3′ Untranslated Region-Mediated Regulation of Luteinizing Hormone/Human Chorionic Gonadotropin Receptor Expression†

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ABSTRACT: Multiple luteinizing hormone/human chorionic gonadotropin (LH/hCG) receptor mRNAs (6.7, 4.4, 2.6, and 1.8 kb) have been identified in the rat ovary. Our laboratory has previously cloned a 3.5 kb cDNA that corresponds to the 3′ untranslated region (3′ UTR) of the 6.7 kb transcript, the major LH/hCG receptor mRNA in rat ovary. In order to determine the effects of the 3′ UTR on receptor expression, we have constructed cDNAs corresponding to the open reading frame of LH/hCG receptor or luciferase, plus these constructs with the addition of the 3′ UTRs associated with the short (4.4 kb) and long (6.7 kb) LH/hCG receptor transcripts, and measured receptor or luciferase expression in 293 cells transformed with large T antigen (293T). Ligand binding analysis with ^{125}I-hCG revealed that the 3′ UTR inhibited receptor expression, which occurs through posttranscriptional events. First, the 3′ UTRs reduced receptor mRNA half-life in actinomycin D-arrested cells, as compared to the open reading frame alone. Second, LH/hCG receptor mRNAs with the long 3′ UTR associated with significantly fewer ribosomes. The effect of the LH/hCG receptor 3′ UTRs on luciferase expression was also determined. The short 3′ UTR increased luciferase activity, whereas the long 3′ UTR decreased luciferase expression. Thus, the short 3′ UTR exerts opposite effects on receptor and luciferase expression. However, sequences in the long 3′ UTR are sufficient to inhibit both receptor and luciferase expression in 293T cells.

The interaction of luteinizing hormone (LH)[1] or its placental counterpart human chorionic gonadotropin (hCG) with its specific receptor leads to activation of adenylate cyclase, which stimulates a number of intracellular functions, including steroidogenesis (Menon & Gunaga, 1974; Clark et al., 1976; Dufau, 1988). The rat LH/hCG receptor gene spans 75−96 kb, contains 11 exons (Tsai-Morris et al., 1991; Koo et al., 1991), and is processed into multiple mRNAs in all target tissues (Wang et al., 1991). In rat ovary, one major 6.7 kb transcript, two minor transcripts of 4.4 kb and 2.6 kb, and a less abundant 1.8 kb transcript have been reported (Wang et al., 1991; Lu et al., 1993; LaPolt et al., 1990; McFarland et al., 1989).

The nucleotide sequences of the 6.7, 4.4, and 2.6 kb transcripts contain the complete 2.2 kb open reading frame plus varying 3′ untranslated regions (3′ UTRs). A 2.9 kb cDNA, which contains a 0.8 kb 3′ UTR, was identified as the 4.4 kb transcript (McFarland et al., 1989). Our laboratory has previously cloned a novel 3.5 kb cDNA that encodes the 3′ UTR of the 6.7 kb mRNA transcript (Lu & Menon, 1994). Thus, the 6.7 kb mRNA is composed of the open reading frame plus a long 3′ UTR. This newly identified 3′ UTR is an extension of the 3′ UTR found in the 4.4 kb transcript. The 2.6 kb transcript is thought to contain an alternate short 3′ UTR (Hu et al., 1994). The nucleotide identity of the 1.8 kb transcript remains unknown, but is postulated to encode an LH/hCG receptor lacking the putative transmembrane domain (Wang et al., 1991).

The mechanisms responsible for the expression of the multiple LH/hCG receptor transcripts have not been fully determined. Previously, our laboratory has demonstrated that the steady-state levels of receptor mRNA decline during ligand-induced down-regulation. As observed by Northern blot hybridization, the transcripts decline coordinately during down-regulation and reappear during subsequent recovery (Hoffman et al., 1991). The decline in steady-state mRNA was found to result from increased mRNA degradation and not decreased gene transcription (Lu et al., 1993). Thus, during homologous down-regulation, LH/hCG receptor expression is primarily regulated at a posttranscriptional level.

All three larger transcripts in rat ovary (6.7, 4.4, and 2.6 kb) appear to contain full-length open reading frames and varying 3′ UTRs. It is not known whether the 3′ UTRs present in the larger mRNAs confer differential regulation on LH/hCG receptor expression. In other systems, 3′ UTRs have been implicated in posttranscriptional regulation, specifically mRNA localization, stability, and translational efficiency (Jackson, 1993; Decker & Parker, 1995). It is possible that specific sequences in the different LH/hCG receptor 3′ UTRs may be important for differential regulation of the multiple receptor mRNAs.

To determine the possible function(s) of the 3′ UTRs, we have constructed cDNAs corresponding to the LH receptor (LHR) open reading frame, the 4.4 kb and 6.7 kb LHR mRNAs, and transfected them into human embryonic kidney (293) cells expressing T antigen (293T). We have also constructed cDNAs containing the luciferase open reading

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[1] Abbreviations: LH, luteinizing hormone; hCG, human chorionic gonadotropin; kb, kilobase(s); 3′ UTR, 3′ untranslated region; LHR, luteinizing hormone receptor.

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frame plus LHR 3′ UTRs found in the endogenous 4.4 kb and 6.7 kb transcripts and transfected them into 293T cells. Our results demonstrate that the longer 3′ UTR confers an inhibitory effect on both LH/hCG receptor and luciferase expression in 293T cells.

EXPERIMENTAL PROCEDURES

cDNA Constructs. The 3.5 kb cDNA encoding the 3′ UTR of the 6.7 kb LH/hCG receptor transcript in rat ovary was isolated and characterized as previously described (Lu & Menon, 1994). The clone containing the open reading frame plus 28 nucleotides of the 5′ UTR (2.2 kb cDNA) was obtained from Dr. William Moyle at the Robert Wood Johnson (Rutgers) Medical School (Bernard et al., 1990). A 2.9 kb cDNA resembling a previously cloned cDNA (McFarland et al., 1989) was constructed by ligating a partial cDNA clone encoding the entire 3′ UTR of the 4.4 kb transcript to the 2.2 kb cDNA clone (described above) through a *Hpa*I site. The 3.5 kb cDNA was ligated to the 2.9 kb cDNA clone through an *Eco*RI site to generate a 5.7 kb cDNA clone containing the open reading frame and the longer 3′ UTR. The 2.2, 2.9, and 5.7 kb LH/hCG receptor cDNA clones were ligated into pBK-CMV vectors (Stratagene) between the *Not*I and *Cla*I sites in the multiple cloning site.

The chimeric luciferase:LH/hCG receptor 3′ UTR cDNAs were constructed as follows. A plasmid containing a luciferase cDNA (pGEM-luc) was purchased from Promega. LH/hCG receptor 3′ UTRs present in the 4.4 and 6.7 kb mRNAs were restriction-digested with *Hpa*I and *Sal*I, resulting in the excision of the entire 3′ UTR sequence plus 7 nucleotides of the carboxy-terminal end of the open reading frame and polylinker sequences at the 3′ end. The LH/hCG receptor 3′ UTRs were subcloned into pGEM-luc between the *Stu*I and *Sal*I sites. The resultant luciferase:LH/hCG receptor 3′ UTR chimeras contain an intervening sequence consisting of 12 nucleotides of pGEM and 7 nucleotides of LH/hCG receptor open reading frame between the luciferase cDNA and LH/hCG receptor 3′ UTRs. The resultant cDNAs were cloned into expression vectors in two steps. First, the pBK-CMV vector was modified by subcloning in a cDNA containing the pBluescript SK+ polylinker between the *Sac*I/*Apa*I sites, resulting in an altered pBK-CMV multicloning site. Second, the luciferase and luciferase:LHR 3′ UTRs were directionally cloned between the *Not*I and *Sal*I sites.

Transient Transfection of 293T Cells. The method for transient calcium phosphate-mediated transfection was similar to one previously described (Sambrook et al., 1989; Kawate & Menon, 1994). Human embryonic kidney cells expressing large T antigen (293T) were a gift from Dr. G. P. Nolan at Stanford University. The cells were maintained in Dulbecco's modified Eagle's medium (Sigma) containing 10 mM HEPES, 50 μg/mL gentamicin, 2 units/mL nystatin, and 10% fetal bovine serum at 37 °C in a humidified environment containing 5% CO_2. The 293T cells were plated in 35, 60, 100, or 150 mm dishes to 30−60% confluency and transfected with 0.25, 0.75, 1.5, or 5 nmol of plasmid DNA, respectively, and harvested 60−72 h after transfection. The mRNA transcribed from the neomycin phosphotransferase gene in the pBK-CMV vector was quantitated and used as an internal control to normalize for cell number and transfection efficiency.

^{125}I-hCG Binding Assay. HCG, a gift from the National Hormone and Pituitary Program, NIH, was radiolabeled using the chloramine-T method (Catt et al., 1972) to a specific activity of 40−60 cpm/pg. Specific binding of ^{125}I-hCG to intact 293T cells was performed using a procedure previously described by this laboratory (Kawate & Menon, 1994). Nonspecific binding was determined by the addition of a 1000-fold excess of unlabeled hCG. After a 20 h incubation at 4 °C, cells were rinsed and centrifuged, and the radioactivity in the pellet was counted in a gamma counter. Scatchard analysis was performed using the EBDA/Ligand program (Munson & Rodbard, 1980). All points in the Scatchard plot were the average of duplicate determinations, and the B_{max} values were normalized to neomycin phosphotransferase mRNA, as determined by dot blot analysis.

RNA Extraction, Northern, and Dot Blot Analysis. RNA was isolated by lysing cells in guanidine isothiocyanate, 2 M sodium acetate, pH 4.0, and extracted with water-saturated phenol and chloroform/isoamyl alcohol (49:1) (Chomczynski & Sacchi, 1987). Cytoplasmic RNA was also isolated from cell lysates used for the luciferase assays as previously described (Sambrook et al., 1989). In both instances, RNA was precipitated overnight with 3 volumes of ethanol and spectrophotometrically quantified.

The procedure for the Northern blot was similar to one previously described (Davis et al., 1986). Total RNA was separated by electrophoresis in a 1.2% agarose gel containing 3% formaldehyde. RNA was blotted to Duralon-UV membranes (Stratagene) using 10× SSC (20× stock solution is 3 M NaCl and 0.3 M sodium citrate, pH 7). For dot blot analysis, RNA samples were fixed onto Duralon-UV membranes (Stratagene) using a Bio-Dot SF dot blot apparatus (Bio-Rad) as described (Sambrook et al., 1989). The RNA was cross-linked to the membrane in a UV-Stratalinker (Stratagene) at 12 mJ.

Blots were prehybridized in a solution containing 0.75 M NaCl, 0.05 M TES, 0.05 M EDTA (pH 7.1), 1× Denhardt's solution, 50% deionized formamide, and 100 μg/mL salmon sperm DNA (Sambrook et al., 1989). cDNA probes for LH/hCG receptor, neomycin phosphotransferase, and actin were radiolabeled using [α-^{32}P]dCTP (ICN) and the Klenow fragment of DNA polymerase (Feinberg & Vogelstein, 1983) and hybridized to blots overnight in fresh buffer. Blots were washed twice with 2× SSC, 0.1% SDS at room temperature for 20 min and once at 60 °C for 30 min and exposed at −80 °C to Kodak XAR film in a cassette containing intensifying screens. When indicated, blots were stripped by rinsing 3 times with boiling H_2O for 15 min.

mRNA Half-Life Measurements. Cells were originally plated at a density of 6×10^6 cells/100 mm dish and transiently transfected with LHR expression vectors. The cells were evenly divided between four 35 mm dishes 48 h after transfection. The medium was removed 12 h later and replaced with serum-free DMEM plus HEPES containing 5 μg/mL actinomycin D (Sigma). Cells were harvested at varying intervals post-actinomycin D addition, and RNA was extracted as described above. LH/hCG receptor mRNA signals from the autoradiogram were quantified in densitometric units and expressed as a percentage of mRNA remaining after actinomycin D treatment, with time 0 set as 100%. The half-life was calculated using $t_{1/2} = \ln (2/k)$, where k is the slope derived from the linear equation, $\ln [\text{LHR mRNA}] = \ln [\text{LHR mRNA}]_0 - kt$.

Isolation of Polysomes and Preparation of RNA. Polysomes were isolated as described (Chiorini et al., 1993). Cells were originally plated at a density of 6×10^6 cells/100 mm dish and transfected with LHR expression vectors. The cells were rinsed with PBS/EDTA, pH 7.2, containing 100 μg/mL cycloheximide (Sigma) 60−72 h after transfection. The cells were then resuspended in a buffer [20 mM MOPS (pH 7.2), 20 mM NaCl, 3 mM $MgCl_2$, and 150 mM sucrose] containing 50 units of RNasin (Promega) in a final volume of 450 μL and placed on ice for 10 min; 150 μL of lysis buffer [2% deoxycholate, 4% Triton X-100, 4% Tween 40, 25 mM MOPS (pH 7.2), 100 mM KCl, 5 mM $MgCl_2$, 0.1 M EDTA (pH 7.2), 1 mM DTT, and 20 μg/mL cycloheximide] was added to the mixture and incubated for 10 min at 4 °C. The cell lysate was clarified in a microfuge at maximum speed for 2 min.

The supernatant was layered over a 5 mL linear 15−40% sucrose gradient containing 25 mM MOPS (pH 7.2), 100 mM KCl, 5 mM $MgCl_2$, and 0.1 mM EDTA (pH 7.2) and centrifuged at 42K in an SW 50.1 rotor for 85 min. Samples (0.5 mL) were collected from the bottom of the gradient and fractionated through a UV detector (Gilson, Model 111B) and microfractionator (Gilson FC-80). To each sample were added 5 μL of 20% SDS and 5 μL of 10 mg/mL proteinase K, incubated at 37 °C for 30 min, and extracted with phenol/chloroform. After the addition of 0.1 volume of 3 M sodium acetate (pH 5.4), RNA was precipitated overnight with an equal volume of 2-propanol and quantified spectrophotometrically.

Luciferase Assays. Luciferase assays were performed using the Promega Luciferase Assay System. Cells were harvested 48 h posttransfection by adding 85 μL of cell culture lysis reagent buffer/35 mm plate. The supernatant was collected after a high-speed microfuge spin at 4 °C for 5 min, and assayed for protein content using the BCA reagent (Pierce). The cell lysates were then diluted to 1 mg/mL. Luminescence measurements were made over 30 s in an MGM Optocomp I luminometer, using 10 μL of cell lysate mixture diluted 1:5 (0.2 mg/mL protein) added to 100 μL of luciferase assay buffer. The relative light units (RLUs) of luciferase activity were normalized to neomycin phosphotransferase mRNA levels.

RESULTS

Effect of 3′ UTR Length on LH/hCG Receptor Expression in 293T Cells. The LH/hCG receptor cDNA constructs used in the present studies are shown in Figure 1A. The cDNAs correspond to the open reading frame plus a long 3.5 kb 3′ UTR (LHR:L3), a short 0.8 kb 3′ UTR (LHR:S3), or lacking receptor 3′ UTR (LHR). The open reading frame plus short (0.8 kb) and long (3.5 kb) 3′ UTR cDNAs correspond to the endogenous 4.4 and 6.7 kb mRNAs in rat ovary, respectively.

To determine whether the 3′ UTR confers an effect on LH/hCG receptor expression, 293T cells were transiently transfected with the cDNA constructs shown in Figure 1A, and the specific binding of ^{125}I-hCG was determined 72 h later. The data were normalized to neomycin phosphotransferase mRNA levels to account for differences in transfection efficiency and cell number. A representative Scatchard plot in Figure 2 reveals that the maximal receptor number expressed was inversely proportional to the length of the 3′ UTR. As shown in Table 1, the shorter 3′ UTR (LHR:S3) decreased the B_{max} by 70%, and the longer 3′ UTR (LHR:L3) resulted in an additional 30% decline in the B_{max}. The dissociation constants (K_ds) for the expressed receptors were comparable to those previously reported (Kawate & Menon, 1994; Segaloff & Ascoli, 1993). Thus, the 3′ UTR appears to have an inhibitory effect on the number of receptors expressed on the cell surface without affecting the binding affinity.

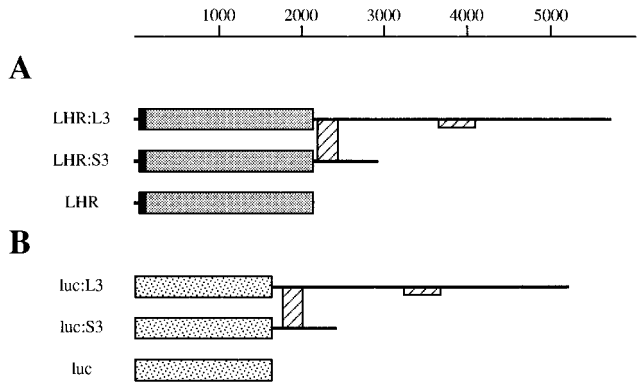

FIGURE 1: Diagram of cDNAs. cDNAs were constructed as described under Experimental Procedures. The stippled boxes correspond to open reading frames, and the solid bars within the LH/hCG receptor open reading frames represent signal peptides. Untranslated regions are depicted as straight lines. The hatched boxes indicate the locations of the AUUUA motifs. A scale in bases is shown above the left-aligned cDNAs. (A) LHR cDNAs, (B) luc:LHR 3′ UTR cDNAs.

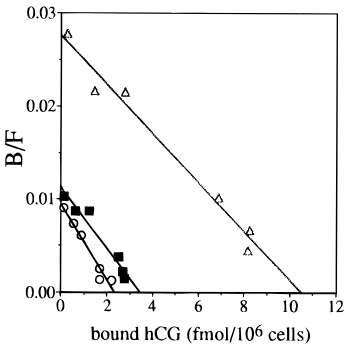

FIGURE 2: Scatchard analysis of 293T cells transfected with LH/hCG receptor constructs. Cells transfected with vectors containing the LH/hCG receptor open reading frame plus long 3′ UTR (○), plus short 3′ UTR (■), and open reading frame only (△) were incubated with varying amounts of ^{125}I-hCG to reach saturation. Each point represents the average of duplicate determinations. All points were normalized to neomycin phosphotransferase receptor mRNA, as determined by dot blot analysis. A representative result of three independent experiments is shown.

Table 1: LH/hCG Receptor Binding Constants in Transfected Cells[a]

construct	K_d (nM)	B_{max} (fmol/10^6 cells)
LHR:L3	0.34 ± 0.06	2.32 ± 0.24
LHR:S3	0.32 ± 0.01	3.23 ± 0.34
LHR	0.42 ± 0.02	10.81 ± 0.20

[a] Diagrams for LHRs are shown in Figure 1A. Binding data were calculated from three independent experiments. A representative Scatchard plot is shown in Figure 2.

Steady-State Levels of LH/hCG Receptor mRNAs in 293T Cells. To determine if the 3′ UTRs affect LH/hCG receptor mRNA processing, Northern blot analysis was performed on RNA isolated from 293T cells transfected with the cDNAs depicted in Figure 1A. The blot was hybridized with an N-terminal (NT) cDNA probe (Figure 1), and the results are

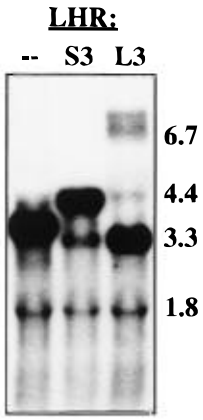

FIGURE 3: Northern blot analysis of 293T cells transfected with LH/hCG receptor constructs. Cells were transfected with vector LHR cDNAs shown in Figure 1A, and total RNA was extracted 60−72 h later. 20 μg of RNA was loaded per lane, and the Northern blot was hybridized with a cDNA probe corresponding to nucleotides 1−550 of the open reading frame. Transcript sizes of the transfected cells are shown in kb, on the right.

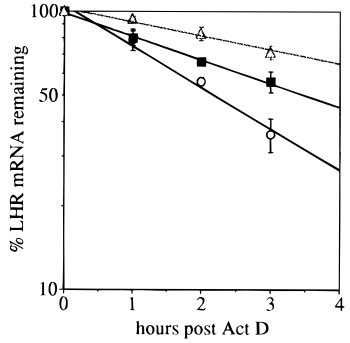

FIGURE 4: Half-life determination of LH/hCG receptor mRNAs in transfected cells. Cells were transfected with vectors containing the LH/hCG receptor open reading frame plus long 3′ UTR (○), plus short 3′ UTR (■), and open reading frame only (△). cDNA probes corresponding to nucleotides 5052−5686, 2176−3124, and 1−550 of the open reading frame plus long 3′ UTR construct were used for dot blot analysis of the LH/hCG receptor open reading frame plus long 3′ UTR (LHR:L3), plus short 3′ UTR (LHR:S3), and open reading frame only (LHR) mRNAs, respectively. The decay of LHR mRNAs is plotted as the logarithmic function of percent mRNA remaining after actinomycin D addition. Each point represents average values calculated from four independent experiments.

shown in Figure 3. As previously observed with Northern blots of LHR-transfected cells (Wang et al., 1991), multiple transcripts were observed. RNA from mock-transfected cells (mk) did not hybridize to the LH/hCG receptor cDNA probe, as expected (data not presented).

Despite the heterogeneity of mRNAs within the transfected cells, some interesting observations can be made. The most abundant transcript in 293T cells is the open reading frame (3.3 kb), followed by the mRNAs containing the short 3′ UTR (4.4 kb) and the long 3′ UTR (6.7 kb). The 6.7 kb transcript is significantly less abundant than both the open reading frame alone and its shorter 3′ UTR counterpart. Thus, the differences in steady-state levels of these transcripts may account for the decreased receptor expression observed in the ligand binding assays.

It is likely that functional polyadenylation sites for the 4.4 kb and 6.7 kb mRNAs are recognized by 293T cells, since these transcripts are similar in size in both 293T cells and rat ovary. The fact that the expression of the 6.7 kb transcript was lower than the 4.4 and 3.3 kb transcripts suggests that the long 3′ UTR may contain *cis* acting elements that are involved in decreased mRNA expression.

To ensure that posttranscriptional effects are attributed to the specific transcript encoding the short and long 3′ UTRs of interest, transcript-specific cDNA probes that correspond to the 3′ end of the mRNA were used in subsequent dot blot analysis. The specificity of these probes has previously been verified by Northern blot hybridization (Lu & Menon, 1994).

Effect of 3′ UTRs on LH/hCG Receptor mRNA Half-Life. The decrease in receptor expression in cells transfected with the open reading frame plus 3′ UTRs may be due, in part, to decreased mRNA stability. To examine this possibility, the half-lives of the LH/hCG receptor mRNAs (cDNA diagrams shown in Figure 1A) were determined in actinomycin D-arrested cells. The expression of LH/hCG receptor mRNAs as a function of the percentage of mRNA remaining after actinomycin D treatment is shown in Figure 4. Each value represents an average of four independent experiments, with 100% representing the mRNA content at the start of the experiment (time 0). The average half-lives of the mRNAs were calculated as 2.0 h (LHR:L3), 3.6 h (LHR:S3), and 6.0 h (LHR) in 293T cells. The difference in the mRNA half-lives may, in part, explain the changes in receptor expression.

Polysomal and Subpolysomal Fractionation of LH/hCG Receptor mRNAs in Transfected Cells. Another possibility that may account for the 3′ UTR-mediated decline in LH/hCG receptor expression is a decrease in translational efficiency, since previous studies have demonstrated that 3′ UTRs may play such a role in other systems (Dickey et al., 1988; Kruys et al., 1987). To examine this possibility, polysomal and subpolysomal fractions were separated on sucrose density gradients to determine ribosomal coupling to LH/hCG receptor mRNAs. A sucrose density gradient fractionation that illustrates the representative distribution of the polysomal and subpolysomal fractions is shown in Figure 5A. RNA was extracted from each of the eight fractions, transferred to nylon membranes, and hybridized with LHR cDNAs. The LHR mRNA distribution in polysomal and subpolysomal fractions is shown in Figure 5B. The polysome profiles of LH/hCG receptor mRNA distribution were repeated 3 times with similar results, and the data from a representative experiment are presented in Figure 5.

As shown in Figure 5B, the 6.7 kb mRNA (LHR:L3) peaked in fractions 5 and 6, the lighter polyribosome fractions. Conversely, the 4.4 kb (LHR:S3) and open reading frame (LHR) mRNAs shifted to the heavier polyribosome fractions. Since the 6.7 kb mRNA was found to bind fewer ribosomes, it is less efficiently translated than its counterparts with shorter 3′ UTRs. In order to ensure that the changes in ribosomal coupling seen with the longer 3′ UTR were specific to LH/hCG receptor mRNA, we examined the translational efficiency of endogenous actin mRNA since it has been shown to be independent of translational regulation (Degen et al., 1983). The dot blots from Figure 5B were stripped and rehybridized with an actin cDNA. All three constructs show similar polysome profiles for actin (Figure 5C).

The polysome profiles reveal that the LH/hCG receptor open reading frame plus short 3′ UTR (LHR:S3) and the open reading frame (LHR) mRNAs show similar translational

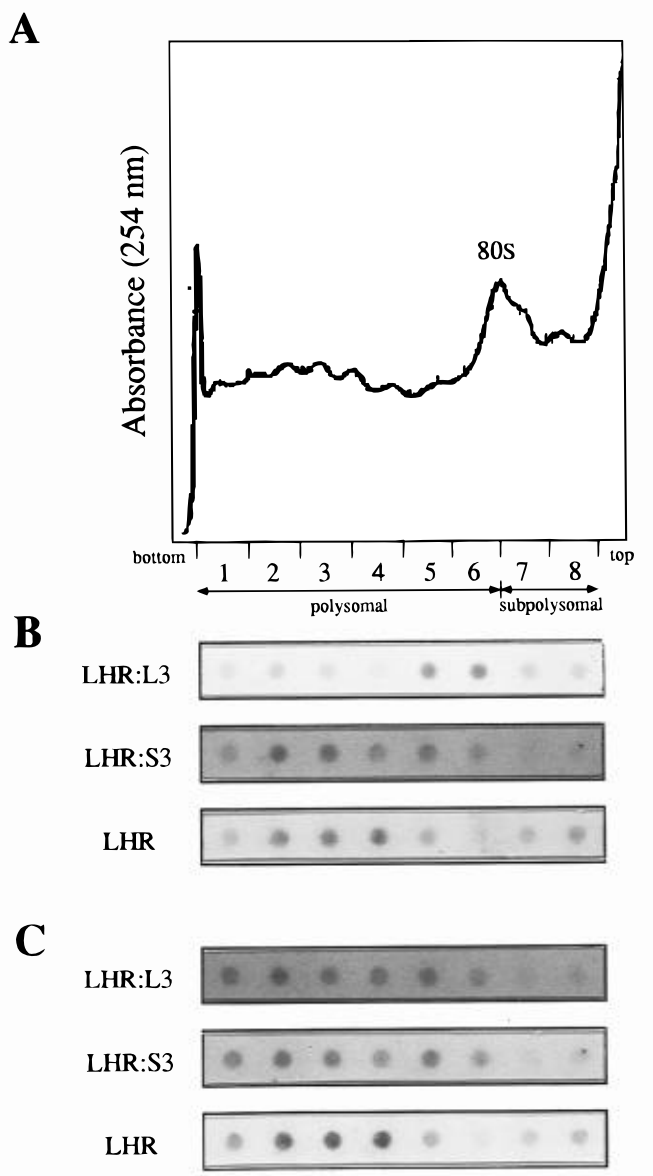

FIGURE 5: Polysome distribution of LHR and actin mRNAs in transfected cells. (A) Representative absorbance profile of polysomes and subpolysomal particles fractionated on sucrose gradients. Supernatant was isolated from 293T cells 60−72 h after transfection. A_{254} was continuously monitored, and fractions were collected from the bottom to the top of the gradient. Fractions 1−6 represent polysomal fractions, and fractions 7−8 represent subpolysomal fractions. The monosome peak (80S) is indicated. (B) Distribution of LHR mRNAs in polysomal (1−6) and subpolysomal (7−8) fractions. Cells were transfected with vectors containing the LH/hCG receptor open reading frame plus long 3′ UTR (LHR:L3), plus short 3′ UTR (LHR:S3), and open reading frame only (LHR). RNA was isolated from polysomal and subpolysomal fractions, and hybridized with the same cDNA probes used in Figure 3. (C) Distribution of actin mRNAs in polysomal (1−6) and subpolysomal (7−8) fractions. Dot blots from panel B were stripped and rehybridized with actin.

efficiencies whereas the open reading frame plus long 3′ UTR (LHR:L3) mRNA is shifted toward smaller polysomes. Thus, it appears that the long LHR 3′ UTR mediates a decrease in translational efficiency by associating with fewer ribosomes. Together with the decrease in mRNA half life, the decline in receptor expression seen with the long 3′ UTR may be attributed to both decreased mRNA stability and translational efficiency.

Effect of the 3′ UTRs on Luciferase Expression. In addition to examining the polysome profiles of LH/hCG receptor mRNAs, we also examined the effect of the 3′ UTR sequences on the expression of a reporter gene, luciferase. Luciferase was used as a reporter gene because it requires no posttranslational modification for activation, and its assay is highly sensitive. Furthermore, luciferase remains stable even when overexpressed, so the enzymatic activity becomes a direct measure of the relative efficiencies of the translation of the chimeric mRNAs (Grens & Scheffler, 1990).

Table 2: Luciferase Activity in 293T Cells Transiently Transfected with Constructs Containing Luciferase plus LH/hCG Receptor 3′ UTRs[a]

construct	RLUs ($\times 10^6$/mg of protein)	% of control
luc:L3	2.03 ± 0.97	40
luc:S3	12.53 ± 2.01	247
luc	5.07 ± 1.05	100

[a] Diagrams for luc constructs are shown in Figure 1B. Luciferase activity is expressed as relative light units (RLUs) normalized to neomycin phosphotransferase RNA per milligram of protein in the cell lysate. The values are averaged from triplicate determinations and are representative of three independent experiments.

To determine whether the long 3′ UTR confers inhibitory effects on luciferase, we constructed chimeras containing luciferase plus the short and long LH/hCG receptor 3′ UTRs, as shown in Figure 1B. The effect of the LH/hCG receptor 3′ UTRs on luciferase expression in 293T cells is shown in Table 2. The long 3′ UTR (luc:L3) decreased luciferase expression as compared to both luciferase (luc) alone and luciferase plus short 3′ UTR (luc:S3).

In contrast to the ligand binding data, the short 3′ UTR (luc:S3) stimulated luciferase expression, as compared to luciferase (luc) alone. It is likely specific interaction(s) between sequences present in the short 3′ UTR and the LH/hCG receptor open reading frame and/or 5′ UTR resulted in decreased receptor expression. These specific interactions would not be present with the luciferase construct, which could explain why the short 3′ UTR alone stimulates luciferase expression. However, sequences present in the long 3′ UTR are sufficient to confer an inhibitory effect on luciferase expression. Thus, the difference in translational control mediated by the short and long 3′ UTRs on LH/hCG receptor expression extends to luciferase as well.

DISCUSSION

The largest and most predominant LH/hCG receptor mRNA in rat ovary (6.7 kb) contains a long 3′ UTR. Here we demonstrate that the 3′ UTR exerts an inhibitory influence on the expression of cell surface receptors in transfected cells. Furthermore, mRNA transcribed from the LH/hCG receptor construct containing the open reading frame plus the long 3′ UTR (representing the endogenous 6.7 kb transcript in rat ovary) has a shorter mRNA half-life and associates with significantly fewer ribosomes. The long 3′ UTR also decreases luciferase expression as compared to the shorter 3′ UTR of the 4.4 kb transcript. Our studies provide insight into the posttranscriptional regulation of the LH/hCG receptor, especially with regard to the 3′ UTR and its inhibitory effect on receptor expression.

The decrease in the number of receptors on the cell surface seen in cells transfected with receptor cDNAs containing the long 3′ UTR could be regulated at the gene, mRNA, or protein level. Since the expression of all the LH/hCG receptor cDNAs is mediated by the same promoters (CMV

and SV40), it is likely that transcription of all the cDNAs occurs at a similar rate. This assertion has been indirectly verified by hybridization of the Northern blot in Figure 3 with the neomycin phosphotransferase cDNA probe. Since the neomycin phosphotransferase gene is encoded on the same vector as the LH/hCG receptor cDNAs, it serves as a measure for the relative transcription rate of the plasmid. The signals for neomycin phosphotransferase are consistent in 293T cells transfected with the varying constructs (data not shown), suggesting that the transcription rates of the different cDNAs are comparable.

Conversely, since all the transcripts contain the same open reading frame, once the protein is translated, it is unlikely that the 3′ UTRs would be involved in posttranslational regulation of the newly synthesized receptor. Thus, 3′ UTR-mediated regulation most likely occurs posttranscriptionally. Our data show that the 3′ UTR mediates its inhibitory effects on receptor expression through decreased mRNA stability and ribosomal coupling.

The shorter half-life conferred by the long 3′ UTR, as compared to the short 3′ UTR, may be due to the abundance of AUUUA motifs present in the longer 3′ UTR. In LH/hCG receptor mRNAs, the 3′ UTRs contain several AUUUA motifs, 4 in the short 3′ UTR and 11 in the long 3′ UTR (Lu & Menon, 1994). Thus, there are seven AUUUA motifs unique to the longer 3′ UTR. Since AUUUA motifs have been shown to be involved in the regulation of mRNA stability (Shaw & Kamen, 1986), the presence of additional AUUUA motifs in the longer 3′ UTR may confer greater instability to its mRNA.

The construction of chimeric luciferase:LH/hCG receptor 3′ UTR cDNAs and the subsequent measurement of luciferase activity enable the direct measurement of the posttranscriptional control exerted by the 3′ UTRs. The 6-fold change in luciferase expression conferred by the short and long LH/hCG receptor 3′ UTRs illustrates the differential control mediated by the two 3′ UTRs. The stimulation of luciferase expression by the short 3′ UTR and the inhibition of receptor expression exerted by the same 3′ UTR suggest that sequences within the short 3′ UTR may interact with LH/hCG receptor-specific sequences to decrease receptor expression. However, the inhibitory effect of the long 3′ UTR on LH/hCG receptor expression also extends to a decrease in luciferase activity. Thus, it appears that sequences in the long 3′ UTR are sufficient to decrease both LH/hCG receptor and luciferase expression.

Our finding that the longer 3′ UTR confers increased destabilization and decreased translational efficiency may be compatible with a model where the destabilization of short-lived mRNAs requires ongoing translation of that message (Sachs, 1993). It has been observed that protein synthesis inhibitors can stabilize short-lived mRNAs, leading many to conclude that translation is a requirement for mRNA degradation, or that mRNA destabilization requires the continuous synthesis of a protein required for degradation (Sachs, 1993).

In the case of an mRNA containing the hepatitis B virus S-antigen open reading frame plus a 3′ UTR of the AU-rich region of GM-CSF mRNA, AUUUA-mediated mRNA degradation proceeds only when the message is undergoing active translation (Aharon & Schneider, 1993). Alternately, the degradation of c-*fos* may not involve a corequisite for translation (Koeller et al., 1991). Although there appears to be no general correlation between mRNA decay rates and the efficiency of translational initiation, these two events may be related in some systems (Sachs, 1993). It has also been determined that 3′ UTR length plays an important role in determining both the translational efficiency and stability of an mRNA (Tanguay & Gallie, 1996), which is consistent with our results. It is possible that decreased mRNA−ribosome associations may result in increased mRNA turnover or vice versa, since the interrelatedness of these two processes has been demonstrated in other systems.

Only a few examples where translational inhibition by the 3′ UTR involves decreased ribosome coupling have been reported. In addition to the long LH/hCG receptor mRNA, interferon β and *C. elegans* fem3 mRNA share a similar phenomenon (Kruys et al., 1990; Goodwin et al., 1993). The critical sequence in the long LH/hCG receptor 3′ UTR that mediates translational efficiency has not yet been determined, which may enable a further understanding of how 3′ UTRs can influence translation.

In rat ovary, differences in LH/hCG receptor number seen during follicular development, ovulation, and luteinization involve concomitant changes in receptor mRNA levels (Segaloff & Ascoli, 1993). Since levels of LH/hCG receptor mRNA closely parallel receptor number, it is likely that posttranscriptional regulation has a pivotal role in mediating physiological changes in receptor expression seen during the ovarian cycle. Moreover, rapid changes in LH/hCG receptor expression have been shown to occur following hormonal stimulation (Segaloff & Ascoli, 1993). An example of the dramatic, posttranscriptional change in LH/hCG receptor mRNA expression in response to high hormone levels has already been demonstrated. We have previously shown that ligand-induced down-regulation of the receptor involves increased mRNA degradation and not decreased gene transcription (Lu et al., 1993).

In conclusion, we have shown that the 3′ UTR of the 6.7 kb transcript in rat ovary exerts an inhibitory effect on both LH/hCG receptor and luciferase expression in 293T cells. This 3′ UTR-mediated effect on receptor expression appears to be due to a decrease in both ribosomal coupling and mRNA stability.

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REFERENCES

Aharon, T., & Schneider, R. J. (1993) *Mol. Cell. Biol. 13*, 1971−1980.

Bernard, M. P., Myers, R. V., & Moyle, W. R. (1990) *Mol. Cell. Endocrinol. 71*, R19−R23.

Catt, K. J., Dufau, M. L., & Tsuruhara, T. (1972) *J. Clin. Endocrinol. Metab. 34*, 123−132.

Chiorini, J. A., Boal, T. R., Miyamoto, S., & Safer, B. (1993) *J. Biol. Chem. 268*, 13748−13755.

Chomczynski, P., & Sacchi, N. (1987) *Anal. Biochem. 162*, 156−159.

Clark, M., Azhar, S., & Menon, K. M. J. (1976) *Biochem. J. 158*, 175−182.

Davis, L. G., Dibner, M. D., & Battey, J. F. (1986) *Methods in Molecular Biology*, Elsevier, New York.

Decker, C. J., & Parker, R. (1995) *Curr. Opin. Cell Biol. 7*, 386−392.

Degen, J. L., Neubauer, M. G., Degen, S. J., Seyfried, C. E., & Morris, D. R. (1983) *J. Biol. Chem. 258*, 12153−12162.

Dickey, L. F., Wang, Y. H., Shull, G. E., Wortman, I. A., & Theil, E. C. (1988) *J. Biol. Chem. 263*, 3071−3074.
Dufau, M. L. (1988) *Annu. Rev. Physiol. 50*, 483−508.
Feinberg, A. P., & Vogelstein, B. (1983) *Anal. Biochem. 132*, 6−13.
Goodwin, E. B., Okkema, P. G., Evans, T. C., & Kimble, J. (1993) *Cell 75*, 329−339.
Grens, A., & Scheffler, I. E. (1990) *J. Biol. Chem. 265*, 11810−11816.
Hoffman, Y. M., Peegel, H., Sprock, M. J. E., Zhang, Q. Y., & Menon, K. M. J. (1991) *Endocrinology 128*, 388−393.
Hu, Z. Z., Buczko, E., Zhuang, L., & Dufau, M. L. (1994) *Biochim. Biophys. Acta 1220*, 333−337.
Jackson, R. J. (1993) *Cell 74*, 9−14.
Kawate, N., & Menon, K. M. J. (1994) *J. Biol. Chem. 269*, 30651−30658.
Koeller, D. M., Horowitz, J. A., Casey, J. L., Klausner, R. D., & Harford, J. B. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7778−7782.
Koo, Y. B., Ji, I., Slaughter, R. G., & Ji, T. H. (1991) *Endocrinology 128*, 2297−2308.
Kruys, V., Wathelet, M., Poupart, P., Contreras, R., Fiers, W., Content, J., & Huez, G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6030−6034.
Kruys, V., Beutler, B., & Huez, G. (1990) *Enzyme 44*, 193−202.
LaPolt, P. S., Oikawa, M., Jia, X. C., Dargan, C., & Hsueh, A. J. W. (1990) *Endocrinology 126*, 3277−3279.
Lu, D. L., & Menon, K. M. J. (1994) *Eur. J. Biochem. 222*, 753−760.
Lu, D. L., Peegel, H., Mosier, S. M., & Menon, K. M. J. (1993) *Endocrinology 132*, 235−240.
McFarland, K. C., Sprengel, R., Phillips, H. S., Kohler, M., Rosemblit, N., Nikolics, K., Segaloff, D. L., & Seeburg, P. H. (1989) *Science 245*, 494−499.
Menon, K. M. J., & Gunaga, K. P. (1974) *Fertil. Steril. 25*, 732−750.
Munson, P. J., & Rodbard, D. (1980) *Anal. Biochem. 107*, 220−239.
Sachs, A. (1993) *Cell 74*, 413−421.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning—A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Segaloff, D. L., & Ascoli, M. (1993) *Endocr. Rev. 14*, 324−347.
Shaw, G., & Kamen, R. (1986) *Cell 46*, 659−667.
Tanguay, R. L., & Gallie, D. R. (1996) *Mol. Cell. Biol. 16*, 146−156.
Tsai-Morris, C. H., Buczko, E., Wang, W., Xie, X.-Z., & Dufau, M. L. (1991) *J. Biol. Chem. 266*, 11355−11359.
Wang, H., Ascoli, M., & Segaloff, D. L. (1991) *Endocrinology 129*, 133−138.

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