Developmental and Hormonal Regulation of Murine Scavenger Receptor, Class B, Type 1

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The scavenger receptor, class B, type I (SR-BI), is the predominant receptor that supplies plasma cholesterol to steroidogenic tissues in rodents. We showed previously that steroidogenic factor-1 (SF-1) binds a sequence in the human SR-BI promoter whose integrity is required for high-level SR-BI expression in cultured adrenocortical tumor cells. We now provide *in vivo* evidence that SF-1 regulates SR-BI. During mouse embryogenesis, SR-BI mRNA was initially expressed in the genital ridge of both sexes and persisted in the developing testes but not ovary. This sexually dimorphic expression profile of SR-BI expression in the gonads mirrors that of SF-1. No SR-BI mRNA was detected in the gonadal ridge of day 11.5 SF-1 knockout embryos. Both SR-BI and SF-1 mRNA were expressed in the cortical cells of the nascent adrenal glands. These studies directly support SF-1 participating in the regulation of SR-BI *in vivo*. We examined the effect of cAMP on SR-BI mRNA and protein in mouse adrenocortical (Y1-BS1) and testicular carcinoma Leydig (MA-10) cells. The time courses of induction were strikingly similar to those described for other cAMP- and SF-1-regulated genes. Addition of lipoproteins reduced SR-BI expression in Y1-BS1 cells, an effect that was reversed by administration of cAMP analogs. SR-BI mRNA and protein were expressed at high levels in the adrenal glands of knockout mice lacking the steroidogenic acute regulatory protein; these mice have extensive lipid deposits in the adrenocortical cells and high circulating levels of ACTH. Taken together, these studies suggest that trophic hormones can override the suppressive effect of cholesterol on SR-BI expression, thus ensuring that steroidogenesis is maintained during stress. (Molecular Endocrinology 13: 1460–1473, 1999

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INTRODUCTION

Steroidogenic cells require cholesterol to maintain their plasma membranes and support the synthesis of steroid hormones. Cholesterol for steroidogenesis is preferentially obtained from circulating lipoproteins, but also can be acquired from endogenously synthesized cholesterol or hydrolysis of intracellular cholesterol esters (1–3). Both low-density lipoproteins (LDL) and high-density lipoproteins (HDL) can deliver cholesterol to support steroidogenesis, with the relative contributions of these two lipoproteins differing among species.

More than 20 yr ago, HDL was found to be the major source of cholesterol for steroidogenesis in rodents (4–6). The dominant role of HDL in maintaining cholesterol ester stores in steroidogenic tissues is reflected by the marked lipid depletion seen in the adrenocortical cells of mice lacking apolipoprotein AI, the major apolipoprotein of HDL (7). The adrenal cortical cells of rats made hypolipidemic by treatment with either high-dose estrogen or 4-aminopyrazolopyrimidine are also lipid depleted (5, 8, 9).

Cholesterol delivery to steroidogenic tissues from HDL differs from the well characterized LDL receptor pathway (5, 6). The cholesterol uptake from HDL is selective; lipids are transported into the cell without the concomitant uptake and degradation of the apolipoproteins (10). In contrast, after LDL binds to its cell surface receptor, the entire particle is taken up by receptor-mediated endocytosis and delivered to lysosomes, where the apoproteins are degraded and the cholesterol esters are enzymatically hydrolyzed to release cholesterol (11).

The protein that mediates the selective uptake of lipids from HDL was identified 3 yr ago by Krieger and colleagues (12) and named scavenger receptor, class B, type I (SR-BI) (12). SR-BI is expressed at highest levels in those tissues and cell types most active in selective uptake *in vivo*: the liver, the zona fasciculata and zona reticularis of the adrenal glands, the theca cells and corpus luteum of the ovaries, and the Leydig cells of the testes (12–16). Antibodies to the extracellular domain of

SR-BI block HDL-cholesterol ester uptake and HDL-stimulated synthesis of steroids in cultured mouse adrenocortical cells (17). This finding is consistent with SR-BI playing a major role in supplying steroidogenic cells with cholesterol. Furthermore, SR-BI knockout mice have lipid-depleted adrenal glands and a 2- to 3-fold increase in plasma levels of HDL-cholesterol (18). Mice with a reduced amount of SR-BI showed decreased selective uptake of cholesterol esters (19).

Trophic hormones, acting by a cAMP-dependent protein kinase pathway (20), induce the expression of both the LDL receptor and SR-BI (9, 13, 21, 22). Trophic hormones fail to increase LDL receptor activity in adrenocortical cells when steroidogenesis is inhibited and the intracellular cholesterol content is maintained by the addition of exogenous lipoproteins (9). These observations are consistent with the model in which trophic hormones deplete intracellular cholesterol stores by stimulating steroidogenesis and thereby indirectly increase LDL receptor activity (23).

The mechanism by which trophic hormones up-regulate SR-BI expression is not known. SR-BI levels are elevated in the adrenal glands of multiple strains of genetically manipulated mice that are hypolipidemic, including some *apoAI*$^{-/-}$ mice (24) and mice in which the lecithin-cholesterol acyl transferase or the hepatic lipase genes have been inactivated (24, 25). These observations suggest that the levels of SR-BI, like the LDL receptor, may be regulated by the intracellular content of cholesterol.

cAMP acts synergistically with the nuclear hormone receptor steroidogenic factor 1 (SF-1) to activate the genes encoding multiple components of the steroidogenic pathway (26). All cytochrome P450 steroid hydroxylases and the steroidogenic acute regulatory protein (StAR), which mediates transport of cholesterol from the cytoplasm to the inner mitochondrial membrane, are regulated by SF-1 via SF-1-responsive promoter elements (27, 28). The human *CLA-1/SR-BI* gene also contains a consensus SF-1 binding motif in its promoter region (29). We previously showed that SF-1 binds to this site in a sequence-dependent manner and that this element is required for high-level expression of SR-BI promoter constructs in cultured adrenocortical cells (29).

In this paper, we explore the regulation of SR-BI mRNA expression in steroidogenic tissues of the mouse and examine the relative roles of trophic hormones (via cAMP and SF-1) and the intracellular concentration of cholesterol in regulating the levels of SR-BI in cultured mouse YI adrenocortical cells.

RESULTS

SR-BI mRNA Is Expressed in the Undifferentiated Urogenital Ridge

To determine the ontogeny of SR-BI expression, *in situ* mRNA hybridization studies were performed in sections from mouse embryos. The antisense and sense SR-BI probes used in these studies were generated from a 2.4-kb mouse SR-BI cDNA fragment. The sensitivity and specificity of the probes were confirmed with sections of adult mouse adrenal tissue (Fig. 1A). An intense signal was seen in the cortical region of the adrenal gland using the antisense probe, but not the sense probe. In all subsequent *in situ* mRNA hybridization studies, no signal was apparent using the sense SR-BI probe (data not shown).

To examine the relationship between SR-BI and SF-1 gene expression, *in situ* mRNA hybridization studies were performed during early embryonic development. During mouse development, the urogenital ridge, which is the anlage for ovaries, testes, adrenal cortex, and part of the kidney, first appears as a mesenchymal thickening on embryonic day 9.5 (E9.5). Shortly thereafter, the genital ridge emerges as a structure distinct from the mesonephros. Serial sagittal sections of male and female mouse embryos were analyzed by *in situ* mRNA hybridization using antisense probes to murine SR-BI and SF-I. Bright-field views of male and female embryos from E10.5 and E11.5 are shown in the *left panels* of Fig. 1B. Hybridization with the SF-1 antisense probe revealed a discrete linear band of staining in the urogenital ridges of both female and male embryos (*middle panels*). This is consistent with prior studies showing that SF-1 mRNA can be detected as soon as the urogenital ridge forms (30).

Adjacent sections from E10.5 and E11.5 embryos were hybridized with the SR-BI antisense probe. No staining was seen in the urogenital ridge on E10.5 in either male or female embryos (panels C and F); the only tissue that stains at this early time point is the premordial liver. On E11.5, a signal for SR-BI mRNA was present in the genital ridge of the male embryo (panel I), but only a marginal signal was apparent in the female embryo (panel L). Thus, SR-BI mRNA is first expressed in the genital ridge of the male embryo at E11.5, which is approximately 2 days after the first appearance of SF-1 mRNA and 1 day after the initial appearance of *StAR* (31) and the cholesterol side chain cleavage enzyme (SCC) (30). The level of SR-BI mRNA expression in the genital ridge at this stage was lower in female than male mice. In contrast to SR-BI, no sex-dependent differences in the expression levels of StAR or SCC mRNA were apparent at this time point (30, 31).

No SR-BI mRNA was detected in the hepatic primordia at day 9.5, which is the first day this structure can be identified (data not shown). In both the male and female embryos, low-level staining was apparent at E10.5 within the septum transversum of the hepatic/biliary primordia. Significant levels of SR-BI mRNA expression were detected in the liver primordia on E11.5 of the male and female embryos (panels I and L).

Sexually Dimorphic Tissue Expression of SR-BI Mirrors SF-I during Fetal Development

In male mice, the testes become histologically distinct at approximately E12.5 as they organize into round, cord-like structures (the testicular cords), which contain both Sertoli cells and primordial germ cells and

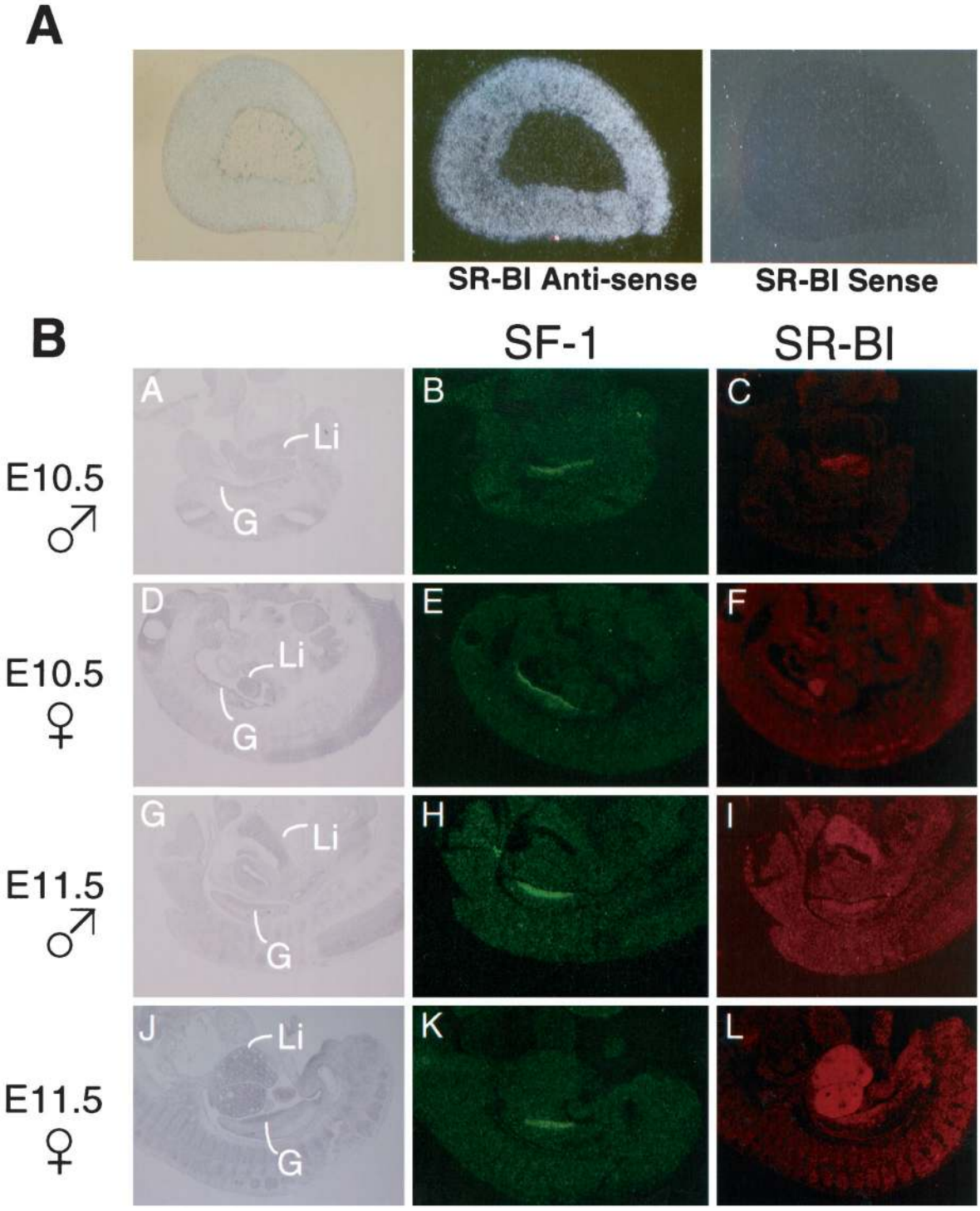

Fig. 1. *In Situ* Hybridization of E10.5 and E11.5 Male and Female Wild-Type Embryos to Examine the Expansion of SF-1 and SR-BI mRNA

A, Localization of SR-BI mRNA in sections of adult mouse adrenal glands. A bright-field view (left panel) and dark-field microscopy (*middle and right panel*) of mouse adrenal gland after hybridization with a SR-BI antisense (*middle panel*) and sense (*right panel*) probes are shown. Serial sagittal sections of the adrenal gland from an adult mouse were prepared and hybridized with radiolabeled antisense and sense probes derived from the murine SR-BI cDNA as described in *Materials and Methods*. The sections were exposed to emulsion for 2 weeks before development. B, Localization of SF-1 and SR-BI mRNA in male and female murine embryos on E10.5 and E11.5. Serial sagittal sections of mouse embryos obtained at days 10.5 and 11.5 of pregnancy were prepared as described in *Materials and Methods*. The sections were incubated with antisense SF-1 or SR-BI radiolabeled RNA probes. Panels A, D, G, and J are bright-field views of the embryos. Panels B, E, H, and K and panels C, F, I, and L are dark-field views of adjacent sections hybridized with SF-1 or SR-BI antisense RNA probes, respectively. The sections were incubated with emulsion for 3 weeks before development. Green and red fluorescent filters were used for the sections hybridized with the SF-1 and SR-BI probes, respectively. G, Gonadal ridge; Li, liver primordia.

ultimately develop into seminiferous tubules. The Leydig cells, which reside between the testicular cords in the interstitial region, synthesize testosterone. High levels of expression of both SF-1 and SR-BI mRNAs are seen within the testes during this time period (Fig. 2A). The patterns of expression of SF-1 and SR-BI mRNAs within the testes were overlapping but not identical (Fig. 2A). SR-BI mRNA has a more restricted and punctate pattern of distribution, with patches of intense signal corresponding to the Leydig cells, thus resembling patterns previously reported for SCC and StAR (30, 31). The signal for SF-1 was more generalized, consistent with expression in both fetal Sertoli and Leydig cells at this stage of development (30).

In contrast to the testis, the ovary does not produce significant quantities of steroid hormones before puberty. SF-1 is expressed in the indifferent gonad of both sexes, and expression persists in the murine ovary at E12.5 (Fig. 2B, panel B). Thereafter, expression levels decline, so that only a trace signal was seen in the E16.5 ovary. Little or no signal for SR-BI was present in the ovaries of the embryos at E12.5, E14.5, and E16.5 (panels C, F, and I). Once again, the pattern of expression of SR-BI in the embryonic ovary resembles those described previously for SCC and StAR, as neither SCC nor StAR mRNA is detected in the murine ovary from E12.5 to E16.5 (30, 31).

The expression of SR-BI in the liver was similar in the male and female embryos. SR-BI was expressed in the liver at E12.5 and E14.5 (panels C and F in Fig. 2) but the levels declined by E16.5. In contrast, prior immunocytochemical studies failed to detect SR-BI in the murine fetal liver through E17.5 (32). The apparent discrepancy between the *in situ* hybridization and immunocytochemical studies most likely reflects different sensitivities of the assays. It also is possible that an alternatively spliced form of SR-BI, which is not recognized by the antibody used for the immunocytochemical studies, is expressed in the liver at these time points. The SR-BI gene is alternatively spliced at its 3′-end (33). The antibody used for the immunocytochemical studies only detects the major form of SR-BI (referred to as SR-BI or SR-BI.1) (12), whereas our antisense probe detects both SR-BI and SR-BII (also called SR-BI.2). More likely, the absence of any immunodetectable hepatic SR-BI in murine embryos at these time points reflects a level of expression that is too low or too diffuse to detect by immunostaining.

From these studies, we conclude that SR-BI expression in the developing gonads is sexually dimorphic and generally correlates with the expression of SF-I. The pattern and timing of expression of SR-BI in the developing embryo closely resemble those described for the transcripts of two other key participants in the steroid biosynthetic pathway, SCC and StAR (30, 31).

SR-BI is Expressed at High Levels in the Fetal Adrenal Gland

SF-1 transcripts can be detected at E12.5 in the cells that comprise the adrenal primordium (30). Within 24 h, high levels of expression of both SF-1 and SR-BI transcripts were seen throughout the adrenal gland (Fig. 3, panels A–C). These results correlate well with prior immunocytochemical studies that revealed the first expression of immunodetectable SR-BI in the adrenal at E14.5 (32). By E16.5 the chromaffin cell precursors have migrated into the central portion of the gland to form the adrenal medulla. SF-1 and SR-BI are not expressed in the adrenal medulla (13), so the staining pattern of the adrenal gland by E16.5 is doughnut shaped.

SR-BI is Not Expressed in the Genital Ridge of SF-1 Knockout Mice, but Is Expressed in the Liver

The expression of SR-BI was also examined in E11.5 male SF-I knockout embryos (*Ftz-F1*$^{-/-}$) (34). As shown in Fig. 4, comparable levels of SR-BI mRNA were detected in the developing liver of the *Ftz-F1*$^{-/-}$ and wild-type embryos, consistent with the fact that SF-1 is not expressed in the liver (30) and thus cannot regulate SR-BI expression in this tissue. A weak SR-BI signal was apparent in the gonadal ridge of the wild-type embryo (*bottom panel, left*). In contrast, no SR-BI signal was present in the genital ridge of the *Ftz-F1*$^{-/-}$ embryo (*bottom panel, right*). To the extent that the developing gonads are still intact at this relatively early stage of development, this finding suggests that SF-I plays an important role in the regulation of SR-BI expression in this tissue *in vivo*.

Regulation of SR-BI mRNA and Protein Levels by cAMP Analogs in Cultured Murine Steroidogenic Cells

ACTH administration to mice dramatically increases the expression of immunodetectable SR-BI protein in the adrenal cortex, especially in the zonae fasiculata and reticularis (18). Human CG (hCG) administration causes a similar increase in SR-BI expression in the Leydig cells of the rat testes (13). Both ACTH and hCG exert their effects by activating protein kinase A, and their effects can be mimicked by cAMP analogs (20). The time course of the up-regulation of SR-BI protein expression by cAMP was examined in mouse adrenocortical tumor cells (Y1-BS1) and in a murine testicular Leydig carcinoma cell line (MA-10). Immunoblot analysis was performed using a polyclonal antibody directed against the last 14 amino acids of murine SR-BI (12). A 2-fold increase in the level of immunodetectable SR-BI protein was seen in the Y1-BS1 cells within 4 h, reaching a maximum 5-fold induction at 24 h (Fig. 5A).

Only a trace amount of immunodetectable SR-BI protein was seen in the MA-10 cells before the addition of $(Bu)_2$cAMP (Fig. 5A), which is compatible with the prior observation that MA-10 cells produce almost no steroids unless stimulated with cAMP (35). An increase in SR-BI protein levels was detected within 4 h of the addition of the cAMP analog, and by 24 h the

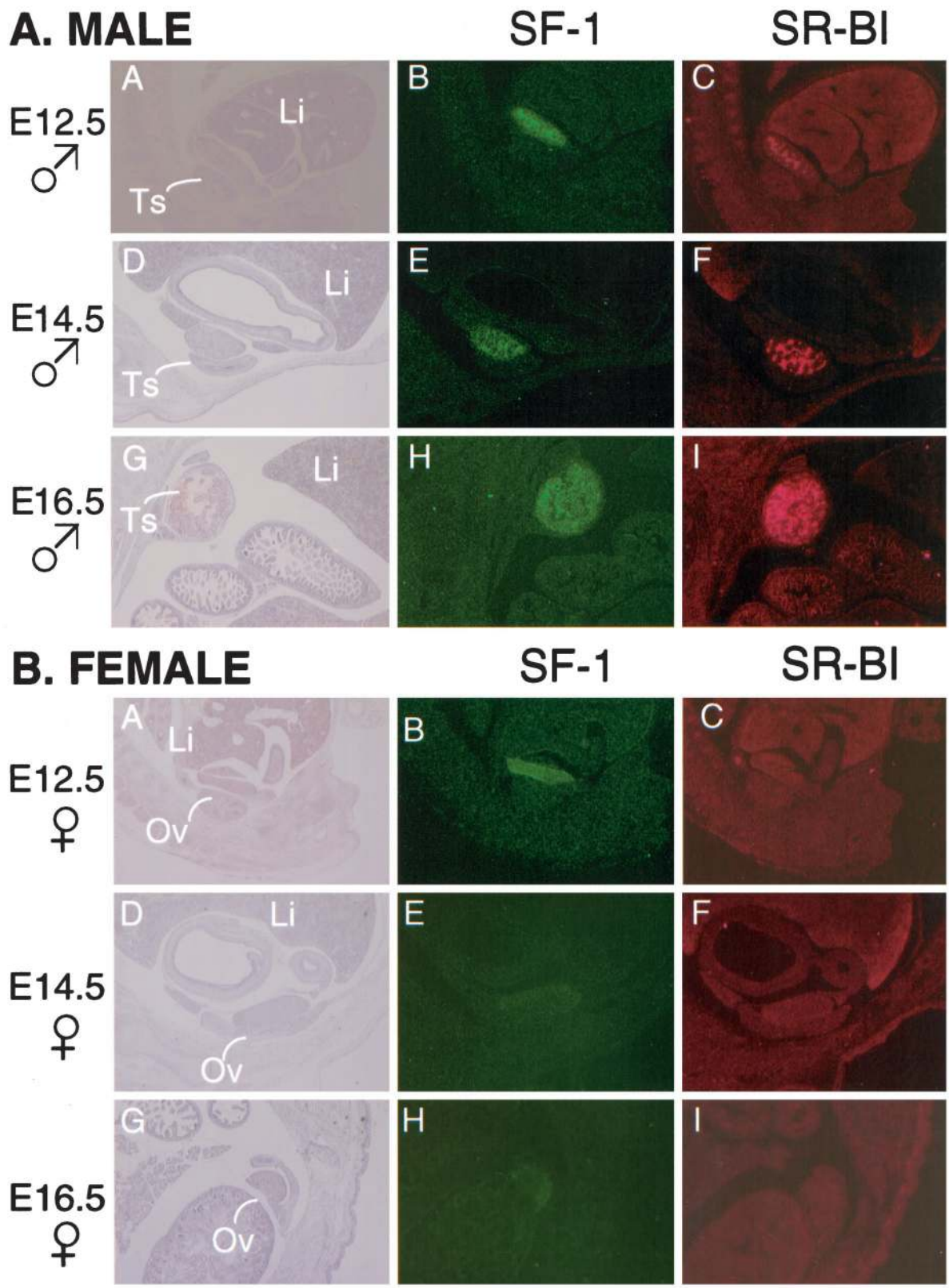

Fig. 2. *In Situ* Hybridization of E12.5, E14.5, and E16.5 Male (A) and Female (B) Wild-Type Embryos to Examine Expression of the SF-1 and SR-BI mRNA

The embryos were prepared and incubated with antisense SF-1 and SR-BI probes as described in Fig. 1. The sections were exposed to emulsion for 3 weeks before development. Bright-field (*left panel*) and dark-field views (*middle and right panels*) of the sections are shown. Ts, Testis; Li, liver; Ov, ovary.

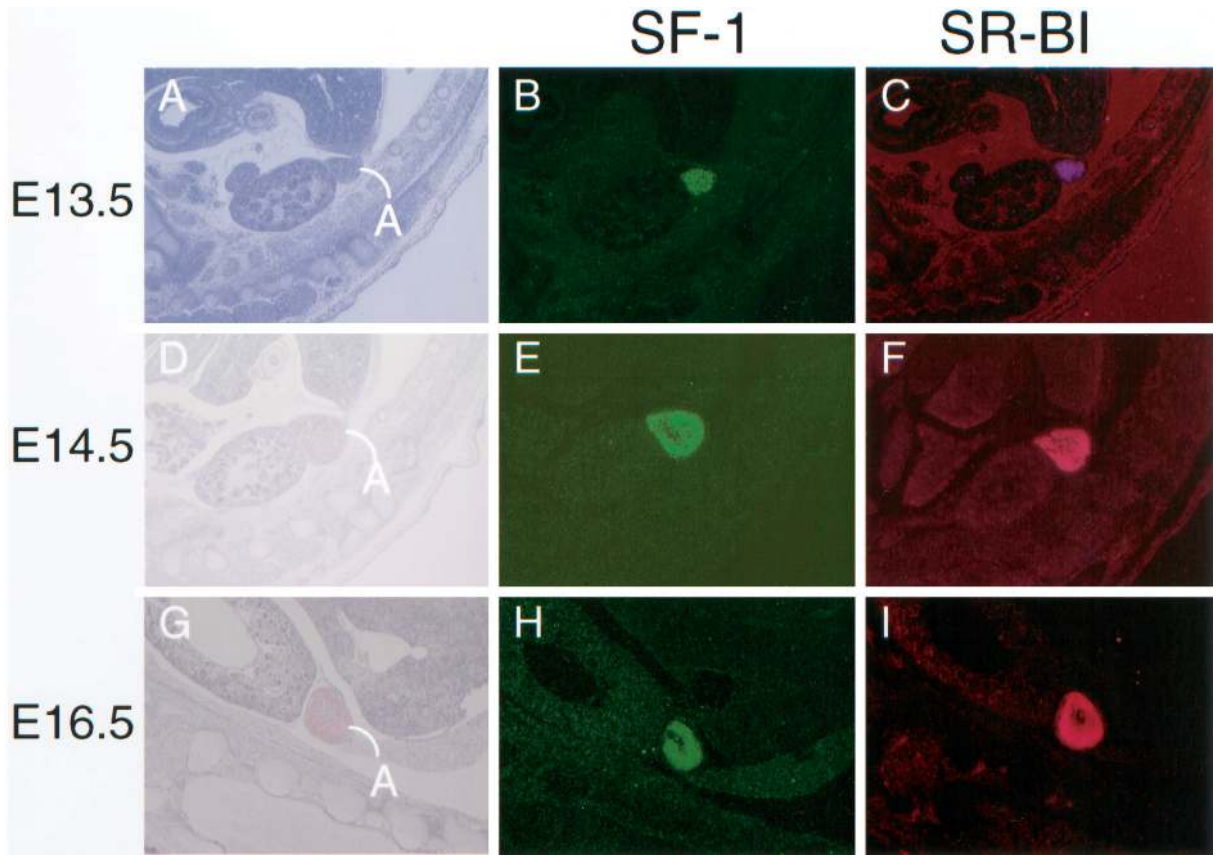

Fig. 3. *In Situ* Hybridization Studies of Wild-Type Mouse Embryos at E13.5, E14.5, and E16.5 Using Antisense SR-BI and SF-1 Probes

Serial sagittal sections of E13.5, E14.5, and E16.5 mouse embryos were prepared and processed as described in Fig. 1. The E13.5 sections were stained with toluene blue, whereas the E14.5 and E16.5 sections were stained with hematoxylin, thus accounting for the *purple* signal at E13.5 *vs*. the *red* signals at E14.5 and E16.5. The sections were exposed to emulsion for 3 weeks before development. Bright-field views are shown on the *left*, and dark-field views are shown in the *middle and right panels*. A, Adrenal.

level of SR-BI protein had increased more than 20-fold.

The antibody used in these studies detects only the SR-BI/SR-BI.I transcript, which is the predominant mRNA transcript in steroidogenic cells (36). The other mRNA transcript, SR-BII (or SR-BI.2), differs in sequence in the last 39 amino acids and thus is not detected by our antibody (33). To determine whether $(Bu)_2$ cAMP increases the levels of both the SR-BI and SR-BII transcripts, we used an RNase protection assay to assess the relative levels of the two transcripts in the Y1-BS1 and MA-10 cells. When we used this assay to assess the relative proportion of SR-BI and SR-BII in the testes, approximately 75% of the total SR-BI mRNA was SR-BII (data not shown), which is similar to that previously reported (36). At all time points, more than 95% of the total mRNA (SR-BI plus SR-BII) in both Y1 cells and MA-10 cells was SR-BI (Fig. 5B). Moreover, a pronounced increase in the levels of SR-BI mRNA was seen in both cell lines, with a time course congruent with that seen for SR-BI protein (Fig. 5A). Thus, there is no evidence for differential regulation of the two mRNA transcripts in response to $(Bu)_2$ cAMP in the Y1 and MA-10 cells.

SR-BI Levels Are Regulated by Intracellular Cholesterol Levels Independently of Trophic Hormones in YI-BSI Cells at Both a Transcriptional and Posttranscriptional Level

Cholesterol substrate for adrenal steroidogenesis comes from three sources: endogenous synthesis from acetyl CoA, hydrolysis of intracellular cholesterol esters, and uptake from circulating lipoproteins. ACTH dramatically up-regulates expression of SR-BI in the adrenocortical cells of the mouse adrenal gland (18). Is the up-regulation of SR-BI a direct effect of trophic hormones, or the result of depletion of intracellular cholesterol due to the induction of steroidogenesis?

To determine whether SR-BI levels are regulated by changes in the intracellular concentration of cholesterol, we inhibited endogenous cholesterol synthesis with compactin (10 μM), and steroidogenesis with aminoglutethimide (5 μg/ml). To document that steroid

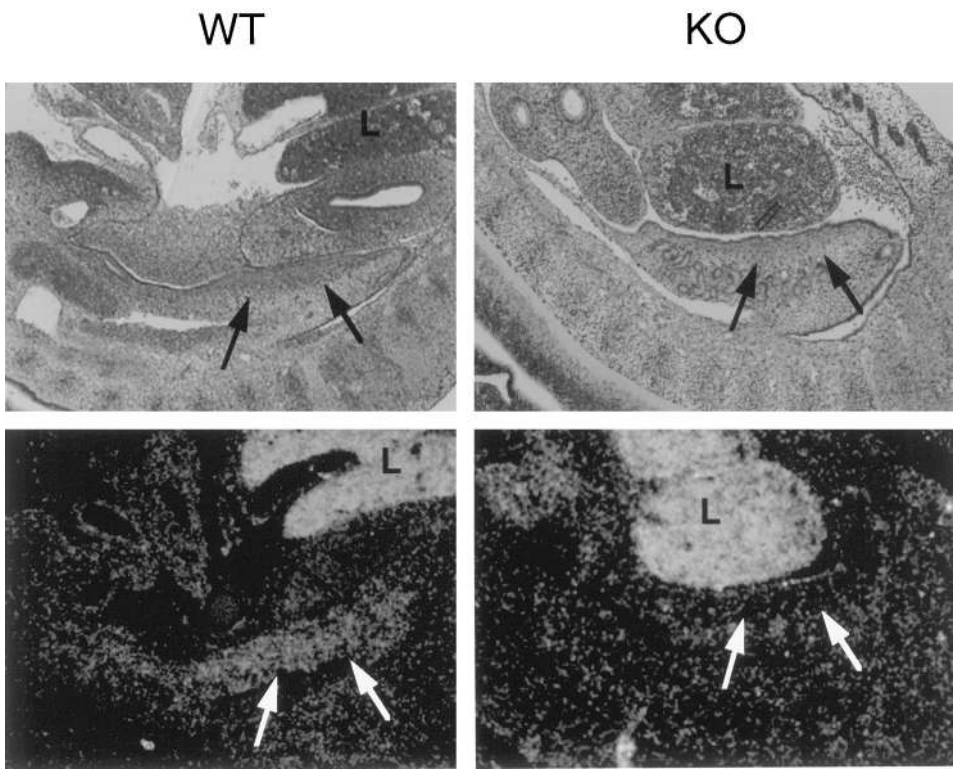

Fig. 4. SR-BI Transcripts Are Not Detected in the Gonadal Ridge of Knockout Mice Lacking SF-1

E11.5 embryos were harvested and genotyped by PCR analysis. Serial transverse sections were prepared from wild-type (WT, *left panels*) and SF-1 knockout (KO, *right panels*) embryos and analyzed by *in situ* hybridization with antisense cRNA probe specific for SR-BI as described in *Materials and Methods*. Low-magnification, bright-field views (*top panel*) and dark-field views (*bottom panels*) are shown. The *arrows* point to the genital ridge. L, Liver.

hormone production was effectively inhibited, we measured the level of progesterone in the medium, which fell into the undetectable range (data not shown). Aminoglutethimide treatment without the addition of lipoproteins did not alter the level of SR-BI (data not shown). Addition of 50 μg/ml LDL to the media of aminoglutethimide-treated cells resulted in a 4-fold decrease in the amount of immunodetectable SR-BI protein (Fig. 6A). No further decrease in the level of SR-BI was seen when the concentration of LDL in the media was increased to 100 μg/ml.

To indirectly assess the concentration of cholesterol in the endoplasmic reticulum of the cells, we examined the level of 3-hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase protein. HMG-CoA reductase is regulated by sterols both at the transcriptional and posttranslational levels (37). The protein was readily detected in the cholesterol-depleted YI-BSI cells. No change in the level of HMG-CoA reductase was seen upon addition of aminoglutethimide in the absence of lipoproteins (data not shown). Addition of LDL to the medium was associated with a marked reduction in the level of immunodetectable HMG-CoA reductase as well as LDL receptor, reflecting an increase in the delivery of cholesterol from the lipoproteins to the endoplasmic reticulum (Fig. 6A).

To determine whether the decrease in SR-BI protein was due to a reduction in the level of SR-BI mRNA, we used our RNase protection assay to assess the effect of aminoglutethimide and LDL on the amount of SR-BI and SR-BII mRNA. The levels of both SR-BI and SR-BII fell by approximately 20% with the addition of LDL (Fig. 6B). Under these conditions, the reduction in the level of SR-BI mRNA was less than that of immunodetectable SR-BI (Fig. 6A), suggesting posttranscriptional regulation of SR-BI. This could be caused by a decrease in the efficiency of mRNA translation or an increase in the degradation of SR-BI protein. Alternatively, the epitope that binds the anti-SR-BI antibody may be masked under these conditions.

To differentiate between these models, we assessed the half-life of SR-BI by performing a pulse-chase experiment. After a 1-h pulse, the level of immunoprecipitable SR-BI declined over the 12-h chase at the same rate in the cells incubated in the presence of absence of aminoglutethimide and LDL. The $t^{1/2}$ in both is 7 h (Fig. 7), which is slightly longer than what was previously reported for recombinant SR-BI that was stably expressed in Chinese hamster ovary cells (38). These results suggest that LDL and aminoglutethimide treatment does not decrease SR-BI protein by increasing the rate of degradation of SR-BI.

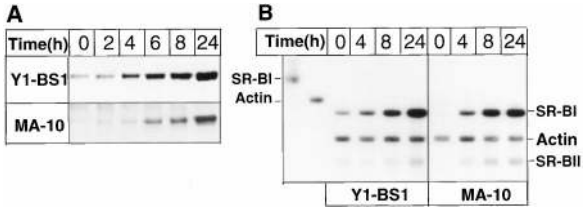

Fig. 5. Time Course of the Effect of $(Bu)_2$cAMP on SR-BI Protein (A) and mRNA (B) Levels in Y1-BSI and MA-10 Cells

A, Y1 adrenocortical cells were plated in 60-mm dishes at 400,000 cells per dish in medium A supplemented with 15% horse serum and 2% FCS and refed on day 3. On day 4 the medium was changed to medium A with 10% NLPPS, and on day 5 the cells were treated with $(Bu)_2$cAMP (1 mM) for the indicated time periods. The MA-10 cells were set up in 100-mm dishes at 500,000 cells per dish and grown in Waymouth's MB 782/l medium with 15% horse serum. On day 3 the medium was changed to Ham's F-12 medium with 10% NCLPPS and on day 4 $(Bu)_2$cAMP was added to the cells to a final concentration of 1 mM. The cells were scraped from the dish after 24 h and cellular lysates were made. A total of 50 μg of protein was subjected to SDS-PAGE, and immunoblot analysis was performed using rabbit polyclonal antibodies directed against the last 14 amino acids of murine SR-BI (13). On day 4, cells were treated with 1 mM $(Bu)_2$cAMP for the times indicated. The cells were collected and immunoblot analysis was performed as described above. B, Total cellular RNA was prepared from the Y1-BS1 and MA-10 cells, and an RNase protection assay was performed using a probe that can differentiate the SR-BI and SR-BII splice variant, as described in *Materials and Methods*. A total of 10 μg of RNA was incubated with ^{32}P-labeled probes from the mouse SR-BI and actin cDNA. The protected RNA fragments were size fractionated on a 6% denaturing PAGE, and the gel was exposed to Reflection NEF film at −80 C with an intensifiying screen for 16 h. The sizes of the intact probes and protected fragments are indicated on the *left* and *right*, respectively. The signals were quantified for SR-BI and actin using a phosphoimager (Fuji Photo Film Co., Ltd.).

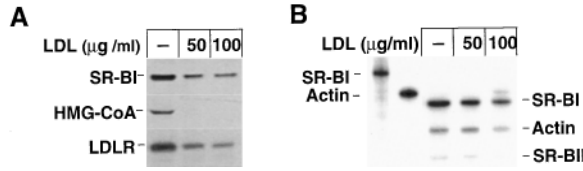

Fig. 6. Down-Regulation of SR-BI in Y1-BS1 Cells Treated with Aminoglutethimide and LDL

A, Immunoblot analysis of SR-BI, HMG-CoA reductase, and the LDL receptor in Y1-BS1 cells grown in the presence of LDL and aminoglutethimide (5 μg/ml). Cells were grown according to the protocol outlined in Fig. 5. On day 4, after a 24-h incubation in medium A with 10% NLPPS and 10 μM compactin, 5 μg/ml aminoglutethimide and LDL were added to the cells at the indicated concentrations. After 24 h, the cells were collected, cellular lysates were prepared, and immunoblot analysis was performed as described in the legend to Fig. 5. B, Total RNA was prepared from the cells and 10 μg RNA were subjected to the RNase protection assays to assess the amount of SR-BI mRNA as described in the legend to Fig. 5.

cAMP-Mediated Regulation of SR-BI

To determine the hierarchy of regulation of SR-BI expression, we examined the levels of immunodetectable SR-BI in YI-BSI cells in the presence of both a cAMP analog and lipoproteins. Y1-BS1 cells were incubated in newborn calf lipoprotein-deficient serum (NLPPS), 10 μM compactin, and 5 μg/ml of aminoglutethimide to block steroidogenesis. Increasing concentrations of LDL were added to the cells. After 24 h, the levels of immunodetectable SR-BI, HMG-CoA reductase, and LDL receptor were assessed by immunoblotting. As shown in Fig. 8, addition of LDL to the cells progressively decreased the immunodetectable levels of all three proteins.

Treatment with 8-Br-cAMP resulted in 11- and 2-fold increases in SR-BI and HMG-CoA reductase, respectively. No change was seen in the level of LDL receptor under these conditions. Increasing concentrations of LDL progressively decreased the levels of all three proteins, although the magnitude of the changes in protein levels differed. HMG-CoA reductase and LDL receptor levels fell by 94% and 68%, respectively, when 100 μg/ml of LDL were added to the cells for 24 h. SR-BI levels remained significantly elevated, decreasing by only 20% under these same conditions.

Taken together, these experiments demonstrate that SR-BI is regulated by both cAMP analogs and the intracellular content of cholesterol; the effect of the cAMP analog appears to largely override the suppressive effect of the increase in intracellular cholesterol.

SR-BI Expression in the Adrenal Glands of Newborn StAR$^{-/-}$ Knockout (*StAR*$^{-/-}$) Mice

StAR$^{-/-}$ mice are unable to efficiently transport cholesterol from the cytoplasm across the mitochondria membrane and thus accumulate cholesterol within their steroidogenic cells (39). These mice have elevated circulating levels of ACTH due to their adrenal insufficiency (39). *In situ* hybridization studies were conducted to compare the levels of SR-BI transcripts in newborn *StAR*$^{-/-}$ and wild-type mice (Fig. 9A). Although the adrenocortical architecture is distorted by the abundant lipid deposits in the *StAR*$^{-/-}$ adrenals (*top panels*), the level of SR-BI transcripts is high (*bottom panels*). Thus, the levels of SR-BI mRNA are high in the *StAR*$^{-/-}$ adrenocortical cells despite their increased cellular content of cholesterol, presumably due to the elevated circulating levels of ACTH.

DISCUSSION

Previously we showed that the tissue- and cell-specific distribution of SR-BI protein and mRNA overlaps with that of SF-I in adult rodents and humans (13, 29).

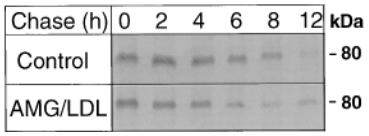

Fig. 7. Pulse-Chase Analysis of SR-BI Protein in Y1-BS1 Cells Treated with 50 μg/ml of LDL and 5 μg/ml of Aminoglutethimide

The cells grown in standard conditions until day 2, when the medium was switched to medium A plus 10% NLPPS. After 24 h, the cells were grown in methionine- and cysteine-free medium for 30 min before the addition of 200 μCi/ml of [^{35}S]methionine for 1 h. Then the medium was changed to medium A with 2 mM cold methionine with or without 50 μg/ml LDL, 10 μM compactin, and 5 μg/ml aminoglutethimide. The cells were lysed at the indicated time points and immunoprecipitated using a rabbit antimouse SR-BI polyclonal antibody directed against the last 14 amino acids, as described in *Materials and Methods*. A total of 35% of the immunoprecipitate was size fractionated on an 8% SDS-polyacrylamide gel. The gel was subjected to Kokak Safety film (Eastman Kodak Co., Rochester, NY) with an intensifying screen for 2 weeks.

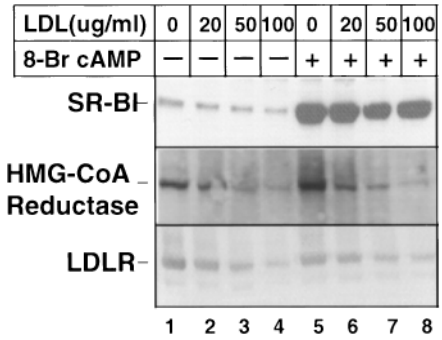

Fig. 8. Immunoblot Analysis of SR-BI, HMG-CoA Reductase, and the LDL Receptor (LDLR) in Y1-BS1 Cells

Y1-BS1 cells were grown under standard conditions until day 2, when they were switched to medium A containing NLPPS and grown for 24 h before 10 μM compactin, aminoglutethimide (5 mg/ml), and LDL were added to the dishes. Where indicated, 8-Br-cAMP (1 mM final concentration) was added to the dishes. After 24 h, the cells were collected. Immunoblot analysis was performed using cell lysates as described in Fig. 5 to examine the levels of SR-BI, HMG-CoA reductase, and the LDL receptor. *Scale bar*, 50 mm.

We now extend these studies by demonstrating that the temporal and spatial patterns of expression of SF-1 and SR-BI mRNA are similar in the steroidogenic tissues of developing mouse embryos. SR-BI mRNA is expressed in the urogenital ridge shortly after the first appearance of SF-1. SR-BI is expressed in the embryonic testes, which are active in steroidogenesis, but not in the early developing ovaries, which do not initiate steroidogenesis until late in gestation. This sexually dimorphic pattern of SR-BI expression during mouse embryogenesis mirrors that of SF-1. In the adrenal gland, the time course and pattern of expression of SR-BI mRNA and SF-1 were indistinguishable and exhibited no sexual dimorphism.

The timing and cell-specific distribution of expression of SR-BI within the murine embryonic adrenal glands and gonads are similar to those of two other genes that encode proteins in the steroidogenic pathway, *StAR* and *SCC* (30, 31). StAR plays a key role in the acute response of steroidogenic cells to trophic hormones by mediating transport of cholesterol from cytoplasm to the inner mitochondrial membrane where SCC catalyzes the first reaction in steroidogenesis (40). The mRNA transcripts for StAR and SCC are first detected in murine embryos 1–2 days after the initial appearance of SF-1 transcripts in urogenital ridge (30, 31). Therefore, the SCC and StAR transcripts are both present in the early developing testes and adrenal glands, but not the ovaries, again resembling the expression pattern of SR-BI. The ontogeny of expression of SR-BI mRNA in the developing embryo, and its similarity to the pattern of expression of two other SF-1-regulated genes (StAR and SCC), strongly suggest that SR-BI is part of the coordinated response of steroidogenesis to trophic hormones and plays an important role in embryonic, as well as adult, steroidogenic tissues.

If SF-1 is responsible for activating transcription of SR-BI, then it would be expected that SR-BI expression would not be present in the steroidogenic tissues of mice that do not express SF-1. We could only examine SR-BI expression in SF-1 knockout mice before E12 because, after this time point, gonads and adrenal primordium undergo apoptosis (30, 35). As shown in Fig. 4, comparable levels of SR-BI transcripts were detected in the developing liver of wild-type and SF-1 knockout embryos at E11.5, consistent with the fact that SF-1 is not expressed in this organ and thus cannot regulate SR-BI. In contrast, SR-BI was expressed in the genital ridge of the wild-type embryo, but not SF-1 knockout mice. To the extent that the developing gonads are still intact at this relatively early developmental stage, this finding suggests that SF-1 plays important roles in SR-B1 expression *in vivo*.

Although the expression patterns of SF-1 and SR-BI mRNA in the developing rodent are similar, they are not identical. In the testes, SF-1 is expressed in the fetal Sertoli cells as well as Leydig cells. SR-BI has an expression pattern in the testes that is similar to that of SCC and StAR (30, 31); all three transcripts are detected only in the steroidogenic cells of the interstitium. In other embryonic tissues where SF-1 mRNA is present, like the pituitary and ventromedial hypothalamus (30), we found no detectable SR-BI transcripts (data not shown). Conversely, SR-BI is expressed at significant levels in some tissues that do not express SF-1, such as the embryonic liver. As discussed in the results, the most likely explanation for the absence of any immunodetectable hepatic SR-BI in murine embryos is that the level of expression is either too low or too diffuse to be detected by immunostaining.

To further define the mechanisms that regulate SR-BI in steroidogenic tissues, we examined its ex-

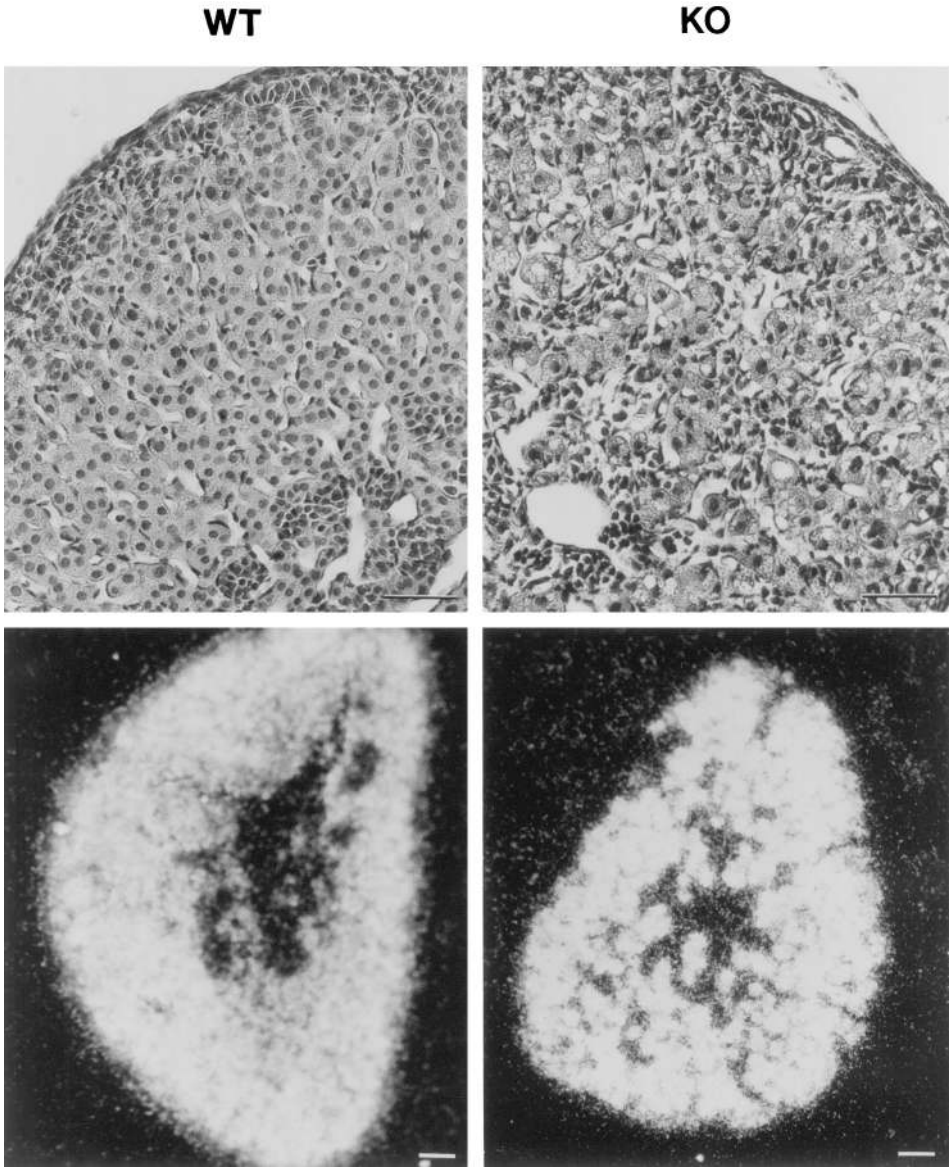

Fig. 9. *In Situ* Hybridization Analysis of SR-BI Expression in Wild-Type and StAR Knockout Mice

Adrenal glands were isolated from wild-type (WT, *left panels*) and StAR knockout newborn mice (KO, *right panels*). Hematoxylin-eosin staining of wild-type and StAR knockout adrenal sections (*top panels*). Dark-field views of *in situ* hybridization with an antisense cRNA probe specific for SR-BI are shown (*bottom panels*). *Bars*, 50 μm.

pression in cAMP-stimulated cultured murine adrenocortical (Y1-BS1) and testicular carcinoma (MA-10) cells. SR-BI mRNA and protein levels were dramatically increased upon cAMP stimulation in both cell lines. The kinetics of SR-BI induction by cAMP analogs in these cell lines differed somewhat from that which was previously reported for StAR (31). The increase in SR-BI levels lags behind that of StAR by approximately 2 h. The time course of SR-BI induction in response to cAMP analogs in MA-10 cells is similar to that seen in rat granulosa cells (42). In the unstimulated state, these two cell lines are similar in that they synthesize no steroid hormones and express no SR-BI; after cAMP stimulation it takes approximately 6 h before any detectable increase in SR-BI protein is apparent.

In the absence of any trophic hormones, SR-BI levels appear to be regulated by the intracellular content of cholesterol. Our results are compatible with the demonstration that SR-BI expression remains high in desensitized, lipid-depleted, rat luteal cells, which cannot respond to trophic hormones (16); these lipid-depleted cells have high levels of immunodetectable SR-BI protein, as well as HMG CoA reductase and the LDL receptor. In our studies with Y1-BS1 cells, the levels of immunodetectable SR-BI fall into the nondetectable range when cells are cultured for 24 h in the presence of LDL (100 μg/ml). The reduction in SR-BI

protein mass was greater than the fall in the level of SR-BI mRNA, which is consistent with a posttranscriptional mechanism. We found no evidence that the decrease in SR-BI in the lipoprotein-supplemented Y1-BS1 cells is due to an increased SR-BI degradation. Further studies will be required to define the posttranscriptional mechanism responsible for the observed dissociation between the levels of SR-BI mRNA and protein.

Even when Y1-BS1 cells are provided sufficient exogenous LDL to reduce HMG-CoA reductase to trace levels, administration of cAMP resulted in a considerable increase in SR-BI. These results suggest that trophic hormones up-regulate SR-BI expression directly rather than by depleting intracellular cholesterol stores. These data are consistent with the studies of Dexter *et al.* (2) who showed that ACTH stimulates the uptake of cholesterol from lipoproteins in the adrenal glands of hypophysectomized rat, even if steroidogenesis is completely inhibited. The results of our studies in cultured cells are similar to the findings of Gwynne *et al.* (4), who examined the effect of aminoglutethimide on the ACTH-stimulated uptake of radiolabeled cholesterol in rat adrenal slices. They showed that aminoglutethimide did not affect cholesterol uptake despite increasing the cellular cholesterol content by 5-fold. In granulosa cells, like the Y1-BSI cells, high levels of SR-BI were maintained even in the presence of high concentrations of lipoproteins (41).

Further evidence that trophic hormones override the effect of intracellular cholesterol concentrations on SR-BI expression is the finding that SR-BI levels are not decreased in the adrenal glands of StAR knockout mice (39). These mice are unable to efficiently transport cholesterol into the mitochondria of steroidogenic tissues and thus fail to synthesize sufficient steroid hormones to suppress pituitary ACTH secretion. As a consequence, the *StAR*$^{-/-}$ mice accumulate massive amounts of cholesterol in their adrenal glands and have elevated plasma levels of ACTH (39). Despite having cholesterol-laden adrenocortical cells, these mice have normal to elevated levels of SR-BI, which presumably are maintained by the high levels of circulating trophic hormones.

Taken together, the results of these studies are consistent with SR-BI being part of the repertoire of SF-1-responsive genes in steroidogenic tissues and the major pathway by which cholesterol is delivered for steroid hormone biosynthesis in the mouse. The physiological importance of this regulation may be to ensure that SR-BI will be up-regulated during times of stress, even if the adrenal gland is replete with cholesterol. This regulatory mechanism presumably ensures that the organism always has a sufficient supply of cholesterol available for maximal steroidogenesis in times of stress. This formulation is consistent with the extensive and careful studies of Reaven *et al.* (42), who showed that the selective uptake pathway is optimally designed for the dramatic increase in cholesterol delivery that is required upon stimulation.

Finally, it is important to note that multiple lines of mouse Y1 adrenocortical cells have been developed (20). These cell lines differ in their responsiveness to trophic hormones and are likely to differ in their expression levels of SR-BI. In some of the YI adrenal cell lines, exogenous HDL fails to reduce HMG-CoA reductase activity or incorporation of HDL-cholesterol into steroids (23). It is likely that these cell lines have a dysfunctional SR-BI receptor pathway.

MATERIALS AND METHODS

Materials

^{33}P-UTP and ^{32}P-CTP were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL). Y1-BS1 cells were kindly provided by Bernard Schimmer (University of Toronto, Toronto, Ontario, Canada), and the mouse testicular carcinoma Leydig cells (MA-10) were a kind gift from Mario Ascoli (Department of Pharmacology, University of Iowa, Iowa City, IA). Cell culture media (DMEM/F-12, and Waymouth's MB 782/1) were purchased from Life Technologies, Inc. (Gaithersburg, MD), FCS, horse serum, (Bu)2cAMP, 8-bromo cAMP, and aminoglutethimide were purchased from Sigma Chemical Co. (St. Louis, MO). The LDL and HDL were prepared by ultracentrifugation of human plasma as described (43). A RIA (CT Progesterone Kit) was purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA) and used according to the recommendations of the manufacturer.

Animals

Timed pregnant NIH Swiss mice were purchased from Harlan Laboratories (Indianapolis, IN). Noon of the day on which the copulatory plug was detected was designated 0.5 day of the gestation (E0.5). Pregnant mice were killed by cervical dislocation at the time intervals indicated, and the embryos were dissected, fixed in 4% paraformaldehyde, and embedded in paraffin. Serial sagittal sections of 4 μm thickness were prepared using a microtome (44). To determine the sex of each embryo, a small aliquot of tissue was taken from the yolk sac and placed in 500 μl of 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris HCl, pH 8.5, 0.01% (wt/vol) gelatin, 0.45% (vol/vol) NP40, 0.45% (vol/vol)Tween 20, and 100 μg/ml proteinase K (Sigma Chemical Co.) at 55 C overnight. PCR was used to amplify a fragment from the mouse SRY gene (45). The amplification reaction included 1 μl of the yolk sac lysate as template and two oligonucleotides (5′-TCATGAGACTGCCAACCACAG-3′ and 5′-CATGACCACCACCACCACCAA-3′). The PCR products were size fractionated on a 1% (wt/vol) agarose gel, the gel was stained with ethidium bromide, and the bands were visualized using UV light.

In Situ Hybridization

In situ hybridization was performed as previously described (44). A 2.4-kb fragment containing the mouse SR-BI cDNA (kindly provided by Dr. Monty Krieger, Massachusetts Institute of Technology, Cambridge, MA) was subcloned into pcDNA3 (Invitrogen, Carlsbad, CA). The plasmid was linearized using *Hin*dIII or *Xho*I to generate labeled antisense and sense RNA probes, respectively, employing the *In Vitro* Transcription System (Promega Corp., Madison, WI). The probes were partially hydrolyzed by incubating them with 200 mM Na_2CO_3, pH 10.2, at 60 C for 25 min. Serial sections were deparaffinized and then allowed to hybridize with the probes (1×10 (6)cpm/ml) using Hybridization Cocktail (Amresco,

Solon, OH) at 55 C overnight. The sections were washed and the slides were dipped in NTB2 liquid emulsion (Eastman Kodak Co., Rochester,NY) diluted 1:1 in H_20. The slides were incubated at 4 C for 23 days and placed in Dektol developing solution (Eastman Kodak Co.) and counterstained with hematoxylin. Photographs of the slides were taken using a Eclipse E1000M microscope (Nikon, Melville, NY) linked to a video system (Media Cybernetics, Silver Spring, MD). Green and red fluorescent filters was used under dark field illumination for the sections incubated with the SF-1 and SR-BI probe, respectively.

Immunoblot Analysis of SR-BI, LDL Receptor, and HMG-CoA Reductase

The Y1-BS1 cells were maintained in medium A (1:1 mixture of DMEM and Ham's F-12 medium, plus 100 U/ml penicillin and 100 μg/ml streptomycin sulfate) with 15% horse and 2% FCS. On day 4 the medium was switched to medium A plus 10% NLPPS. After 24 h, the medium was supplemented with 1 mM $(Bu)_2$cAMP. The same protocol was used for MA-10 cells except that the cells were maintained in Waymouths MB 752/1 medium plus 15% horse serum. Cultured cells were washed twice with ice-cold PBS before being collected in 2 ml of PBS. The cells were isolated by centrifugation at 1300 $\times$ *g* for 5 min and resuspended in lysis buffer [1% (vol/vol) Triton, 50 mM Tris, 2 mM $CaCl_2$, 80 mM NaCl, pH 8.2] containing protease inhibitors (0.5 mM phenylmethylsulfonylfluoride, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, and 2 μg/ml aprotinin). After a 15-min incubation on ice, the samples were centrifuged at 16,000 $\times$ *g* for 10 min. The protein concentration of the lysates was determined using a BCA Protein Assay kit (Pierce Chemical Co., Rockford, IL). Cell lysates for immunoblots to detect HMG-CoA reductase were prepared as described previously (37). Approximately 50 μg of each cell lysate were reduced by the addition of β-mercaptoethanol to 1.5% and then size fractionated on a 6.5% SDS-polyacrylamide gel. The proteins were transferred to Hybond C Extra Transfer membrane (Amersham Pharmacia Biotech), and immunoblot analysis was performed using a rabbit antibovine LDL receptor antiserum (1:1000) (46), a rabbit antipeptide polyclonal antibody directed against the last 14 amino acids of mouse SR-BI (10 μg/ml), and IgG-A9, a monoclonal antibody to HMG-CoA reductase (5 μg/ml) (37). Immunoblot analyses were performed using the Enhanced Chemiluminescence Western Blotting Detection Kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions, and then the filters were exposed to Reflection NEF film (DuPont NEN, Wilmington, DE). The images were scanned into a Power 7500/100 MacIntosh computer, and the relative intensities of the bands were quantified using NIH Image 1.61 (http://rsb.info.nih.gov/nih-image/download.html).

Immunoprecipitation

On day 0, Y1-BS1 cells were plated at 500,000 cells per well in a 6-well dish and grown for 2 days in medium A plus 15% horse serum and 2% FCS. On day 3 the medium was changed to medium A with 10% NLPPS. After 24 h the cells were incubated in methionine- and cysteine-free DMEM medium (ICN Biochemicals, Inc., Costa Mesa, CA) for 30 min and then pulsed with Trans-label methionine-cysteine (200 μCi/ml)(ICN Biochemicals, Inc.) for 1 h. The cells were then chased in medium A plus 10% NLPPS plus 2 mM cold methionine with or without 50 μg/ml of LDL, compactin (10 μM), and 5 μg/ml of aminoglutethimide. Cells were lysed at the indicated time points and SR-BI was immunoprecipitated exactly as described (38) except that the Protein A Sepharose was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

RNase Protection Assay

A 307-bp PCR fragment containing the sequence that encodes amino acids 397–499 of the mouse SR-BI cDNA was amplified from total murine hepatic RNA using RT-PCR (Stratagene, La Jolla, CA) and two oppositely oriented oligonucleotides with the following sequences: 5′-GGGCAAACAGGGAAGATCGAGCCA-3′ and 5′-ACCGTGCCCTTGGCAGCTGGTGAC-3′. The PCR product was subcloned into pGEMT Easy vector (Promega Corp.) and the insert was sequenced. The plasmid was linearized using *Nco*I, and an *in vitro* transcription reaction was carried out in the presence of [α-^{32}P]-CTP and SP6 polymerase (Promega Corp.) for 1 h at 37 C using the Riboprobe *in vitro* Transcription System (Promega Corp.). The reaction product was incubated in 1 U of RQ DNase (Promega Corp.) for 15 min at 37 C to digest the DNA template. The reaction mixture was then diluted with RNase-free water to a final volume of 50 ml, extracted once with 50 ml phenol/chloroform (1:1), and then purified using a G50 spin column (5 Prime→3 Prime, Inc., Boulder, CO). A HybSpeed kit from Ambion, Inc. (Austin, TX) was used for the RNase protection assay. A total of 1×10^5 cpm were mixed with 10 μg of total cellular RNA that was isolated from the cultured cells using RNA STAT (Tel-Test, Friendswood, TX). The RNase protection assay was performed as recommended by the manufacturer. The protected fragments were resolved on a 6% denaturing polyacrylamide gel, dried, and exposed to Reflection NEM film for the indicated times. The bands were quantified using a phosphoimager (Fuji Photo Film Co., Ltd., Stamford, CT).

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