Rat Cholesterol Side-chain Cleavage Cytochrome P-450 (P-450<sub>sec</sub>) Gene
STRUCTURE AND REGULATION BY CAMP IN VITRO

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The entire rat P-450<sub>sec</sub> gene has been cloned, positions/sequences of the exon-intron boundaries (I-IX) are described and 940 base pairs (bp) of 5'-flanking DNA have been sequenced, compared to mouse, bovine and human genes, and analyzed by functional assays. Primer extension analysis mapped the transcription start site 32 bp upstream of the initiator methionine codon. The rat P-450<sub>sec</sub> promoter was ligated to the human growth hormone (GH) gene yielding p-940RecGH. This rat P-450<sub>sec</sub> fusion gene and a mouse gene (p-1500MsecGH) were transiently transfected into primary cultures of rat granulosa cells and Y1 adrenal cells. In the Y1 cells primer extension analysis showed that the rat P-450<sub>sec</sub>GH gene was expressed at lower basal levels than that of the mouse gene but showed greater stimulation (4-8-fold) in response to 8-bromo-cyclic AMP than the mouse (3-4-fold). Similar results were obtained when the fusion genes were transfected into primary cultures of rat granulosa cells and production of GH was measured in the media of the cells stimulated with forskolin. Furthermore, we document that gonadotropins (follicle-stimulating hormone/luteinizing hormone) can induce luteinization of granulosa cells in vitro, that this process is associated with constitutive maintenance of P-450<sub>sec</sub> mRNA in the absence of hormones/cAMP, that these events associated with luteinization are differentiation-stage specific and occur only in granulosa cells of preovulatory follicles but not in small antral follicles, that the process can be inhibited by cycloheximide if the protein synthesis inhibitor is present during the first 6 h of exposure to luteinizing hormone but not if added for short durations (3-5 h) later, and that once luteinization is induced P-450<sub>sec</sub> mRNA expression and progesterone biosynthesis are not strictly dependent on cAMP. Thus, the P-450<sub>sec</sub> gene is regulated by both cAMP-dependent and cAMP-independent mechanisms, each of which are associated with a specific stage of granulosa cell differentiation. The DNA domains involved in regulating these two diverse processes remain to be determined. Although there was remarkable sequence homology among rat, mouse, bovine, and human genes within 70 bp of the transcriptional start site, no other sequence similarities revealed conserved functional domains among the four genes. Although some cAMP-responsive element-like sequences are present in the rat gene, these were not conserved in the other species; including the mouse which showed high sequence homology with the rat throughout 900 bp of 5'-flanking DNA. Thus, the cAMP domains specific to this and other steroidogenic genes remain to be clearly identified.

The biosynthesis of progesterone by the mammalian ovary is a hormonally regulated process essential for reproductive cyclicity (1) and maintenance of pregnancy (2, 3). Cholesterol side-chain cleavage cytochrome P-450 (P-450<sub>sec</sub>) is the mitochondrial enzyme which together with adrenodoxin and adrenodoxin reductase (4) catalyzes the first rate-limiting step in progesterone biosynthesis (5). For this reason, numerous investigations have been done to determine what hormones/factors regulate the induction and activity of this enzyme in developing follicles and corpora lutea, as well as what factors lead to the demise of the corpus luteum and loss of P-450<sub>sec</sub> (6-9).

In the ovary, P-450<sub>sec</sub> mRNA (10-12) and protein (10-15) are induced in a developmental specific manner. P-450<sub>sec</sub> mRNA is low in theca and granulosa cells of small antral (SA) follicles and is increased marginally in these cells of preovulatory (PO) follicles (10). Whereas LH is primarily responsible for regulating P-450<sub>sec</sub> in theca cells, FSH and estradiol (in the rat) have been shown to be the principle regulators of the enzyme in granulosa cells (10). The major stimulus for the induction of this enzyme in all mammalian species appears to be the midcycle LH surge (16). High concentrations of LH/CG have been shown to rapidly and maximally induce P-450<sub>sec</sub> mRNA in rat PO follicles prior to ovulation (10, 11). Once induced as a consequence of elevated gonadotropin/cAMP, expression of P-450<sub>sec</sub> mRNA appears to be maintained in luteinized cells in vivo (10, 12) and in vitro (11) by mechanisms which are largely independent of CAMP. This has been observed not only for luteinized rat follicles but also in PO follicles (10-12) and PO follicles prior to ovulation (10, 11).

1 The abbreviations used are: SA, small antral; bp, base pair(s); P-450<sub>sec</sub>, cholesterol side-chain cleavage cytochrome P-450; (o)FSH, (ovine) follicle-stimulating hormone; (h)CG, (human) chorionic gonadotropin; LH, luteinizing hormone; PO, preovulatory; PRL, prolactin; (h)GH, (human) growth hormone; H8, N-[2-(methylamino)ethyl] isoquinolinesulfonamide dihydrochloride; CRE, cAMP-responsive element; kb, kilobase pair(s); FBS, fetal bovine serum; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid.
granolosa cells but also is supported by in vitro (17, 18) and in vivo (19) evidence in primate cells. Rat luteal cells, however, unlike luteal cells in the primate and many other mammals, are also regulated by estradiol and prolactin (PRL) (2, 3, 7).

To understand the diverse molecular mechanisms controlling the expression of this key steroidogenic enzyme in gonadal, adrenal, and placental (primate) cells, transacting factors and cis-acting DNA sequences need to be identified. To this end, the entire human P-450c,, gene (20) and 5' regulatory/promoter regions of the mouse (21) and bovine (22) genes have been cloned. The 5'-flanking regions of the mouse (22), human (23, 24), and bovine (22) genes all show cell-specific (23, 24) and cAMP-dependent (21-23) regulation of reporter genes when transfected into various cell lines.

The exact sequences mediating cell-specific and cAMP-dependent regulation of P-450c,, have not yet been determined. The CRE consensus so well described and characterized in other genes (25-27) is not present in the mouse, human, or bovine P-450c,, sequences analyzed to date. Site-directed mutagenesis of specific regions within the mouse promoter proportionally reduced basal and induced expression of the GH reporter gene but did not decrease the fold induction by cAMP (21). Although AF-1 sites have been identified in the mouse (21) and bovine (22) regulatory regions, functional activity of these sites has not yet been unequivocally associated with these regions.

The following study was undertaken to determine the structure of the rat P-450c,, gene and to characterize the 5'-flanking regulatory/promoter region of this gene by using a chimeric expression vector transfected into primary cultures of granulosa cells and by comparing genomic sequence similarities among the four genes for which nucleotide information is now available. Furthermore, a series of in vitro studies were designed to determine if constitutive expression of P-450c,, mRNA and progesterone biosynthesis could be induced in granulosa cells in culture and if this response was dependent on the stage of granulosa cell differentiation in vivo or to the removal of the granulosa cells from an environment containing inhibitory molecules. By establishing specific granulosa cell cultures that exhibit cAMP-dependent versus cAMP-independent regulation of P-450c,, mRNA, we will have the opportunity to use these cultures to identify stage-specific regulation of cis-acting DNA elements.

**EXPERIMENTAL PROCEDURES**

Isolation of the Rat P-450c,, Genomic Clone—A library of partially BamHI-digested rat liver genomic DNA in phage Charon 35 (kindly provided by Dr. C. B. Kasper, University of Wisconsin, Madison, WI) was screened with a 32P-labeled 5' 352-bp AvaI fragment of human P-450c,, cDNA (24) and a 57-mer oligonucleotide (11). Recombinant phage plaques were amplified in Escherichia coli K802 grown on nitrocellulose filters. For plaque hybridization (32), the random primer labeled (33) human P-450c,, cDNA fragment and the end-labeled (22) 57-oligomer were used as probes. One clone of >20 kb in size was consistently positive and plaque purified.

Subcloning and Sequence Analysis—The P-450c,, genomic clone contained two internal BamHI sites. Three genomic fragments subcloned in pGEM3Z (Promega, Madison, WI) were digested with different restriction enzymes. Southern blots of these digests were hybridized with three rat P-450c,, cDNA clones (11), a 200-bp PstI-EcoRI 3' rat cDNA fragment, the 57-oligomer used for screening (11), and four 20-mer oligonucleotides with sequences based on human P-450c,, cDNA sequence (bp 942-961, exon 5) (20, 24) and rat P-450c,, cDNA sequences (bp 1030-1049, bp 1150-1169, bp 1357-1376) (11). Positive restriction fragments were subcloned into pGEM3Z for double-stranded sequence analysis by the dideoxy chain termination method (34) using a Pharmacia sequencing kit (Pharmacia LKB Biotechnology Inc.). Sequences were compared to the full-length rat P-450c,, cDNA (11) to identify exons and exon-intron boundaries.

The splice-junctions were also compared to the consensus sequence (35).

**Construction of Plasmids**—The human growth hormone (hGH) gene was used as a reporter gene in transient transfections of primary cultures of granulosa cells (36). All plasmids contained a 2.1-kb BamHI-EcoRI fragment encompassing structural GH sequences from the start of transcription at the BamHI site to +1000 bp (p40GH) (40), a polyadenylation signal (17), and a rat 5' promoter (p40RecGH) (11). Plasmids were transfected into primary cultures of granulosa cells (36) on day 3 of culture with 20 µg total of plasmid DNA, using the calcium phosphate precipitation technique (39). After 4 h of transfection, the cells were incubated in culture medium with or without 7.5 µM forskolin, and hGH secretion was measured after 60-72 h. RNA was isolated from the transfected cells as described above. In some cultures, protein content was measured (40).

**Transfection Procedures**—Granulosa cell cultures were transfected with regimens of deoxynucleotide mix (10 mM each of dCTP, dATP, dGTP, and dTTP), 1 µl of actinomycin D (1 mg/ml), and 1 µl of reverse transcriptase (Bethesda Research Laboratories, Md-MV, 200 units/ml) were added. The reactions were incubated at 42 °C for 1 h, extracted with phenol/chloroform, precipitated with ethanol, and analyzed by electrophoresis on a 6% denaturing polyacrylamide gel. The size of the extended product in each reaction was determined by comparison with a sequencing reaction of the mouse P-450c,, promoter region, from which the G reaction is shown. Levels of correctly initiated transcripts in the transfected Yl adrenocortical cells were also determined by primer extension analysis using a deoxynucleotide complementary to bases +47 to +70 of the hGH gene (21).

**Animals**—Intact immature rats (day 26 of age) were obtained from Holtzmann (Madison, WI) and maintained under a 16 h light-8 h dark regimen with food and water ad libitum.

**Isolation and Culture of Non-luteinizing and Luteinizing Granulosa Cells**—Small antral (SA) follicles were isolated from ovaries of immature rats at day 26 of age. Maturation of preovulatory (PO) follicles was stimulated by treatment of immature rats at day 28 of age with a low dose of hCG (0.15 IU) given twice daily for 2 days (17, 28). Ovulation and luteinization of these PO follicles was subsequently achieved by an intravenous injection of an ovulatory dose (10 IU) of hCG at day 35 of age. Non-luteinized granulosa cells were isolated from SA follicles and PO follicles of 30-day-old rats treated with the low dose of hCG. Luteinizing granulosa cells were harvested from PO follicles isolated 7 h after the intravenous injection of 10 IU hCG (PO + hCG). Cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12, 1:1, containing 1% fetal bovine serum (FBS), and 16 nM testosterone, as described previously (29). Other additions to the media are indicated in the legends of the figures.

**Incubations in Vitro**—For some experiments, follicles isolated from PO or PO + hCG ovaries were incubated at 37 °C under 95% O2, 5% CO2 in culture medium in the presence or absence of 1.0 (500 ng/ml) and/or the presence or absence of inhibitors of translation or transcription. After incubation, the follicles were homogenized, and RNA was prepared from the follicles or granulosa cells. The RNA was isolated from these follicles and cultured for 3-7 days in the absence of inhibitors.

**Granulosa Cell Cultures for Transfection**—For the transfection experiments, granulosa cells were isolated from the ovaries of un-
treated, immature rats (27–28 days old) by expressing granulosa cells from fragments of ovaries that had been incubated in 6.8 mM EGTA and 0.2% bovine serum albumin in medium 199 followed by incubation in 0.5 M sucrose, 1.8 mM EGTA and 0.2% bovine serum albumin in medium 199, as described (30). This procedure yields a single cell suspension which was plated at a density of 30,000 cells/cm², and cultured in medium containing 50 ng/ml NIH-oFSH-16 and 10 nM testosterone.

RNA Isolation and Northern Blotting—Total RNA was extracted from follicles and cultures of granulosa/luteal cells using a buffer containing 1% Nonidet P 40 (31). All RNA was phenol/chloroform extracted, ethanol precipitated, and quantitated by absorbance at A260. Preparation and hybridizations of Northern blots were done as described previously (11).

Radioimmunoassays—For the measurement of progesterone biosynthesis media samples were collected, boiled, and stored at -20 °C. Radioimmunoassays were subsequently performed as described by Richards et al. (15).

Materials—Human chorionic gonadotropin (2800 IU/mg) was purchased from Organon Special Chemicals, West Orange, NJ. Ovine follicle-stimulating hormone (NIH-oFSH-16, 20 × NIH-FSH-S1; LH 0.04 × NIH-LH-S1), and ovine prolactin (NIH-oPRL-15, 30.5 IU/mg) were kindly provided through the National Hormone and Pituitary Program, Baltimore, MD. Oligonucleotides were obtained from Genetic Design, Houston, TX. [α-32P]dCTP and [γ-32P]ATP were purchased from ICN Radiochemicals, Irvine, CA. H8, N-[α-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride, was from Seikagaku America, St. Petersburg, FL. Tissue culture materials were purchased from GIBCO. All electrophoresis materials were from Bio-Rad.

RESULTS

Cloning and Structure of the Rat P-450, Subgene—A >20-kb genomic clone containing the entire rat P-450, subgene was identified in and isolated from a rat liver genomic library using a 5′-5′-2-bp human AXL cDNA fragment (24) as a probe (Fig. 1A). BamHI digestion of plaque-purified P-450, genomic DNA produced three fragments >17, 4.0, and 3.7 kb in size. A 6-kb subfragment of the largest BamHI fragment and the two smaller fragments were subcloned into pGEM3Z. These fragments were then used for restriction mapping and additional subcloning to identify exons I–IX (Fig. 1B) and to determine the positions/sequences of the exon-intron boundaries (Figs. 1C and 2). The rat P-450, subgene structure, including the nine exons, the intron-exon boundaries and the unusual sequences delineating the exon-intron boundary of exon VI, is highly conserved with that of the human gene (20).

Sequence analysis of the 940 bp of 5′-flanking DNA of the rat gene revealed a putative TATA box (TATAAA) beginning 71 bp upstream from the translational ATG start codon (Fig. 2). Putative regulatory domains identified in the rat P-450, 5′-flanking region are indicated in Fig. 2. These include a CAAT box conserved in the mouse (21) but not in the human (20) or bovine (22) genes, a GC box, and several CRE-like sequences not conserved in the regulatory/promoter regions of the mouse, human, or bovine genes (20–22). The transcriptional start site of the rat P-450, subgene was mapped by primer extension analysis. As shown in Fig. 3, the predominant extended product had a size of 103 nucleotides, which mapped the transcriptional start site to 32 bp upstream of the initiator methionine codon and 24 bp downstream from the TATA box (Fig. 2). An extended product of the same size was seen with mouse adrenal RNA, whereas no extended product was seen with mouse liver RNA, confirming the specificity of the reaction. A second less abundant product was also observed at 72 nucleotides in the rat granulosa cell reaction lane. The significance of this additional band is not known at this time, but may reflect rare use of an alternative start site or the presence of pause sites in the reverse transcription reaction. The lower intensity of the signal with the mouse adrenal RNA may reflect the minor sequence differences between the rat and the mouse P-450, mRNA.²

This promoter region was highly conserved among rat, mouse (21), human (20), and bovine sequences (22) as shown in Figs. 4 and 5. Computer-derived sequence similarities revealed, in particular, that the region extending 70 bp upstream from the transcriptional start site was the only region highly conserved among all species (Fig. 4), whereas the rat and mouse sequences exhibited remarkable similarity in the 800–900 bp of 5′-flanking sequence analyzed (Fig. 5). Sequence similarity between human and bovine genes was greater than either of these was with the rat and mouse genes (Fig. 4).

Functional Characteristics of the P-450, 5′-Flanking Region—We next determined if the 5′-flanking region of the rat P-450, subgene contained regulatory domains capable of directing the expression of a reporter gene (hGH) in primary cultures of rat granulosa cells and if expression could be

² K. Parker, unpublished results.
regulated by cAMP, the major intracellular transducer of gonadotropin action in granulosa cells. Accordingly, the 940-bp fragment of the rat P-450, 5'-flanking sequence was ligated to the hGH gene via a BamHI site created to use the GH ATG initiation codon (see "Experimental Procedures"). Expression of the p-940RsccGH chimeric gene was compared to p-1500MsccGH, a vector containing 1500 bp of mouse P-450, 5'-flanking sequence (21) and to a control vector, pXGH5, containing the mouse metallothionein (MT) promoter plus the GH gene (36). All transfections were done using primary cultures of granulosa cells harvested from ovaries of immature rats. Previous studies have shown that expression of endogenous P-450, mRNA (29, 30) are induced in these cells by forskolin, a non-hormonal stimulator of CAMP. Furthermore, we (30) have shown previously that forskolin can regulate the expression of an (YCG-CAT chimeric gene (46) when transiently transfected into similar cultures, indicating that these primary cultures of granulosa cells can be used effectively for transfections and that they contain factors capable of regulating transcription involving the CRE consensus domain.

An extensive series of transfection experiments were done to determine the optimal amount of vector DNA to be transfected, time of culture, duration of transfection, necessity of glycerol shock, dose of forskolin, and time course of GH induction for each of the three vectors (data not shown). In experiments presented herein, on day 3 of culture 10 and 20 pg of DNA were transfected for 4h after which fresh medium containing 1% FBS with or without forskolin was added for 65 h. As shown in Fig. 6A, basal expression of p-940RsccGH was low in transient transfections of granulosa cells maintained in the absence of forskolin but was induced 4-8-fold with forskolin. The p-1500MsccGH vector (5 pg/transfection) had a higher basal activity but was not stimulated as much (3-fold) by forskolin (Fig. 6A). Similar results were obtained in three additional experiments (p-94ORsccGH, 5.7 ± 0.9-fold...
increase with forskolin; p-1500MscGH, 3.2 ± 0.7-fold increase with forskolin). Fig. 6A also shows the relative activity of the rat P450sec promoter compared to the high basal activity of the mouse MT promoter. The high basal activity of the pmMTGH vector was especially fortuitous for standardizing the results of different experiments.

The p-940RscGH gene was also expressed and regulated by cAMP in Y1 adrenal cells as determined by primer extension analysis. As shown in Fig. 6B, p-940RscGH mRNA was lower than that of p-1500MscGH in the absence of 8-Br-CAMP but was induced to a greater extent in the presence of 8-Br-cAMP. These results confirm those obtained in the primary cultures of rat granulosa cells and also provide further evidence to verify the correct transcriptional start site for the rat P-450sec gene. The difference in length between the rat and mouse extension products originates from the different size of the P-450sec fragments ligated to the GH gene via a BamHI site created by site-directed mutagenesis at +37 bp in the rat and at +28 bp in the mouse 5'-flanking regions.

Hormonal Regulation of P-450 mRNA in Granulosa Cells—In previous studies we have shown that P-450sec mRNA is low in granulosa cells of SA follicles, is increased marginally in granulosa cells of PO follicles, and is maximally induced in vivo within 7 h of an LH/CG surge (10). Once induced by the LH surge, P-450sec mRNA appears to be constitutively maintained in granulosa cells that either luteinize in vivo (10, 12) or in vitro (11). The following experiments were done to determine if a stable luteinized phenotype could be induced in vitro by exposing PO granulosa cells to elevated levels of gonadotropins in culture. Accordingly, granulosa cells were harvested from PO follicles and cultured in the presence of 1% FBS and 100-1000 ng/ml of FSH. On day 3 of culture, this medium was removed, and the cells were then cultured for an additional 6 days in medium containing 1% FBS alone or 1% FBS with either FSH (100 ng/ml), LH (1 µg/ml), or FSH, LH and PRL (1 µg/ml). As shown in Fig. 7A, PO granulosa cells exposed to FSH for 3 days underwent morphological and functional luteinization. The cells were similar to other luteinized granulosa cells in culture (29) by having an epithelial-like appearance and an abundance of lipid droplets. In addition, these cells were capable of expressing elevated levels of P-450sec mRNA in the absence of hormones on days 3–9 (Fig. 7A, 1% FBS). The continuous presence of FSH or the addition of LH had little effect on either cell morphology or P-450sec mRNA levels. PRL, however, did demonstrably increase P-
sequences. Dashes indicate identical nucleotides; dots indicate gaps in these cells, a response similar to that of luteal cells in Go. These changes in \( \text{P-450}_{\text{\alpha\beta}} \) mRNA were associated with parallel changes in progesterone biosynthesis. On day 9, progesterone concentrations in cultures containing PRL (17 \( \pm \) 2 rig/ml) were twice as high as those in cultures maintained in 1% FBS, FSH, or LH (1 \( \times \) 0.4, 8 \( \pm \) 2, 7 \( \pm \) 0.4 rig/ml, respectively). Although the lack of a response by these SA follicles might be explained by the absence of LH receptors on immature granulosa cells (7, 28), their response to gonadotropins. Therefore, we also determined if granulosa cells of SA follicles could respond to gonadotropins in vitro. SA follicles isolated from ovaries of hormonally primed immature rats and incubated 6 h with 500 rig/ml LH. Cycloheximide was added either at the initiation of the incubations (t = 0) or 2 h after the addition of LH. As shown in Fig. 8, \( \text{P-450}_{\text{\alpha\beta}} \) mRNA was increased rather than reduced by exposure to LH + cycloheximide added either at t = 0 or t = 2 h. Cycloheximide alone had no effect (data not shown). These results suggest that protein synthesis is not required for the effect of LH on \( \text{P-450}_{\text{\alpha\beta}} \) mRNA in PO follicles, but in contrast show that the cycloheximide-related increase in LH-induced \( \text{P-450}_{\text{\alpha\beta}} \) mRNA is submaximal in the presence of ongoing protein synthesis. The cycloheximide-related increase in LH-induced \( \text{P-450}_{\text{\alpha\beta}} \) mRNA, however, is not associated with an enhancement of luteinization. Specifically, when granulosa cells were isolated from ovaries of hormonally primed immature rats and incubated with ovulatory doses of FSH (1 \( \mu \)g/ml) and LH (500 ng/ml) for 6 h, these cells luteinized in culture and maintained elevated levels of progesterone biosynthesis (30 \( \pm \) 2 ng/ml) and \( \text{P-450}_{\text{\alpha\beta}} \) mRNA (data not shown) for 8 days in the absence of hormones. In contrast, granulosa cells isolated from PO follicles that had
Evidence for constitutive maintenance of P-450\textsubscript{sec} mRNA in the absence of hormones on days 3-9. B, lack of induction of luteinization from PO follicles (A) or SA follicles (B) and cultured in DMEM/F-12 containing 1% FBS and 100-1000 ng/ml of FSH and 10 ng/ml testosterone for 3 days. From days 3-9 cells were cultured in medium with 1% FBS, or 1% FBS plus either FSH (100 ng/ml), LH (1 μg/ml) or FSH + LH + PRL (1 μg/ml). On day 9 of culture, media were collected for analysis of progesterone biosynthesis and RNA was extracted from the cells. For Northern blot analysis 20 ¼g total RNA were used/lane (A) or as indicated (B).

Fig. 7. A, induction of luteinization of PO granulosa cells in vitro. Evidence for constitutive maintenance of P-450\textsubscript{sec} mRNA in the absence of hormones on days 3-9. B, lack of induction of luteinization in granulosa cells of SA follicles. Granulosa cells were isolated from PO follicles and cultured in DMEM/F-12 containing 1% FBS and 100-1000 ng/ml of FSH and 10 ng/ml testosterone for 3 days. From days 3-9 cells were cultured in medium with 1% FBS, or 1% FBS plus either FSH (100 ng/ml), LH (1 μg/ml) or FSH + LH + PRL (1 μg/ml). On day 9 of culture, media were collected for analysis of progesterone biosynthesis and RNA was extracted from the cells. For Northern blot analysis 20 ¼g total RNA were used/lane (A) or as indicated (B).

These PO + hCG follicles were then incubated for 1 or 3 h with actinomycin D or for 3 and 5 h with cycloheximide. Following these short incubations, granulosa cells were harvested and cultured in 1% FBS for 9 days. As shown in Fig. 9A (right panel), actinomycin D for 3 h did impair the expression of P-450\textsubscript{sec} mRNA observed on day 9 of culture whereas 1 h had little effect. Cycloheximide did not alter the expression of P-450\textsubscript{sec} mRNA (Fig. 9A, left and right). Addition of H8 (200 µM), an inhibitor of cAMP-dependent protein kinase, to the cultures of luteinized granulosa cells on days 3-9 also had no effect on P-450\textsubscript{sec} mRNA (Fig. 9A, left), suggesting that P-450\textsubscript{sec} mRNA in these cells was being maintained by mechanisms independent of basal activation of protein kinase A.

To determine if the PO granulosa cells that luteinized in vitro (Fig. 9B) required protein kinase A to maintain their stable luteinized phenotype, increasing concentrations of H8 were added to these cultures in the presence of FSH or forskolin. High concentrations of H8 reduced but did not completely block expression of P-450\textsubscript{sec} mRNA (Fig. 9A) and progesterone biosynthesis in these cells (Table I).

**DISCUSSION**

We report herein the cloning and structure of the rat P-450\textsubscript{sec} gene, including 940 bp of 5'-flanking DNA. Computer comparisons of the rat P-450\textsubscript{sec} gene with ~885 bp of mouse P-450\textsubscript{sec} regulatory/promoter sequences (21) show striking similarities throughout the entire 5' region. In marked contrast, the rat P-450\textsubscript{sec} 5'-flanking DNA shows only limited similarity with the 5' regions of the human (20) and bovine (22) P-450\textsubscript{sec} genes. The regions of similarity include a TATA box, a transcriptional start site 24 bp downstream of the TATA box and a promoter region corresponding to -60 to -36 bp (rat), -56 to -32 bp (mouse), -154 to -94 bp (human) and -58 to -33 bp (bovine). Deletions in this region of mouse (21), human (23, 24), and bovine (22) promoters have been shown to abolish transcriptional activity of reporter genes.

**Fig. 8.** Effect of cycloheximide on LH-mediated induction of P-450\textsubscript{sec} mRNA in PO follicles. PO follicles were isolated from immature rats primed with low doses of hCG ("Experimental Procedures") and incubated for 6 h at 37°C in 5% CO\textsubscript{2}, 95% O\textsubscript{2} with 500 ng/ml LH. Cycloheximide (CHX, 10 μg/ml) was added at t = 0 or at t = 2 h of the incubation. After 6 h of incubation, RNA was isolated from the follicles. For Northern blot analysis 20 ¼g total RNA were used/lane.

**Fig. 9.** A, effects of cycloheximide and actinomycin D on P-450\textsubscript{sec} mRNA in luteinized granulosa cells in culture. PO + hCG (7 h) follicles were isolated and cultured in DMEM/F-12 containing 1% FBS and 10 ng/ml testosterone for 1, 3, or 5 h in the presence or absence of cycloheximide (CHX, 10 μg/ml) or actinomycin D (Act D, 1 μg/ml). Subsequently, granulosa cells were isolated and plated in the same medium without inhibitors or hormones and cultured for 3, 5, or 7 days. Fresh medium as added on days 3 and 5. B, effects of H8 on PO granulosa cells cultured in the presence of FSH (100 ng/ml) and forskolin (10 µM). Granulosa cells were isolated from PO follicles. The cells were cultured and treated as described in Table I. On day 9 of culture media were collected for analysis of progesterone biosynthesis and RNA was extracted from the cells. Northern blots were as described in legends of Figs. 7 and 8.
Footprint analyses of this region in the mouse promoter reveal two motifs, CAAGGCT (the reverse complement of AGCCTTG, 21) and AGGTCA, which bind factors designated SF-1 and SF-2 (21). SF-1 appears to be specific for endocrine (Y1 and MA-10) cells and thereby may be part of a tissue-specific element.

Somewhat surprising is the total absence of sequence similarities between the 5'-flanking regions of these P-450,cr genes in regions which have been implicated as major sites of hormone and cAMP regulation based on transient expression of chimeric genes. For example, sequences -183 to -83 of the bovine gene can confer cAMP regulation in Y1 adrenal cells (22) whereas sequences -425 to -362 of the mouse gene also appear to modulate the fold induction of the GH fusion gene although the quantitation was not absolute (21). AP-1 sites have been identified in each gene and may confer cAMP regulation. However, their positions have not been conserved and mutations of the AP-1 element in the mouse gene had no effect on cAMP-induced P-450,cr-GH expression (21). Although we have identified several CRE-like motifs in the rat P-450,cr gene, none of these is conserved in the mouse sequence despite the high degree of overall similarity between the DNA of these two genes. Thus, the transacting factors and cis-acting DNA elements that specifically confer cAMP regulation and cell-specific expression of the P-450,cr gene in each species remain to be determined. In addition, the rat and mouse P-450,cr genes are presumed to have a PRL regulatory domain. Perhaps, the greater sequence homology between rat and mouse than between murine and human or bovine is indicative of other mechanisms of regulation.

At least some of the domains responsive to cAMP in the rat P-450,cr gene appear to be localized within the -940-bp fragment of the 5'-flanking region that has been cloned. For example, forskolin (cAMP)-increased expression of the p 940RaccGH gene when it was transfected into either primary cultures of granulosa cells or Y1 adrenal cells. The fold stimulation by forskolin was similar to that reported for chimeric genes containing mouse (21), human (23), and bovine (22) P-450,cr promoters. When expression of both rat granulosa cells and mouse Y1 adrenal cells, basal activity of the rat vector was consistently less than that of the mouse but showed greater fold stimulation by forskolin or 8-Br-cAMP. Reasons for these differences are not entirely clear. The greater basal activity of the mouse sequence may be associated with additional nucleotides upstream of those present in the rat sequence which could contain other transcriptional enhancing elements and/or cell-specific elements. Expression vectors containing longer regions of the human gene (−2500 bp (24)) and −5400 bp (23)) also exhibit high basal activity, especially in Y1 cells (24). Deletion analysis indicated the presence of adrenal specific elements in the human P-450,cr gene between −573 and −2500 bp (24). Additional sequences may also be required for expression in Leydig cells because in MA-10 Leydig cells the endogenous gene but not the transfected mouse P-450,cr gene was regulated by cAMP (21). The greater fold stimulation by forskolin/8-Br-cAMP of the rat P-450,cr gene compared with the mouse P-450,cr gene may be associated with some of the putative CRE-like sequences which are present in the rat but not in the mouse promoter. These results indicate that there may be one or more domains regulated by cAMP that are specific for steroidogenic genes, as suggested by Ahlgren et al. (22). Precedence for this is not surprising based on the recent complexity and increased number of factors belonging to the CREB/JUN/ATF families (47-49) as well as on the ability of at least one transcriptional factor, COUP, to bind to more than one sequence motif via a different set of contact points on the DNA (50).

Previous studies have shown that 5-7 h of exposure of PO follicles to high concentrations of LH/hCG in vivo are sufficient to induce P-450,cr mRNA and progesterone biosynthesis to a maximal level which subsequently is constitutively maintained by luteinized cells in vivo and in vitro. Results of the hormonal regulation studies in vitro document that PO granulosa cells can respond to FSH/cAMP in culture and undergo differentiation to a stable luteal cell phenotype that constitutively maintains progesterone biosynthesis and expression of P-450,cr mRNA. Furthermore, these PO granulosa cells that have luteinized in vitro respond to PRL, a functional index of luteal cell activity in the rat (2, 3). The ability of granulosa cells to luteinize in vitro is dependent on their stage of differentiation in vivo. Granulosa cells isolated from SA follicles failed to luteinize in culture and did not maintain either P-450,cr mRNA or progesterone biosynthesis in the absence of hormones. These results suggest that the failure of SA granulosa cells to luteinize in vivo is not compromised by the presence of inhibitory factors but rather is due to a lack of an appropriate developmental repertoire. PO granulosa cells, which do luteinize in vitro, have more LH receptors, produce more cAMP in response to LH and FSH and can synthesize more estradiol (7, 51), an important autocrine regulator in the rat (6, 7). Any one or all of these functions may enhance the ability of PO granulosa cells to luteinize.

LH-induced luteinization of PO granulosa cells could be blocked if a protein synthesis inhibitor (cycloheximide) was present during the entire time (6 h) the PO follicles were incubated with the ovulatory dose of LH or if the inhibitor was added for the last 3-4 h. However, at the time the granulosa cells were harvested for culture (6 h), P-450,cr mRNA was higher in the PO follicles exposed to LH and cycloheximide than in PO follicles exposed to LH alone. Taken together, these results indicate that the enhanced levels of P-450,cr mRNA observed in the presence of LH plus inhibitor were not indicative of an overall enhancement of the luteinization process but rather to altered cellular processing of the induced mRNA. Furthermore, the data indicate that luteinization is dependent on the synthesis of specific proteins during the first few hours of exposure to LH.

In contrast, once the luteinization process is initiated in granulosa cells as a consequence of the LH surge in vivo, the process is not easily disrupted by short term exposure of granulosa cells to cycloheximide or actinomycin D. Once
granulosa cells are luteinized, they constitutively express P-450m mRNA in the absence of hormones (11), even in the presence of H8, a selective inhibitor of protein kinase A. Thus, the regulation of P-450m expression shifts from cAMP-dependent mechanisms in granulosa cells to cAMP-independent controls in luteal cells. This shift appears to be more complete in PO granulosa cells exposed to the LH/CG surge in vivo than in PO granulosa cells exposed to elevated levels of FSH in vitro, based on the greater effectiveness of H8 in reducing P-450m mRNA and progesterone biosynthesis in the PO cells that luteinize in vitro. Nevertheless, the PO granulosa cells that are luteinized by hormones in vitro do subsequently acquire the ability to constitutively express P-450m mRNA in the absence of hormones. Because it is more difficult to identify and isolate authentic PO follicles (80–100% accuracy) compared with PO follicles exposed in vivo to hCG (100% accuracy; these are exclusively hyperemic), it is possible that there is greater heterogeneity of the PO granulosa cells that are isolated and cultured.

These cAMP-dependent and -independent stages of regulation of P-450m mRNA in granulosa/luteal cells share some similarities to the regulation of P-450c mRNA expression in adrenal cells. For example, corticotropin increases P-450c transcription in adrenal cells by a cAMP-dependent mechanism (21, 38). However, this process in the adrenal is inhibited by cycloheximide and appears to involve de novo protein synthesis (22). In contrast, cycloheximide did not immediately block the LH-mediated increase in P-450m mRNA in PO granulosa cells. Rather, the effect of LH on P-450m mRNA was greater when protein synthesis was inhibited, suggesting ongoing synthesis of a putative inhibitory factor which down-regulates P-450m mRNA. Thus, there appear to be tissue-specific and hormone-specific mechanisms regulating P-450m mRNA in both gonadal and adrenal cells.

In summary, we have cloned the entire rat P-450m gene and 940 bp of regulatory/promoter sequences. The promoter region (70 bp) of the P-450m gene is highly conserved across species (rat, mouse, human, bovine). Other 5′ regions in the rat and mouse are highly conserved whereas those of the human and bovine exhibit less obvious similarities based on the computer analyses done. Because of the rigorous cell-specific expression of the P-450m gene and its regulation by hormones/cAMP at defined stages of granulosa cell differentiation in all species, one would predict that some underlying similarities will emerge once more detailed functional studies are done on each of the 5′-flanking regions and in cells at specific stages of differentiation. The marked similarity between the rat and mouse genes may provide insight into regions that respond to PRL as well.

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