CCAAT Enhancer-binding Protein β and GATA-4 Binding Regions within the Promoter of the Steroidogenic Acute Regulatory Protein (StAR) Gene Are Required for Transcription in Rat Ovarian Cells*

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Steroidogenic acute regulatory protein (StAR) is a vital accessory protein required for biosynthesis of steroid hormones from cholesterol. The present study shows that in primary granulosa cells from prepubertal rat ovary, StAR transcript and protein are acutely induced by gonadotropin (FSH). To determine the sequence elements required for hormone inducibility of the StAR promoter, truncated regions of the −1002/+6 sequence of the mouse gene were ligated to pCAT-Basic plasmid and transfected by electroporation to freshly prepared cells. FSH inducibility determined over a 6-h incubation was 10–40-fold above basal levels of chloramphenicol acetyltransferase activity. These functional studies, supported by electrophoretic mobility shift assays indicated that two sites were sufficient for transcription of the StAR promoter constructs: a non-consensus binding sequence (−81/−72) for CCAAT enhancer-binding protein β (C/EBPβ) and a consensus motif for GATA-4 binding (−61/−66). Western analyses showed that GATA-4 is constitutively expressed in the granulosa cells, while all isoforms of C/EBPβ were markedly inducible by FSH. Site-directed mutations of both binding sequences practically ablated both basal and hormone-driven chloramphenicol acetyltransferase activities to less than 5% of the parental −96/+6 construct. Unlike earlier notions, elimination of potential binding sites for steroidogenic factor-1, a well known tissue-specific transcription factor, did not impair StAR transcription. Consequently, we propose that C/EBPβ and GATA-4 represent a novel combination of transcription factors capable of conferring an acute response to hormones upon their concomitant binding to the StAR promoter.

The first and key reaction in the enzymatic cascade of steroid hormone biosynthesis is catalyzed in the mitochondria by cholesterol side chain cleavage cytochrome P450 (P450scc) (1–3). In the presence of atmospheric oxygen and reducing power provided by associated proteins, P450scc converts cholesterol substrate to the first steroid prototype molecule, pregnenolone (1). In order to do so, a supply of cholesterol is required to be transferred from cytosolic pools into the inner membranes of the mitochondrion, where P450scc resides (4–7). Recently, it was found that cholesterol delivery into the mitochondria is enhanced by a novel protein (8, 9) designated steroidogenic acute regulatory (StAR) protein (reviewed in Refs. 10–12). More studies have established the fact that StAR is a vital protein essential for steroidogenesis in the adrenal cortex and the gonads (13, 14). In rodents, StAR is also expressed in steroidogenic brain cells (15) and placenta.2 Interestingly, StAR is not expressed in human placenta, where its role is probably assumed by a less efficient StAR substitute called MLN64 (16). Perhaps the most compelling evidence for the critical role of StAR in steroidogenesis was the discovery that various mutations of the StAR gene encoding a functionally impaired protein (14) cause a syndrome known as lipid congenital adrenal hyperplasia (17). Affected individuals die shortly after birth in the absence of adrenal steroids, unless treated with steroid hormone replacement therapy. Similar patterns were also observed in StAR null mice (14).

Trophic hormones, such as gonadotropins and ACTH, trigger up-regulation of StAR expression by cAMP signaling (18–20). Additionally, Ca2+ changes evoke StAR expression in glomerulosa cells of the adrenal cortex (21, 22). Very little is known about the factors controlling StAR expression at the transcriptional level, downstream to the signal transduction pathways. Special attention has been devoted to examine the potential involvement of the steroidogenic factor-1 (SF-1, or Ad4BP), which is a pivotal tissue-specific orphan nuclear receptor essential for regulation of many steroid hydroxylases in steroid-producing tissues (23, 24). In light of the fact that StAR promoter includes several putative recognition sites for SF-1 binding, several attempts have been made to determine if the latter factor is involved in StAR regulation. At present, the available results are somewhat inconsistent. Using the human, mouse, and rat promoters, an apparent up-regulation of STAR transcription by SF-1 could be demonstrated upon co-transfection of SF-1 cDNA and promoter-reporter plasmids in non-steroidogenic cells (25–29). However, other studies analyzing the activity of StAR promoter in SF-1-expressing cells did not support a role for SF-1 in a cAMP-inducible fashion (30). These data suggested that SF-1 may not confer cAMP responsiveness in authentic steroidogenic cells and, therefore, cannot be an exclusive transcription factor controlling the acute regulation of StAR in such cells.

In search for alternative regulatory elements that can mediate the acute response of StAR to hormones, we undertook to study the inducibility of the mouse promoter in ovarian gran-

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1 The abbreviations used are: P450scc, cholesterol side chain cleavage cytochrome P450; StAR, steroidogenic acute regulatory protein; SF-1, steroidogenic factor-1; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; RT, reverse transcription; EMSA, electrophoretic mobility shift assay; PEPCK, phosphoenolpyruvate carboxykinase; PAGE, polyacrylamide gel electrophoresis; hCG, human chorionic gonadotropin; PMSG, pregnant mare serum gonadotropin; FSH, follitropin.

2 Y. Arensburg and J. Orly, unpublished data.
ula cells from prepubertal rats. Earlier studies have unambiguously shown that endogenous SF-1 in these cells is critical for the induction of P450scC and P450aromatase by follicle-stimulating hormone (FSH) (31–34). In contrast, the present study suggests that SF-1 is probably not involved in hormonal activation of StAR promoter. We also demonstrate that promoter regions capable of C/EBPβ and GATA-4 binding are required for activation of StAR transcription in FSH-treated cells. Thus, StAR provides the first example of a steroidogenesis-associated protein that is transcriptionally controlled by C/EBPβ and/or GATA-4.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ovine FSH (NIDDK-fFSH-20) was kindly provided by the National Institute of Health NIAMD (Bethesda, MD.). Acetyl-CoA, poly(dI-dC), RNase A, indomethacin, proteinase K, sodium orthovanadate, aprotinin, NaF, pepstatin, phenylmethylsulfonyl fluoride, peroxidase-conjugated goat anti-rabbit and peroxidase-conjugated rabbit anti-goat sera were obtained from Sigma. Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium were from Grand Island Biological, New York. Polyacrylamide gels to C/EBPβ were used to separate cells from Sigma. Dounce homogenizer using 2–3 volumes of buffer A contain- ing 100 mM KCl, 10 mM NaOH, 100 mM NaF, 1 mM sodium orthovanadate, 5 μM aprotinin, 2 μM pepstatin, and 1 mM phenylmethylsulfonyl fluoride. Following homogenization, the protein slurry was freeze-thawed three times in liquid nitrogen and finally centrifuged for 2 min at 14,000 × g. After determination of the protein content, the supernatant was aliquoted and kept at −70 °C until use.

**Electrophoretic Mobility Shift Assay**—Electrophoretic mobility shift assay (EMSA) was performed as described before (37). Briefly, whole cell extracts (3–15 μg) were incubated with 2 ng of double-stranded DNA, previously labeled by a fill-in reaction using Klenow fragment (Promega, Madison, WI) and [α-32P]dCTP (Amersham International, Little Chalfont, United Kingdom). Incubation was performed at room temperature for 1 h with a final volume of 30 μl of buffer containing 100 mM KCl, 15 mM Tris-HCl (pH 7.5), 10 mM dithiothreitol, 1 mM EDTA, 5 mM MgCl2, 12% glycerol, and 4.5 μg of poly(dI-dC). After incubation for 35 min at room temperature, the binding reactions were resolved on pre-run 5% acrylamide gel as described previously for quantitative RT-PCR analyses (38). When competition experiments were conducted in the presence of sodium orthovanadate, the protein extracts were added last to the reaction mixture. When antibodies were used for supershift (or ablation) of a given protein-DNA complex, the protein extracts were preincubated for 25 min at room temperature with 2–8 μg of the antiserum, prior to the addition of the DNA-labeled probe. The following oligonucleotide probes used for EMSA included overhanging restriction site sequences: S1Cl/S1P-1 (32) (upper strand, 5′-GATGCCCTCCTCTTCAAGCCTGA- CTA GTTA; consensus Sp1 (upper strand, 5′-GATCCGATGCCGCCCCGC- GGGACG); −148/−127 StAR (upper strand, 5′-TGCTCCTCCCACCTTGGCCGAG- CACATTCAAGTCGATGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGACTGAC...
**Fig. 1.** Putative binding sites for trans-acting proteins potentially involved in regulation of the mouse and rat StAR promoter. The $-178/+6$ region in the murine StAR promoter (30) harbors the following potential recognition sites (boxed) for trans-acting proteins, which were examined in this study: Sp1 ($-146/–137$), SF-1 ($-139/–132$, SF-1, AddIIP), C/EBP-$\beta$ ($-117/–108$), C/EBP-$\beta$-3 ($-81/–72$), and GATA ($-66/–61$). Additional SF-1 and C/EBP-$\beta$ sites (broken line boxes) were proposed by other investigators: SF-1-a ($-102/–95$); C/EBP-$\beta$-2 ($-90/–81$) and SF-1b ($-46/–39$). A TATA-like element is underlined. Mismatched nucleotides of the rat promoter (72) are indicated by highlighted superscripts.

(1:10,000 dilution). Specific signals were detected by chemiluminescence utilizing the LumiGlo substrate (New England Biolabs). Quantitation of chemiluminescence signals on x-ray films was performed as described previously (40).

Total RNA was extracted by dissolving the granulosa cells in 0.5 ml of RNAzol B (Tel-Test, Inc., Friendwood, TX) added to each culture well (16 mm). Further steps followed the manufacturer’s instructions. Semiquantitative RT-PCR analysis of total RNA extracts from granulosa cells was performed exactly as described previously (40).

**Statistical Analysis**—Student’s unpaired two-tailed $t$ test was performed using Microsoft Excel 97 statistical analysis functions. Differences between the activities of the indicated constructs were considered statistically significant at $p < 0.05$.

**RESULTS**

**Is SF-1 Involved in Regulation of StAR Promoter?**—Aiming to identify the regulatory elements controlling StAR expression, we have applied transient expression assays of the mouse StAR promoter by use of granulosa cells from prepubertal rat ovaries. To this end, a $-1002$ to $+6$ fragment of the StAR gene was cloned by PCR and ligated to a promoterless pCAT-Basic plasmid. We reasoned that the expression of the mouse promoter in rat cells is justified by the fact that the proximal regions of the rat and mouse promoters are almost identical, in particular through the first 150 base pairs upstream to the transcription start site (Fig. 1). Testing the hormonal inducibility of the promoter constructs was performed following a 6-h incubation with FSH added shortly after transfection by electroporation. Semiquantitative RT-PCR and Western blot analyses confirmed that under similar experimental conditions the levels of StAR mRNA and protein rise acutely upon the addition of FSH (Fig. 2).

At large, the activity values obtained by transfecting a series of progressive deletions of the promoter showed that hormone inducibility remained high in all constructs pruned down to $-96/+6$ (Fig. 3). The latter region exhibited the highest-fold induction by FSH (44-fold), suggesting that two potential upstream binding sites for SF-1 ($-139/–132$ and $-102/–95$), are not necessarily required for the FSH activation of the promoter. These results did not agree with earlier reports, which strongly advocated the notion that SF-1 is implicated in regulation of StAR expression (26–28, 30, 41). This inconsistency, together with the fact that deletion of the $-139/–132$ SF-1 site significantly reduced the basal activity of $-152$ StAR (Fig. 3), urged us to cautiously reassess the importance of this element by site-directed mutations and EMSAs. To our surprise, SF-1 did not bind to a $-148/–127$ probe (Fig. 4), previously shown to be capable of SF-1 binding using extracts of Y-1 adrenocortical cell line (30). Instead, the rat cell extracts generated a slower migrating protein complex, which was not affected by antisera to SF-1 (Fig. 4A, lanes 2 and 4). A closer examination of this sequence revealed a potential Sp1 site, which is overlapping the SF-1 binding element to create an “Sp1”/SF-1 motif (see Fig. 5B, probe 2). This G-rich element ($-146$, 5’-TGG-GAGGGAG, lower strand) is nearly identical to an Sp1-like binding sequence previously reported to be involved in cAMP-dependent regulation of the bovine P450scc transcription (34, 42). In StAR promoter, this Sp1-like site, termed “Sp1,” binds a protein that is antigenerically cross-reactive with Sp1 antisera (Fig. 4B, lanes 6 and 8). Moreover, molar excess of Sp1 consensus DNA can compete for the binding of “Sp1” to its site in StAR promoter (Fig. 5A, lanes 4 and 5). Finally, a site-directed mutation replacing GG with ca (Fig. 5B, lane 16) resulted in the loss of “Sp1” band shift and rendered the SF-1 site available for a typical SF-1 binding (Fig. 5B, lane 16). Noteworthy, the “Sp1”/SF-1 element could bind both proteins, providing the extracts were prepared from the mouse MA-10 cells (Fig. 5B, lane 14), which are highly enriched with SF-1 content. These results suggest that the $-148/–127$ region has a dual capacity to bind both “Sp1” and SF-1, which compete with each other depending on their relative content in a given cell type.

The apparent cooperativity of Sp1 and SF-1 in mediating cAMP-driven expression of steroidogenic genes (34, 42) could have suggested that a similar concept might be functionally
Progressive deletions of StAR promoter when compared with the respective values. Activity levels were statistically significant results in Fig. 6 show that a 5'-CAAGGTGG mutation to 5'-CAAtaTGG (-152mutSF-1), did not affect the response of StAR promoter to hormones, but instead improved it. The same mutation was previously shown to ablate SF-1 binding and transcriptional activation of the P450scc and P450aromatase promoters (31, 32). The notion that binding of SF-1 is irrelevant for transcriptional activation of StAR was further strengthened by the fact that a mutant of the “Sp1” site (-152mut2”Sp1”), which capacitated SF-1 binding (Fig. 5, lane 16), did not improve any of the construct performances (Fig. 6). Finally, when the “Sp1” site was mutated (-152mut1“Sp1”), as previously done to critically impaired its activity when harboring the bovine P450scc promoter (42), no significant loss of hormonal inducibility or basal activity were noticed (Fig. 6).

FSH Responsiveness Is Determined by the −93/−51 Region: A Role for C/EBPβ—Further 5' deletions of StAR promoter finally resulted in a severe loss of FSH responsiveness. Fig. 7 shows that the −73/+6 and −51/+6 CAT constructs retained only 12% and 0.3% of the FSH-driven activity exhibited by the −96/+6 construct. To verify that no additional sequences located downstream to −51 are potentially involved in hormonal regulation of this promoter, we have randomized 17 base pairs constituting the −49/−33 region. Clearly, this mutation performed within the context of the −96/+6 construct did not affect its activity (Fig. 7), suggesting that no important elements reside immediately upstream to the TATA box.

Sequence analysis of the −96/−51 region depicts a putative near-consensus C/EBPβ site (C/EBPβ-2), residing upstream to a conserved consensus GATA binding motif (Fig. 1). To test if the C/EBPβ-2 site may have a regulatory role in the rat granulosa cells, we mutated this site in the context of −96StAR, as shown in Fig. 8. However, the mutated construct (−96mut2-2) did not affect its 55-fold response to FSH, which was not much different from the parental plasmid (Fig. 8). Further EMSA studies provided an explanation for this observation by showing that a specific C/EBPβ-2 probe (−96/−75) did not bind to any protein (data not shown). Instead, a downstream adjacent element reminiscent of an AP-1 site, if anything else (5'-TGACTGA), was found capable of C/EBPβ binding. We designated this non-consensus element C/EBPβ-1 (see Fig. 1). EMSAs showed that a C/EBPβ-3 oligonucleotide probe (−87/−70) binds C/EBPβ in a specific fashion; antisera to C/EBPβ caused a supershift and ablation of the typical triplet bands relevant for FSH induction of StAR. Therefore, CAT reporter transgenes containing point mutations in the −132/−146 region were created in the context of −152StAR, as an alternative approach for the deletion strategy described before. The results in Fig. 6 show that a 5'-CAAGGTGG mutation to 5'-CAAtaTGG (-152mutSF-1), did not affect the response of StAR promoter to hormones, but instead improved it. The same
bound to C/EBPβ DNA (Fig. 9, lanes 1 and 3), and a specific antiserum to C/EBPα supershifted the upper band, suggesting that it consists of C/EBPα/C/EBPβ heterodimer (Fig. 9, lane 2). Finally, three sera served for negative controls, including antiserum to sterol-responsive element-binding protein (SREBP, Fig. 9, lanes 5 and 6), previously shown to activate STAR transcription in human granulosa cells (29), and ineffective antisera to c-Fos and c-Jun (Fig. 9, lanes 8 and 9).

Despite the fact that the sequence of C/EBPβ-3 site (TGACTGATGA) is so remotely different from a consensus C/EBPβ motif, it is absolutely required for C/EBPβ binding, which was lost upon a TGAtcgTGA mutation of the probe (Fig. 9b, lane 16). Yet, as could be expected, the C/EBPβ-3 motif does not necessarily exhibit the best affinity for C/EBPβ binding, as we learn from competitive EMSAs mixing a 32P-C/EBPβ-3 probe (-87/-70) with a 10–100-fold molar excess of the near-consensus C/EBPβ-1 sequence (-125/-100); in doing so, a 10-fold excess of C/EBPβ-1 was enough to displace 90% of the labeled C/EBPβ-3 probe (Fig. 9, lane 14). The reason for using the C/EBPβ-1 sequence for competitor DNA lies in the curious fact that its motif (ATGGCGCAT) specifically binds immunocross-reactive C/EBPβ (Fig. 9C). However, mutating or deleting the C/EBPβ-1 site does not impair transcriptional activation of the StAR promoter (Fig. 3, bottom two constructs). By contrast, functional analysis of the C/EBPβ-3 sequence by site-directed mutagenesis (Fig. 8, -96mutβ-3) suggested that an intact C/EBPβ-3 site is, indeed, required for the activity of STAR promoter.

**The Role of GATA-4**—However, the moderate attenuating effect caused by mutating the C/EBPβ-3 site could not account for the severely impaired activity of the -51StAR construct (Fig. 3). Therefore, the involvement of an additional regulatory element downstream of C/EBPβ-3 was likely to be found. To test this possibility, we examined the candidacy of a perfect GATA binding site located at -61/-66 (Fig. 1). Like -96mutβ-3, modification at this GATA site (-96mutGATA) resulted in no more than a partial attenuation of the construct activity (Fig. 8). However, double mutation of both C/EBPβ-3 and GATA sites (-96doublemut) resulted in a nearly complete loss of CAT activity (Fig. 8). A similar marked impairment of the FSH responsiveness was also observed when the GATA site was mutated in the context of -73mutGATA, from which the C/EBPβ-3 motif was deleted. In our view, the residual but not negligible FSH -fold induction observed in cells transfected with the double-mutated construct (-96double mut) is probably meaningless and reflects a pleiotropic effect FSH has on the basal activity by acting as a trophic hormone. In fact, the}
expression of the constructs in cells treated with or without FSH were performed as described in Fig. 3. CAT activities were determined using 5 

illustrated in the left panel. The bottom construct depicts the 17 bases −49/−33 were randomized. Transient expression of the constructs in cells treated with or without FSH were performed as described in Fig. 3. CAT activities were determined using 5 μg of protein for a 5-h assay. The results are presented as the mean ± S.E. of percentage of [14C]chloramphenicol converted to the acetylated products. Hatched bars represent the FSH -fold induction above basal activity. Multiple independent transfections (n) were performed for each construct. Activity levels were statistically significant when compared with the respective values obtained for the −96StAR construct: a, p < 0.05; b, p < 0.005; c, p < 0.001.

Fig. 8. Recognition elements for C/EBP β and GATA-4 binding confer transcriptional activation to StAR promoter. The left panel illustrates a series of −96StAR constructs including mutated sequences (lowercase letters) for binding of either C/EBP proteins (−96mutβ-2, −96mutβ-3), or the GATA factors (−96mutGATA). Additionally, double-mutated constructs were prepared as either −96double mut, or −73mut-GATA. Transfection and activity assays were conducted as described in Fig. 3. The results are presented as the mean ± S.E. of percentage of [14C]chloramphenicol converted to the acetylated products. Hatched bars represent the FSH -fold induction above basal activity. Multiple independent transfections (n) were performed for each construct. Activity levels were statistically significant when compared with the respective values obtained for the −96StAR construct: a, p < 0.05; b, p < 0.001; c, p < 0.001.

activity performances of the double mutant were similar to those obtained for the maximally trimmed promoter construct −51StAR, which also exhibited a significant 5-fold induction of CAT activity by FSH (Fig. 7). However, this trophic improvement of the basal activity was no higher than 3% of the FSH activity measured for the parental −96StAR construct (Fig. 7) and, therefore, could not represent other than a misleading basal activity.

Fig. 10 shows a single protein complex labeled by either of the two DNA probes, −73/−42, or −73/+6 (Fig. 10, A and B, respectively). These results confirm that no other protein complexes can bind to the entire region residing between −73 and +6. The binding specificity was demonstrated by use of anti-serum to GATA-4, which ablated and supershifted the DNA-protein complex. The latter remained unaffected in the presence of anti-GATA-6 serum (Fig. 10A, lanes 1 and 2). Additionally, mutating the GATA site in the context of the −73/+6 probe (5′-TATCTC−5′-TTAGT) associated with loss of binding capacities, as evident by competition (Fig. 10B, lanes 7 and 8) and direct binding studies using radiolabeled probe (Fig. 10B, lane 9).

Granulosa Cell Expression of C/EBP β and GATA-4—We also aimed to study by Western blot analysis the levels of C/EBP β and GATA-4 proteins in our tissue and cell preparations. First, we tested granulosa cell extracts prepared at identical time points previously used for determination of CAT activity. Fig. 11A shows that the C/EBP β antiserum cross-reacted with three protein bands of 45, 39, and 22 kDa, known as C/EBP β isoforms (43). The levels of those proteins were barely detectable in vivo (t0), but seeding the cells for a few hours in culture substantially elevated the levels of the higher molecular weight forms. The 22-kDa isoform of C/EBP β was not affected by seeding to culture (Fig. 11A). A 6-h treatment with FSH generated an increase of all the isoforms of C/EBP β up to 7-fold. In agreement with our EMSAs (Fig. 9A), Western blot analysis of the ovarian cells detected C/EBP β protein bands (data not shown), which were also reported by earlier studies of these proteins during follicular development (44).

By contrast to the profile of C/EBP β expression, neither seeding nor FSH treatment had any effect on the high level of the GATA-4 content, which is probably constitutively expressed in the rat granulosa cells (Fig. 11B). It should be noted
FIG. 9. The −87/−70 StAR promoter region binds C/EBPβ proteins. A, electrophoretic mobility shift assays using PMSG/hCG ovary extract were performed as described in Fig. 4 using a −87/−70 32P-labeled probe (lanes 1–15). The formation of the three DNA-protein complexes (arrows, lanes 1 and 4) was examined in the presence of the following antisera: C/EBPα (lane 2), C/EBPβ (lanes 3 and 7), sterol-responsive element-binding protein, SREBP (lane 5 and 6), c-Fos (lane 8), and c-Jun (lane 9). B, competition studies using the −87/−70 as a probe (lane 11) and molar excess of unlabeled self-competitor DNA (lanes 12 and 13), or unlabeled C/EBPβ-1 DNA (lanes 14 and 15). Lane 16 demonstrates lack of binding to a mutated C/EBPβ-3 labeled probe. C, characterization of C/EBPβ binding to a C/EBPβ-1 probe (−125/−100) was performed in the presence of either an antiserum to C/EBPβ (5 μg, lane 19), or anti-SF-1 serum as a negative control (4 μg, lane 18).

that, in correlation to our EMSA data, we could not detect the GATA-6 protein by this Western blot analysis (data not shown). Fig. 11C shows that the C/EBPβ and GATA-4 proteins also exist at high levels in the ovarian extracts we selected, for practical reasons, as a source for our EMSAs. Those extracts were expected to express the necessary factors since StAR expression during the post-hCG period is in its prime response (40).

DISCUSSION

The objective of the present study was to reveal the principal sequence elements that render the promoter of StAR gene responsive to FSH in cells of the rodent ovary. Based on previously published sequence (30), we cloned a −1002 to +6 fragment of the 5′-flanking region of the mouse StAR gene and placed it upstream of CAT gene in a promoterless pCAT-Basic reporter plasmid. Then, by contrast to most of the previous STAR studies expressing promoter-reporter plasmids in established cell lines (26–30, 41), we conducted our functional assays using primary naive granulosa cells from prepubertal rats. Following electroporation of these cells in the presence of plasmid DNA, a 6-h incubation in culture resulted in a robust activation (up to 50-fold) of the promoter by FSH. Having such
a sensitive assay in hand, we combined a progressive deletion strategy with site directed mutations aiming to validate, or eliminate, the potential involvement of candidate cis-acting regulatory elements in StAR expression. This approach led us to identification of two novel elements, C/EBPβ and GATA-4, which were never known before to be required for transcriptional activation of genes encoding steroidogenic proteins.

High responsiveness to FSH still remained if the promoter was pruned down to position −96 from the transcription start site. However, we were interested in performing a limited analysis upstream of the −96 regions, in particular addressing those sites that were previously suggested to be functionally involved in regulation of StAR expression. For example, between −100 and −1000 of the StAR promoter reside at least two putative binding sites for the tissue-specific orphan receptor SF-1 (45), also known as Ad4BP (24). This factor is required for activation of many genes expressed in steroidogenic tissues (23). Therefore, a host of studies have recently proposed that SF-1 is also essential for expression of StAR gene (26–30, 41). Using the naive granulosa cell model, our findings do not support this notion since the removal of all putative SF-1 sites (28, 30) hardly affected the promoter performance (−96StAR and −96rStAR, Figs. 2 and 7, respectively). A possible explanation for the apparent discrepancy between the present findings and earlier works may reflect species-dependent differences between the human (26) and the murine (30) promoters. However, one cannot exclude the possibility that the StAR promoter was never examined in steroidogenically committed primary cells which contain normal levels of SF-1. Instead, the role of SF-1 was demonstrated in luteal cells (27, 46) and steroidogenic cell lines expressing exceptionally high endogenous levels of the orphan receptor (30, 41), or by use of non-steroidogenic cells overexpressing transfected SF-1 plasmids (26–29, 41). Therefore, the role of SF-1 was always examined under conditions favoring its interaction with StAR promoter, but did not necessarily simulate the physiologically compatible requirements for regulation of StAR in normal cells.

Upstream of position −96 in the mouse promoter resides an additional element of considerable interest, i.e. a non-consensus putative binding site for Sp1 (−137/−146). A similar sequence for binding of an Sp1-like protein (“Sp1”) was found important for cAMP induced transcription of the bovine P450scc gene (42). Moreover, an analogous Sp1 site seemed to be important for activation of the human StAR promoter (26). In rodents, however, this “Sp1” element is overlapping a well studied SF-1 binding motif, thus creating a mixed “Sp1”/SF-1 site. This composite sequence can bind SF-1 from Y-1 (30) or MA-10 cells (Fig. 4B), but is apparently incapable of doing so when tested with granulosa cell extracts. We may, therefore, conclude that “Sp1” and SF-1 compete for binding to this site, so that SF-1 band shift in EMSAs is demonstrable only in cell lines expressing extremely high levels of SF-1, as discussed above. However, at present it is not clear if activation of the StAR promoter in granulosa cells involves “Sp1” action, since removal of the “Sp1”/SF-1 site down to position −123 attenuated the basal activity, but retained the hormonal responsiveness intact. Interestingly, further pruning of the promoter down to −96 kept suppressing the basal activity of the promoter by removing a near-consensus C/EBPβ (C/EBPβ-1) binding moiety at −116/−107. Thus, the C/EBPβ-1 site is irrelevant for activation of the promoter despite its apparent excellence in binding typical C/EBPβ proteins (Fig. 9). Collectively, these results have suggested that the critical element(s) controlling the inducibility of StAR promoter must have resided further downstream, within the first 80–90 base pairs of the promoter.

Indeed, deletion mutants and site-directed mutation within the −87 to −51 region revealed the involvement of trans-acting proteins that bind two sites located 10 base pairs apart: an upstream sequence interacting with C/EBPβ (C/EBPβ-3) and a consensus site specifically binding a GATA-4 protein. Contrary to our earlier expectations, the sequence core subserving for C/EBPβ binding (TGACTGA) seemed more like an AP-1 site, so that a typical c-Jun/c-Fos binding was demonstrable upon a single base substitution to TGACTcA (data not shown). Even more confusing was the fact that overlapping with this odd C/EBPβ-3 site resides another putative recognition element for C/EBPβ, designated C/EBPβ-2. However, mutating the core sequence of C/EBPβ-2 (ACATA to tcctc) did not affect the inducibility of the −96StAR construct, while a four-base mutation of C/EBPβ-3 reduced the basal and FSH-responsive activity by 50%. This shy effect of the mutated C/EBPβ-3 did not impress much until after the elucidation of the GATA binding site at −66/−61. Mutation of the GATA site resulted in a 45% drop of the promoter activity, but a double mutation of both GATA-4 and C/EBPβ-3 resulted in a dramatic 97% loss of the construct activity. These results suggest that a concurrent binding of the two regulatory proteins is required for activation of StAR promoter.

The present study shows that the prepubertal granulosa cells already express GATA-4 in vivo, and the level of this protein remained unaffected under any culture manipulations. By contrast, the C/EBPβ proteins are absent in vivo, but their expression in culture was markedly promoted by FSH. A similar induction of C/EBPβ expression was recently documented in testicular Leydig cells responding to cAMP (47). These results suggest that the level of C/EBPβ might determine the rate-limiting regulatory switch of StAR transcription, while the constitutively expressed GATA-4 plays an essential, yet more permissive role for that matter. It is noteworthy that, other than StAR, members of the C/EBP family have been described as regulators of acute responses, such as the control of certain inflammatory functions (48). Moreover, the suggested involvement of C/EBPβ proteins in the acute response of StAR to cAMP (8, 20) and trophic hormones (40, 49, 50) is also well accepted in light of a well studied example where C/EBP plays a critical role in transcriptional regulation of the phosphoenolpyruvate carboxykinase (PEPCK) gene (51). Similar to the emerging scenario in StAR promoter, it has been shown that cAMP and probably protein kinase A drive PEPCK transcription via a complex interactions of C/EBP with other activators and co-activators (51); also, the binding of C/EBPβ to a non-consensus recognition motif in the StAR promoter is reminiscent of the C/EBP binding to non-dyad-symmetric sequences in the PEPCK promoter, a cyclic AMP-responsive element-like site (52) and a site termed P3(I) (53).

Our EMSAs showed that the C/EBPβ-1 and C/EBPβ-3 probes formed three DNA-protein complexes, which were identical to those obtained when binding capacities of rat granulosa cell extracts were previously examined with a C/EBPβ motif located in the hormone-inducible prostaglandin synthase-2 promoter (54). The alternative approach testing the potential cross-reactivity of the C/EBPβ proteins with specific antibodies tested by Western blot analysis revealed that the rat granulosa cells express three isoforms, previously identified as liver-enriched activating proteins (LAPs) and liver-enriched inhibiting protein (LIP) (43, 55). Clearly, LIP and LAP-I were markedly elevated as a result of FSH treatment, while LAP-II level rose by merely seeding the cells into culture. Therefore, it is conceivable to propose that the onset of StAR transcription is
controlled by a two-step mechanism: first, the seeding-induced rise of LAP-II capacitizes the immediate response of the granulosa cells to FSH, and a follow-up activation of the promoter may proceed thanks to the hormone-elevated levels of LAP-I. The likelihood of such a mechanism is not too speculative since the granulosa cells express high levels of FSH-activated cyclic AMP-responsive element-binding protein (56, 57), which in turn, is known to up-regulate LAP transcription (58, 59). Further studies should address this hypothesis and reveal even more about the potential involvement of the C/EBPβ isoforms in regulation of STAR expression. Also, it is not unlikely that other C/EBP proteins, such as C/EBPα, can interact with the –87/-70STAR site. If truly so, C/EBPα should be considered as potential substitute for C/EBPβ in the C/EBPβ-deficient mouse ovary (60–63). It has been shown that the ovaries of the latter null mice do not ovulate (60, 64) despite normal production of gonadal steroids and expression of steroidogenic enzymes, probably including STAR.3

Like C/EBPβ, GATA-4 had not been described previously as a potential regulator of genes encoding steroidogenic enzymes, or their accessory proteins. Gata4-null mice die in utero by 9.5 days postcoitum (65, 66). However, recent studies have suggested that this transcription factor is involved in control of gonadal development and sex differentiation in rodents (67–69). Moreover, in adult mouse tissues, high levels of GATA-4 are observed in the ovary, testis, and heart. Interestingly, little or no GATA-4 protein was found in the cells of the mouse corpus luteum (68), which do express record levels of STAR (20, 40, 70, 71). One way to reconcile this apparent inconsistency is provided by assuming that the role of GATA-4 during follicular phase could be fulfilled by another member of this family, like GATA-6, which is highly abundant in the corpus luteum (68). Alternatively, we may propose that the mode of STAR expression in the corpus luteum may not necessarily resemble its regulatory pattern in granulosa cells of the follicular phase. Accordingly, SF-1, which is extremely high in the corpus luteum, may control the gland expression of STAR expression after all. This tempting speculation implies that activation of STAR transcription may be achieved by more than one set of cis-acting proteins, depending on the origin of the cells and tissue under study. In this regard, the present findings raise the question: what tissue-specific factor(s) can potentially replace SF-1 in our stereoidogenically committed, but yet undifferentiated cell model? Alternative experimental approaches addressing this challenging question are currently under study.

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