Regulation of Luteinizing Hormone Receptor Expression

EVIDENCE OF TRANSLATIONAL SUPPRESSION IN VITRO BY A HORMONALLY REGULATED mRNA-BINDING PROTEIN AND ITS ENDOGENOUS ASSOCIATION WITH LUTEINIZING HORMONE RECEPTOR mRNA IN THE OVARY

Received for publication, March 22, 2005, and in revised form, September 29, 2005 Published, JBC Papers in Press, November 1, 2005, DOI 10.1074/jbc.M503154200

Anil K. Nair and K. M. J. Menon

From the Departments of Obstetrics/Gynecology and Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan 48109-0617

Our previous studies have identified a luteinizing hormone receptor (LHR) mRNA-binding protein (LRBP) that binds to the coding region (LBS) of rat LHR mRNA. The identity of LRBP was later established as mevalonate kinase (MVK). The present study examined if LRBP binding to LHR mRNA impairs translation. A full-length FLAG-tagged rat LHR mRNA was synthesized and translated in vitro. The translation product was immunoprecipitated and analyzed on SDS-PAGE. The addition of LRBP inhibited LHR mRNA translation. This inhibitory effect was reversed by an excess of wild type (wt) LBS. To determine whether this reversal of the inhibitory effect of LRBP was indeed due to the sequestration of LRBP by the wtLBS, a translation reaction was performed in the presence of mutated LBS in which all the cytidine in the wtLBS was mutated to uridine. This mutation of LBS has been shown to render it incapable of interacting with LRBP. Unlike wtLBS, the mutated LBS was unable to reverse the inhibitory effect of LRBP on LHR mRNA translation. The addition of mevalonate, which has been shown to compete for LHR mRNA binding to LRBP, also reduced the extent of translation inhibition by LRBP. Endogenous association of LHR mRNA with MVK was assessed by immunoprecipitation of the ribonucleoprotein complex with MVK antibody followed by reverse transcription-PCR. Amplification of LHR mRNA, if any, associated with the immunoprecipitate obtained from ovarian ribonucleoprotein complex with gene-specific primers confirmed the association of LHR mRNA with MVK. Collectively, the present data support the novel function of LRBP as a translational inhibitor of LHR mRNA in the ovary.

Biological actions of luteinizing hormone (LH), a glycoprotein hormone crucial in regulating gonadal functions in mammals, are mediated by its interaction with specific cell surface receptors expressed primarily on the cell membranes of reproductive organs such as testis and ovary. Luteinizing hormone receptor (LHR) belongs to the rhodopsin/β2-adrenergic receptor subfamily A of G-protein-coupled receptors. In the ovary interaction of LH with LHR leads to increased production of cAMP, which in turn stimulates steroidogenesis and other specialized functions of the ovary such as ovulation. The expression of LHR on the cell surface varies during different stages of the ovarian cycle, thereby regulating the actions of LH. In rat ovarian granulosa and luteal cells, the expression of LHR is greatly decreased by the endogenous preovulatory LH surge or by the administration of a pharmacological dose of human chorionic gonadotropin (hCG), a placental counterpart of LH. Previous studies from our laboratory have shown that the decline in cell surface expression of ovarian LHR seen after hCG administration is paralleled by a specific, transient loss of all four LHR mRNA transcripts. We have shown that this transient loss of LHR mRNA does not result from decreased transcription but occurs post-transcriptionally with a 3-fold decrease in mRNA half-life.

Further studies have shown that a specific LH receptor mRNA-binding protein (LRBP), identified in the ovary cytosolic fraction, plays a regulatory role in the expression of LHR mRNA by binding to a polypyrimidine-rich, bi-partite sequence in the coding region of LHR mRNA. LRBP exhibits characteristics of a functional mRNA-binding protein with respect to expression in target cells, induction of its expression in the ovary when LHR mRNA levels are down-regulated, and also by demonstration of its ability to increase the degradation of LHR mRNA in an in vitro decay system. Subsequent studies led to the purification and identification of this trans-acting factor as mevalonate kinase (MVK). In vivo studies showed an increase in mevalonate kinase expression in rat corpus luteum before ligand-induced loss of LHR mRNA, consistent with its role as an endogenous regulator of LHR mRNA expression. Because the mRNA binding characteristics of this protein revealed specificity for sequences in the LHR mRNA coding region, the present studies were undertaken to examine if binding of LRBP to the coding region of LHR mRNA impairs translation. The results presented here show that in a cell-free in vitro translation system, LRBP binds to the coding region of LHR mRNA, and the resulting ribonucleoprotein complex prevents LHR mRNA translation. This was further supported by the fact that immunoprecipitation of ribonucleoprotein (RNP) complex from ovarian homogenate showed the association of LH receptor mRNA with mevalonate kinase in vivo during hCG-induced LH receptor down-regulation in the ovary.

MATERIALS AND METHODS

Received for publication, March 22, 2005, and in revised form, September 29, 2005 Published, JBC Papers in Press, November 1, 2005, DOI 10.1074/jbc.M503154200

Anil K. Nair and K. M. J. Menon

From the Departments of Obstetrics/Gynecology and Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan 48109-0617

Our previous studies have identified a luteinizing hormone receptor (LHR) mRNA-binding protein (LRBP) that binds to the coding region (LBS) of rat LHR mRNA. The identity of LRBP was later established as mevalonate kinase (MVK). The present study examined if LRBP binding to LHR mRNA impairs translation. A full-length FLAG-tagged rat LHR mRNA was synthesized and translated in vitro. The translation product was immunoprecipitated and analyzed on SDS-PAGE. The addition of LRBP inhibited LHR mRNA translation. This inhibitory effect was reversed by an excess of wild type (wt) LBS. To determine whether this reversal of the inhibitory effect of LRBP was indeed due to the sequestration of LRBP by the wtLBS, a translation reaction was performed in the presence of mutated LBS in which all the cytidine in the wtLBS was mutated to uridine. This mutation of LBS has been shown to render it incapable of interacting with LRBP. Unlike wtLBS, the mutated LBS was unable to reverse the inhibitory effect of LRBP on LHR mRNA translation. The addition of mevalonate, which has been shown to compete for LHR mRNA binding to LRBP, also reduced the extent of translation inhibition by LRBP. Endogenous association of LHR mRNA with MVK was assessed by immunoprecipitation of the ribonucleoprotein complex with MVK antibody followed by reverse transcription-PCR of the RNA associated with the immune complex. Amplification of LHR mRNA, if any, associated with the immunoprecipitate obtained from ovarian ribonucleoprotein complex with gene-specific primers confirmed the association of LHR mRNA with MVK. Collectively, the present data support the novel function of LRBP as a translational inhibitor of LHR mRNA in the ovary.

Biological actions of luteinizing hormone (LH), a glycoprotein hormone crucial in regulating gonadal functions in mammals, are mediated by its interaction with specific cell surface receptors expressed primarily on the cell membranes of reproductive organs such as testis and ovary. Luteinizing hormone receptor (LHR) belongs to the rhodopsin/β2-adrenergic receptor subfamily A of G-protein-coupled receptors. In the ovary interaction of LH with LHR leads to increased production of cAMP, which in turn stimulates steroidogenesis and other specialized functions of the ovary such as ovulation.

The expression of LHR on the cell surface varies during different stages of the ovarian cycle, thereby regulating the actions of LH. In rat ovarian granulosa and luteal cells, the expression of LHR is greatly decreased by the endogenous preovulatory LH surge or by the administration of a pharmacological dose of human chorionic gonadotropin (hCG), a placental counterpart of LH. Previous studies from our laboratory have shown that the decline in cell surface expression of ovarian LHR seen after hCG administration is paralleled by a specific, transient loss of all four LHR mRNA transcripts. We have shown that this transient loss of LHR mRNA does not result from decreased transcription but occurs post-transcriptionally with a 3-fold decrease in mRNA half-life.

Further studies have shown that a specific LH receptor mRNA-binding protein (LRBP), identified in the ovary cytosolic fraction, plays a regulatory role in the expression of LHR mRNA by binding to a polypyrimidine-rich, bi-partite sequence in the coding region of LHR mRNA. LRBP exhibits characteristics of a functional mRNA-binding protein with respect to expression in target cells, induction of its expression in the ovary when LHR mRNA levels are down-regulated, and also by demonstration of its ability to increase the degradation of LHR mRNA in an in vitro decay system. Subsequent studies led to the purification and identification of this trans-acting factor as mevalonate kinase (MVK). In vivo studies showed an increase in mevalonate kinase expression in rat corpus luteum before ligand-induced loss of LHR mRNA, consistent with its role as an endogenous regulator of LHR mRNA expression. Because the mRNA binding characteristics of this protein revealed specificity for sequences in the LHR mRNA coding region, the present studies were undertaken to examine if binding of LRBP to the coding region of LHR mRNA impairs translation. The results presented here show that in a cell-free in vitro translation system, LRBP binds to the coding region of LHR mRNA, and the resulting ribonucleoprotein complex prevents LHR mRNA translation. This was further supported by the fact that immunoprecipitation of ribonucleoprotein (RNP) complex from ovarian homogenate showed the association of LH receptor mRNA with mevalonate kinase in vivo during hCG-induced LH receptor down-regulation in the ovary.

MATERIALS AND METHODS

Chemicals—Pregnant mare serum gonadotropin was purchased from Calbiochem. Highly purified human chorionic gonadotropin (CR 127) was a gift from the Center for Population Research (NICHD, National Institutes of Health) through the National Hormone and pituitary Program. [α-32P]UTP was from PerkinElmer Life Sciences and Redivue -[35S]methionine (in vitro translation grade) was from Amer sham Biosciences. mMessage mMachine T7 Ultra was a product of...
Translational Regulation of LH Receptor mRNA

Ambion (Austin, TX). EDTA-free protease inhibitor mixture tablets and Quick spin columns (G-50-Sephadex) for radiolabeled RNA purification were obtained from Roche Applied Science. RNasin and Flexi rabbit reticulocyte lysate system were purchased from Promega (Madison, WI). Macro-Prep high S support column was from Bio-Rad. Cen-triplus YM-10, Centricron YM-10, and Microcon YM-10 were products of Millipore Corp. (Bedford, MA). Anti-FLAG M2-agarose affinity gel and DL-mevalonic acid lactone were purchased from Sigma. BCA reagent was from Pierce. Enlightening (rapid autoradiography enhancer) reagent was a product of PerkinElmer Life Sciences.

Animals and Tissues—Pseudopregnancy was induced in 21-day-old Sprague-Dawley female rats by subcutaneous injection of 50 IU of pregnant mare serum gonadotropin followed by 25 IU of hCG 56 h later. The day of hCG injection was taken as day 0. LH receptor down-regulation was induced by the injection of 50 IU of hCG on the fifth day of pseudopregnancy. Ovaries were collected 6 and 12 h after hCG injection and were processed immediately for immunoprecipitation of RNP complex and LRBP purification.

Purification of LRBP—LRBP from rat ovary was purified as described previously (12). Partial purification of LRBP was performed by homogenizing rat ovaries in buffer A (10 mM Hepes, pH 7.9, 0.5 mM MgCl2, 50 μM EDTA, 5 mM dithiothreitol, and 10% glycerol) containing 50 mM KCl and protease inhibitor mixture at 4 °C. The cytoplasmic proteins were collected by centrifuging the homogenate at 100,000 × g for 90 min at 4 °C. The supernatant containing the cytoplasmic proteins (S100 fraction) was collected and applied to a 10% PAGE and eluting the LRBP from the gel and renaturing as described above in the presence or absence of LRBP. The translation reaction was stopped at 0, 20, and 40 min by adding 475,000 cpm/reaction using rabbit reticulocyte lysate system as translational reaction mixtures (25–µl reaction volume) were performed using a Flexi rabbit reticulocyte lysate system as described by the manufacturer. Proteins synthesized in vitro were labeled with [35S]methionine and separated by 10% SDS-PAGE (Bio-Rad mini gel) according to the method of Laemmli. The gel was fixed in 40% methanol (v/v) and 10% acetic acid (v/v) for 20 min and then incubated in Enlightening reagent for another 30 min. The gel was then dried under vacuum for 20 min at 80 °C and exposed to x-ray film for autoradiography.

Immunoprecipitation—FLAG-tagged in vitro translated rat LH receptor was immunoprecipitated using anti-FLAG M2-agarose affinity gel. 25 µl of the in vitro translation reaction mixture was diluted to 500 µl with dilution buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100). Anti-FLAG M2-agarose affinity gel was washed 3 times with wash buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) and added to the diluted translation reaction mixture (40 µl of gel suspension per 500 µl of translated translation reaction mixture) and incubated overnight in an end-over-end shaker at 4 °C. The sample was centrifuged for 5 s at 10,600 × g at room temperature, and supernatant was removed. The beads were washed 3 times with wash buffer, and 30 µl of 2× SDS-PAGE sample buffer was added. The beads with sample buffer were heated at 65 °C for 10 min and centrifuged at 10,600 × g for 5–10 s, and the supernatant was collected. The supernatant was then applied onto a 10% SDS-PAGE.

mRNA Decay Coupled to in Vivo Translation—In vitro translation of LHR mRNA was performed with 200 ng of α-[32P]-labeled LHR mRNA (100,000 cpm/reaction) using rabbit reticulocyte lysate system as described above in the presence or absence of LRBP. The in vitro translation reaction was stopped at 0, 20, and 40 min by adding 475 µl of high salt cleaning buffer (25 mM Tris-HCl, pH 7.6, 0.1% SDS, and 400 mM NaCl) (16) to 25 µl of reaction mixture. Yeast tRNA (10 µg) was added per reaction to allow quantitative recovery of LHR mRNA during precipitation. The remaining RNA in the reaction mixture was extracted with an equal volume of nonsense-water-saturated phenol/chloroform/isoamyl alcohol (50:49:1). Unincorporated nucleotides were removed using Quick spin columns (G-50 Sephadex). The RNA was precipitated with an equal volume of isopropl alcohol at −20 °C. The precipitated RNA was washed 3 times with 75% ethanol, air-dried, and dissolved in nuclease-free water. Both radiolabeled and unlabeled RNAs were quantitated spectrophotometrically at 260 nm.

In Vitro Translation—Unlabeled and α-[32P]-labeled RNAs were in vitro transcribed from cDNA templates using Ambion in vitro transcription kits. The full-length capped and FLAG-tagged rat LHR mRNA and human β-actin mRNA were synthesized using Message mMachine T7 Ultra kit. The wild type and mutant LBSs were synthesized using MAXIscript kit. The radiolabeled LHR mRNA was prepared using 60 µCi of α-[32P]UTP in the reaction mixture. After transcription, the RNAs were treated with RNase-free DNase 1 and extracted with nuclease-free water-saturated phenol/chloroform/isoamyl alcohol (50:49:1). Unincorporated nucleotides were removed using Quick spin columns (G-50 Sephadex). The RNA was precipitated with an equal volume of isopropl alcohol at −20 °C. The precipitated RNA was washed 3 times with 75% ethanol, air-dried, and dissolved in nuclease-free water. Both radiolabeled and unlabeled RNAs were quantitated spectrophotometrically at 260 nm.
**Immunoprecipitation of RNP Complex**—Immunoprecipitation of RNP complex was performed as described by Chu et al. (18). This technique is based on the immunoprecipitation procedure developed by Lerner and Steitz (19). Rat ovaries were homogenized in NET-2 buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Nonidet P-40) containing RNasin (100 units/0.5 ml of buffer) and protease inhibitor. The homogenate was centrifuged at 10,000 × g for 10 min at 4 °C. Supernatant was collected, and 1–1.5 mg of protein/ml of the extract was precleared with 300 µl of protein A-agarose beads for 30 min at 4 °C in an end-over-end shaker and centrifuged at the end of the incubation period to remove the protein A-agarose beads. The cleared supernatant was then incubated with antibody for mevalonate kinase (12) along with 50 µg of yeast tRNA and RNasin (100 units/0.5 ml) for 1 h at 4 °C. Pre-immune rabbit IgG and high density lipoprotein-binding protein (HBP) antibody were used as negative controls. Protein A-agarose beads were then added at the end of the 1-h period and incubated further for another 30 min. The protein A-agarose immune complex was collected by centrifuging at 10,000 × g for 3 min at 4 °C. The beads were washed 5 times with 400 µl of NET-2 buffer. After the final wash 400 µl of NET-2 buffer was added, and the immunoprecipitate was subjected to phenol-chloroform extraction to isolate the nucleic acids associated with the immune complex. The extract was then incubated with 10 units of RNase-free DNase at 37 °C for 15 min followed by phenol-chloroform extraction again to isolate the RNA remaining in the extract. The RNA was then precipitated in the presence of 20 µg of glycogen by isopropyl alcohol at −20 °C. The RNA was washed 2 times with 80% ethanol and dissolved in 15 µl of nuclease-free water.

**RT-PCR of Precipitated RNA**—Full-length rat LH receptor DNA was synthesized by RT-PCR using Invitrogen SuperScript One-Step RT-PCR with Platinum Taq (40 cycles of PCR). Five µl of the immunoprecipitated RNA was used for the amplification with the oligonucleotide primers synthesized by Invitrogen. Their sequences were as follows: sense primer, 5′-GCATGCTAATACGACTCACTATAGGGATGGGGCGGCGAGTCCCAG-3′; antisense primer, 5′-CTAGTGAGTTAACGCTCTCGGTTG-3′.

**Analysis of the Amplified DNA Sequence**—RT-PCR products were separated on a 0.8% agarose gel, and the DNA bands were isolated by QIAquick gel extraction kit. The purified DNA was subjected to PCR (40 cycles) using Invitrogen PCR Platinum SuperMix for amplifying the first nearly 300 nt of rat LH receptor cDNA. The sequences of primers used were as follows: sense primer, 5′-GCATGCTAATACGACTCAGATGAACTAGGATTAG-3′ (underlined sequences represent the T7 RNA polymerase promoter); antisense primer, 5′-CCTTTGCAAGGAATCCTC-3′. The amplified DNA products were isolated after separation on a 0.8% agarose gel, and the purified products were subjected to PstI nuclease digestion. The digested DNA products were analyzed by agarose gel electrophoresis.

**RESULTS**

**In Vitro Translation of FLAG-tagged Rat LHR mRNA**—A FLAG sequence was introduced at the 3′ end of rat LH receptor cDNA, and the corresponding mRNA was transcribed for the _in vitro_ translation and subsequent immunoprecipitation of the translated protein as described under “Materials and Methods.” To examine if the FLAG-tagged rat LH receptor mRNA could be translated _in vitro_, varying concentrations of FLAG-tagged LH receptor mRNA were used in the rabbit reticulocyte lysate system using 15 µCi of [35S]methionine in a 25-µl reaction volume. The reaction was performed for 90 min at 30 °C. The translation product was then immunoprecipitated using anti-FLAG M2-agarose affinity gel, separated on a 10% SDS-polyacrylamide gel, and subjected to autoradiography. The autoradiogram (Fig. 1a) revealed a single prominent band at about 63 kDa, and the intensity of this band increased with increasing mRNA concentrations from 50 to 200 ng in the translation reaction. A negative control reaction, performed with no RNA added to the translation reaction, yielded no protein band. The expected size of the non-glycosylated rat LH receptor is about 62 kDa (20). Because the FLAG epitope introduced contained 8 amino acids,
the size of the non-glycosylated LH receptor was expected to be about 63 kDa. To further confirm that the band seen on the autoradiogram was indeed LHR protein and not a nonspecific translation product, an in vitro translation was performed with LHR mRNA with and without FLAG tag at the 3’ end. LHR protein was then immunoprecipitated from the reaction mixtures, separated by SDS-PAGE, and then subjected to autoradiography. The results presented in Fig. 2b show that the LHR protein band was visible only in the translated products of FLAG-tagged LHR mRNA (lane 3). No LHR protein-specific band (lane 1 and 2) was seen when translation products were immunoprecipitated from reaction mixtures either containing no RNA or non-FLAG-tagged LHR mRNA.

**Effect of LRBP on the Translation of LHR mRNA in Vitro**—We have previously shown that LRBP specifically binds to the coding region of LH receptor mRNA (10). To examine if binding of LRBP to LH receptor mRNA in the coding region has any effect on the synthesis of LH receptor protein, the translation reaction of LHR mRNA was also conducted in the presence of varying concentrations of gel-purified rat LRBP. The resulting LHR protein was immunoprecipitated and processed for developing autoradiogram. c, 1 μg of capped FLAG-tagged rat LHR mRNA was in vitro translated in a 125-μl reaction system containing 5 μg of purified LRBP. 25-μl aliquots were withdrawn at 20, 30, 40, and 60 min of reaction and were processed for immunoprecipitation of LHR protein and autoradiogram. A control reaction was performed in the absence of purified LRBP.

To examine if this inhibition of LHR mRNA translation by LRBP was specific to LHR mRNA, human β-actin mRNA was in vitro translated in the presence of rat LRBP, and the resulting translation products were examined by SDS-PAGE, as described above. As shown in Fig. 3, there was no change in β-actin protein synthesis in the presence of LRBP, thus indicating that the inhibitory effect of LRBP was specific to the translation of LHR mRNA.

**Effect of LRBP on the Translation of Rat LHR mRNA in the Presence of wtLBS and mLBS**—We have previously shown that LRBP binds to a bipartite, polypurimidine-rich sequence in the coding region of rat LHR mRNA, and all the cytidine residues in this region are required for the binding of LRBP (11). This 40-nt (188–228) region of the rat LHR mRNA was designated as the LBS.

To further examine if the translation inhibition caused by LRBP was indeed due to the binding of LRBP to the LHR mRNA, an in vitro translation reaction was performed in the presence of wild type LRBP binding site (wtLBS) or mutated (all the cytidines mutated to uridines) LRBP binding site (mLBS) of rat LHR mRNA (11) in molar excess to the full-length rat LHR mRNA. The resulting translation products were then immunoprecipitated and analyzed as described above. The results (Fig. 4) clearly indicate that the LRBP caused an inhibition of LHR mRNA translation (lanes 3 and 4) when compared with translation reactions performed in the absence of LRBP (lanes 1 and 2). The inhibition in translation produced by LRBP was decreased by the inclusion of mLBS in the reaction in a concentration-dependent manner (lanes 5, 6, and 7). The inclusion of mLBS, which does not bind to LRBP (11), did not cause any change in the inhibition of translation of LHR mRNA produced by LRBP (lanes 8–10). These data indicate that the translation inhibition caused by LRBP was due to the binding of LRBP to the LHR mRNA.

**Effect of Mevalonate on the Translation of LHR mRNA**—We have recently established the identity of LRBP as MVK (12). Additionally, mevalonate, the substrate for MVK, inhibited the binding of LRBP to LH receptor mRNA (12). We, therefore, examined the effect of mevalonate on the inhibition of LHR mRNA translation produced by LRBP. To test this, translation of LHR mRNA was performed in the presence of mevalonate at 0.5 and 1.00 mM concentrations, and the translation products were processed for immunoprecipitation of LHR protein and autoradiogram. A control reaction was performed in the absence of purified LRBP.

To further examine if the translation inhibition caused by LRBP was due to the binding of LRBP to the LHR mRNA, an in vitro translation reaction was performed in the presence of wild type LRBP binding site (wtLBS) or mutated (all the cytidines mutated to uridines) LRBP binding site (mLBS) of rat LHR mRNA (11) in molar excess to the full-length rat LHR mRNA. The resulting translation products were then immunoprecipitated and analyzed as described above. The results (Fig. 4) clearly indicate that the LRBP caused an inhibition of LHR mRNA translation (lanes 3 and 4) when compared with translation reactions performed in the absence of LRBP (lanes 1 and 2). The inhibition in translation produced by LRBP was decreased by the inclusion of mLBS in the reaction in a concentration-dependent manner (lanes 5, 6, and 7). The addition of mLBS, which does not bind to LRBP (11), did not cause any change in the inhibition of translation of LHR mRNA produced by LRBP (lanes 8–10). These data indicate that the translation inhibition caused by LRBP was due to the binding of LRBP to the LHR mRNA.

Effect of LRBP on the Translation of Rat LHR mRNA in the Presence of wtLBS and mLBS—We have previously shown that LRBP binds to a bipartite, polypurimidine-rich sequence in the coding region of rat LHR mRNA, and all the cytidine residues in this region are required for the binding of LRBP (11). This 40-nt (188–228) region of the rat LHR mRNA was designated as the LBS.

To further examine if the translation inhibition caused by LRBP was indeed due to the binding of LRBP to the LHR mRNA, an in vitro translation reaction was performed in the presence of wild type LRBP binding site (wtLBS) or mutated (all the cytidines mutated to uridines) LRBP binding site (mLBS) of rat LHR mRNA (11) in molar excess to the full-length rat LHR mRNA. The resulting translation products were then immunoprecipitated and analyzed as described above. The results (Fig. 4) clearly indicate that the LRBP caused an inhibition of LHR mRNA translation (lanes 3 and 4) when compared with translation reactions performed in the absence of LRBP (lanes 1 and 2). The inhibition in translation produced by LRBP was decreased by the inclusion of mLBS in the reaction in a concentration-dependent manner (lanes 5, 6, and 7). The addition of mLBS, which does not bind to LRBP (11), did not cause any change in the inhibition of translation of LHR mRNA produced by LRBP (lanes 8–10). These data indicate that the translation inhibition caused by LRBP was due to the binding of LRBP to the LHR mRNA.

To examine if this inhibition of LHR mRNA translation by LRBP was specific to LHR mRNA, human β-actin mRNA was in vitro translated in the presence of rat LRBP, and the resulting translation products were examined by SDS-PAGE, as described above. As shown in Fig. 3, there was no change in β-actin protein synthesis in the presence of LRBP, thus indicating that the inhibitory effect of LRBP was specific to the translation of LHR mRNA.

Effect of LRBP on the Translation of Rat LHR mRNA in the Presence of wtLBS and mLBS—We have previously shown that LRBP binds to a bipartite, polypurimidine-rich sequence in the coding region of rat LHR mRNA, and all the cytidine residues in this region are required for the binding of LRBP (11). This 40-nt (188–228) region of the rat LHR mRNA was designated as the LBS.

To further examine if the translation inhibition caused by LRBP was indeed due to the binding of LRBP to the LHR mRNA, an in vitro translation reaction was performed in the presence of wild type LRBP binding site (wtLBS) or mutated (all the cytidines mutated to uridines) LRBP binding site (mLBS) of rat LHR mRNA (11) in molar excess to the full-length rat LHR mRNA. The resulting translation products were then immunoprecipitated and analyzed as described above. The results (Fig. 4) clearly indicate that the LRBP caused an inhibition of LHR mRNA translation (lanes 3 and 4) when compared with translation reactions performed in the absence of LRBP (lanes 1 and 2). The inhibition in translation produced by LRBP was decreased by the inclusion of mLBS in the reaction in a concentration-dependent manner (lanes 5, 6, and 7). The addition of mLBS, which does not bind to LRBP (11), did not cause any change in the inhibition of translation of LHR mRNA produced by LRBP (lanes 8–10). These data indicate that the translation inhibition caused by LRBP was due to the binding of LRBP to the LHR mRNA.

Effect of Mevalonate on the Translation of LHR mRNA—We have recently established the identity of LRBP as MVK (12). Additionally, mevalonate, the substrate for MVK, inhibited the binding of LRBP to LH receptor mRNA (12). We, therefore, examined the effect of mevalonate on the inhibition of LHR mRNA translation produced by LRBP. To test this, translation of LHR mRNA was performed in the presence of mevalonate at 0.5 and 1.00 mM concentrations, and the translation products were processed for immunoprecipitation of LHR protein and autoradiogram. A control reaction was performed in the absence of purified LRBP.

The results clearly showed an inhibition of translation of LHR mRNA by as low as one μg of gel-purified rat LRBP and produced almost complete inhibition at the 2-μg level. A time-course of in vitro translation of LHR mRNA was then performed in the absence or presence of 1 μg of gel-purified rat LRBP. The results (Fig. 4) showed translation inhibition by LRBP at all time intervals starting at 20 min of translation reaction when compared with the respective control reactions. It should be noted that there was a slight increase in the translation of LH receptor mRNA with increase in incubation time, which is due to continued translation of LH receptor mRNA with the progress in incubation time.
Translational Regulation of LH Receptor mRNA

**FIGURE 3. In vitro translation of β-actin mRNA; effect of LRBP.** β-Actin mRNA (200 ng) was translated in vitro by Flexi rabbit reticulocyte lysate system using 15 μCi of [35S]methionine in the presence or absence of 5.0 μg of partially purified rat LRBP from the ovary. A 5-μl aliquot from a 25-μl reaction system was analyzed on 10% SDS-PAGE, and the gel was fixed, incubated in Enlightning reagent, dried, and exposed to x-ray film.

**FIGURE 4. In vitro translation of LH receptor mRNA; effect of wtLBS and mLBS.** 200 ng of FLAG-tagged rat LH receptor mRNA was in vitro translated using 15 μCi of [35S]methionine in the presence of increasing concentrations (molar excess to LH receptor mRNA) of wtLBS and mLBS in the reaction mixture. The translated LH receptor protein was immunoprecipitated and processed for developing the autoradiogram. 2.5 μg of LRBP was used for the translation reaction.

were examined by autoradiography. Because the reported K_m for mevalonate of purified rat liver MVK is 0.271 ± 0.031 mM (21), mevalonate at the 0.5–1.0 mM range was used in the translation reaction to saturate the enzyme. We have also shown earlier that the addition of mevalonate at 0.05, 0.5, and 1.0 mM levels causes a decrease in binding of LRBP to LH receptor mRNA in a concentration-dependent manner (12). As shown in the Fig. 5, inclusion of mevalonate in the translation reaction also caused a concentration-dependent reversal of the inhibitory effect produced by LRBP (lanes 3 and 4) compared with that produced by LRBP alone (lane 2) in the reaction mixture. Mevalonate alone did not have any effect on the translation of LH receptor mRNA (lane 5). This further confirmed that the translation inhibition caused by LRBP was indeed due to the binding of LRBP to LH receptor mRNA.

**Stability of Rat LH receptor mRNA in Rabbit Reticulocyte Lysate System in the Presence of LRBP**—To further clarify that the inhibition in translation of rat LH receptor mRNA seen in the presence of LRBP was not due to an increased degradation of LH receptor mRNA, *in vitro* translation reactions of [32P]-labeled LH receptor mRNA were performed in the absence or presence of LRBP. The translation reactions were stopped at different time intervals (0, 20, 40 min), and the LH receptor mRNA remaining in the reaction mixture was extracted as described under “Materials and Methods.” The extracted RNA was then separated by applying onto a 5% PAGE, 8 M urea gel and dried for 20 min at 80°C, and the autoradiogram was developed. The results shown in Fig. 6 indicate that no increased degradation of LH receptor mRNA occurred in the presence of LRBP (L0, L20, and L40) when compared with control (C0, C20, and C40). This observation confirmed that the inhibition of LH receptor protein synthesis *in vitro* was due to inhibition of translation of LH receptor mRNA and not due to increased degradation of mRNA.

**FIGURE 5. Translation of LH receptor mRNA; effect of mevalonate.** FLAG-tagged rat LH receptor mRNA (200 ng) was *in vitro* translated using 15 μCi of [35S]methionine in the presence of 0.5 or 1.0 mM mevalonate in the reaction mixture. 5.0 μg of LRBP was used in the translation reaction. The translated LH receptor protein was immunoprecipitated and processed for developing the autoradiogram.

**FIGURE 6. LH receptor mRNA decay associated with *in vitro* translation.** *In vitro* translation of LH receptor mRNA was performed with 200 ng of ω-[32P]-labeled LH receptor mRNA (100,000 cpm/reaction) using rabbit reticulocyte lysate system in the presence (1) or absence (C) of LRBP as described under “Materials and Methods.” The reaction was stopped at different time intervals (0, 20, and 40 min), and the remaining LH receptor mRNA was extracted and separated on a 3% polyacrylamide, 8 M urea gel as described under “Materials and Methods.” The gel was then dried and exposed to x-ray film for the autoradiogram.

Mevalonate Kinase Binds to LH Receptor mRNA Endogenously in the Ovary—Earlier studies from our laboratory have shown that hCG-induced down-regulation of rat LH receptor mRNA is paralleled with an increase in mevalonate kinase expression in the ovary (14). To verify that mevalonate kinase endogenously remain associated with LH receptor mRNA in the ovary during LH receptor down-regulated state, hCG-induced LH receptor down-regulated oocytes were homogenized in NET-2 buffer, and the RNP complex was immunoprecipitated using rat mevalonate kinase antibody as described under "Materials and Methods." The RNA isolated from the RNP complex was analyzed by primer extension using LH receptor cDNA-specific primers to amplify the coding region. The results shown in Fig. 6a show that immunoprecipitation using MVK antibody followed by RT-PCR amplification of the total RNA isolated from the...
immune complex resulted in two DNA bands (lane 1 and 5). The band B1 was similar in size to the cDNA band obtained from a vector (pCMV4) containing the rat LH receptor cDNA by PCR amplification (positive control) (lane 3 and 6). The shorter band B2 resolved at a position between the 1.6- and 2.0-kilobase DNA markers. Preimmune rabbit IgG and an irrelevant antibody (high density lipoprotein-binding protein antibody) did not produce any DNA bands (lane 2 and lane 7).

To further verify the identity of these two bands as LH receptor cDNAs, the bands B1 and B2 were eluted from the agarose gel and subjected to PCR amplification with a different set of LH receptor cDNA-specific primers for amplifying approximately the first 300 nt. As shown in Fig. 7b, both B1 and B2 produced the expected size LH receptor cDNA bands (lanes 1 and 3). The amplified bands were then isolated and digested with PstI, and the digested products were separated on native agarose gel stained with ethidium bromide (lanes 2, 4, and 6).

**DISCUSSION**

In addition to the regulation of the rate of RNA synthesis, controlling the stability of mRNA is another effective means of regulating gene expression in all organisms. The half-life of mRNA is influenced by its own regulatory cis-acting elements present in the 5'-untranslated region, coding region, or 3'-untranslated region (22). In general, the stability of mRNA is governed by the interaction of various cytoplasmic/nuclear proteins (trans-acting factors) with the cis-acting regulatory regions in the mRNA (22). The formation and/or disruption of these RNP complexes in response to various cellular stimuli controls the stability of mRNA. A number of trans-acting factors have been identified and characterized as mRNA-stabilizing, destabilizing, or translational regulatory proteins (23–28). In the case of LHR mRNA, the interaction of the mRNA-binding protein, LRBP, occurs in the coding region, and the net result of this interaction is a decrease of the cell surface expression of LHR and its transcripts in the ovary.

The present study examined the role of LRBP on the translation of LH receptor mRNA. The decrease in LHR protein translation observed in the presence of partially purified LRBP and not in the presence of an irrelevant protein (bovine serum albumin) indicates a translation inhibitory effect of LRBP. The inhibitory effect was found to be LHR
mRNA-specific as translation of another, unrelated mRNA (β-actin) transcript remains unaffected by LRBP. Restoration of translation in the presence of wtLBS and mevalonate indicates that the inhibitory effect of LRBP on LHR mRNA translation was in fact due to the binding of LRBP to the coding region of LHR mRNA. Our previous studies have clearly shown that the interaction of LRBP with LHR mRNA was inhibited by mevalonate and by excess amounts of LBS (12). Thus, the demonstration of the reversal of the translation inhibitory effect of LRBP by mevalonate and the wtLBS underscores their ability to disrupt the formation of ribonucleoprotein complex between LHR mRNA and LRBP. The lack of reversal of the translation inhibition by mLBS, in which all cytidines were mutated to uridine residues, further argues for the exquisite specificity of LRBP to interact with a defined coding region sequence of the LHR mRNA to cause translation inhibition. No appreciable change in mRNA stability in the presence of LRBP in the Flexi rabbit reticulocyte lysate system further confirmed that the inhibition of LHR protein synthesis was in fact due to translation inhibition. The lack of a good in vitro model system for silencing MVK RNA hampered the demonstration of the role of mevalonate kinase as a trans factor in the ligand-induced down-regulation of LH receptor. However, the demonstration of the endogenous association of mevalonate kinase with LH receptor mRNA by immunoprecipitation of LH receptor RNP complex from the ovary using mevalonate kinase antibody (Fig. 7) strongly supports a functional role for mevalonate kinase in receptor-down-regulation.

A number of mRNA binding proteins (trans factors) have been implicated in translational regulation of eukaryotic mRNAs. For example, in the case of acid β-glucosidase a mammalian cytoplasmic protein, TCP80/NF90, binds to the coding region of β-glucosidase mRNA and inhibits its translation (29, 30). Similarly heterogeneous nuclear ribonucleoprotein K, poly(pC)-binding protein 1 (PCBP-1), and PCBP-2 bind to the coding region of human Papillomavirus Type 16 L2 mRNA and inhibit its translation in vitro (31). In some cases translation of mRNA is controlled by its own protein, product. For example, human thymidylate synthase and dihydrofolate reductase are regulated in this manner. Thymidylate synthase binds to its own mRNA in the coding region and represses its translation in vitro (27). The existence of thymidylate synthase ribonucleoprotein complex has been shown in human colon cancer cells (32). Thymidylate synthase binds not only to its own mRNA but also binds to the coding regions of c-Myc and p53 mRNAs and inhibits their translation (33, 34). Thus, thymidylate synthase functions as an mRNA-binding protein in addition to its role as an enzyme in the synthesis of thymidine. Human dihydrofolate reductase enzyme has also been shown to bind to its own mRNA and inhibits its translation in vitro (28). Translational repression has also been reported in prokaryotes. For instance, bacteriophage R17 coat protein binds to the translational start site of its own mRNA and represses its translation (35). Similarly, our data presented here demonstrate that mevalonate kinase, in addition to its role as an enzyme in the de novo synthesis of cholesterol, has a dual function of regulating LH receptor mRNA translation.

Translation of eukaryotic mRNA is a complex and highly regulated process. Once transported to the cytoplasm, the fully processed mRNA can exist either in a polysomal-associated, translationally active form or in an inactive form as a ribonucleoprotein (messenger RNP) complex (29, 30, 36, 37). The exchange of mRNA between the polysomal and mRNP compartments modulates the quantity of mRNA available for translation (38). Several studies have implicated a clear link between translation and mRNA stability, since the factors and processes required for mRNA translation and decay are intimately connected (22, 36, 39). It has been shown that translational arrest in general alters the degradation rate of most eukaryotic mRNAs (22, 36, 39, 40). Several reports have shown that inhibition of translation or aberrant translational termination of some mRNAs destabilizes the transcripts (36, 39, 41). The mechanism by which translation affects mRNA stability can vary depending on the transcript, cis elements and trans factors and the physiological needs of the cell.

MVK is a member of galactokinase, homoserine kinase, mevalonate kinase, and phosphomevalonate kinase (GHMP) kinase superfamily of enzymes that are known to have a left-handed β-α-β fold, termed the ribosomal protein S5 domain 2-like fold. A similar β-α-β fold exists in elongation factor G, ribonuclease P and other RNA/DNA binding proteins (42). Thus, it is conceivable that this fold could be the primary target of MVK interaction with LH receptor mRNA. Although MVK does not posses any intrinsic ribonuclease activity, we have observed an increased degradation of LHR mRNA by LRBP in an in vitro mRNA decay assay using ovarian polysomes (13). In the present study we clearly demonstrate that MVK inhibits LHR mRNA translation and, thus, leads to the formation of an untranslatable mRNP complex. This complex might go into an RNA degradation pathway with the help of other ovarian polysome-associated factors, causing rapid LHR mRNA degradation as we have previously reported (13).

Translational suppression of LHR mRNA by converting it to an untranslatable or less translatable form by interaction with LRBP could regulate the LHR expression both by controlling translation and stability. The formation of an untranslatable LHR mRNA complex instantly reduces the LHR protein synthesis. As a second stage of this translation inhibition, other tissue-specific cytosolic factors capable of recognizing this untranslatable mRNP complex might trigger the decay of LHR mRNA. The ability to regulate the expression of LHR at the translational level might be a quick and efficient way to control receptor expression, especially in response to the constantly changing hormonal milieu during the ovarian cycle.

REFERENCES
1. Dufau, M. L. (1998) Annu. Rev. Physiol. 60, 461–496
2. McFarland, K., Sprengel, R., Phillips, H., Kohler, M., Rosembliit, N., Nikolaikis, K., Seagaloff, D., and Seeburg, P. (1989) Science 245, 494–499
3. Ascoli, A., Fanelli, F., and Segaloff, D. (2002) Endocr. Rev. 23, 141–174
4. Menon, K. M. J., and Gunaga, K. P. (1974) Fertil. Steril. 25, 732–750
5. Lapolt, P., Oikawa, M., Jia, X., Dargan, C., and Hsueh, A. (1990) Endocrinology 126, 3277–3279
6. Hoffman, Y. M., Peegel, H., Sprock, M. J., Zhang, Q. Y., and Menon, K. M. J. (1991) Endocrinology 128, 388–393
7. Peegel, H., Randolph, J., Jr., Midgley, A. Jr., and Menon, K. M. J. (1994) Endocrinology 135, 1044–1051
8. Lu, D., Peegel, H., Mosier, S., and Menon, K. M. J. (1993) Endocrinology 132, 235–240
9. Segaloff, D., Wang, H., and Richards, J. (1999) Mol. Endocrinol. 4, 1856–1865
10. Kash, J. C., and Menon, K. M. J. (1998) J. Biol. Chem. 273, 10658–10666
11. Kash, J. C., and Menon, K. M. J. (1999) Biochemistry 38, 16889–16897
12. Nair, A. K., and Menon, K. M. J. (2004) J. Biol. Chem. 279, 14937–14944
13. Nair, A. K., Kash, J. C., Peegel, H., and Menon, K. M. J. (2002) J. Biol. Chem. 277, 21468–21473
14. Wang, L., and Menon, K. M. J. (2005) Endocrinology 146, 423–431
15. Bradbury, F., Kawate, N., Foster, C., and Menon, K. M. J. (1997) J. Biol. Chem. 272, 5921–5926
16. Fritz, D. T., Ford, L. P., and Wilusz, J. (2000) Sci. STKE 2000, RE1–13
17. Chornockyoznski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
18. Chu, E., Schmutz, J. C., Ju, J., and Copur, S. M. (1999) in RNA-Protein Interaction Protocols (Haynes, S. R., ed) Vol. 118, pp. 265–274, Humana Press Inc., Totowa, NJ
19. Lerner, M. R., and Steitz, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 5495–5499
20. Keinanen, K. P. (1988) Biochim. J. 256, 719–724
21. Tanaka, R. D., Schara, B. L., Lee, L. Y., Freudenberger, J. J., and Mosley, S. T. (1990) J. Biol. Chem. 265, 2391–2398
22. Ross, J. (1995) Microbiol. Rev. 59, 423–450
23. Peng, S. S.-Y., Chen, C.-Y. A., Xu, N., and Shyu, A.-B. (1998) EMBO J. 17, 3461–3470
24. Klauser, R. D., Rouault, T. A., and Harford, J. B. (1993) Cell 72, 19–28
25. Fan, X. C., and Steitz, J. (1998) EMBO J. 17, 3448–3460
26. Burd, C. G., and Dreyfuss, G. (1994) Science 265, 615–621
Translational Regulation of LH Receptor mRNA

27. Chu, E., Koeller, D. M., Casey, J. L., Drake, J. C., Chabner, B. A., Elwood, P. C., Zinn, S., and Allegra, C. J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8977–8981
28. Chu, E., Takimoto, C. H., Voeller, D., Grem, J. L., and Allegra, C. J. (1993) Biochemistry 32, 4756–4760
29. Xu, Y., Busald, C., and Grabowski, G. (2000) Mol. Genet. Metab. 70, 106–115
30. Xu, Y., and Grabowski, G. (1999) Mol. Genet. Metab. 68, 441–454
31. Collier, B., Larsson, L., Sokolowski, M., and Schwartz, S. (1998) J. Biol. Chem. 273, 22648–22656
32. Chu, E., Voeller, D. M., Jones, K. L., Takechi, T., Maley, G. F., Maley, F., Segal, S., and Allegra, C. J. (1994) Mol. Cell. Biol. 14, 207–213
33. Chu, E., Takechi, T., Jones, K. L., Voeller, D., Copur, S. M., Maley, F., Segal, S., and Allegra, C. J. (1995) Mol. Cell. Biol. 15, 179–185
34. Chu, E., Copur, S. M., Ju, J., Chen, T.-M., Khleif, S., Voeller, D., Mizunuma, N., Patel, M., Maley, F. G., Maley, F., and Allegra, C. J. (1999) Mol. Cell. Biol. 19, 1582–1594
35. Bernadi, A., and Spahr, P. F. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 3033–3037
36. Mathews, M., Soenberg, N., and Hershey, J. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J., and Mathews, M., eds) pp. 1–32, Cold Spring Harbor Laboratory Press, New York
37. Hershey, J. (1991) Annu. Rev. Biochem. 60, 717–755
38. Kozak, M. (1989) J. Cell Biol. 108, 229–241
39. Jacobson, A., and Peltz, S. (1996) Annu. Rev. Biochem. 65, 693–739
40. Patcher, J., Yen, T., and Cleveland, D. (1987) Cell 51, 283–292
41. Blume, J., and Shapiro, D. (1989) Nucleic Acids Res. 17, 9003–9014
42. Zhou, T., Daugherty, M., Grishin, N., Osterman, A., and Zhang, H. (2000) Structure Fold Des. 8, 1247–1257