Regulated Expression of Sterol Carrier Protein 2 in the Ovary: A Key Role for Cyclic AMP†

Hanna Rennert,‡ Abraham Amsterdam,§ Jeffrey T. Billheimer,∥ and Jerome F. Strauss, III*,‡

Department of Obstetrics and Gynecology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, Department of Hormone Research, Weizmann Institute of Science, Rehovot 76100, Israel, and Medical Products Division, Du Pont Merck Pharmaceutical Company, Experimental Station, Wilmington, Delaware 19880

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ABSTRACT: Sterol carrier protein 2 (SCP_2) is believed to play an important role in the intracellular movement of cholesterol in steroidogenic cells. We examined the distribution of SCP_2 gene expression in the rat ovary and the role of gonadotropins and cyclic AMP in the regulation of SCP_2 mRNA levels. In situ hybridization revealed that the most steroidogenically active ovarian compartments (e.g., corpora lutea and theca cells) contain significant amounts of SCP_2 mRNA whereas granulosa cells have modest levels. Gonadotropins, which promote follicular growth and luteinization, increased the ovarian content of SCP_2 mRNA as assessed by Northern blotting along with increases in cytochrome P450scc mRNA. Using steroidogenic transformed rat granulosa cells (Grs-21), a cyclic AMP analogue (8-Br-cAMP) was found to increase SCP_2 mRNA and protein levels within 24 h of treatment. P450scc mRNA was also induced whereas actin mRNA levels were not affected. The 8-Br-cAMP stimulation of SCP_2 mRNA accumulation was completely inhibited by actinomycin D and cycloheximide. The cyclic AMP analogue also increased SCP_2 mRNA levels in a non-steroid hormone producing transformed rat granulosa cell line Gs-8. We conclude that SCP_2 gene expression in the ovary is correlated with the state of differentiation of granulosa cells. Gonadotropic hormones which stimulate luteinization of the cells increase SCP_2 gene expression. These actions of gonadotropins appear to be mediated at least in part by cyclic AMP through a mechanism requiring ongoing RNA and protein synthesis. However, SCP_2 gene expression is not obligatorily coupled to steroidogenic activity, as cyclic AMP analogues can increase SCP_2 mRNA in a line of transformed ovarian granulosa cells incapable of synthesizing hormones.

Sterol carrier protein 2 (SCP_2; also called nonspecific lipid-transfer protein) is a 13.2-kDa basic protein that is believed to play a role in the intracellular movement of cholesterol (Vahouny et al., 1987). On the basis of cDNA cloning and Western blot analysis of tissue extracts with specific antisera, it appears that SCP_2 is a member of a family of proteins which share a common carboxy terminus (Billheimer et al., 1990; Ossendorp et al., 1990; Van Heusden et al., 1990; Yamamoto et al., 1991; Seedorf & Assmann, 1991; He et al., 1991). cDNAs encoding proteins of 58, 30, and 15.3 kDa have been described. The mature SCP_2 molecule is apparently derived from processing of the 15.3-kDa protein by removal of a 20 amino acid N-terminal extension (Fujiki et al., 1989; Yamamoto et al., 1991). The 58-kDa protein is localized in peroxisomes (Van der Krift et al., 1985), but its function is not known. The subcellular location of the 30-kDa protein, its function, and its relationship to SCP_2 also remain obscure. Northern blot analyses reveal multiple mRNAs with SCP_2 sequences ranging in size from 0.8 to 3.2 kb that could encode the three proteins (Billheimer et al., 1990; Yamamoto et al., 1991; Seedorf & Assmann, 1991).

In steroid hormone producing tissues, one of the postulated functions of SCP_2 is to enhance the transport of cholesterol to mitochondria where the first committed step in steroid hormone synthesis takes place (Chanderbhan et al., 1983; Tanaka et al., 1984; Vahouny et al., 1987). Evidence supporting a role for SCP_2 in steroidogenesis includes the ability of exogenous SCP_2 to facilitate movement of cholesterol to mitochondria from isolated lipid droplets and the ability of SCP_2 to stimulate cholesterol side-chain cleavage by isolated mitochondria (Vahouny et al., 1987). The introduction of anti-SCP_2 antibodies into rat adrenal cells via liposomes results in a significant reduction in steroid secretion (Chanderbhan et al., 1986), and the coexpression of SCP_2 and cholesterol side-chain cleavage enzyme in monkey COS cells results in enhanced synthesis of progestins (Yamamoto et al., 1991).

SCP_2 levels and synthesis in steroidogenic cells, assessed by Western blotting and incorporation of labeled amino acids into immunoprecipitable protein, appear to be under the regulation of tropic factors which stimulate hormone synthesis (Trzeciak et al., 1987; Van Noort et al., 1988; McLean et al., 1989). The availability of cloned SCP_2 cDNAs provides opportunities to further explore the regulation of SCP_2 expression. Here we describe the pattern and regulation of SCP_2 gene expression in the rat ovary, which was selected for study for two reasons. First, the growth and differentiation of the ovarian follicle and its luteinization provides a unique system for the study of the expression of steroidogenic machinery during cellular differentiation (Richards & Hedin, 1988). Second, immortalized granulosa cell lines are available, which facilitates in vitro studies (Amsterdam et al., 1988).

Materials and Methods

Animals. Immature female Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were housed

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* Address correspondence to this author at Department of Obstetrics and Gynecology, 771 Clinical Research Building, 422 Curie Blvd., Philadelphia, PA 19104.

‡ University of Pennsylvania School of Medicine.

§ Weizmann Institute of Science.

∥ Du Pont Merck Pharmaceutical Co.

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in a controlled environment with food and water available ad libitum. Some immature animals (24 days of age) were treated with 50 IU of pregnant mare's serum gonadotropin (PMSG) (Dynosynth, Chicago, IL) sc and killed at various times after treatment. Other animals were used after they displayed regular estrus cycles.

Cell Culture. Grs-21 cells were derived by transformation of rat granulosa cells with SV-40 and the Ha-*ras* oncogene, whereas Gs-8 cells were transformed with SV-40 alone (Amsterdam et al., 1988). These cells were cultivated in Dulbecco's-modified Eagle's medium/F-12 (1:1 v/v) supplemented with 5% fetal bovine serum as described by Amsterdam et al. Some cultures were treated with 8-Br-cAMP (Sigma Chemical Co., St. Louis, MO) in the absence or presence of actinomycin D (2 μg/mL) or cycloheximide (20 μg/mL) (Sigma Chemical Co.) for the indicated times.

Molecular Probes. A 850-bp fragment derived from a 1.4-kb cDNA encoding rat SCP_2 by digestion with *Eco*RI and *Sac*I (Billheimer et al., 1990) was used for in situ hybridization histochemistry and Northern blot analyses. This cDNA encompasses the coding sequence of the 30-kDa protein and the 15.3-kDa pre-SCP_2 molecule. Some blots were also probed with cDNAs encoding rat cytochrome P450scc (McMasters et al., 1987) and human β-actin (Gunning et al., 1983). The P450scc cDNA was generously provided by Dr. William Moyle. The actin cDNA was kindly provided by Dr. Peter Gunning.

In Situ Hybridization. Ovaries were removed from immature PMSG-treated animals and adult cycling females, trimmed of adherent tissue, and frozen in liquid nitrogen until sectioned. Cryostat sections (10 μm in thickness) were prepared and fixed for 10 min with 4% paraformaldehyde in phosphate-buffered saline. Sections were prehybridized for 1 h at room temperature in a solution consisting of 4× SSC, 50% formamide, 1× Denhardt's solution, 5% dextran sulfate, 0.5 mg/mL salmon sperm DNA, and 0.25 mg/mL yeast t-RNA. Following prehybridization, fresh solution was added containing 30 ng/mL of heat-denatured SCP_2 cDNA insert or pBluescript vector DNA labeled with digoxigenin-11-dUTP using the Genius system (Boehringer Mannheim Biochemicals, Indianapolis, IN). Slides were incubated with probes at 37 °C for 18 h in a humidified chamber. Following hybridization, slides were rinsed twice for 1 h with 2× SCC at room temperature, once with 1× SSC for 1 h, and once with 0.5× SSC for 30 min. Following a wash in 100 mM Tris-HCl buffer, pH 7.5, the slides were incubated with 2% normal sheep serum and 0.3% Triton X-100 in this buffer for 2 h at room temperature, then washed for 15 min twice with the Tris buffer and incubated with color development solution, treated with graded ethanol solutions, and mounted for viewing.

Northern Blot Analyses. Total RNA was prepared from rat ovaries by the method of Feramisco et al. (1982) with the exception that the procedure was carried out at room temperature. Poly(A)-tailed RNA from cultured cells was isolated using oligo(dT)-cellulose as described by Hartman et al. (1990). Briefly, cells were collected and resuspended in lysis buffer (0.5 M NaCl, 10 mM Tris, 10 mM EDTA, 1% SDS, and 0.2 mg/mL proteinase K), followed by careful shearing through needles. Proteinase K was then added at a concentration of 0.3 mg/mL. After the suspension was incubated at 37 °C for 3 h, it was added to washed resuspended oligo-(dT)-cellulose. The mixture was then incubated overnight with gentle rocking at room temperature. It was then loaded into a disposable column and washed with 0.1 M NaCl/10 mM Tris/0.1 mM EDTA/0.2% SDS. Poly(A)-tailed RNA was eluted with 10 mM Tris/0.1 mM EDTA/0.2% SDS. A total of 20 μL of 1 mg/mL glycogen was added as carrier to each milliliter of eluate followed by a standard precipitation. Poly(A)-tailed RNA was then resuspended and quantitated by absorbance at 260 nm.

A total of 20 μg of total RNA or 2–5 μg of poly(A+) RNA per lane was subjected to size fractionation in 0.8% agarose/formaldehyde gels and transferred to Nytran membranes (Schleicher & Schuell, Keene, NH).

Filters were hybridized with ^{32}P-labeled cDNA probes prepared either by nick translation or by random primer labeling. The dried filters were moistened with 6× SCC (1× SCC = 0.15 M NaCl and 0.015 M sodium citrate) and prehybridized with a mixture of 5× SSC, 50% formamide, 0.1% each of Ficoll, poly(vinylpyrrolidone), bovine serum albumin, and SDS, and 250 mg/mL denatured salmon sperm DNA at 42 °C for 4 h. Hybridization solution was then added containing labeled probe [(5–10) × 10^6 cpm/mL], and the filters were incubated at 42 °C for 18 h. They were washed in 2× SSC and 0.1% SDS twice for 30 min at room temperature and then twice for 45 min at 65 °C in 1× SSC and 0.1% SDS. The filters were then blotted dry and placed in plastic wrap against X-ray film (Kodak X-Omat) and stored at −70 °C.

The autoradiograms were subjected to densitometric analysis using a Resources Technology (Nashville, TN) image analysis system. The SCP_2 mRNA signal was normalized to actin mRNA for determination of changes in relative SCP_2 mRNA abundance.

Western Blot Analysis. Grs-21 cells were solubilized as previously described (Yamamoto et al., 1991). The cell extracts were subjected to SDS–PAGE in 18% acrylamide gels. The separated proteins were transferred to nitrocellulose filters, and the filters were probed with a specific anti-rat liver SCP_2 antiserum as previously described (Yamamoto et al., 1991).

Results and Discussion

The rat ovary contains several compartments with varying steroidogenic activity, including follicles in various stages of development which are comprised of thecal and granulosa cells, interstitial tissue, and corpora lutea. In situ hybridization studies with the SCP_2 cDNA probe on sections of ovaries from immature PMSG-treated animals as well as adult cycling rats revealed that SCP_2 mRNA is primarily localized in luteinized tissue (i.e., corpora lutea, Figure 1A). It was also detected in the theca of developing follicles (Figure 1A) and interstitial tissue (not shown). The luteal cells, theca, and interstitial cells are the most steroidogenic active cells in the rat ovary. In contrast, granulosa cells of the small and large antral follicles displayed only a modest signal. Hybridization was not seen in connective tissue separating luteal bodies. Control sections hybridized with vector DNA gave no signal (Figure 1B). The pattern of SCP_2 gene expression revealed by in situ hybridization histochemistry is similar to the distribution of cholesterol side-chain cleavage enzyme in the rat ovary (Goldring et al., 1987). The apparent coordination of expression of SCP_2 and cytochrome P450scc in ovarian compartments is consistent with the notion that SCP_2 plays an important role in steroid hormone synthesis. The in situ hybridization studies revealing low levels of SCP2 mRNA in granulosa cells and high levels in granulosa lutein cells also document for the first time luteinization-dependent expression of the SCP_2 gene.

Follicular growth and luteinization are stimulated by gonadotropic hormones. Treatment of immature rats with a high dose (50 IU) of pregnant mare's serum gonadotropin (PMSG) promotes time-dependent follicular growth and luteinization.

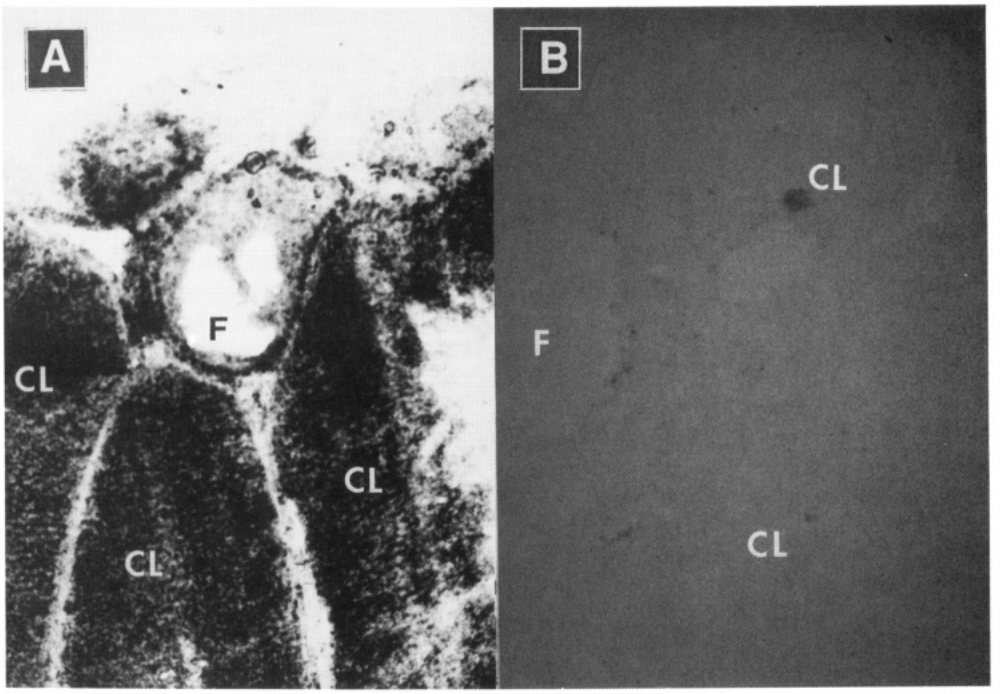

FIGURE 1: Distribution of SCP_2 mRNA in the ovary of a diestrus rat as demonstrated by in situ hybridization histochemistry. Sections of the ovary were subjected to hybridization with the SCP_2 cDNA (A) or vector DNA (B) as described in the text. Corpora lutea (CL) and follicles (F) are indicated. A strong SCP_2 hybridization signal is seen in CL and theca cells but not in granulosa cells. The vector DNA gives no hybridization signal. Controls in which peroxidase-conjugated antibody was omitted gave no detectable signal. The general pattern of SCP_2 mRNA distribution seen in this section of a diestrus ovary was found in preparations from immature PMSG-treated rats with SCP_2 mRNA being concentrated in luteinized structures. (Magnification is 165×.)

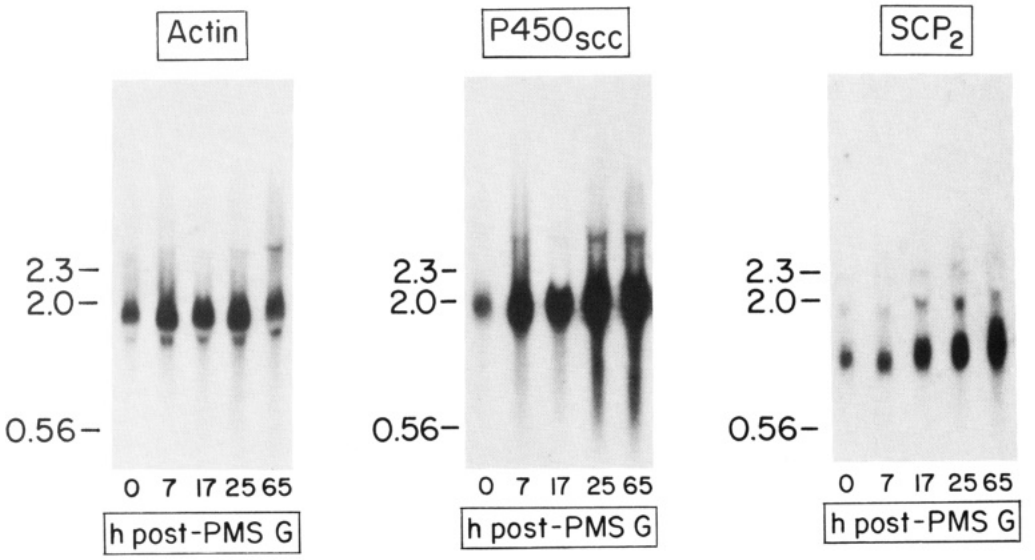

FIGURE 2: Gonadotropin regulation of ovarian SCP_2 mRNA. Immature female rats, 23 days of age, were treated sc with 50 IU of PMSG as described in the text. Animals were killed at the indicated times after treatment, and total RNA was isolated from ovaries and subjected to Northern blotting (20 μg/lane) as described in the text. The filter was hybridized sequentially with rat SCP_2 cDNA, rat P450scc cDNA, and human actin cDNA. This is a representative blot revealing results which were confirmed in a separate experiment.

Northern blot analysis of total RNA prepared from ovaries of animals at various times after PMSG treatment reveals a substantial accumulation of SCP_2 and cytochrome P450scc mRNAs as follicles grow and luteinize without a significant change in actin mRNA (Figure 2). The accumulation of SCP_2 and cytochrome P450scc mRNAs as follicles luteinize is consistent with the in situ hybridization histochemistry studies.

The Northern blots reveal several species of SCP_2 mRNA. The most abundant signal is produced by mRNAs in the range of 0.8–1.4 kb, with smaller signals from mRNAs of about 2.1 and 2.8 kb. The smaller mRNAs encode SCP_2, whereas the larger mRNAs code for the 58-kDa SCP_2-related protein called SCPx (Seedorf & Assmann, 1991; He et al., 1991). PMSG treatment promotes the accumulation of all these mRNAs. The 3′ untranslated regions of the SCP_2 cDNAs have been reported to contain several possible polyadenylation sites (Yamamoto et al., 1991; Seedorf & Assmann, 1991). Utilization of several of these sites and differences in poly(A)-tail length appear to account for some of the diversity in mRNA size.

The gonadotropic hormones act upon ovarian cells via a variety of second messenger systems including those functioning through cAMP-dependent protein kinases (Trzeciak et al., 1986). To determine if SCP_2 gene expression is affected by cyclic AMP, we examined the effects of a cyclic AMP

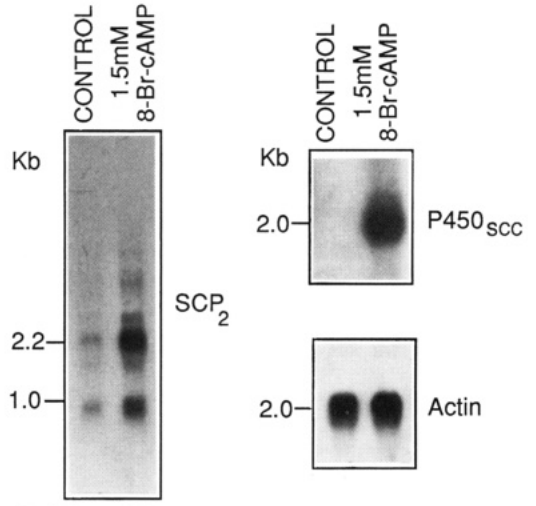

FIGURE 3: 8-Br-cAMP regulation of SCP_2 mRNA. Grs-21 cells were treated with 1.5 mM 8-Br-cAMP for 24 h and poly(A+) RNA was isolated and subjected to Northern blotting (5 μg/lane). Blots were probed with SCP_2, P450scc, and actin probes.

analogue, 8-Br-cAMP, on SCP_2 mRNA using a transformed rat granulosa cell line that displays steroidogenic activity (Grs-21). Although these cells do not express gonadotropin receptors, they do respond to agents that raise cyclic AMP levels with increased progestin production resulting from the de novo synthesis of steroidogenic enzymes including P450scc (Amsterdam et al., 1988; Suh & Amsterdam, 1990; Hanukoglu et al., 1990). Treatment of Grs-21 cells with 1.5 mM 8-Br-cAMP for 24 h resulted in approximately a 5-fold increase in SCP_2 mRNA and no significant change in actin mRNA (Figure 3). Several SCP_2 mRNAs were detected, yielding a more complex hybridization pattern than that found when total RNA from whole ovaries was examined. Unlike the normal rat ovary, comparable levels of ~1- and 2.1-kb mRNAs were observed with several weaker signals from mRNAs in the range of 2.8 and $\geq$3.2 kb. The cyclic AMP analogue increased the level of all these mRNAs. It also induced the appearance of P450scc mRNA, which was expected on the basis of previous studies in which P450scc protein accumulated in the transformed cells following stimulation with agents which raise cellular cyclic AMP (Hanukoglu et al., 1990).

The concentration of 8-Br-cAMP required to produce a maximal response in SCP_2 mRNA after 24 h of treatment was found to be between 100 and 1000 μM (Figure 4). An analysis of the time course of the response to 8-Br-cAMP indicated that a small increase in SCP_2 mRNA could be detected after 6 h of treatment with 1.5 mM 8-Br-cAMP with the maximal increase after 24 h (Figure 5). In three separate experiments, the peak response of SCP_2 mRNA was found at 24 h of 8-Br-cAMP treatment with a mean increment above control SCP_2 mRNA levels of 5.4-fold.

Western blot analysis of the Grs-21 cell protein documented that the SCP_2 protein was increased approximately 4-fold in response to 8-Br-cAMP treatment for 24 h (Figure 6). Of note is the fact that the 58-kDa and 30-kDa SCP_2-related proteins were not detectable, indicating a selective accumulation of SCP_2 despite the increase in mRNAs which presumably encode the larger SCP_2-related molecules.

To determine if ongoing RNA synthesis and protein synthesis are required for the 8-Br-cAMP stimulation of SCP_2 mRNA accumulation, we examined the effects of actinomycin D and cycloheximide at concentrations which inhibit RNA synthesis and protein synthesis by >95%, respectively, on the response to the cyclic nucleotide analogue. Both actinomycin D and cycloheximide completely inhibited the 8-Br-cAMP-induced increase in the usual SCP_2 mRNAs detected in the Grs-21 cells (Figure 7). However, cycloheximide treatment (with and without 8-Br cAMP) caused the appearance of several large RNAs which hybridized with the SCP_2 cDNA. The significance of these hybridization signals is not yet known. In contrast to the effects on SCP_2 mRNA, neither actinomycin D nor cycloheximide treatment significantly altered the 2-kb actin mRNA levels (Figure 7). Thus, RNA formation and protein formation appear to be needed for 8-Br-cAMP to affect SCP_2 mRNA levels.

These findings reveal that SCP_2 gene expression in the ovary is regulated by cyclic AMP, and we presume, therefore, that the gonadotropin-induced accumulation of SCP_2 in the ovary is due fully, or at least in part, to the action of cyclic AMP. Our results are consonant with the studies of Trzeciak et al. (1987), who found that treatment of rat adrenal cells with

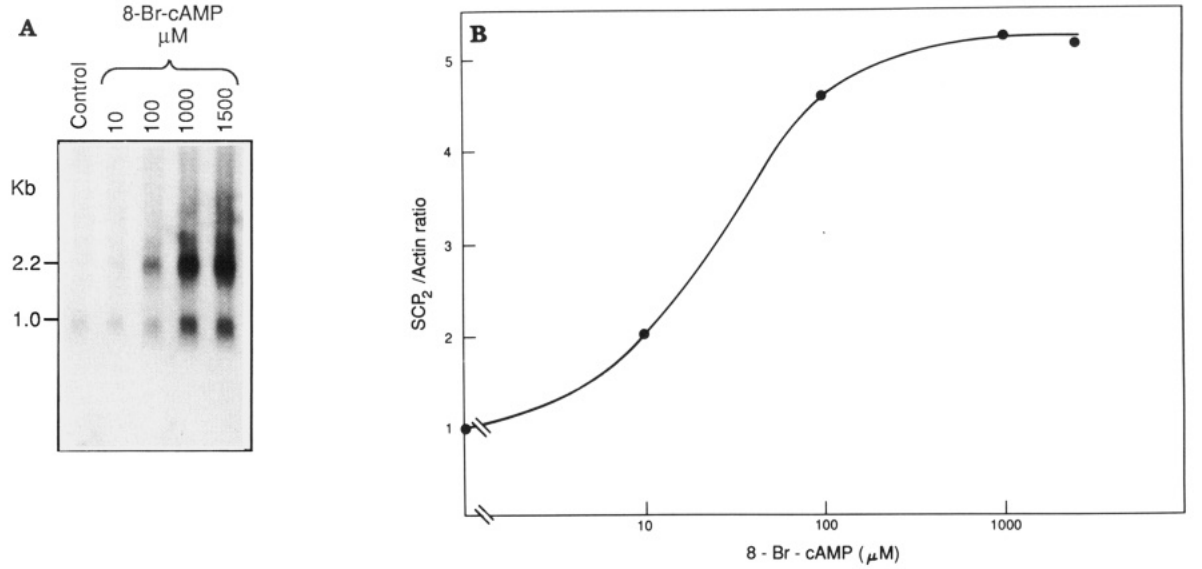

FIGURE 4: Dose response of Grs-21 cells to 8-Br-cAMP. Cells were treated with the indicated concentrations of 8-Br-cAMP for 24 h, and poly(A+) RNA (2 μg/lane) was analyzed as described in the text. (A) Northern blot analysis of SCP_2 mRNA. (B) Quantitative analysis of Northern blot data from another experiment. Relative changes in the ratio of SCP_2:actin mRNA are presented as determined by densitometric scanning of autoradiograms. The SCP_2:actin ratio for the control group has been arbitrarily set to 1.0.

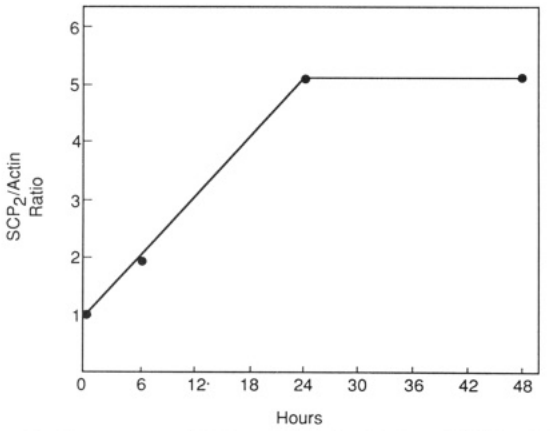

FIGURE 5: Time course of 8-Br-cAMP stimulation of SCP_2 mRNA accumulation. Grs-21 cells were exposed to 1.5 mM 8-Br-cAMP for up to 48 h, and SCP_2 and actin mRNAs were assessed by Northern blotting. Quantitative analysis of relative changes in the ratio of SCP_2:actin mRNA were determined by densitometric scanning of a representative experiment. The SCP_2:actin ratio of the zero time point has been arbitrarily set to 1.0.

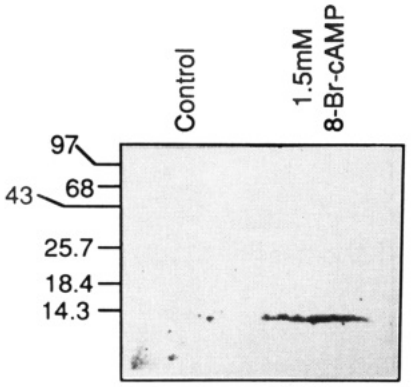

FIGURE 6: 8-Br-cAMP treatment increases SCP_2 protein in Grs-21 cells. Grs-21 cells were exposed to 1.5 mM 8-Br-cAMP for 24 h, and SCP_2 was demonstrated in cell extracts (200 μg of protein/lane) by Western blotting. Molecular masses are indicated on the left (in kilodaltons).

ACTH or dibutyryl-cAMP increased SCP_2 synthesis as measured by incorporation of labeled amino acids into immunoprecipitable proteins.

The transcripts which presumably code for the entire family of SCP proteins are regulated in concert. The cAMP-induced accumulation of these mRNAs may be the result, at least in part, of increased transcription. The observation that actinomycin D inhibits the action of 8-Br-cAMP on SCP_2 mRNA and preliminary nuclear runoff studies that indicate that 8-Br-cAMP increases SCP_2 gene transcription by 4-fold are consistent with this notion. However, effects on mRNA processing and stability cannot be ruled out at this time. Notably, there is a selective increase in SCP_2 protein in ovarian cells despite the accumulation of mRNAs which could encode the larger SCP_2-related proteins. This finding is consistent with the metabolic labeling studies of Trzeciak et al. (1987), who found that ACTH or dibutyryl-cAMP stimulated synthesis of SCP_2 by rat adrenal cells with little apparent increase in the 58-kDa protein. This differential response may reflect translational or posttranslational control (including protein turnover) mechanisms which may determine steady-state levels of the members of the SCP family of proteins in various cell types. It is noteworthy that SCP_2, which is believed to have a role in steroidogenesis, accumulates in the steroidogenic Grs-21 cells, rather than the 30- and 58-kDa proteins whose functions remain to be determined.

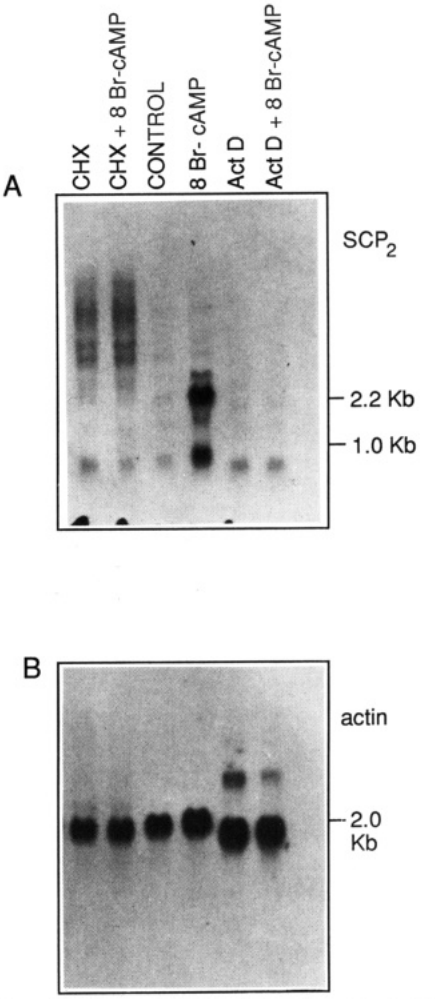

FIGURE 7: Effect of actinomycin D (Act D) and cycloheximide (CHX) on 8-Br-cAMP stimulation of SCP_2 mRNA. Grs-21 cells were treated with actinomycin D (2 μg/mL), cycloheximide (20 μg/mL), 8-Br-cAMP (1 mM), or a combination of 8-Br-cAMP and actinomycin D or cycloheximide. Actinomycin D and cycloheximide were added 30 min before 8-Br-cAMP, and cells were cultured for 18 h. Northern analysis for SCP_2 (panel A) and actin (panel B) mRNAs [5 μg of poly(A+) RNA/lane] was then carried out. Results of a representative study are presented. Identical results were obtained in two separate experiments.

Using Western blot analysis of subcellular fractions and electron microscope immunocytochemistry, other authors have described acute changes in the subcellular distribution of SCP_2-immunoreactive proteins in steroid-producing cells stimulated with tropic hormones (Van Noort et al., 1988; McLean et al., 1989; Mendis-Handagama et al., 1990). These changes seem to occur within minutes of treatment and probably represent a level of control of SCP_2 (presumably posttranslational) other than that identified in this report.

Because of the increase in both SCP_2 and P450scc mRNAs observed in our studies of normal ovaries and Grs-21 cells, we were interested in determining if regulated expression of these genes was linked. To this end, we examined Gs-8 cells, a line of SV-40-transformed rat granulosa cells which cannot synthesize steroid hormones because they do not express the P450scc gene (Amsterdam et al., 1988). Whereas treatment of Grs-21 cells with 1.5 mM 8-Br-cAMP for 24 h increased SCP_2 mRNA levels and induced the appearance of P450scc mRNA, Gs-8 cells did not have detectable levels of P450scc mRNA on Northern analysis of poly(A+)-selected RNA (data not shown). However, SCP_2 mRNA was detected in these cells and treatment with the cyclic AMP analogue increased

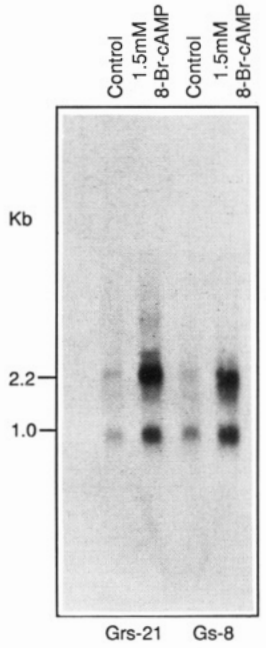

FIGURE 8: Effect of 8-Br-cAMP on SCP_2 mRNA levels in Grs-21 and Gs-8 cells. Cells were cultured with or without 8-Br-cAMP (1.5 mM) for 24 h. Five micrograms of poly(A+) RNA from each treatment group was subjected to Northern analysis for SCP_2. The increases in SCP_2:actin mRNA ratios for the Grs-21 and Gs-8 cells in this experiment were 6.35-fold and 6.47-fold, respectively.

SCP_2 mRNA levels as in the steroid-producing Grs-21 cells (Figure 8). These observations document that SCP_2 gene expression can be completely uncoupled from P450scc gene expression. Since Gs-8 cells do not produce steroid hormones in response to 8-Br-cAMP, we can also claim that a steroidogenic response is not required for increased SCP_2 mRNA accumulation. This conclusion is also consistent with the observations of McLean et al. (1989), who found that estradiol regulated SCP_2 and P450scc protein levels independently in the rat corpus luteum.

In summary, we have shown that (1) SCP_2 transcript levels display a differentiation-dependent pattern in the rat ovarian follicle; (2) SCP_2 gene expression is regulated by gonadotropins, apparently via a cAMP second message; (3) the actions of cAMP on SCP_2 mRNA accumulation require ongoing RNA and protein synthesis; and (4) SCP_2 can be regulated in ovarian cells independently of steroidogenesis and P450scc. The increase in SCP_2 levels in response to tropic stimulation may be important in the facilitation of steroidogenesis.

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