to the +3′-UTR-AT_{1A} mRNA. No signal was observed in non-transfected CHO-K1 cells at either length of exposure. (**B**) Ethidium bromide stained gel demonstrating equal loading. The arrows indicate the positions of 28S and 18S ribosomal RNA.

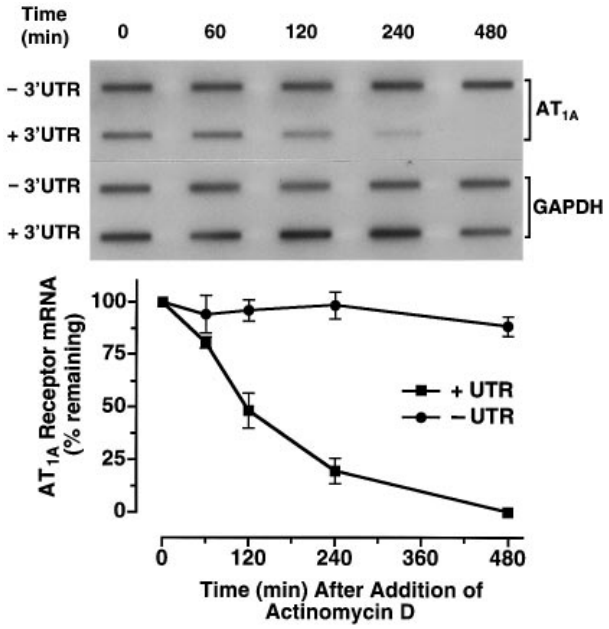

Figure 4 Actinomycin D treatment of cells revealed a different rate in decay of +3′-UTR and −3′-UTR AT_{1A}-receptor mRNA

Slot-blot analyses were performed on total RNA prepared from CHO-K1 cells expressing the AT_{1A}-receptor +3′-UTR and −3′-UTR at the indicated time points. Upper panel, results of a representative experiment. The top two lanes represent the amount of +3′-UTR and −3′-UTR AT_{1A}-receptor mRNA remaining after actinomycin D treatment. The bottom two lanes show the reprobing of the blot with GAPDH as an internal standard. Lower panel, densitometeric analysis of the slot-blot plotted for the decay of +3′-UTR and −3′-UTR AT_{1A}-receptor mRNA (means ± S.E.M. of three separate experiments).

analysis showed that the receptor mRNA expression is significantly less in +3′-UTR cells compared with the −3′-UTR cells (Figure 3), even though binding studies using ^{125}I-labelled Ang II demonstrated that receptor expression on the cell surface of these two cell lines was similar (Figure 2). Non-transfected CHO-K1 cells showed no bands, suggesting the absence of Northern-blot detectable receptor mRNA in these cells, consistent with our binding data, which showed no detectable AT_1s in the plasma membranes of non-transfected cells [17]. To measure the half-life of AT_{1A}-receptor mRNA with and without the 3′-UTR, the cells were treated with actinomycin D and cytoplasmic mRNAs from both cell types were assayed at various times after addition. The results of the slot-blot analysis, shown in Figure 4, indicate that in transfected CHO-K1 cells AT_{1A}-receptor mRNA +3′-UTR decayed with a half-life of less than 2 h to a undetectable level at 8 h, whereas AT_{1A} receptor −3′-UTR remained stable for the 8 h incubation period.

Agonist-mediated change in cAMP accumulation

Endogenous AT_1s couple positively or negatively to cAMP accumulation in a tissue specific manner [26,27]. We have shown previously that Ang II stimulates cAMP in CHO-K1 cells overexpressing the AT_{1A} receptor [24]. Therefore we determined the effect of Ang II on cAMP accumulation in cells expressing +3′-UTR and −3′-UTR receptors (Table 1). In TF27CHO/AT_{1A} cells (+3′-UTR), no cAMP stimulation was observed in response to Ang II. In T24CHO/AT_{1A} cells (−3′-UTR), Ang II stimulated an 11-fold accumulation of cAMP, which was blocked by EXP3174, confirming our previous observation [24]. In both cell lines, forskolin stimulated similar levels of cAMP accumulation. We confirmed these results in two additional cell lines (+3′-UTR cell lines: TF22CHO/AT_{1A}, basal = 2.09 ± 0.07, Ang II = 2.0 ± 0.22 and TF32CHO/AT_{1A}, basal = 4.06 ± 0.06, Ang II = 3.81 ± 0.28; −3′-UTR cell lines: T5CHO/AT_{1A}, basal = 2.09 ± 0.16, Ang II = 34.57 ± 6.9 and T11CHO/AT_{1A}, basal = 8.31 ± 1.15, Ang II = 27.33 ± 0.51; all values are pmol cAMP/mg of protein, means ± S.E.M.). Moreover, similar results were observed in every cell line tested. Thus compared with +3′-UTR-expressing cell lines, −3′-UTR-expressing cell lines dem-

Table 1 Ang II-stimulated cAMP accumulation in CHO-K1 cells expressing the −3′-UTR AT_{1A} but not the +3′-UTR AT_{1A}

The Ang II mediated accumulation of cAMP in TF27CHO/AT_{1A} (+3′-UTR) and T24CHO/AT_{1A} (−3′-UTR) cells was determined in serum-deprived (12 h) cells exposed to Ang II, Ang II + EXP3174 or forskolin. Stimulation was performed in the presence of the phosphodiesterase inhibitor Ro 20-1724 to prevent cAMP degradation. Data are expressed as the means ± S.E.M. (n = 3).

	cAMP produced (pmol/mg of protein)	
Treatment	+3′-UTR	−3′-UTR
Basal	2.04 ± 0.11	2.51 ± 0.27
Ang II (1 μM)	3.78 ± 0.36	41.48 ± 2.68
Ang II (1 μM) + EXP3174 (100 μM)	4.48 ± 0.31	9.06 ± 1.12
Forskolin (10 μM)	848.67 ± 39.78	1017.50 ± 78.46

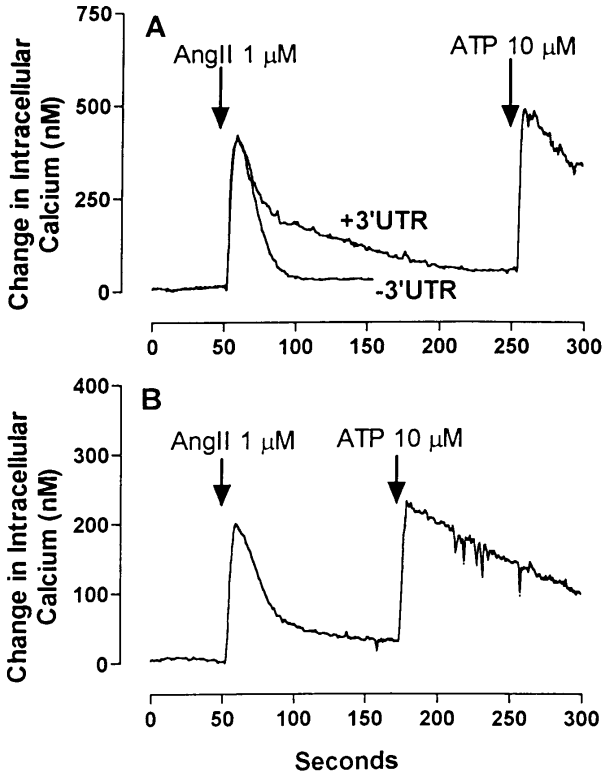

Figure 5 Ang II-mediated intracellular calcium transients in TF27CHO/AT_{1A} (+3′-UTR) and T24CHO/AT_{1A} (−3′-UTR) cells

(**A**) Cells were loaded with the fluorescent dye Fura-2/AM and intracellular calcium transients measured in the presence of Ang II (1 μM) and ATP (10 μM; positive control). In +3′-UTR cells the initial calcium spike was followed by an extended plateau phase but in −3′-UTR cells the calcium response was transient, returning to baseline within 50 s. (**B**) +3′-UTR cells were chelated with 20 mM EGTA to remove extracellular calcium before Ang II stimulation. The traces are representative of three separate experiments.

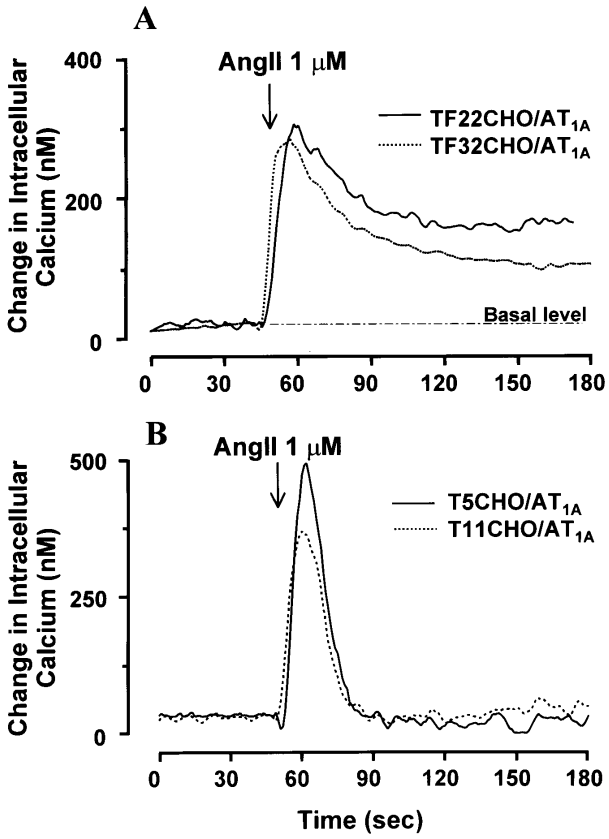

Figure 6 Ang II-mediated intracellular calcium transients in two additional cell lines from +3′-UTR- and −3′-UTR-expressing cells

The experiments were carried out as described in the legend to Figure 5. (**A**) Intracellular calcium transients in cells expressing +3′-UTR AT_{1A} (TF22CHO/AT_{1A} and TF32CHO/AT_{1A}). (**B**) Calcium transients in cells expressing −3′-UTR AT_{1A} (T5CHO/AT_{1A} and T11CHO/AT_{1A}). The traces are representative of three separate experiments.

onstrated a significant Ang II-mediated increase in cAMP accumulation. This observation suggests that the 3′-UTR has a role in modulating AT_{1A}-receptor coupling to this signal transduction pathway.

Angiotensin-II-mediated change in intracellular calcium

One of the earliest AT_1-mediated second messenger signalling events is the stimulated increase in intracellular calcium through enhanced production of Ins(1,4,5)P_3. Ang II generally increases intracellular calcium within seconds of the agonist coupling to the receptor [15–17]. We measured the Ang II-mediated change in intracellular calcium in both TF27CHO/AT_{1A} (+3′-UTR) and T24CHO/AT_{1A} (−3′-UTR) cells (Figure 5A). Both cell lines demonstrated a similar level of calcium stimulation in response to Ang II but the transient in +3′-UTR cells was sustained, whereas it quickly returned to basal levels in −3′-UTR cells. Depletion of extracellular calcium using EGTA had no significant effect on the profile of calcium release in +3′-UTR cells (Figure 5B), suggesting that the Ang II-mediated change in calcium transients originated mostly from intracellular stores. The studies were repeated in two additional cell lines from each group expressing +3′-UTR (Figure 6A) and −3′-UTR (Figure 6B) AT_{1A}. The levels of Ang II-mediated increase in calcium transients differed because there were different levels of plasma membrane receptor expression in these cell lines. However, +3′-UTR cell lines demonstrated the sustained phase of calcium transient but in −3′-UTR cells cytosolic calcium quickly returned to basal levels.

Table 2 [^{3}H]Thymidine incorporation in TF27CHO/AT_{1A} (+3′-UTR) and T24CHO/AT_{1A} (−3′-UTR) cells

Serum depleted (48 h) cells were incubated for 20 h in the presence of Ang II and Ang II + EXP3174. [^{3}H]thymidine was added to the medium during the last 2 h of incubation and [^{3}H]thymidine incorporation was determined as described in the Experimental section. The data are expressed as the means ± S.E.M. ($n = 3$).

	[^{3}H]Thymidine incorporation ($\times 10^{-3}$ c.p.m.)	
Treatment	+3′-UTR	−3′-UTR
Basal	38.81 ± 5.22	28.81 ± 5.27
Ang II (1 μM)	41.80 ± 2.62	9.70 ± 1.43
Ang II (1 μM) + EXP3174 (100 μM)	33.83 ± 1.41	26.11 ± 5.60

Agonist-induced change in [^{3}H]thymidine incorporation

Ang II has been shown to be bifunctional, coupling to growth stimulatory and inhibitory pathways in a tissue specific manner [28]. Therefore we determined if these receptors would couple to

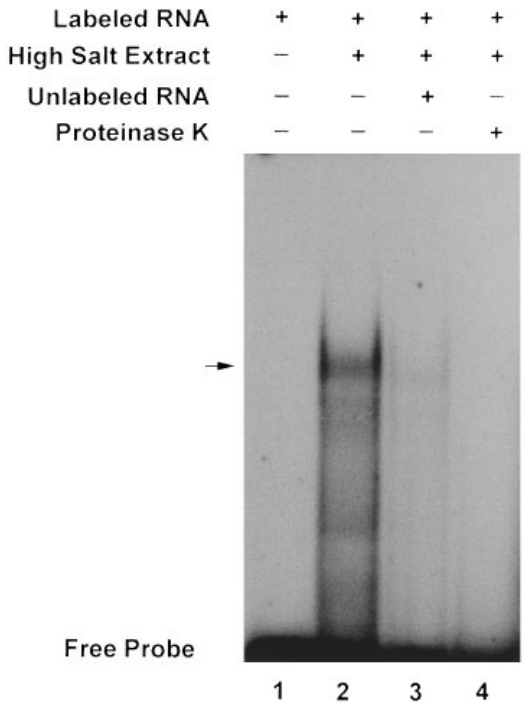

Figure 7 Demonstration of RNA binding proteins that specifically recognize the 3′-UTR of the AT_{1A} receptor

[^{32}P]-labelled 3′-UTR mRNA of the AT_{1A} receptor was incubated with high salt total cellular extract, digested with RNase T1 to remove unbound RNA, and treated with heparin to eliminate non-specific protein binding. The samples were analysed after electrophoresis on a 4% non-denaturing polyacrylamide gel and autoradiography of the gel. Labelled transcript in the absence of extract (lane 1), in the presence of extract (lane 2), in the presence of extract and a 20-fold excess of unlabelled mRNA 3′-UTR transcript (lane 3), and in the presence of extract treated with Proteinase K to digest proteins (lane 4). The arrow indicates the position of the cellular factor–RNA complex.

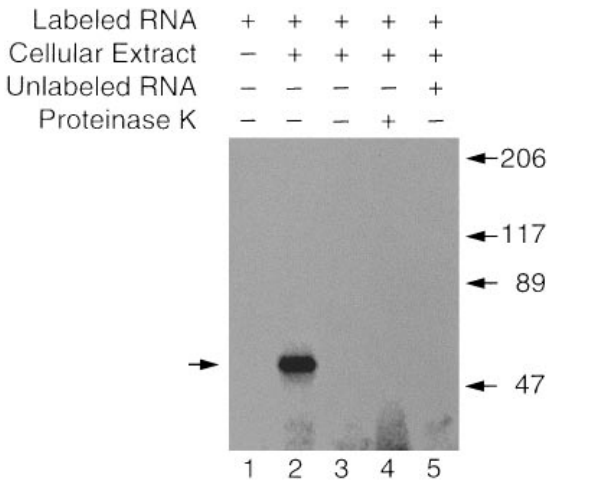

Figure 8 Identification of factors binding to the 3′-UTR of the AT_{1A} receptor and apparent molecular mass

[^{32}P]-labelled mRNA 3′-UTR of the AT_{1A} receptor was incubated with equal amounts of cellular extract from CHO-K1 cells, digested with RNase T1 (100 units) to remove unbound RNA and treated with heparin to eliminate non-specific protein binding. Samples were UV crosslinked and separated by SDS/PAGE (8% gel). The gel was dried and bands were revealed by autoradiography. Labelled Ang II mRNA 3′-UTR transcript: lane 1, in the absence of cellular extract; lane 2, in the presence of cellular extract; lane 4, in the presence of cellular extract treated with Proteinase K; lane 5, in the presence of cellular extract and a 20-fold excess of unlabelled mRNA 3′-UTR transcript. In lane 3, the cellular extract was incubated with the bovine growth hormone mRNA 3′-UTR transcript, a very stable and unrelated message. The arrow on the left indicates the position of the 55 kDa protein–RNA complex. The positions of molecular-mass markers are shown on the right.

growth using Ang-II-mediated [^{3}H]thymidine incorporation as an indicator of DNA synthesis (Table 2). Ang II had no effect on growth in +3′-UTR cells, whereas it significantly inhibited [^{3}H]thymidine incorporation in −3′-UTR cells. In −3′-UTR cells, pretreatment with EXP3174 blocked Ang-II-mediated inhibition of [^{3}H]thymidine incorporation, indicating that the inhibitory response was mediated through the transfected AT_{1A}. Similar results were obtained in additional clones (+3′-UTR cell lines: TF22CHO/AT_{1A}, basal = 27.47±1.05, Ang II = 30.23±2.48 and TF32CHO/AT_{1A}, basal = 31.45±1.63, Ang II = 30.47±1.26; −3′-UTR cell lines: T5CHO/AT_{1A}, basal = 28.71±2.79, Ang II = 3.8±0.5 and T11CHO/AT_{1A}, basal = 26.47±2.93, Ang II = 11.86±0.19; the values are [^{3}H]thymidine incorporation $\times 10^3$ c.p.m., means±S.E.M.).

Identification of specific RNA binding to the 3′-UTR of the AT_{1A}

We performed an RNA mobility-shift assay using a [^{32}P]-labelled 848 bp mRNA transcribed *in vitro* corresponding to the 3′-UTR of the AT_{1A}. Figure 7 (lane 1) contains labelled probe incubated in the presence of RNase T1 (100 units) to show that the concentration and conditions are sufficient for complete digestion of the probe in the absence of cellular extract. Figure 7 (lane 2) shows that, at least, a major cellular-factor–RNA complex was formed with high salt cellular extracts from CHO-K1 cells. The complex was effectively competed with a 20-fold excess of unlabelled 3′-UTR RNA (Figure 7, lane 3). Data from the mobility shift assay indicate that a cellular transacting factor binds specifically to the 3′-UTR of the AT_{1A}. The addition of Proteinase K to the incubation reaction completely abolished the complex formation (Figure 7, lane 4), demonstrating that the factor bound to the 3′-UTR of the receptor is an RNA-binding protein.

To further characterize these factors and to identify their apparent molecular mass, we performed UV crosslinking and SDS/PAGE analysis. Following successive incubations with RNase T1 and heparan sulphate, the reaction mixtures were UV crosslinked and separated by SDS/PAGE (8% gel). The results obtained in the UV crosslinking assays were consistent with those obtained in mobility-shift experiments. The results showed that a major mRNA protein complex (55 kDa) was UV crosslinked and detected in the extracts from CHO-K1 cells when the 848 bp 3′-UTR of the AT_{1A} was used as a probe (Figure 8, lane 2). The 55 kDa protein–RNA complex formation was completely abolished when Proteinase K was added to the incubation reaction (Figure 8, lane 4) and the complex was competed out by a 20-fold excess of unlabelled 3′-UTR of the AT_{1A} (Figure 8, lane 5). The specificity for AT_{1A} 3′-UTR binding activity in the cellular extract was confirmed in Figure 8 (lane 3), since bovine growth hormone 3′-UTR (a 231 bp fragment which also contains the polyadenylation signal) was unable to detect the 55 kDa protein–RNA complex formation in the UV crosslinking assay.

DISCUSSION

Recent evidence suggests that the 3′-UTR of mRNA plays a significant role in regulating gene expression and cellular responses [4]. We report, in the present study, that the AT_{1A} receptor without its 3′-UTR, when transfected into CHO-K1

cells, couples to cAMP stimulation and inhibition of DNA synthesis. In contrast, when transfected with its 3′-UTR, the AT_{1A} did not couple to these two responses, which has been observed in many tissues and cells where native (+3′-UTR) receptors are expressed in reponse to Ang II [11,12,16]. Previously, in CHO-K1 cells overexpressing the AT_{1A} (−3′-UTR) we demonstrated that the stimulation of cAMP is concentration- and time-dependent ($EC_{50} = 3.3$ nM and $t_{1/2} = 2.5$ min) [24]. Activation was initiated within seconds and reached a maximum in 7 min. Although it is possible that there is a titrating effect, it does not seem likely because in −3′-UTR cells Ang II stimulated cAMP within a very short time. Our results suggest that the 3′-UTR of the AT_{1A} receptor may regulate effector pathways by post-transcriptional modification of the receptor, probably by altering its affinity to specific G-protein(s). Ang-II-mediated stimulation of DNA synthesis has been shown in rat AT_{1A} receptor transfected CHO cells [29]. Although this observation may appear difficult to reconcile with our finding, the cell specificity could account for such differences. There are many different CHO cell lines available and cell-specific alterations in Ang II receptor expression and function have been well documented [11,16]. We also observed differences in the profile of agonist-stimulated calcium transients between +3′-UTR and −3′-UTR expressing cells. In +3′-UTR expressing cells, Ang II stimulated an intracellular calcium transient with a sustained phase, which mimics Ang II stimulated calcium transients in cells isolated from tissues expressing endogenous AT_1 receptors (e.g. vascular smooth muscle cells, adrenal zona glomerulosa cells and cardiac fibroblasts) [16,30,31]. In contrast, Ang II stimulation of −3′-UTR cells resulted in a more transient calcium response which returned to basal levels within 50 s. Together, these observations suggest that the 3′-UTR of the AT_{1A} receptor has a role in cell signalling and growth, and that the cell is able to distinguish between the +3′-UTR and −3′-UTR transcripts. A role for the 3′-UTR in cell growth has been described for the muscle structural genes, troponin, tropomyosin and α-cardiac actin, where the ability of the cDNAs for these genes to suppress proliferation and promote differentiation was mapped to the 3′-UTR [5]. Moreover, expression of the α-tropomyosin 3′-UTR suppressed anchorage-independent growth and tumour formation in a non-differentiating myogenic cell line [32]. Our observation that the presence of the AT_{1A} 3′-UTR can modulate the growth response of transfected cells to Ang II is consistent with these studies.

Previous studies have shown that RNA-binding proteins interact with sequence motifs within the 3′-UTR of many mRNAs and that these may mediate 3′-UTR specific responses [33–35]. We used an RNA gel mobility-shift assay to demonstrate interaction of proteins from CHO-K1 cells with the 3′-UTR of the AT_{1A} receptor. Under stringent binding conditions and in non-denaturing gels, a complex was detected, the specificity of which was confirmed by competition-binding studies with unlabelled 3′-UTR and binding studies with labelled but unrelated bovine growth hormone 3′-UTR. This interaction was abolished in the presence of Proteinase K which confirmed that the binding factors were proteins. UV crosslinking studies and SDS/PAGE analysis identified a major complex of 55 kDa. It is possible that these binding proteins, through interaction with the 3′-UTR, mediate the opposing responses to Ang II observed between +3′-UTR and −3′-UTR receptor-expressing cells. The resolution of this possibility awaits the identification and full characterization of this protein, as well as the delineation of its specific recognition sequences within the AT_{1A} receptor 3′-UTR.

The steady-state levels of highly regulated mRNAs are markedly influenced by the rate of degradation [1,36,37], and there is evidence that G-protein-coupled receptors, such as the AT_{1A} receptor, are regulated by agonist-induced mRNA destabilization. This has been shown previously for two G-protein coupled receptors, the M1 muscarinic acetylcholine receptor [38] and the β-adrenergic receptor [39]. For the latter, agonist-induced destabilization has been correlated with the presence of the AUUUA destablization pentamer in the 3′-UTR and the recognition of the 3′-UTR by a 35 kDa RNA-binding protein [40]. As noted by Tholanikunnel et al. [40], various G-protein-coupled receptors possess one or more AUUUA pentamers within the 3′-UTR, suggesting varying susceptibility to degradation. The rat AT_{1A} receptor 3′-UTR has four repeats of the AUUUA sequence suggesting a high degradation rate for this mRNA, which may explain the observation that Ang II treatment leads to a reduction in AT_{1A}-receptor mRNA content in vascular smooth muscle cells and glomerular mesangial cells [41–43]. We have shown that the expression of the AT_{1A} receptor in CHO-K1 cells, devoid of its cognate 3′-UTR, allowed for significant accumulation of receptor transcript, whereas minimal accumulation was seen when the receptor was expressed with its 3′-UTR. Interestingly, this difference in accumulation was present in the absence of agonist stimulation suggesting that these destabilization processes are occurring constitutively. Removal of the 3′-UTR increased the half life of AT_{1A} receptor mRNA transcripts indicating a role for post-transcriptional regulation of gene expression. Whether these changes in mRNA stability are due to the AUUUA sequences and the possible role of mRNA-binding proteins (as demonstrated in this study), needs to be determined.

mRNA stability, however, is unlikely to be the reason for the differential coupling that we observed between the +3′-UTR and −3′-UTR AT_{1A}-expressing cells because, despite large differences in steady-state levels of mRNA, both receptors were expressed at similar densities on the cell surface and with similar ^{125}I-labelled Ang II-binding characteristics. This observation implies that the highly accumulated −3′-UTR AT_{1A} mRNA is either inefficiently translated or conversely that the low-abundance +3′-UTR AT_{1A} mRNA is very efficiently translated. The latter postulate is supported by the observation that a 60 nt motif at the 3′-UTR of protamine mRNA is necessary and sufficient for the control of translation [4]. Efficient translation may also result from 3′-UTR-directed targeting of AT_{1A} mRNA to a specific compartment for translation and subsequent translocation to the plasma membrane. Conversely, incorrectly localized mRNA may be translationally (or functionally) incompetent or may prevent specific conformation for efficient trafficking of receptor protein to the cell surface. To determine between these possibilities, an AT_{1A}-receptor specific antibody for immunohistochemical localization of the receptor to various cell compartments would be required. To our knowledge, such an antibody is not currently available.

The idea of a functional correlate for 3′-UTR-directed localization of the AT_{1A} mRNA is interesting given the recent identification of mRNA localization motifs (zipcodes) in the 3′-UTR of the β-actin mRNA [44]. These evolutionarily conserved zipcodes are sequences of approx. 40–50 nt flanked by the two motifs GGACT and AATGC. These zipcodes are responsible for the specific localization of β-actin mRNA and, by analogy, any mRNA containing these codes. Removal of the β-actin zipcode resulted in disordering of the actin filaments, loss of cell structure and alteration of normal cell function [44]. Sequence analysis revealed the presence of such a zipcode in the rat AT_{1A} 3′-UTR (Figure 9). The GGACT and AATGC flanking motifs are present in the zipcode of the AT_{1A} receptor 3′-UTR and compared with the β-actin 3′-UTR zipcode, there is an overall sequence similarity of 69 %. Similar motifs can be identified in the 3′-UTR

Figure 9 Nucleotide sequence of the putative AT_{1A} 3′-UTR zipcode

Comparison of the putative zipcode sequence of the rat AT_{1A} receptor 3′-UTR (top line) and the chicken β-actin 3′-UTR zipcode [44] (bottom line). The closed triangle above the sequences highlights the GGACT flanking motif, and the open triangle highlights the AATGC flanking motif. Nucleotides common to both zipcodes are indicated with vertical lines.

of the human AT_{1A} receptor but, interestingly, the rat AT_{1B} receptor lacks a readily apparent zipcode within its 3′-UTR, the significance of this is not known. Just how, or if, this zipcode functions to localize the AT_{1A}-receptor mRNA, as well as having a possible role in receptor function remains to be established. There is evidence that 3′-UTR-directed mRNA localization is not unique to β-actin [4]. For example, correct spatial regulation and activity of the nos protein is essential for normal morphology in the *Drosophila* embryo and is controlled by 3′-UTR-mediated mRNA localization [45]. Other genes associated with the nos protein (*oskar* and *vasa*) are also localized by their 3′-UTRs. In somatic cells, structural as well as functional genes are localized to specific regions within the cell and it has been suggested that localization-dependent translation provides a mechanism to ensure the restriction of the activity of a protein to specific regions of the cell or embryo.

Even assuming a localizing effect of the 3′-UTR on AT_{1A} mRNA, it is still difficult to imagine how mRNA localization would promote differential coupling to effector pathways. Perhaps correctly targeted mRNA translation allows for appropriate post-translational modifications to occur. For example, most G-protein-coupled receptors are glycosylated at N-terminal asparagine residues and this modification can contibute significantly to the molecular mass of the mature receptor protein [46,47]. The Ang II receptor is glycosylated [16] and, although the function of this modification is not known, it may allow for correct folding and/or insertion of the receptor into the membrane. Incorrectly targeted AT_{1A} mRNA translation could prevent such modification and as a consequence the receptor may be incorrectly folded or inserted into the membrane, resulting in altered coupling to effector systems. Similarly, G-protein coupled receptors are very hydrophobic and probably require specific chaperones for correct folding and transport to the membrane. Localization by the 3′-UTR may be necessary for correct chaperoning and function of the translated receptor. Interestingly, tissue-distribution studies show that relative levels of the 55 kDa mRNA-binding protein expressed differentially in various target tissues of Ang II (T. J. Thekkumkara, unpublished work), indicating the possibility of mRNA-binding-protein-dependent tissue-specific regulation of AT_{1A}-receptor expression and function.

In summary, transfecting the AT_{1A} receptor without the 3′-UTR results in the expression of high-affinity AT_{1A} receptors on the cell surface, and in coupling to intracellular signaling which differs from that of the AT_{1A} receptor with the 3′-UTR. The reported capacity of the AT_{1A} receptor to couple differentially, in a tissue specific manner, to cAMP and cellular growth may provide a physiological correlate for the 3′-UTR-dictated coupling identified in the present study. It is possible that these responses are mediated by tissue-specific expression of RNA binding proteins that recognize the AT_{1A} 3′-UTR. Future studies will be directed toward identifying and characterizing the 55 kDa binding protein that we have shown to specifically recognize the AT_{1A} 3′-UTR and its possible involvement in mRNA stability and localization. Moreover, it will be important to establish a function for the zipcode that we have identified within the AT_{1A} 3′-UTR.

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REFERENCES

1 Decker, C. J. and Parker, R. (1994) Trends Biochem. Sci. **19**, 336–340
2 Brewer, G. (1991) Mol. Cell. Biol. **11**, 2460–2466
3 Zubiaga, A. M., Belasco, J. G. and Greenberg, M. E. (1995) Mol. Cell. Biol. **15**, 2219–2230
4 Jackson, R. J. (1993) Cell **74**, 9–14
5 Rastinejad, F. and Blau, H. M. (1993) Cell **72**, 903–917
6 Macdonald, P. M. (1992) Semin. Dev. Biol. **3**, 413–424
7 Kislauskis, E. H., Li, Z., Singer, R. H. and Taneja, K. L. (1993) J. Cell Biol. **123**, 165–172
8 Withelm, J. E. and Vale, R. D. (1993) J. Cell Biol. **123**, 269–274
9 Ostareck-Lederer, A., Ostareck, D. H., Standart, N. and Thiele, B. J. (1994) EMBO J. **13**, 1476–1481
10 Baker, K. M., Booz, G. W. and Dostal, D. E. (1992) Annu. Rev. Physiol. **54**, 227–241
11 Lassegue, B., Griendling, K. K. and Alexander, R. W. (1994) in Angiotensin Receptors (Saavedra, J. M. and Timmermans, P. B. M. W. M., eds.), pp. 17–48, Plenum Press, New York
12 Catt, KJ. (1993) in The Renin-Angiotensin System, vol. 1 (Robertson, J. I. S. and Nicholls, M. J., eds.), pp. 12.1–12.14, Gower Medical Publishing, London
13 Murphy, T. J., Alexander, R. W., Griendling, K. K., Runge, M. S. and Bernstein, K. E. (1991) Nature (London) **351**, 233–236
14 Iwai, N. and Inagami, T. (1992) FEBS Lett. **298**, 257–260
15 Gasparo, M. D., Husain, A., Alexander, W., Catt, K. J., Chiu, A. T., Drew, M., Goodfriend, T., Harding, J. W., Inagami, T. and Timmermans, P. B. M. W. M. (1995) Hypertension **25**, 924–927
16 Catt, K. J., Sandberg, K. and Balla, T. (1993) in Cellular and Molecular Biology of the Renin-Angiotensin System (Raizada, M. K., Phillips, M. I. and Summers, C., eds.), pp. 307–356, CRC Press, Boca Raton
17 Thekkumkara, T. J., Du, J., Dostal, D. E., Motel, T. J., Thomas, W. G. and Baker, K. M. (1995) Mol. Cell. Biochem. **146**, 79–89
18 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
19 Swillens, S. (1992) Trends Pharmacol. Sci. **13**, 430–434
20 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
21 Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1987) Current Protocols in Molecular Biology, John Wiley and Sons, New York
22 Brooker, G., Terasaki, W. L. and Price, M. G. (1976) Science **194**, 270–276
23 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
24 Thekkumkara, T. J., Du, J., Zwaagstra, J. C., Conrad, K. M., Krupinski, J. and Baker, K. M. (1995) Mol. Cell. Biochem. **152**, 77–86
25 Alberta, J. A., Rundell, K. and Stiles, C. D. (1994) J. Biol. Chem. **269**, 4532–4538
26 Johnson, M. C. and Aguilera, G. (1992) Endocrinology **131**, 2404–2412
27 Sadoshima, J. and Izumo, S. (1993) Circulation Res. **73**, 424–438
28 Simon, G. and Aitman, S. (1992) J. Hypertens. **10**, 1165–1171
29 Martin, M. M., White, C. R., Li, H., Miller, P. J. and Elton, T. S. (1995) Reg. Pept. **60**, 135–147

30 Dostal, D. E., Murahashi, T. and Peach, M. J. (1990) Hypertension **15**, 815–822
31 Schorb, W., Booz, G. W., Dostal, D. E., Conrad, K. M., Chang, K. C. and Baker, K. M. (1993) Circ. Res. **72**, 1245–1254, 1993
32 Rastinejad, F., Conboy, M. J., Rando, T. A. and Blau, H. M. (1995) Cell **75**, 1107–1117
33 Mullner, E. W., Nupert, B. and Kuhn, L. C. (1989) Cell **58**, 373–382
34 Zaidi, S. H. E., Denman, R. and Malter, J. S. (1994) J. Biol. Chem. **269**, 24000–24006
35 Henics, T., Sanfridson, A., Hamilton, B. J., Nagy, E. and Rigby, W. F. C. (1994) J. Biol. Chem. **269**, 5377–5353
36 Housman, D. E. and Kabnick, K. S. (1988) Mol. Cell. Biol. **8**, 3244–3250
37 Sachs, A. B. (1993) Cell **74**, 413–421
38 Lee, N. H., Earle-Hughes, J. and Fraser, C. M. (1994) J. Biol. Chem. **269**, 4291–4298
39 Port, J. D., Huang, L.-Y. and Malbon, C. C. (1992) J. Biol. Chem. **267**, 24103–24108
40 Tholanikunnel, B. G., Granneman, J. G. and Malbon, C. C. (1995) J. Biol. Chem. **270**, 12787–12793
41 Inagami, T., Iwai, N., Sasaki, K., Yamamo, Y., Bardhan, S., Chaki, S., Guo, D.-F. and Furuta, H. (1992) J. Hypertens. **10**, 713–716
42 Nickenig, G. and Murphy, T. J. (1996) Mol. Pharmacol. **50**, 743–751
43 Makita, N., Iwai, N., Inagami, T. and Badr, K. F. (1992) Biochem. Biophys. Res. Commun. **185**, 142–146
44 Kislauskis, E. H., Zhu, X. and Singer, R. H. (1994) J. Cell Biol. **127**, 441–451
45 Wharton, R. P. and Struhl, G. (1991) Cell **67**, 955–967
46 Chochola, J., Fabre, C., Bellan, C., Luis, J., Bourgerie, S., Abadie, B., Champion, S., Marvaldi, J. and Battari, A. (1993) J. Biol. Chem. **268**, 2312–2318
47 Frost, G. H., Bergmann, J. S. and Carney, D. H. (1991) Biochem. Biophys. Res. Commun. **180**, 349–355

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