

Regulatory role of the 3' untranslated region of luteinizing hormone receptor: effect on mRNA stability

Anil K. Nair, K.M.J. Menon*

Departments of Obstetrics/Gynecology and Biological Chemistry, University of Michigan Medical School, 6428 Medical Sciences Building I, 1301 Catherine Street, Ann Arbor, MI 48109-0617, USA

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Abstract Posttranscriptional regulation of luteinizing hormone receptor (LHR) mRNA has a significant role in regulating cell surface receptor expression during ovarian cycle. In order to gain insight into the mechanism of posttranscriptional regulation, the *cis*-acting elements in the 3' untranslated region (3' UTR) of LHR mRNA were examined by transfection studies followed by measurements of the receptor expression and receptor mRNA half-life. The results show that the inhibitory effect exerted by the 3.51 kb 3' UTR of the LHR resides in the distal 1.98 kb region. Half-life measurement of LHR mRNA showed that the inhibitory effect of the 3' UTR was due to a decrease in receptor mRNA stability. Deletion studies revealed that the entire 1.98 kb region is required for LHR mRNA destabilization. Although the 3.51 kb 3' UTR of LHR contains 11 AUUUA motifs, their removal had no effect on mRNA stability or receptor expression. Thus, although AUUUA motifs have been implicated in mRNA stability, these motifs do not appear to play an inhibitory role in LHR expression. The 3' UTR of LHR was also able to decrease the expression of a reporter gene indicating that the inhibitory effect of 3' UTR is not unique to the open reading frame of LHR. The present studies show that the distal 1.98 kb portion of the 3' UTR exerts an inhibitory effect on the expression of LHR by decreasing the receptor mRNA half-life. The inhibitory effect of 3' UTR might play a role in the maintenance of the steady state levels of the receptor mRNA under different physiological states.

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1. Introduction

Luteinizing hormone (LH) plays a crucial role in regulating gonadal function in all vertebrates through its interaction with a specific, high affinity cell surface receptor. Interaction of ligand with cell surface receptor leads to the activation of adenylate cyclase resulting in an increase in the intracellular cyclic AMP levels. These in turn evoke a sequence of molecular events, which control ovulation, luteinization and steroidogenesis [1,2]. The luteinizing hormone receptor (LHR) belongs to the family of guanine nucleotide binding protein (Gs) coupled receptors [3,4]. In the ovary, multiple transcripts of LHR have been observed [5,6]. In rat ovary, four LHR mRNA transcripts have been identified: a major 6.7 kb transcript and 4.3 and 2.6 kb forms and a less abundant 1.2 kb

transcript [5–7]. All transcripts except the 1.2 kb contain a 2 kb open reading frame with varying length of 3' untranslated regions (3' UTR). The nucleotide identity of the 1.2 kb transcript remains unclear, although it is postulated to encode an LH receptor lacking the transmembrane domain [8,9].

Rat ovarian LHR expression has been shown to be regulated in physiological states such as during the preovulatory LH surge as well as in response to a pharmacological dose of human chorionic gonadotropin (hCG), a placental counterpart of LH [7,10]. A selective decline in the steady state levels of all four LHR mRNA transcripts has been observed during hormone induced down regulation [6,10,11]. We have shown that LHR mRNA expression starts decreasing 6 h after injection of hCG and completely disappears within 24 h, followed by a recovery between 24 and 48 h [6,11]. This selective decline in the steady state levels of receptor mRNA is not due to decreased transcription, but occurs posttranscriptionally with a rapid degradation of mRNA [6]. The mechanism of the selective degradation of the LHR mRNA is not understood.

In eukaryotes a number of *cis*-acting sequences and *trans*-acting cytoplasmic proteins have been identified as mRNA stability determinants (reviewed in [15]). Most of the well characterized sequence determinants altering the half-life of mRNA are present in the 3' UTR. The 3' terminal stem-loop structure of histone mRNA, the iron-responsive elements in the 3' UTR of transferrin receptor mRNA and the 3' UTR long-range stem-loop structure of insulin-like growth factor II mRNA are some of the well characterized *cis*-acting elements that determine eukaryotic mRNA stability [12–15]. A number of cytoplasmic proteins, some of which shuttle between nucleus and cytoplasm, have been identified as *trans*-acting factors [16–20]. AU-rich elements (ARE), mainly AUUUA motifs, present in 3' UTR of many cytokine, oncogene and transcription factor mRNAs, have been implicated as the determinants of mRNA stability (reviewed in [15]). The destabilization activity of AREs can either be increased or decreased as a result of interaction with *trans*-acting factors [16,19,21]. The most abundant LHR transcript (6.7 kb) in rat ovary contains a 3.51 kb 3' UTR with 11 AUUUA motifs [22]. This long 3' UTR exerts an inhibitory effect on receptor expression [23]. The present studies were carried out to identify the region(s) in the LHR 3' UTR that decreases receptor expression and understand the mechanism(s) of control.

Our results indicate that the presence of distal 1.98 kb 3' UTR of LHR destabilizes the mRNA and thus decreases receptor expression. Furthermore this decrease cannot be attributed to the presence of AUUUA motifs present in the destabilizing region.

*Corresponding author. Fax: (1)-734-936-8617.

2. Materials and methods

2.1. Plasmid construction

cDNAs corresponding to open reading frame (ORF) of rat LHR (O 2.1 kb), ORF of LHR plus entire 0.73 kb 3' UTR of 4.4 kb transcript (S 2.9 kb) and ORF of LHR plus 3.51 kb 3' UTR of 6.7 kb transcript (L 5.7 kb) were ligated into pBK-CMV vector (Stratagene) between *NotI* and *Clal* sites in the multiple cloning site as described previously [23]. Construct D1 has a deletion of 647 nucleotides (nt) (between nt 1528 and 2175) from the 3.51 kb 3' UTR associated with 6.7 kb transcript. Since the 3' UTR has two sites for *AflIII* at positions 1528 and 2175, D1 was generated by digesting the plasmid containing 5.7 kb LHR cDNA (ORF+3.51 kb 3' UTR) with *AflIII*. The digested plasmid was religated with T4-DNA ligase. Construct D2 was generated by digesting the plasmid containing 5.7 kb LHR cDNA (ORF+3.51 kb 3' UTR) with *AflIII* and *Clal*, deleting the distal 1.98 kb region. Protruding ends were made blunt by treating with T4-DNA polymerase. The blunt ended plasmid was religated using T4-DNA ligase.

Construct D3 containing the ORF of rat LHR (2.1 kb) plus the distal 1.98 kb 3' UTR was constructed as follows. Construct L was digested with *HindIII/Clal* to isolate the distal 1.98 kb region, having *HindIII* site at 5' end and *Clal* site at 3' end. This 1.98 kb 3' UTR was then ligated to pBluescript SK(+) between *HindIII/Clal* site. The open reading frame of LHR with 28 nt of 5' UTR was then ligated to the pBluescript SK(+) having the distal 1.98 kb 3' UTR between *NotI* and *BamHI* site. The resultant clone contains an intervening sequence of 24 nt of the multiple cloning site of pBluescript SK(+) between ORF of LHR and distal 1.98 kb 3' UTR. The cDNA thus obtained having ORF of LHR with distal 1.98 kb 3' UTR was then ligated into the expression vector pBK-CMV between *NotI* and *Clal* sites.

2.1.1. Chimeric luciferase. cDNAs of luciferase open reading frame (OL) with the short 3' UTR (SL) of 4.4 kb LHR transcript and long 3' UTR of 6.7 kb transcript (LL) were cloned into pBK-CMV vector as described previously [23]. Deletion constructs DL3, DL4, DL5 and DL6 were made by digesting the construct LL with corresponding restriction enzymes. Constructs DL4 and DL6 were prepared by digesting LL with *BglIII/SmaI* and *SacI/SmaI* enzymes respectively. Constructs DL3 and DL5 were constructed by digesting LL with *KpnI* and *HindIII* respectively. The 5' and 3' protruding ends of DNAs were treated with Klenow fragment or T4-polymerase to make blunt ends. The resulting blunt ends were ligated by treatment with T4-ligase.

2.2. Transient expression of LH receptor or luciferase protein in 293 T cells

Human embryonic kidney cells expressing large T antigen (293 T) 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 atmosphere containing 5% CO₂. Exponentially growing cells were plated at 40 to 50% confluency in 35 mm (1–2 × 10⁶ cells) and 100 mm (4–5 × 10⁶ cells) dishes 6 h before transfection. Plasmids were transiently transfected into 293 T cells at 0.25 (35 mm dish) and 1.5 pmol (100 mm dish) concentrations by calcium phosphate coprecipitation technique [24]. Cells were harvested at 48 h for luciferase activity and 72 h for LHR expression studies.

2.3. ¹²⁵I-hCG binding assay

Radioiodination of hCG was performed using Chloramine-T method [25] to give a specific activity of 40–60 cpm/pg. Binding of ¹²⁵I-hCG to intact cells was performed as described previously [24] by incubating the cells at 4°C for 20 h with increasing concentrations of ¹²⁵I-hCG (3–120 ng/ml). Non-specific binding was determined by 1000-fold excess of unlabelled hCG. Maximal binding capacity (*B*_{max}) and equilibrium dissociation constant (*K*_d) were calculated from the binding data using Scatchard analysis [26]. The data were normalized for cell number by DNA and transfection efficiency by neomycin phosphotransferase mRNA (dot-blot) encoded in the pBK-CMV vector. Specific binding was determined by subtracting the non-specific binding from the total binding. All determinations were performed in duplicate.

2.4. Measurement of luciferase activity

Cells were lysed 48 h after transfection with 85 µl of cell culture lysis buffer (25 mM Tris-phosphate pH 7.8, 2 mM DTT, 2 mM 1,2-

diaminocyclohexamine-*N,N,N',N'*-tetra acetic acid, 10% glycerol and 1% Triton X-100) and centrifuged briefly (5–10 s) at 12 000 × *g* to pellet debris. Five µl of the extract was added to 400 µl of luciferase assay buffer (25 mM glycyl glycine pH 7.8, 15 mM MgSO₄, 15 mM KPO₄, 4 mM EGTA, 1 mM DTT and 1 mM ATP) and reaction was initiated with the injection of 100 µl of 200 µM luciferin in luciferase assay buffer. Luciferase activity in relative light units (RLUs) was measured for 20 s with Monolight 2010 Luminometer (Analytical Luminescence Laboratory). RLUs were normalized to neomycin phosphotransferase mRNA for transfection efficiency. Protein concentration in the cell extract was determined by BCA reagent (Pierce).

2.5. Measurement of LHR mRNA half-life

293 T cells plated in 100 mm dishes were transiently transfected with LHR plasmids at 1.5 pmol concentration. For each construct, approximately 4–5 × 10⁶ cells were evenly divided between five 35 mm dishes 48 h after transfection. After 12 h, cells were exposed to serum-free DMEM containing 5 µg/ml actinomycin D. Treatment of cells with 5 µg/ml actinomycin D blocked transcription without affecting cell viability. The cells were harvested at 0, 1, 2, 3 and 4 h following actinomycin D addition. Following extraction by the method of Chomczynski and Sacchi [27], 5 µg of RNA were fixed to nitrocellulose membranes and hybridized with radiolabelled cDNA probes (cDNA probes corresponding to nucleotides 1559–2206, 2907–3511, 980–1200 and 53–693 of the 3.51 kb 3' UTR and 1951–2697 of construct L were used for mRNAs encoded by constructs L, D1, D2, S and O respectively) as described below. The blots were exposed to Kodak XAR film in a cassette containing intensifying screens at –70°C and signals were quantitated in densitometric units. Signals were normalized to 18S rRNA to account for the variation in quantity of RNA applied. Half-life was calculated using the equation $t_{1/2} = \ln 2/k$ where *k* is the slope derived from the linear equation $\ln C = \ln C_0 - kt$, where *C* is the concentration of mRNA at time '*t*'.

2.6. RNA extraction and dot-blot analysis

RNA was extracted from cell lysates by the method of Chomczynski and Sacchi [27] and quantitated spectrophotometrically. Aliquots of RNA were fixed to nitrocellulose membrane with 10 × SSC using a Bio-Dot SF dot-blot apparatus (Bio-Rad). Blots were cross-linked in a UV stratalinker (Stratagene) at 12 mJ and 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 0.5 mg/ml salmon sperm DNA at 42°C for 2 h. cDNA probes for LH receptor, neomycin phosphotransferase and 18S rRNA were radiolabelled using [α -³²P]dCTP (ICN) and Klenow fragment of DNA polymerase [28] and hybridization was performed overnight at 42°C in fresh buffer. The blots were then washed four times with 2 × SSC containing 0.1% SDS for 10 min each at room temperature and once at 60°C for 30 min. The blots were stripped by rinsing three times with 0.1 × SSC containing 0.1% SDS at boiling temperature for 10 min if they were to be reblotted with a different cDNA probe.

3. Results

3.1. Effect of 3' UTR on the LH receptor expression

In order to identify specific region(s) of the long 3' UTR that is responsible for the inhibitory effect on LH receptor expression, cDNA constructs were made with ORF of LHR containing varying lengths of 3' UTR as represented in Fig. 1. A second goal of the study was to determine if AUUUA motifs play any role in LHR mRNA stability. LHR 3' UTR has 11 AUUUA motifs. Four AUUUA motifs are distributed within the first 270 nucleotides and the remaining seven are present between nucleotides 1497 and 1926. Construct L has the entire 3.51 kb LHR 3' UTR with 11 AUUUA motifs. The construct D1 represents 2.87 kb 3' UTR with five AUUUA motifs. This construct is devoid of 647 nucleotides from the 3.51 kb 3' UTR spanning nucleotides 1528 to 2175 containing six AUUUA motifs. The construct D2 has the first 1.52 kb 3' UTR with five AUUUA motifs. Construct S contains the first 730 nucleotides of 3' UTR with four AUUUA

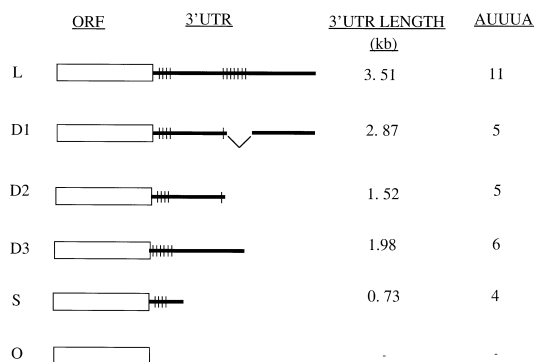


Fig. 1. LHR constructs used for transient transfection. Constructs were made as described in Section 2. Boxes indicate open reading frames and bars represent 3' UTR. The vertical slash represents the AUUUA motif, deleted region as space and V shaped line underneath the space indicates the newly joined sequences. The length of 3' UTR (in kb) and number of AUUUA motifs present in each construct are also shown.

motifs. These constructs were cloned into the mammalian expression vector, pBK-CMV. All constructs contained 28 nucleotides of the LHR 5' UTR.

To examine the role of 3' UTR in LHR expression, the constructs were transiently transfected into 293 T cells and the receptor expression was examined by ^{125}I -hCG binding assay. The results of the binding assay are presented in Fig. 2 as Scatchard plot. The B_{max} and K_{d} values calculated from Fig. 2 are summarized in Table 1. The 3.51 kb 3' UTR produced a decrease in receptor expression (B_{max} 3.0 fmol/ μg DNA) without affecting the K_{d} compared to receptor expression produced by construct O (B_{max} 7.07 fmol/ μg DNA). The construct D2, which lacks distal 1.98 kb 3' UTR, increased receptor expression (B_{max} 6.04 fmol/ μg DNA) compared to cells transfected with the construct L suggesting that the 1.98 kb distal 3' UTR is responsible for decreasing receptor expression. The construct S which contains the first 730 nucleotides of the 3' UTR produced an increase in receptor expression (B_{max} 8.44 fmol/ μg DNA) compared to those transfected with construct O which contains only the open reading frame. These results suggest that the first 0.73 kb 3' UTR has a stimulatory effect on receptor expression.

The LHR expressions produced by constructs L, D1 and S were compared for analyzing the role of AUUUA motifs on receptor expression. The constructs in Fig. 2 show differences in the number of AUUUA motifs. Construct L contains all 11 AUUUA motifs. Constructs D1 and S contain the first five and four AUUUA motifs respectively in their 3' UTR. The removal of six AUUUA motifs present within nucleotides 1528 to 2175 of 3' UTR had no discernable effect on receptor expression as the construct D1, which lacks these AUUUA

Table 1
Binding constants of LH/hCG receptor constructs

Construct	B_{max} (fmol/ μg DNA)	K_{d} (nM)
L	3.00	0.7
D1	4.00	0.6
D2	6.04	0.5
S	8.44	0.5
O	7.07	0.5

The B_{max} and K_{d} were calculated using the binding data from Fig. 2 as described in Section 2.

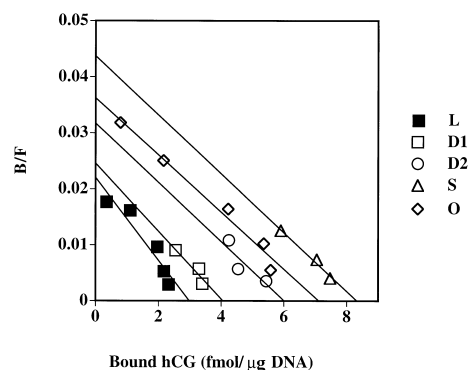


Fig. 2. Scatchard analysis. 293 T cells were transiently transfected with LHR constructs (shown in Fig. 1). Receptor expression was measured by ^{125}I -hCG binding assay, as described in Section 2. Each point represents average of three determinations for total binding and two determinations for non-specific binding. Data were normalized to neomycin phosphotransferase mRNA by dot-blot assay, for transfection efficiency and to DNA for cell number. A representative result of three independent experiments is shown.

motifs and construct L having all 11 AUUUA motifs, produced comparable levels of receptor expression. The lack of inhibitory effect of AUUUA motifs is further evidenced by the absence of an inhibitory effect by construct S which has four AUUUA motifs. Although the construct S contains the first four AUUUA motifs, the receptor expression showed an increase. These observations reveal that the AUUUA motifs present in the 3' UTR do not appear to play any role in decreasing LHR receptor expression.

3.2. Effect of LHR 3' UTR on luciferase expression

To determine the specificity of 3' UTR mediated inhibition of LHR, the effect of the 3' UTR on the expression of a reporter gene was examined. For this purpose, the chimeric constructs containing the ORF of luciferase reporter gene with varying lengths of 3' UTR were prepared (Fig. 3). The chimeric constructs cloned into pBK-CMV expression vectors were then transiently transfected into 293 T cells, and luciferase

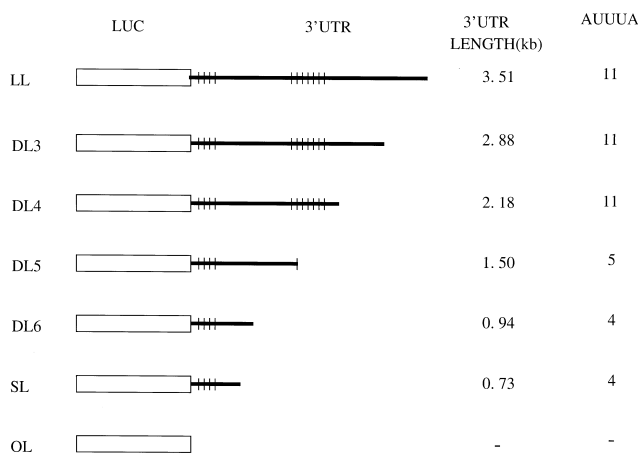


Fig. 3. Chimeric luciferase: LHR 3' UTR constructs used for transient transfection. Constructs were made as described in Section 2. Constructs vary in length of LHR 3' UTR. The length of 3' UTR (in kb) and number of AUUUA motifs present in each construct are also shown. Boxes indicate open reading frame of luciferase and bars represent LHR 3' UTR. The vertical slash represents the AUUUA motif.

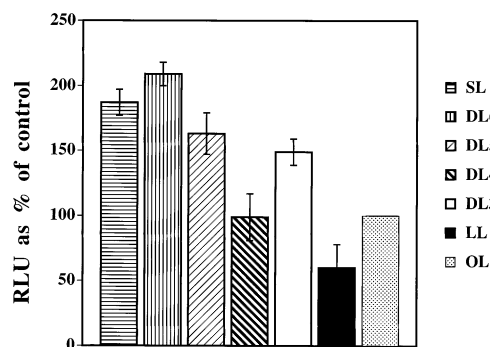


Fig. 4. Luciferase activity in 293 T cells. Cells were transiently transfected with luciferase constructs containing varying lengths of LHR 3' UTR (shown in Fig. 3). Luciferase activity is measured in relative light units (RLUs) as described in Section 2 and expressed as percentage of luciferase control (OL). Data were normalized to neomycin phosphotransferase mRNA for transfection efficiency and to protein for cell number. Values are averages of duplicate determinations from three independent experiments \pm S.D.

ase expression was measured 48 h after transfection. The results are shown in Fig. 4.

Cells transfected with LL exhibited less luciferase activity compared to construct OL which contains only the open reading frame of luciferase. This indicates that the long 3' UTR was also able to decrease the luciferase expression. Constructs SL, DL6 and DL5 containing the first 0.73, 0.94 and 1.5 kb as well as DL3 and DL4 containing 2.88 and 2.18 kb of the 3' UTR respectively, increased luciferase activity when compared to construct LL. Although the constructs DL3 and DL4 contain some portions (DL3 1.34 kb, DL4 0.65 kb) of the 1.98 kb distal 3' UTR, they failed to decrease luciferase activity. This showed the importance of the entire distal 3' UTR for the inhibition. Constructs containing nucleotides corresponding to the first 1 kb region (SL, DL6 and DL5) produced the highest levels of luciferase expression. In a similar fashion, the level of LHR expression using the construct S with 0.73 kb 3' UTR also produced higher level of luciferase expression. These results taken together suggest that the positive *cis*-acting elements may reside in this region.

3.3. Effect of distal 1.98 kb 3' UTR on LHR expression

The studies described above suggest that the distal 1.98 kb region of 3' UTR of LHR confers an inhibitory effect on LHR or reporter gene expression. To determine if this distal 1.98 kb region functions independently, the construct D3, which encodes this region, was transiently transfected into 293 T cells. Cells transfected with construct L containing the long 3' UTR and construct O devoid of 3' UTR were used as controls. The receptor expression was studied by 125 I-hCG binding assay. The B_{\max} and K_d values in Table 2 were calculated from the Scatchard plot shown in Fig. 5. The

Table 2
Binding constants of LH/hCG receptor constructs

Construct	B_{\max} (fmol/ μ g DNA)	K_d (nM)
L	4.8	0.29
D3	8.0	0.35
O	10.26	0.37

The B_{\max} and K_d were calculated using the binding data from Fig. 5 as described in Section 2.

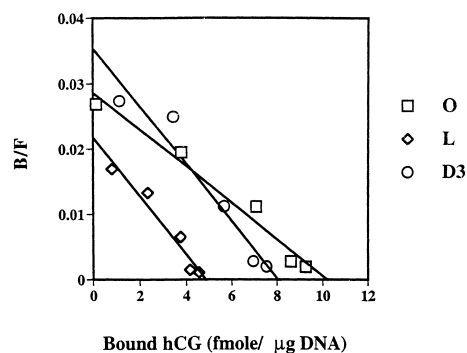


Fig. 5. Scatchard analysis. 293 T cells were transiently transfected with constructs D3, L and O (shown in Fig. 1). Receptor expression was measured by 125 I-hCG binding assay, as described in Section 2. Each point represents average of three determinations for total binding and two determinations for non-specific binding. Data were normalized to DNA for cell number. The transfection efficiency was examined by assaying the neomycin phosphotransferase mRNA expression and was found to be similar for all the constructs. A representative result of three independent experiments is shown.

distal 1.98 kb 3' UTR was able to inhibit the receptor expression (B_{\max} 8.0 fmol/ μ g of DNA) without affecting the binding affinity (K_d 0.35 nM) when compared to construct O (B_{\max} 10.26 fmol/ μ g of DNA, K_d 0.37 nM). The decrease in receptor expression was more pronounced in the case of construct L (B_{\max} 4.8 fmol/ μ g of DNA) when compared to construct D3, indicating that the distal 1.98 kb 3' UTR interacts with other regions of the 3' UTR in order to exert full inhibitory effect. The transfection efficiency as determined by assaying neomycin phosphotransferase mRNA encoded in the same vector (dot-blot assay) was similar for all three constructs (data not shown). Thus although the distal 1.98 kb region was able to exert inhibitory effect, it was functionally more effective in the presence of the remaining 3' UTR sequence.

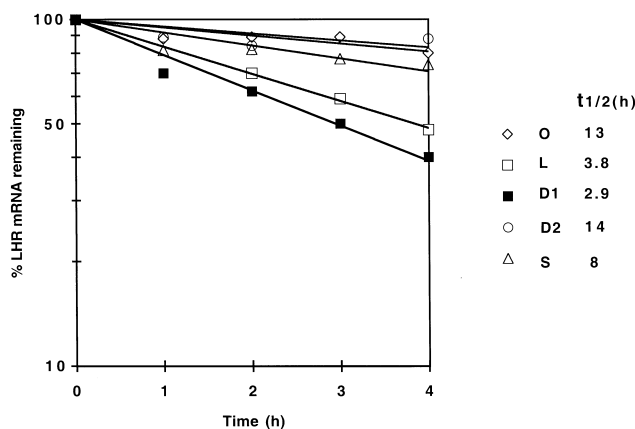


Fig. 6. Half-life determination of LHR mRNA degradation. 293 T cells were transiently transfected with LHR constructs containing varying lengths of 3' UTR (shown in Fig. 1). Cells were harvested at 0, 1, 2, 3 and 4 h intervals after actinomycin D addition. RNA dot-blot analysis and hybridization with radiolabelled probes were performed as described in Section 2. mRNA degradation is plotted as logarithmic function of percent mRNA remaining after actinomycin D addition. Data were normalized for the variation in quantity of RNA applied, to 18S ribosomal RNA. Each point represents average values calculated from two independent experiments.

3.4. Effect of 3' UTR on LHR mRNA stability

To gain insight into mechanism(s) of control of LHR expression by 3' UTR, studies were carried out to examine the possible alteration in mRNA stability. 293 T cells were transiently transfected with LHR constructs shown in Fig. 1 and the half-life of LHR mRNA was measured. Half-life measurements were carried out by measuring LHR mRNA levels remaining at various time intervals in actinomycin D arrested cells. The percentage of LHR mRNA remaining after actinomycin D treatment was plotted in log scale against time as shown in Fig. 6. The quantity of mRNA present at time 0 was taken as 100%. Constructs L, D1, D2 and S had $t_{1/2}$ values 3.8 h, 2.9 h, 14 h and 8 h, respectively. This clearly shows that the transcripts resulting from constructs L and D1 were less stable than those derived from D2 and S. The mRNA formed from construct O containing only the ORF of LHR exhibited stability similar to the mRNAs arising from constructs D2 and S. The $t_{1/2}$ of mRNAs of the constructs L and D1 were markedly different from constructs O and D2. These results demonstrate that the distal 1.98 kb region of 3' UTR of LHR was responsible for the accelerated degradation of mRNAs. This decrease in mRNA stability conferred by the 1.98 kb distal 3' UTR explains the decrease in both receptor expression and luciferase activity. Since the decrease produced by the distal 1.98 kb region alone was small, no attempts were made to determine the $t_{1/2}$ with the construct D3 which encodes this region.

4. Discussion

Previous studies conducted in our laboratory have shown that treatment of pseudopregnant rats with a pharmacological dose of hCG results in down regulation of LH receptor in the ovary [10]. Half-life measurements and nuclear run-off experiments showed that the selective loss of LHR mRNA seen during hCG induced down regulation of the receptor is due to rapid degradation of mRNA and not due to decreased transcription [6]. Further extension of these studies conducted in our laboratory has shown that the long 3' UTR associated with the 6.7 kb transcript causes a decrease in receptor expression in transfected cells [23]. Thus one of the aims of the present studies was to identify the destabilizing region(s) present in the 3' UTR.

Although it has been established that the 3' untranslated region has a major role in mRNA stability (reviewed in [15]), the regulatory role(s) of specific 3' UTR sequences on G protein coupled receptor mRNA stability has not been examined. This is the first report of the regulatory role of specific 3' UTR sequences of a member of a G_s protein coupled receptor family. Many short lived mRNAs contain AU-rich cis elements in the 3' UTR. AUUUA motifs present in AU-rich regions in 3' UTR of many early response gene mRNAs have been identified as the key destabilizing motifs [29]. In the present study, we have shown that the long 3' UTR associated with the 6.7 kb LHR mRNA containing 11 AUUUA motifs inhibited receptor expression compared to construct containing only ORF of LHR. This inhibitory effect observed is not unique to LHR, as the same 3' UTR sequence was also able to inhibit luciferase expression. Deletion of the distal 1.98 kb of this 3' UTR causes an increase in receptor expression to a level almost comparable to that seen in cells transfected with a construct containing ORF of LHR. This observation was

confirmed by the increase of luciferase activity by construct DL5 lacking the entire distal 1.98 kb 3' UTR. Shorter deletions within this 1.98 kb region (constructs D1, DL3 and DL4) have made the region non-functional. This underscores the importance of the entire 1.98 kb region for imparting decrease of receptor/luciferase expression. The extent of inhibition produced by the construct D1 was lower than the construct containing the entire 1.98 kb distal 3' UTR. The changes in receptor expression seen by construct S and luciferase expression by constructs SL, DL6 and DL5 further showed that the inhibitory effect of the 3' UTR is confined to the distal 1.98 kb region. The decrease in receptor expression produced by construct D3 containing the distal 1.98 kb 3' UTR was only 22% whereas the construct L containing the entire 3.5 kb 3' UTR caused a decrease in receptor expression by more than 50%, when compared to ORF of LHR (construct O). This indicates that although the distal 1.98 kb region is important for the 3.5 kb 3' UTR to impart LHR mRNA destabilization, it is not fully functionally independent. This highlights the importance of native conformation of 3' UTR for function. Deletion of the distal 1.98 kb region may cause a distortion of the secondary or tertiary structure of the 3' UTR thereby affecting its function. Because the AUUUA motifs present in the 1.98 kb distal region are found to be non-functional and rest of the region lacks known destabilizing motifs, primary structure alone is insufficient to cause mRNA destabilization. Moreover, an increase of receptor/luciferase expression by the first 1 kb region indicates the possibility that positive *cis*-acting elements may reside within this region.

Another important finding is the non-functional nature of pentanucleotide AUUUA motifs present in the 3' UTR. Our results show that construct D1 which is devoid of six AUUUA motifs present within nucleotides 1528 and 2175 was still able to destabilize receptor mRNA. One of the AUUUA motifs present in construct D1 is a nonamer flanked by UU at 5' end and AA at 3' end (UUAUUUAAA). In the case of *c-fos* mRNA, the nonamer UUAUUUAAU has been shown to be a key ARE that effectively destabilizes mRNA [30]. Similarly, the presence of such nonamers in the destabilizing ARE regions in the 3' UTR of GM-CSF, IL-3 and β -IFN mRNAs has also been reported [31–34]. The LHR mRNA also contains a nonamer in between nucleotides 1496 and 1505 in the 3' UTR. The construct D2 which is devoid of the distal 1.98 kb region containing five AUUUA motifs with this nonamer as the fifth one, caused an increase in receptor expression compared to the construct containing distal 1.98 kb 3' UTR. This suggests that the presence of AUUUA or a nonamer does not cause LHR mRNA destabilization. This observation provides further support for the previous findings with certain labile early response gene mRNAs such as *c-jun*, *krox-20*, *zif-268* and heat stable antigen mRNAs in which non-functional AUUUA motifs have been reported [32,35].

The half-life measurement studies showed an increase in LHR mRNA stability resulting from deletion of the distal 1.98 kb 3' UTR. The constructs (L and D1) containing this destabilizing region caused a decrease (74%) in $t_{1/2}$ compared to that containing ORF of LHR (O). The mRNA produced from construct D1 which is devoid of six AUUUA motifs from the 1.98 kb destabilizing region still showed a shorter $t_{1/2}$ further supporting the non-functional nature of AUUUA

motifs in mRNA destabilization. This suggests that the entire 1.34 kb distal 3' UTR is the minimum essential region required for mRNA destabilization.

The present study indicates that the presence of the distal 1.98 kb 3' UTR of the LHR decreases receptor expression mainly by destabilizing the mRNA, and the AUUUA motifs present in 3' UTR do not confer instability to mRNA. In rat ovary, the LH/CG receptor numbers are regulated throughout follicular development, ovulation and luteinization. The post-transcriptional regulation of LHR mRNA as a means to maintain the steady levels of LHR mRNA during ovarian cycle is advantageous since regulation of mRNA at posttranscriptional level obviates the need for continued reprogramming of the transcriptional system.

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