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Hormonal Regulation of Steroidogenic Acute Regulatory (StAR) Protein Messenger Ribonucleic Acid Expression in the Rat Ovary

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Steroidogenic acute regulatory (StAR) protein is thought to mediate the rapid increase in steroid hormone biosynthesis in response to tropic hormones by facilitating cholesterol transport to the inner mitochondrial membrane where the P450 side-chain cleavage enzyme (P450scc) is located. Since cholesterol delivery is the regulated step in steroidogenesis and is dependent on *de novo* protein synthesis, StAR mRNA levels were examined in response to the tropic hormones, pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG). The results of this investigation revealed that major StAR mRNA transcripts of 3.4 and 1.6 kb and a less abundant transcript of 1.2 kb were detected in the adrenal, ovary, and testis. Within the ovary, StAR mRNA levels were regulated by PMSG and hCG. The two major transcripts were increased in the immature rat ovary following PMSG administration and in the ovary, 8 d after ovulation, in response to stimulation by hCG. Serum progesterone levels were increased following hCG treatment in parallel with the enhanced expression of StAR. Following PMSG treatment, ovarian StAR transcripts at 3.4 and 1.6 kb were each increased twofold. In the ovary, 8 d following ovulation, basal ovarian StAR mRNA levels were elevated up to sixfold relative to the preovulatory StAR mRNA levels. Even with the enhanced basal level of StAR mRNA within the ovary 8 d postovulation, hCG administration still resulted in a 2.5- and 7-fold increase in the 3.4 and 1.6 kb ($p < 0.025$) transcripts, respectively, and a 58% increase in serum progesterone. In contrast to the dramatic alterations in StAR mRNA expression following hormonal stimulation, P450scc mRNA levels remained unchanged in response to hCG stimulation. The levels of serum progesterone paralleled the change in ovarian StAR mRNA in all experiments. This study provides the first evidence that StAR mRNA expression in the rat ovary is mediated by gonadotropins, further supporting its important role in the regulation of steroid hormone biosynthesis.

Key Words: StAR, cholesterol, steroidogenesis, ovary, progesterone

Introduction

The rapid increase in steroid hormone biosynthesis in steroidogenic cells in response to hormone stimulation is well documented (Garren et al., 1965; Mendelson et al., 1975; Cooke et al., 1975). The rate-limiting step of this rapid response appears to be the delivery of the cholesterol substrate to the mitochondrial P450 side-chain cleavage enzyme (P450scc), which is located on the inner mitochondrial membrane (Crivello and Jefcoate, 1980; Privalle et al., 1983a, b; Jefcoate et al., 1987; Lambeth et al., 1987; Iida et al., 1989). Using mouse MA-10 Leydig tumor cells, Stocco and colleagues (Stocco and Sodeman, 1991; Stocco and Chen, 1991; Stocco, 1992; Stocco and Clark, 1993; Stocco and Ascoli, 1993) have described a series of mitochondrial proteins at 37, 32, and 30 kDa, which are synthesized in response to luteinizing hormone (LH), human chorionic gonadotropin (hCG), and in response to stimulation with the cAMP analog dibutyryl-cAMP (Stocco and Kilgore, 1988). Similar mitochondrial proteins were reported to appear in adrenal cells (Pon et al., 1986a; Krueger and Orme-Johnson, 1988; Epstein et al., 1989; Alberta et al., 1989; Epstein and Orme-Johnson, 1991a; Griffin and Orme-Johnson, 1991), Leydig cells (Pon et al., 1986b), and luteal cells (Pon and Orme-Johnson, 1986, 1988) in response to tropic hormone stimulation. Stocco and Sodeman (1991) and Epstein and Orme-Johnson (1991a) have postulated that during the processing of the 37-kDa mitochondrial protein to the 32- and 30-kDa forms, cholesterol may be transferred from the outer to the inner mitochondrial membrane. Recently, Clark et al. (1994) isolated and cloned the 30-kDa LH-induced mitochondrial protein from mouse MA-10 Leydig tumor cells and have

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referred to this protein as the steroidogenic acute regulatory (StAR) protein. StAR enhanced the mitochondrial conversion of cholesterol into pregnenolone in COS-1 cells when cotransfected with vectors encoding P450scc and adrenodoxin (Clark et al., 1994; Lin et al., 1995; Sugawara et al., 1995). This novel protein appears to be required for the acute regulation of hormone-induced steroidogenesis (Clark et al., 1994).

The elegant work by Lin et al. (1995) has recently demonstrated that StAR is mutated in individuals with congenital lipoid adrenal hyperplasia (lipoid CAH), an autosomal recessive disorder that is characterized by impaired synthesis of all adrenal and gonadal steroid hormones. This study demonstrates that StAR is indispensable for normal adrenal and gonadal steroidogenesis. Although the critical role that StAR plays in adrenal and gonadal tissue is evident from the study of lipoid CAH (Lin et al., 1995), hormonal regulation of this protein in the process of normal ovarian tissue development requires analysis. In the ovary and corpus luteum, StAR may be an essential protein that is required to facilitate cholesterol delivery to the inner mitochondrial membrane for steroid hormone synthesis.

To study hormone-mediated regulation of this novel protein in the ovary, StAR messenger ribonucleic acid (mRNA) levels were examined in rat ovarian tissue following stimulation by pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG). This study provides the first evidence that StAR mRNA levels are hormonally regulated in the rat ovary, further supporting its important role in steroid hormone biosynthesis.

Results

A cDNA probe specific for rat StAR was generated using reverse transcription and polymerase chain reaction (RT-PCR) methods. An oligonucleotide primer specific for the 3'-end of mouse StAR was used to reverse-transcribe rat adrenal RNA to cDNA. Using oligonucleotides designed from the mouse StAR sequence, a single PCR product was amplified. This PCR product migrated at a similar molecular mass to that of mouse StAR, which was generated from mouse adrenal RNA using the identical PCR primers (Fig. 1). This 867-bp band was then cloned into the TA vector for sequencing and further propagation. Dideoxy chain termination sequence analysis of the putative rat and mouse StAR cDNAs indicated that the PCR-amplified sequences were similar. To compare the sequence of the rat StAR cDNA obtained by RT-PCR with that of the published mouse and human cDNA sequences, the PC Gene program (IntelliGenetics, Mountain View, CA) was utilized. The rat StAR sequence adjacent to the PCR primers was 92% homologous with the mouse StAR sequence (Fig. 2) (Clark et al., 1994) and 83% homologous with the human StAR sequence (Sugawara et al., 1995). The deduced amino acid sequences indicate that the three amino acid sequences are similar. The amino acid sequences of rat and mouse are 96% identical, and there is 85% identity between rat, mouse, and human showing that this protein is well conserved between species (Fig. 3).

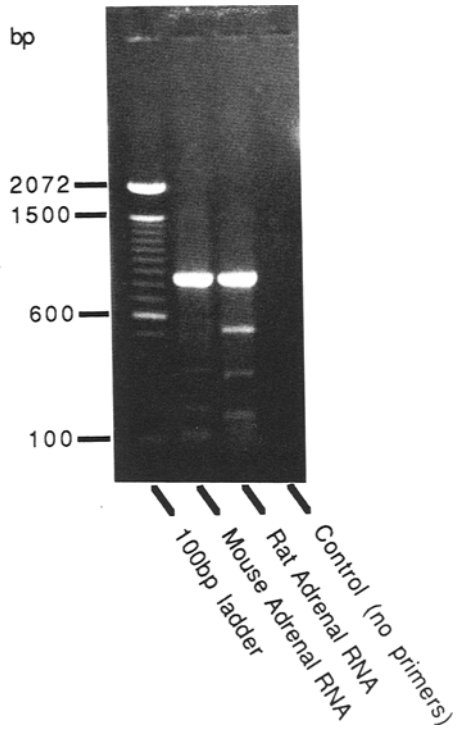

Fig. 1. RT-PCR of the rat StAR cDNA. PCR products were analyzed by 1% agarose gel electrophoresis. Mouse and rat adrenal RNA were reverse-transcribed to cDNA and amplified by PCR, lanes 1 and 2, respectively. Lane 3 is the RT-PCR reaction lacking the StAR-specific primers.

To demonstrate that the rat cDNA probe generated was specific for StAR mRNA, total RNA isolated from mouse adrenal was probed and compared to the StAR cDNA hybridization products detected in the rat adrenal and ovary. The rat StAR cDNA probe hybridized to two major mouse adrenal RNA transcripts at 3.4 and 1.6 kb, and a minor transcript at 2.7 kb (Fig. 4). Similar to the StAR mRNA transcripts in the mouse adrenal, rat adrenal and ovarian mRNA also hybridized to transcripts at 3.4 and 1.6 kb. The 2.7-kb transcript was not detected in the rat tissues; however, a minor 1.2-kb transcript was detected in the rat adrenal and ovary when blots were exposed for periods longer than 24 h.

To demonstrate the tissue specificity of StAR expression in the rat, RNA was isolated from several rat tissues and hybridized with the rat StAR cDNA. Results shown in Fig. 5 indicates that StAR was expressed only in the adrenal, and not in any other tissue examined in this experiment. This suggests that StAR expression is limited to tissues that produce high levels of steroid hormones. In order to determine whether all tissues that produce steroids express StAR, we examined StAR mRNA levels in the ovary, adrenal, testis, placenta, and brain. The results of this analysis (Fig. 6) indicated that StAR expression was limited to the adrenal, ovary, and testis, and that the StAR transcripts

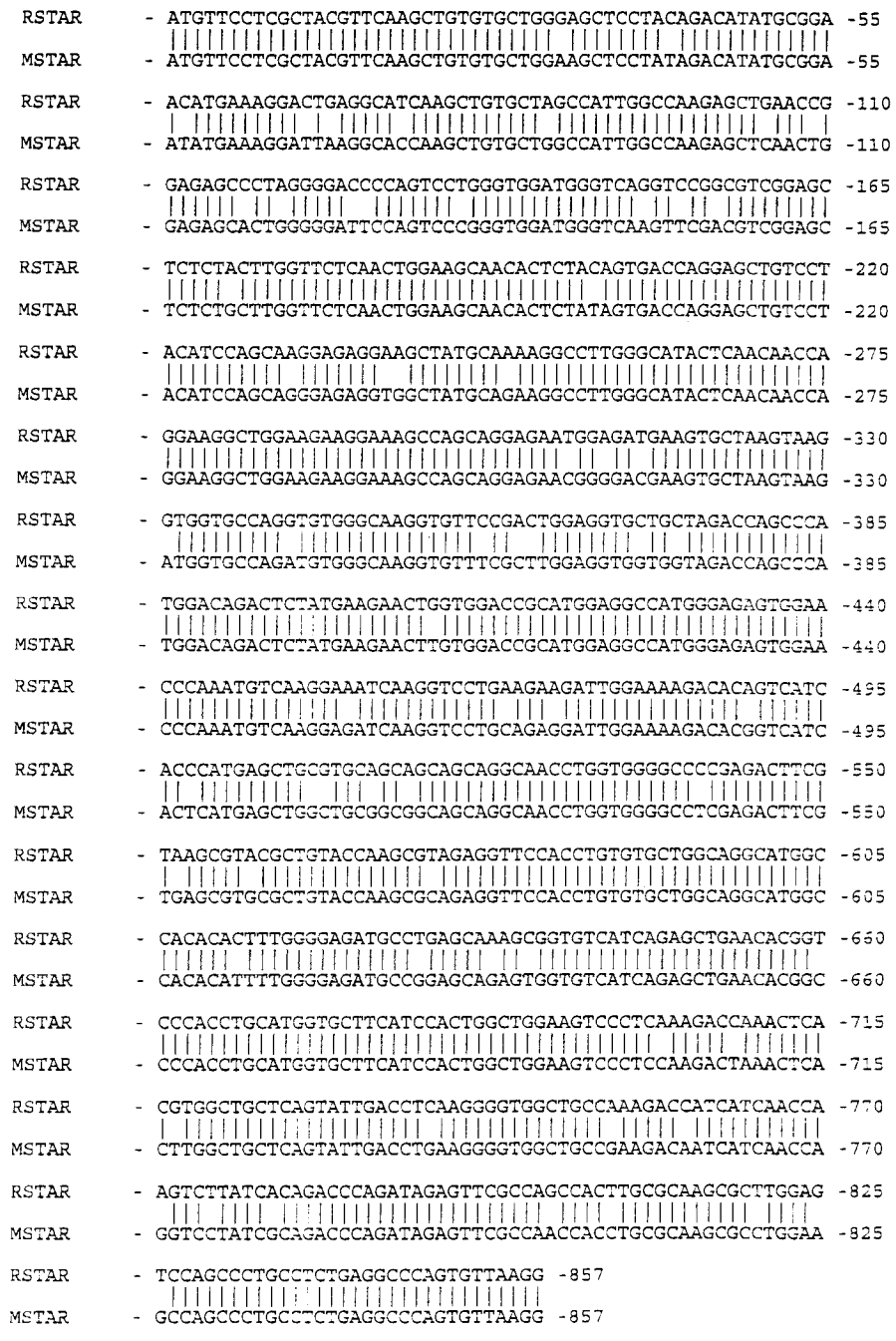

Fig. 2. Comparison of the rat StAR cDNA sequence with the published mouse sequence (Clark et al., 1994). The rat and mouse nucleotide sequences share 96% homology between the two species.

were not expressed in the placenta or brain. StAR was not detected in the testis by Northern blot analysis using total RNA. In order to enhance the level of the testicular StAR transcripts, StAR mRNA was induced by injecting male rats with hCG (50 IU). Testicular StAR mRNA expression was examined 3 h post-hCG injection, and the transcript sizes were similar to those in the adrenal and ovary. To demonstrate whether the absence of StAR mRNA in the brain, kidney, placenta, and spleen was due to our inability to detect StAR using Northern blot analysis, the highly sensitive RT-PCR method was utilized. The RT-PCR results revealed that StAR mRNA was limited to the adrenal (Fig. 7), confirming the tissue distribution observed by Northern blot analysis.

To examine hormonal regulation of ovarian steady-state StAR mRNA levels, rats were treated with PMSG to induce follicular development followed by hCG treatment (50 IU), which induced ovulation and corpus luteum formation. The results of this experiment (Fig. 8) indicate that immature rat ovaries express very low basal StAR mRNA levels. Following PMSG injection, StAR mRNA increased twofold. Serum progesterone levels in these animals were increased 84% following PMSG injection. Following hCG injection, StAR mRNA levels were enhanced further (Fig. 8). The

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MSTARP      MFLATFKLCAGSSYRHMRNMKGLRHQAVLAIGQELNWRALGDSSPG-WMG    49
RSTARP      MFLATFKLCAGSSYRHMRNMKGLRHQAVLAIGQELNRRALGDPSPG-WMG    49
HSTARP      MLLATFKLCAGSSYRHMRNMKGLRQQAVMAISQELNRRALGGPTPSTWIN    50
            *.**********************.***.**.****.****...*. *..

MSTARP      QVRRRSSLLGSQLEATLYSDQELSYIQQGEVAMQKALGILNNQEGWKKES    99
RSTARP      QVRRRSSLLGSQLEATLYSDQELSYIQQGEEAMQKALGILNNQEGWKKES    99
HSTARP      QVRRRSSLLGSRLEETLYSDQELAYLQQGEEAMQKALGILSNQEGWKKES   100
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MSTARP      QQENGDEVLSKMVPDVGKVFRLEVVVDQPMDRLYEELVDRMEAMGEWNPN   149
RSTARP      QQENGDEVLSKVVPGVGKVFRLEVLLDQPMDRLYEELVDRMEAMGEWNPN   149
HSTARP      QQDNGDKVMSKVVPDVGKVFRLEVVVDQPMERLYEELVERMEAMGEWNPN   150
            **.***.*.**.**.*********..****.*******.***********

MSTARP      VKEIKVLQRIGKDTVITHELAAAAAGNLVGPRDFVSVRCTKRRGSTCVLA   199
RSTARP      VKEIKVLKKIGKDTVITHELRAAAAGNLVGPRDFVSVRCTKRRGSTCVLA   199
HSTARP      VKEIKVLQKIGKDTFITHELAAEAAGNLVGPRDFVSVRCAKRRGSTCVLA   200
            *******..***** ***** *.****************.**********

MSTARP      GMATHFGEMPEQSGVIRAEHGPTCMVLHPLAGSPSKTKLTWLLSIDLKGW   249
RSTARP      GMATHFGEMPEQSGVIRAEHGPTCMVLHPLAGSPSKTKLTWLLSIDLKGW   249
HSTARP      GMDTDFGNMPEQKGVIRAEHGPTCMVLHPLAGSPSKTKLTWLLSIDLKGW   250
            **.*.**.****.************************************

MSTARP      LPKTIINQVLSQTQIEFANHLRKRLEASPASEAQC   284
RSTARP      LPKTIINQVLSQTQIEFASHLRKRLESSPASEAQC   284
HSTARP      LPKSIINQVLSQTQVDFANHLRKRLESHPASEARC   285
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Fig. 3. Comparison of the deduced amino acid sequence of rat StAR with the published mouse (Clark et al., 1994) and human (Sugawara et al., 1995) sequences. There is 85% identity among the rat, mouse, and human deduced amino acid sequences, and 96% identity between rat and mouse.

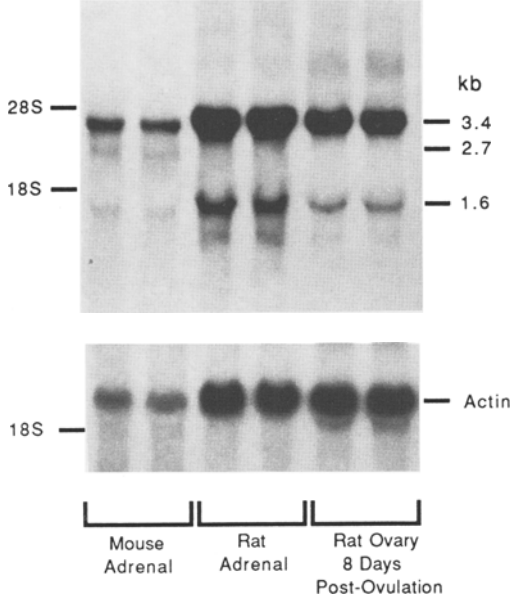

Fig. 4. Comparison of StAR transcripts in mouse and rat tissues. Northern blot containing 20 μg of total RNA isolated from the indicated tissues was probed with the rat StAR cDNA. Both rat and mouse had major StAR transcripts at 3.4 and 1.6 kb. A minor transcript of 2.7 kb appeared in the mouse, whereas in the rat, a minor transcript appeared at 1.2 kb. This blot was exposed for 48 h in order to detect the minor StAR transcripts.

3.4-kb transcript was increased eightfold ($p < 0.005$), and the 1.6-kb transcript was elevated ninefold ($p < 0.001$) at 3 h post-hCG treatment. The levels of both transcripts declined slightly at 6 h, but remained significantly elevated relative to the control StAR mRNA levels. The cytochrome P450scc mRNA level was increased 15-fold ($p < 0.025$) following PMSG treatment and remained elevated throughout the experiment. The results of this experiment indicated that ovarian P450scc mRNA levels were not altered by hCG injection, whereas serum progesterone levels increased (control = 21.5 ± 0.9 ng/mL; 3 h post-hCG = 35.5 ± 2.5 ng/mL, $p < 0.05$) at 3 and 6 h (47.5 ± 2.3 ng/mL, $p < 0.05$) post-hCG injection. The rapid increase in progesterone levels following PMSG and hCG treatments paralleled the increase in StAR mRNA expression in the ovary.

To determine whether hCG regulates ovarian StAR expression after ovulation, ovaries were examined on day 8 of pseudopregnancy. PMSG-primed immature rat ovaries 8 d postovulation consist mainly of corpora lutea. The StAR Northern blot results indicate that ovarian StAR expression was elevated 8 d after ovulation (Fig. 9) and that StAR mRNA levels were increased further (2.5 and sevenfold, respectively) following hCG injection. Both the 3.4- and 1.6-kb transcripts were increased significantly ($p < 0.025$). In contrast to the change in StAR expression, P450scc mRNA was not altered by hCG injection in these animals. Serum progesterone levels were elevated following hCG administration (control = 79.0 ± 3.1 ng/mL; 3 h post-hCG = 112.8 ± 2.8 ng/mL, $p < 0.005$; 6 h post-hCG = 124.4 ± 3.3 ng/mL, $p < 0.005$) in parallel with the increase in StAR mRNA expression.

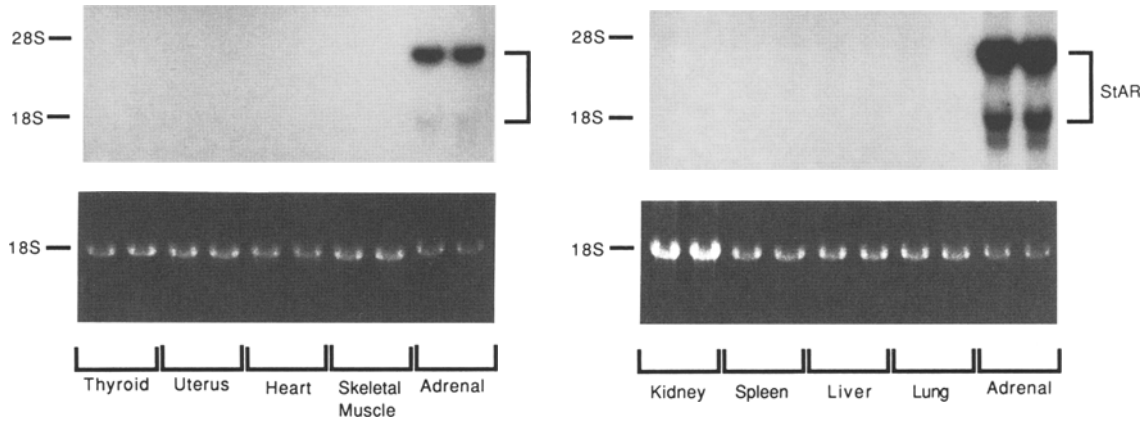

Fig. 5. Expression of StAR mRNA in various rat tissues. Northern blots containing 20 μg of total RNA isolated from the indicated tissues were probed sequentially with StAR and β-actin cDNAs. The autoradiograms for StAR were exposed for 20 h **(A)** and 48 h **(B)**. Ethidium bromide staining of the 18S ribosomal RNA subunit demonstrated equal loading of total RNA.

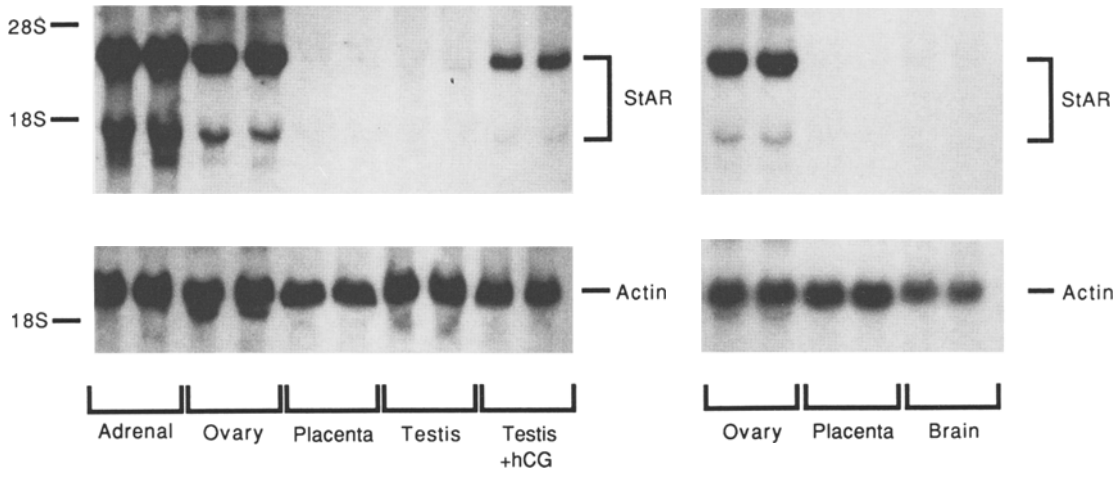

Fig. 6. Expression of StAR mRNA in steroidogenic tissues. Northern blots containing 20 μg of total RNA isolated from the indicated tissues ($n = 4$ separate animals) were probed sequentially with StAR and β-actin cDNAs. Expression was limited to the steroidogenic tissues: adrenal, ovary, and testis. StAR expression in the testis was detected only after hCG injection. The blot for StAR was exposed for 15 h, whereas the blot for actin was exposed for 12 h.

Discussion

This investigation demonstrates that StAR mRNA is expressed in rat tissues that have high steroidogenic capacity. This study shows for the first time that StAR mRNA expression in the rat ovary is controlled by tropic hormones, which are known to regulate steroid hormone biosynthesis. These experiments demonstrate that hCG increases the steady-state levels of StAR mRNA in the ovary during follicular and luteal development. The rise in StAR expression paralleled the rise in serum progesterone levels, consistent with StAR's presumed role in the regulation of steroidogenesis.

The deduced rat StAR sequence was found to be similar to the mouse (Clark et al., 1994), bovine (Hartung et al., 1995), and human (Sugawara et al., 1995) StAR cDNA sequences recently reported. It is unlikely that sequence variation noted in our experiment between the rat and other species was attributable to the PCR, since RT-PCR of the mouse sequence, using identical primers and methods, yielded a StAR sequence that was 100% homologous with the mouse cDNA coding region reported previously (Clark et al., 1994). Although there is 92% homology between rat and mouse nucleotide sequences and 96% identity at the amino acid level, an important difference was noted at amino acids 43 and 44. The deduced rat amino acid sequence contains a proline and serine at amino acids 43 and 44, respectively, whereas the mouse sequence has serine residues at both positions. The serine residue at amino acid 43 is thought to be required for recognition and cleavage by mitochondrial proteases prior to protein import into the mitochondria (Hendrick et al., 1989). However, the serine residue at amino acid 43 in the mouse is not present in the rat, bovine, or human deduced amino acid sequences.

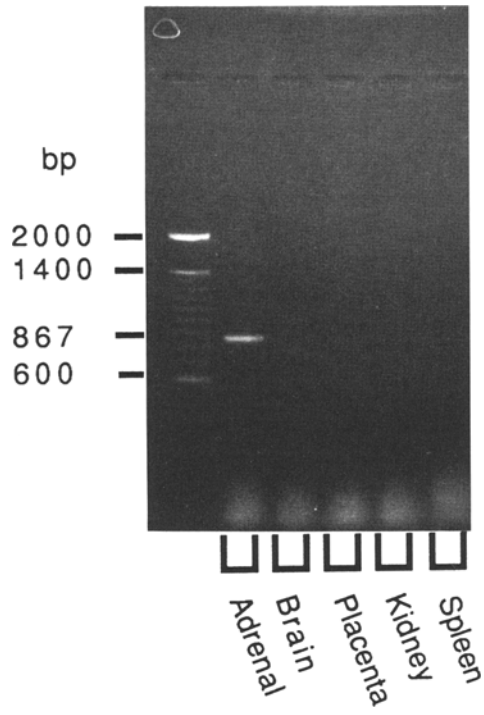

Fig. 7. RT-PCR of mRNA from various rat tissues. One microgram of RNA isolated from various tissues was reverse-transcribed for 1 h at 42°C before PCR using StAR-specific primers. Products were analyzed on a 1% agarose gel containing ethidium bromide. Data are typical of that obtained from two separate animals (n = 2).

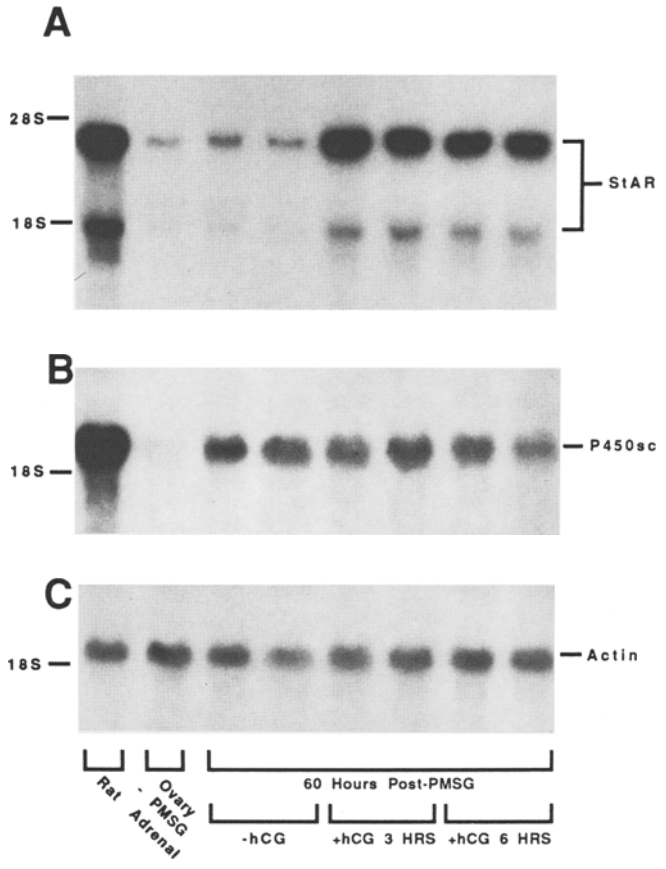

Fig. 8. Regulation of StAR mRNA expression in rat ovaries by hCG. Twenty-eight-d-old Sprague-Dawley rats (n = 4/time-point) were injected with 8 IU PMSG and 60 h later received an injection of hCG (50 U). Ovaries were obtained for control (t_0), 3- and 6-h time-points post-hCG injection. Northern blot analysis of StAR mRNA in the ovary following hCG treatment. This blot was probed sequentially for StAR, P450scc, and β-actin.

Although the exact biochemical mechanisms necessary for mitochondrial StAR import are unclear, we do know that mature StAR is associated exclusively with the mitochondria, and that the 37-kDa form is a cytosolic precursor of StAR and is processed by the mitochondria to all four 30-kDa mature forms (King et al., 1995).

The rat StAR cDNA detected multiple mRNA transcripts in mouse adrenal and in rat adrenal and ovarian tissues. The major transcripts detected in the rat were at 3.4 and 1.6 kb. This is consistent with what has recently been reported in the MA-10 mouse Leydig tumor cell line (Clark et al., 1995). StAR mRNA was detected in the unstimulated rat adrenal and ovary, and in hormone-stimulated testicular tissue. StAR expression in these tissues is consistent with that reported for the human and bovine StAR mRNA transcripts (Sugawara et al., 1995; Hartung et al., 1995). The absence of StAR mRNA in the testis may reflect the fact that this tissue was in a less stimulated state. Our finding may be similar to the results obtained using mouse Leydig cells in which the StAR protein is upregulated only after gonadotropin stimulation (Epstein and Orme-Johnson, 1991b). Since the male animals used in our experiment were 36 d old at the time of analysis, the absence of any basal StAR mRNA levels may also reflect the juvenile state of these rodents. StAR mRNA was not detected in rat placenta or brain, consistent with the findings previously reported by Sugawara et al. (1995). The absence of StAR mRNA in the placenta may be related to the fact that placental progesterone is not under acute cAMP regulation (Sugawara et al., 1995). Unlike the human, StAR was not detected in the rat kidney.

In the rat, the most prominent StAR mRNA species was 3.4 kb in size. Following tropic hormone stimulation, a 1.2-kb transcript was detected. Currently, the relationship between the multiple transcripts in the rat adrenal, ovary, and testis is unclear. However, a recent study has shown that the two different transcripts observed in bovine corpora lutea are due to differential polyadenylation (Hartung et al., 1995). This is further supported by the existence of multiple polyadenylation signals in the 3'-untranslated region of the bovine StAR cDNA. Although the biological relevance of this differential polyadenylation is unknown, our study has clearly demonstrated that both the 3.4- and 1.6-kb rat mRNAs are regulated hormonally. The significant increase in these transcripts following PMSG and hCG administration is consistent with the expression of this protein being rapidly regulated (Clark et al., 1994; Lin et al., 1995; Sugawara et al., 1995). The par-

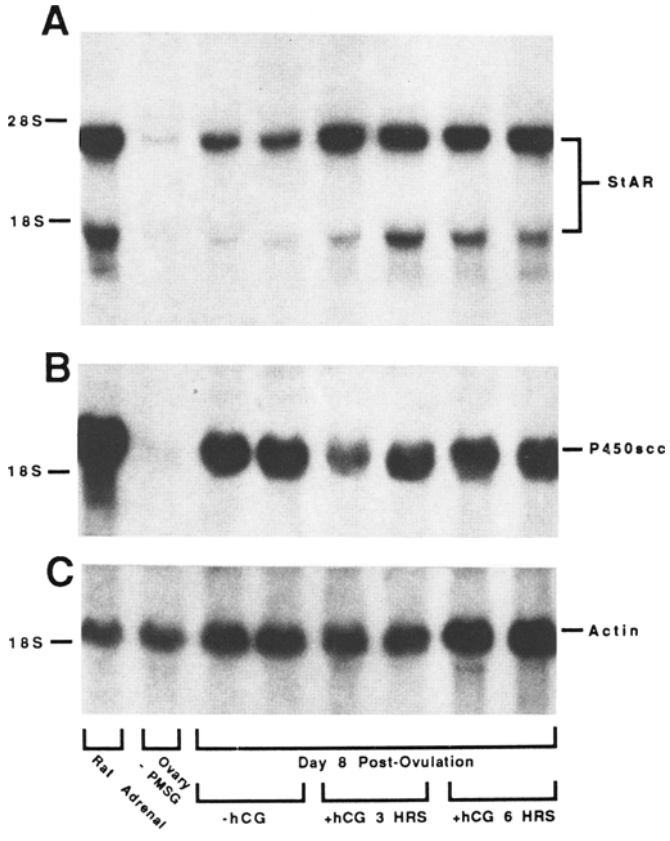

Fig. 9. Regulation of StAR mRNA expression in rat ovary (8 d postovulation) by hCG. Twenty-eight-d-old Sprague-Dawley rats (n = 4/time-point) were injected with 8 IU PMSG and 8 d after ovulation were injected with hCG (50 U). Ovaries were obtained for control (t_0), 3- and 6-h time-points post-hCG injection. Northern blot analysis of StAR mRNA in the ovary 8 d postovulation following hCG treatment. This blot was probed sequentially for StAR, P450scc, and β-actin.

allel increase in the StAR mRNA transcripts in association with the significant rise in serum progesterone further supports StAR's putative role as an essential regulator of steroid hormone synthesis (Clark et al., 1994; Lin et al., 1995; Sugawara et al., 1995). Our experiments are further supported by the fact that the cDNA sequence obtained for rat StAR is very homologous to both mouse and human cDNA sequences, both of which encode for functional proteins necessary for steroidogenesis (Clark et al., 1994; Sugawara et al., 1995).

The results of this study indicate that P450scc is induced in the immature ovary by PMSG and that P450scc mRNA levels remain elevated independent of hCG administration following corpus luteum development. This is consistent with P450scc being constitutively expressed in luteinized ovarian cells following ovulation (Goldring et al., 1987; McLean et al., 1989). Although ovarian P450scc mRNA levels were not altered by hCG treatment, StAR expression was significantly enhanced in parallel with an elevation in serum progesterone. After hCG stimulation, there was a dramatic increase in both the 3.4- and 1.6-kb ovarian transcripts by 3 h, but by 6 h, both the upper and lower transcripts began to decline. By 3 h, the two major transcripts were accompanied by the emergence of a 1.2-kb transcript. Whether the 1.2-kb transcript is also a product of differential polyadenylation remains unclear. Hartung et al. (1995) have suggested that this signal may be a tissue inhibitor of metalloproteinase-1 (TIMP-1) RNA transcript that crosshybridizes to the StAR probe because of an area of high homology to TIMP-1 within the StAR cDNA.

Acute regulation of cholesterol transport in response to tropic hormones is well documented (Garren et al., 1965; Mendelson et al., 1975; Cooke et al., 1975), and studies by several laboratories (Clark et al., 1994; Lin et al., 1995; Sugawara et al., 1995) support the concept that StAR enhances steroid production. The results of this investigation indicate that StAR mRNA is expressed in the adrenal, ovary, and testis. Furthermore, this study indicates that StAR mRNA is rapidly regulated by tropic hormones in the rat ovary and demonstrates that StAR's enhanced expression parallels progesterone production following hormonal stimulation.

Materials and Methods

Chemicals and cDNA Probes

hCG was purchased from Sigma Chemical Co. (St. Louis, MO). PMSG was purchased from Diosynth (Chicago, IL). 1,2,6,7-^{3}H*(N)*-progesterone (104.1 Ci/mmol) and [^{35}*S*]deoxy-ATP (1348 Ci/mmol) were purchased from DuPont-New England Nuclear (Wilmington, DE). [α^{32}P]-deoxy-CTP (3000 Ci/mmol) and the Sequenase DNA sequencing kit were obtained from Amersham Corp. (Arlington Heights, IL). BioMax and XAR-5 films were purchased from Eastman Kodak (Rochester, NY). SeaKem and SeaPlaque agarose were purchased from the FMC Corporation (Rockland, ME), and nylon membrane was obtained from Schleicher and Schuell (Keene, NH). TRI-Reagent, Background Quencher, Formazol, Microcarrier Gel-TR, and High Efficiency Hybridization solution were obtained from Molecular Research Center (Cincinnati, OH). The nick translation DNA labeling kit and all restriction enzymes were obtained from Boehringer Mannheim (Indianapolis, IN). The Wizard Miniprep and Megaprep DNA purification systems were purchased from Promega (Madison, WI). The kit for RT-PCR was purchased from Perkin Elmer (Norwalk, CT). The TA cloning kit was purchased from Invitrogen (San Diego, CA), and the Sephaglas BandPrep Kit was obtained from Pharmacia Biotech (Piscataway, NJ). An RNA mol-wt marker was purchased from Gibco BRL (Grand Island, NY). All other chemicals were reagent-grade, and were obtained from Fisher Scientific (Norcross, GA) or Sigma Chemical Co. The cDNA probe for rat P450scc (Goldring et al., 1987) was obtained from Joanne Richards (Baylor College of Medicine, Houston, TX).

Animals

Twenty-eight-day-old Sprague-Dawley rats were purchased from Harlan Industries of Madison, WI. All procedures for hormone treatment and the methods for tissue and blood sampling were approved by the University of South Florida Animal Care Committee. Throughout the experiment, animals had free access to food and water, and were housed under a 12-h dark, 12-h light cycle. Follicular development and ovulation were induced in rats by injection of 8 IU PMSG (i.m.). Rats ovulate approx 72 h following treatment with 8 IU PMSG (McLean et al., 1995). At 60 h post-PMSG treatment, one set of rats was injected with 50 U of hCG (iv). Ovaries were removed prior to hCG injection (t_0) and at 3 and 6 h post-hCG treatment. In a second experiment, rats were treated with PMSG as indicated above, followed by hCG injection (50 U, iv) on day 8. Ovaries, which consist mainly of luteal tissue using this protocol (McLean et al., 1995), were removed prior to hCG injection (t_0) and at 3 and 6 h post-hCG treatment. The male rats used in this study were 36 d old at the time of hCG administration. Testicular tissue was removed 3 h post-hCG or vehicle injection. Tissue was immediately frozen in liquid nitrogen. Serum samples were obtained by cardiac puncture at the time of tissue removal, and serum was stored at –20°C until progesterone was assayed. Rats were euthanized by clipping the diaphragm while under ether anesthesia. In all experiments, 4 animals were utilized/treatment or time-point.

Serum Progesterone Assay

Progesterone was measured by RIA using 1,2,6,7-^{3}H*(N)*-progesterone. This assay followed the methods previously described (McLean et al., 1989) and used the progesterone antibody GDN 337, which was kindly provided by G. D. Niswender (Colorado State University, Fort Collins, CO). The specificity, validity, and reliability of this RIA have been reported previously (Gibori et al., 1977).

RNA Isolation and Electrophoresis

RNA was prepared from ovaries using a modification of the Chomczynski and Sacchi method (Chomczynski and Sacchi, 1987) (TRI-Reagent Method, Molecular Research Center). This method consistently yields 5–8 μg RNA/mg tissue. Tissue (<200 mg) was homogenized in 3 mL of TRI-Reagent with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY), and centrifuged at 11,000*g* for 15 min at 4°C. RNA was precipitated from the aqueous phase with isopropanol, and the RNA pellet was washed in 75% ethanol and resuspended in Formazol (Molecular Research Center). RNA was quantified by absorbance at 260 nm in a Beckman DU-70 spectrophotometer (Palo Alto, CA).

For Northern blot analysis, total RNA (20 μg) was denatured at 65°C (15 min) and loaded onto 1% agarose gels containing 3% formaldehyde. Following size fractionation, RNA was blotted onto a nylon membrane (0.45-μm pore size) by capillary transfer and RNA was fixed to the membrane by UV crosslinking (0.3 J/cm^2). Ethidium bromide staining of the gel confirmed that the ribosomal RNAs (18S and 28S subunits) were intact, and determined whether equal amounts of RNA were loaded in each lane.

Isolation and Characterization of the Rat StAR cDNA Probe

The isolation and characterization of the rat StAR complementary DNA (cDNA) probe were carried out by engineering primers from the mouse sequence (Clark et al., 1994). The 5'-primer (GGATCCATGTTCCTCGCTAC GTTCAAG) spanned bases 73–92 of the mouse cDNA, and the 3'-primer (AGATCTCCTTAACACTGGGCCTC AGA) spanned bases 910–927 (Clark et al., 1994). Both primers contained additional nucleotides (underlined), which correspond to restriction sites. The 3' (downstream) primer was used to carry out RT-PCR from rat adrenal RNA. One microgram of rat adrenal RNA was reverse transcribed for 1 h at 42°C. This cDNA was then amplified by PCR using both primers. The conditions for PCR were denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min. The 867-bp fragment obtained by RT-PCR was then cloned into the TA vector (Invitrogen, San Diego, CA). The StAR cDNA was sequenced using the dideoxy chain termination method (Sanger et al., 1977) with [^{35}*S*]deoxy-ATP and the Sequenase 2.0 protocol (Amersham, Arlington Heights, IL).

Northern Blot Analysis

Northern blot hybridizations were performed using a 867-bp rat StAR cDNA, a 1.2-kb rat P450scc cDNA (Goldring et al., 1987), or with a 2.0-kb chicken β-actin cDNA. The cDNA inserts were labeled with [α^{32}P] deoxy-CTP using the nick translation DNA labeling method (Rigby et al., 1977). Northern blots were prehybridized at 62°C for at least 3 h in a 1*M* NaCl, 1% SDS solution containing Background Quencher (Molecular Research Center). Hybridization was completed in a High Efficiency Hybridization Solution (Molecular Research Center) containing the ^{32}P-labeled probe (1×10^6 dpm/mL; SA = 2×10^8 dpm/μg DNA) at 62°C for at least 16 h. Blots were washed three times at room temperature (5 min) in 1X SSC/1% SDS and three times at room temperature (10 min) in 0.1X SSC/0.1% SDS. RNA:cDNA hybrids were visualized on BioMax film using two intensifying screens and a 12–48 h exposure period. The RNA transcript size was determined by comparison to an RNA mol-wt marker run adjacent to the sample RNA lanes. Blots were stripped and reprobed with rat P450scc and actin cDNAs. Densitometric analysis was performed on the 2.0-kb β-actin transcript for the standardization of RNA loading.

RT-PCR

Total RNA was isolated from various rat tissues as indicated above. One microgram of total RNA from the different rat tissues was used to carry out RT-PCR using StAR-specific primers (listed above). Using the downstream primer, the RNA was reverse-transcribed for 1 h at 42°C. The cDNA was then amplified by PCR using conditions of denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min. PCR products were analyzed on a 1% agarose gel containing ethidium bromide and compared to a 100-bp ladder.

Data Analysis

The Northern blot results were quantitatively analyzed using a Hoefer Scanning Densitometer (Hoefer Instruments, San Francisco, CA). Minor variations in RNA loading were corrected for using the β-actin cDNA. Serum progesterone was expressed as the mean ± SEM. Data from these individual parameters were compared by analysis of variance (ANOVA) followed by Student-Newman-Keuls multiple comparison test when applicable (Zar, 1974). All analyses were completed using the Statview program with graphics (Abacus Concepts, Berkeley, CA) on a Macintosh IIci computer. A $p < 0.05$ was considered significant for all tests.

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