CCAAT/Enhancer-binding Proteins Regulate Expression of the Human Steroidogenic Acute Regulatory Protein (StAR) Gene*

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Two putative CCAAT/enhancer-binding protein (C/EBP) response elements were identified in the proximal promoter of the human steroidogenic acute regulatory protein (StAR) gene, which encodes a key protein-regulating steroid hormone synthesis. Expression of C/EBPα and -β increased StAR promoter activity in COS-1 and HepG2 cells. Cotransfection of C/EBPα or -β and steroidogenic factor 1, a transcription factor required for cAMP regulation of StAR expression, into COS-1 augmented 8-bromoadenosine 3':5'-cyclic monophosphate (8-Br-cAMP)-stimulated promoter activity. When the putative C/EBP response elements were mutated, individually or together, a pronounced decline in basal StAR promoter activity in human granulosa-lutein cells resulted, but the fold stimulation of promoter activity by 8-Br-cAMP was unaffected. Recombinant C/EBPα and -β bound to the two identified sequences but not the mutated elements. Human granulosa-lutein cell nuclear extracts also bound these elements but not the mutated sequences. An antibody to C/EBPβ, but not C/EBPα, supershifted the nuclear protein complex associated with the more distal element. The complex formed by nuclear extracts with the proximal element was not supershifted by either antibody. Western blot analysis revealed the presence of C/EBPα and C/EBPβ in human granulosa-lutein cell nuclear extracts. C/EBPβ levels were up-regulated 3-fold by 8-Br-cAMP treatment. Our studies demonstrate a role for C/EBPβ as well as yet to be identified proteins, which can bind to C/EBP response elements, in the regulation of StAR gene expression and suggest a mechanism by which C/EBPβ participates in the cAMP regulation of StAR gene transcription.

The translocation of cholesterol from the sterol-rich outer mitochondrial membrane to the cholesterol-poor inner mitochondrial membrane is the rate-limiting step in steroid hormone synthesis (1). The steroidogenic acute regulatory protein (StAR) has an integral role in this cholesterol translocation process in highly steroidogenic tissues such as the ovary, testis, and adrenal gland as evidenced in experiments of nature and mouse gene knockout studies. Congenital lipid adrenal hyperplasia, a disease in which the production of all adrenal and gonadal steroids is severely impaired prior to the synthesis of pregnenolone, is caused by mutations that inactivate the StAR protein (1, 2). Targeted disruption of the mouse StAR gene results in a phenotype identical to human congenital lipid adrenal hyperplasia in nullizygous animals (3).

StAR gene expression as determined by in situ hybridization and Northern blot analysis in gonadal and adrenal cells revealed that the steroidogenic capacity of these cells was tightly linked to the abundance of the StAR transcripts (4, 5). Transcriptional control of the human StAR gene has only recently been examined with initial studies focusing on the orphan nuclear receptor, steroidogenic factor-1 (SF-1) (6) because of its known role in the regulation of other key genes involved in steroidogenesis (e.g. P450ccc, P450c17, aromatase, 3β-hydroxysteroid dehydrogenase) (7). Indeed, the human StAR promoter was shown to have at least three SF-1 binding sites that are functionally important for basal as well as cAMP-stimulated transcription (8). SF-1 has also been shown to be important for transcriptional regulation of the mouse (9), rat (10), and bovine StAR genes (11). Sequence analysis also indicated the presence of a variety of other transcription factor binding sites including multiple putative Sp-1 binding sites, several of which were recently implicated in transcriptional control of StAR gene expression (12). Deletion analysis of the StAR promoter suggested that other transcription factors were also likely to play an important role in StAR gene expression. In addition to the known SF-1 response elements and a novel DAX-1 hairpin loop binding site (13), the StAR promoter contains several putative transcriptional response elements including a series of three sites at –80 to –69 that resemble sterol regulatory element-binding protein (SREBP) motifs, the most proximal of which is similar to a Ying Yang 1 (YY1) transcription factor binding site. The presence of SREBP binding sites is consistent with our recent demonstration that SREBP-1a stimulates StAR reporter activity (14).

Recent observations revealed that ovarian CCAAT/enhancer-binding proteins (C/EBPs), basic leucine zipper transcription factors implicated in the regulation of a variety of genes involved in energy metabolism and cell differentiation pathways (15), are modulated by luteinizing hormone, which acts on ovarian cells through the intermediary of cAMP, and that steroidogenic cell function is altered in mice upon loss of C/EBPα or -β. Gene knockout studies (C/EBPβ) (16) and antisense experiments (C/EBPα) (17) demonstrated that loss of either of these two members of the C/EBP transcription factor family results in the failure of differentiation of the ovulatory follicle as evidenced by the lack of ovulation and formation of the
corpus luteum. The focus of the present studies was to determine if C/EBPs modulate basal and CAMP-stimulated StAR gene expression.

EXPERIMENTAL PROCEDURES

Plasmids—The cDNA for mouse SF-1, a generous gift from Dr. K.L. Parker (Southwestern Medical Center, Dallas, TX), was cloned into the pSV-SPORT vector using standard methods. The pcDNA3 basic vector (Invitrogen) was sequenced and was identical in three of the four sequences. Known SF-1 binding sites in the human StAR promoter and the putative regions that interact with SREBP and YY1 are boxed. The DAX-1 DNA hairpin loop encompasses bases –61 to –21 (not shown). The location of the C/EBP sites and the mutations that block C/EBP binding (bold) are indicated below the human StAR promoter sequence. The sequences of the oligonucleotides used in the electrophoretic mobility shift assay are also shown. All oligonucleotides included 4-base pair extensions that were used for radiolabeling purposes (fill-in reaction).

Identification of Motifs Resembling CCAAT/Enhancer-binding Protein Response Elements—Sequence alignment (Fig. 1) demonstrated that the human, bovine, rat, and mouse StAR gene promoters are identical in three of the four sequences. Known SF-1 binding sites in the human StAR promoter and the putative regions that interact with SREBP and YY1 are boxed. The DAX-1 DNA hairpin loop encompasses bases –61 to –21 (not shown). The location of the C/EBP sites and the mutations that block C/EBP binding (bold bases) are indicated below the human StAR promoter sequence. The sequences of the oligonucleotides used in the electrophoretic mobility shift assay are also shown. All oligonucleotides included 4-base pair extensions that were used for radiolabeling purposes (fill-in reaction).

Sequence homology of the human, bovine, mouse, and rat StAR gene promoters and the known and putative response elements in the human StAR gene are depicted. Shaded boxes mark those bases in the human, bovine, rat, and mouse StAR gene promoters that are identical in three of the four sequences. Known SF-1 binding sites in the human StAR promoter and the putative regions that interact with SREBP and YY1 are boxed. The DAX-1 DNA hairpin loop encompasses bases –61 to –21 (not shown). The location of the C/EBP sites and the mutations that block C/EBP binding (bold bases) are indicated below the human StAR promoter sequence. The sequences of the oligonucleotides used in the electrophoretic mobility shift assay are also shown. All oligonucleotides included 4-base pair extensions that were used for radiolabeling purposes (fill-in reaction).

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if the C/EBPα and -β transcription factors are functionally important for StAR gene expression, we cotransfected COS-1 and HepG2 cells, which do not express StAR endogenously, with StAR promoter-luciferase constructs and plasmids that express these transcription factors. These cells were used to test StAR promoter activity in cells that do not express endogenous SF-1. Fig. 2 shows the results of studies in COS-1 and HepG2 cells transfected with 50 ng (maximal stimulatory dose) of either C/EBPα or -β with either the 1.3-kilobase StAR-luciferase reporter or the DEI-4-luciferase reporter, a control C/EBP-responsive promoter construct. In COS-1 cells, the StAR and DEI-4 promoter activities increased in a dose-dependent manner with expression of both C/EBPα and -β (data not shown). C/EBPα was a more effective stimulator of the promoters than C/EBPβ. In HepG2 cells, C/EBPα and -β were equally potent stimulators of the StAR and DEI-4 promoter activities (Fig. 2).

Studies with the full-length StAR promoter (1.3 kilobases) indicated that coexpression of increasing amounts of C/EBPα expression plasmid (1–50 ng/well) with SF-1 (500 ng/well) had an additive or greater effect on StAR promoter activity (Fig. 3). Fig. 3 depicts the results for the 50-ng doses of C/EBPα, C/EBPβ, or the empty pMEX carrier plasmid in the absence/presence of the SF-1 or its empty pSV-SPORT-1 carrier plasmid. Additionally, each of the six treatment groups was further subdivided, and the cells were cultured in the presence and absence of 8-Br-cAMP (1 μM). Comparison of the mean values (n = 3) for the control group (empty pSV-SPORT-1 + empty pMEX) ± 8-Br-cAMP indicated that StAR promoter activities with and without 8-Br-cAMP were not significantly different (p > 0.1; 28,000 ± 11,000; 40,800 ± 15,900; mean ± S.E.). The remaining data were expressed as fold increases over the mean of these control values, which were given a value of 1. The transient expression of C/EBPα and -β in the COS-1 cells increased basal StAR promoter activity 5.7- and 3.7-fold, respectively, over the control (empty pSV-SPORT-1 + empty pMEX minus 8-Br-cAMP). Promoter activity was not affected by 8-Br-cAMP treatment. In contrast, transient expression of SF-1 alone had little effect on basal StAR promoter activity, whereas it potentiated cAMP responsiveness of the StAR promoter (6.3-fold). Coexpression of C/EBPα and SF-1 had a greater than additive effect on both basal (10.9-fold) and cAMP-dependent (26-fold) StAR promoter activity (Fig. 3). Transient expression of C/EBPα also augmented 8-Br-cAMP-stimulated StAR promoter activity in the presence of SF-1 but to a lesser extent than C/EBPα (Fig. 3).

**StAR Promoter Function in Human Granulosa-Lutein Cell**—To investigate the role of C/EBP in cells that normally express StAR, we transfected human granulosa-lutein cells with a series of StAR promoter deletion constructs (Fig. 4). Basal StAR promoter activity increased (p < 0.05) ~2-fold following the deletion of the distal 400 bases of the 1.3-kilobase StAR promoter, and this level of activity was maintained in the −235 StAR construct. The −150 StAR promoter construct displayed activity similar to the full-length StAR promoter, but all further deletions caused a pronounced decline (p < 0.05) in basal StAR promoter activity with the −95, −60, and −43 constructs exhibiting similar low promoter activity levels. Cells transfected with the shortest of the StAR promoter constructs (−43 to +39) displayed ~2-fold greater luciferase activities than the pGL2-basic plasmid.

Based on the above results, the putative C/EBP response elements were mutated in the context of the −235 promoter construct. Fig. 5 illustrates the activities of the C/EBP mutant constructs in proliferating human granulosa-lutein cells. The −119/−110, −50/−41, and the double C/EBP mutant StAR promoters contained a 3-base pair substitution in each site that disrupted the core C/EBP element. The −235 wild-type StAR promoter construct had activity well above that of the empty pGL2-basic reporter and exhibited a 2-fold increase in response to 24 h of treatment with 8-Br-cAMP. In contrast, there was a pronounced decline (~4-fold, p < 0.05) in basal promoter activity in the −119/−110 and −50/−41 C/EBP mutation constructs. However, these single C/EBP mutant constructs retained their ability to respond to cAMP as evidenced by the 2-fold increase in promoter activity over basal activity in response to 1 μM 8-Br-cAMP treatment. Mutation of both C/EBP sites resulted in a further reduction in basal StAR promoter activity (~90% of the wild type) although not affecting cAMP responsiveness.

**Electrophoretic Mobility Shift Assays**—Recombinant C/EBPα and -β were both able to elicit a mobility shift of the oligonucleotide probe containing the −119 to −110 and −50 to −41 elements (Fig. 6). The gel-shift bands observed for the StAR oligonucleotides were similar to those observed with a consensus C/EBP site probe. Binding of both C/EBPα and -β proteins to the

![COS-1 CELLS](image1)

**Hep-G2 CELLS**

![expression plasmids](image2)

**Fig. 2.** StAR promoter activity in COS-1 and HepG2 cells transiently expressing C/EBPα, C/EBPβ, or the empty expression plasmid (pMEX). Results are the means ± S.D. of a single experiment with three replicates/treatment group. Experiments were replicated at least twice with similar results. RLU, relative luciferase units.
**FIG. 3.** C/EBP and SF-1 transactivation of the human StAR promoter in COS-1 cells. StAR promoter activity is expressed as the fold increase in luciferase activity/β-galactosidase values over the negative controls (cells transfected with empty-pSV-SPORT-1 + empty-pMEX), which were given a value of 1. StAR promoter activities for the negative controls (n = 3) were not different (p > 0.1; 28,000 ± 11,000 and 40,800 ± 15,900; means ± S.E.) for cells cultured in the absence and presence of 8-Br-cAMP, respectively. *, means ± S.E., denotes a significant difference between the (−) 8-Br-cAMP and the (+) 8-Br-cAMP-treated cells within a treatment group (i.e., same expression vector). a–d, means ± S.E. for (−) 8-Br-cAMP or (+) 8-Br-cAMP-treated cells with different superscripts are significantly different (p < 0.05).

**Expression Plasmids**

**FIG. 4.** Human granulosa cell expression of the StAR promoter deletion constructs. Cells were transfected with the indicated StAR promoter-luciferase-based reporter constructs, and the pGL2-basic reporter and luciferase activities were determined 36 h later. Each reporter was tested in triplicate during each experiment and the figure represents the results of 3 independent experiments. a–d, means ± S.E. with different superscripts are significantly different (p < 0.05).

probes could be competitively inhibited by the addition of increasing doses of unlabeled oligonucleotide (highest dose, 20×, is shown). Additionally, the C/EBPα and -β protein-DNA complexes could be supershifted with the cognate antibodies (Fig. 6). These binding interactions were specific, as mutations of the putative C/EBPα and -β binding site in the −55 to −31 StAR oligonucleotide prevented the interaction of this probe with the recombinant C/EBPα and -β proteins. Furthermore, this mutant oligonucleotide failed to inhibit the formation of a protein-DNA complex with either of the recombinant C/EBPs. Additionally, we tested oligonucleotides representing nucleotides −89 to −64 and −96 to −64 of the human StAR promoter, which contains no C/EBP consensus binding sites, and were unable to detect any protein-DNA complexes with the recombinant C/EBPα and -β (data not shown).

Having established that recombinant C/EBPs bind to sequences in the StAR promoter, we next determined if human granulosa-lutein cell nuclear extracts contained proteins capable of interacting with these elements. Fig. 7A shows that a protein-DNA complex is formed when granulosa-lutein cell nuclear extracts are incubated with the −126 to −100 oligonucleotide. Binding specificity was demonstrated by showing that 10–50× molar excess of the cold wild-type competitor abolished the complex between nuclear proteins and the labeled oligonucleotide, whereas a mutant cold oligonucleotide containing the same 3-base mutation used for promoter assays failed to disrupt the protein-DNA interaction. The complex was supershifted by C/EBPβ-specific antibodies, but antibodies to C/EBPα failed to elicit a supershift, indicating that the shift in mobility was because of the interaction of C/EBPβ with the labeled oligonucleotide probe.

**Western Blot Analysis of C/EBPs in Human Granulosa-Lutein Cell Nuclear Extracts**—Granulosa-lutein cell nuclear extracts. A protein-DNA complex of greater molecular weight than that observed for the distal site was observed. Binding specificity was demonstrated by inclusion of 10–50× molar excess of cold wild-type and mutant oligonucleotides, with ablation of the radiolabeled complex with the wild-type oligonucleotide and no effect of the mutant. In contrast to our observations with the distal C/EBP response element, antibodies to C/EBPα and -β did not supershift this DNA-protein complex, indicating that another protein(s) with binding specificity similar to that displayed by C/EBPα or -β was interacting with the oligonucleotide probe.

**Western Blot Analysis of C/EBPs in Human Granulosa-Lutein Cell Nuclear Extracts**—Granulosa-lutein cell nuclear extracts were tested for the presence of C/EBPα and -β by Western blot analysis (Fig. 8). A doublet of ∼42 kDa of C/EBPα was identified, and levels of these proteins were not altered by treatment of the granulosa-lutein cells with 1 mM 8-Br-cAMP. Western blot analysis also indicated that C/EBPβ (38 kDa) was present in granulosa-lutein cell nuclei, and levels of C/EBPβ increased 3-fold (p < 0.05) in response to a 24-h treatment with 1 mM 8-Br-cAMP.
DISCUSSION

This is the first study to demonstrate that members of the family of C/EBP proteins regulate expression of the human StAR gene. We identified two putative C/EBP binding sites within the proximal StAR promoter by sequence homology that were subsequently shown to bind recombinant C/EBPα and -β. Both C/EBPα and -β increased the activity of the StAR reporter construct in COS-1 and HepG2 cells. Furthermore, mutation of the individual C/EBP binding sites markedly reduced basal StAR promoter activity in human granulosa-lutein cells, and combined mutations caused a more pronounced loss in basal StAR promoter function. Although the overall level of promoter activity in response to 8-Br-cAMP was reduced when the C/EBP binding sites were mutated, there was no change in the fold increase in promoter activity in response to 8-Br-cAMP stimulation, demonstrating that C/EBPs, and in particular C/EBPβ, and the cognate response elements regulate the basal level of StAR gene expression and boost the overall response to 8-Br-cAMP.

Our studies with COS-1 cells, a monkey kidney cell line, and HepG2 cells, a liver cell line, demonstrate that C/EBPα and/or -β transactivate the StAR promoter in cells that do not normally express StAR or significant levels of SF-1 (6). This is the second instance in which basal StAR gene promoter activity could be stimulated in SF-1-deficient cell hosts. Our previous studies showed that sterol regulatory element-binding protein-1a increases StAR promoter activity in COS-1 cells in the absence of exogenous SF-1 (14). Collectively, these studies suggest that SF-1 per se is not absolutely required for basal StAR gene expression. It is possible that COS-1 and HepG2 cells contain a homolog(s) of SF-1 that is able to activate SF-1 response elements in the presence of excess exogenous C/EBPs and/or SREBP-1a. However, cAMP responsiveness appears to require SF-1, because StAR promoter activity in COS-1 cells transfected with either C/EBPα or -β did not increase when cells were treated with 8-Br-cAMP unless exogenous SF-1 was present. Consonant with these observations, we found that mutation of either C/EBP site individually caused a pronounced loss in basal StAR promoter activity in granulosa-lutein cells, which express the endogenous StAR gene. However, cAMP-dependent StAR transactivation in the granulosa-lutein cells was not ablated. Mutation of both elements further diminished basal promoter function but again did not prevent 8-Br-cAMP from stimulating promoter activity. Conversely, our previous studies demonstrated that mutation of SF-1 response elements in the StAR promoter reduced basal promoter activity but produced a more profound loss of cAMP responsiveness (8).

The nuclear factors in cells that express StAR that bind to the two motifs we identified as C/EBP response elements appear to be different. The distal element binds C/EBPβ and apparently not C/EBPα, even though C/EBPα is expressed in granulosa-lutein cells. This may reflect binding selectivity of
the distal site for C/EBPβ, differences in the concentrations of the factors in the nuclear extract, or posttranslational modifications that influence binding activity. There has been one previous report suggesting selective binding of C/EBPs to a response element (23) providing a precedent for the first possibility. In contrast, the proximal element binds neither C/EBPα nor -β but rather other protein(s) that evidently have a binding specificity similar to that of C/EBPs. Although recombinant C/EBPα and -β bound specifically to the StAR oligonucleotides representing the two putative response elements, the repertoire of transcription factors and co-activators expressed in granulosa-lutein cells evidently restricts the interaction of C/EBPβ and C/EBPα with the proximal site in favor of other binding proteins that might include other members of the C/EBP family or possibly ATF/CREB, which can bind to elements that recognize C/EBPs (24). The basis for the apparent selectivity of the distal site for C/EBPβ and the proximal site for other factors remains to be elucidated.

The expression of C/EBPs in ovarian cells is regulated providing a potential mechanism for modulating StAR gene transcription. In the immature rat ovary, C/EBPα mRNA and protein are present in granulosa cells of small, antral follicles following the administration of pregnant mare serum gonadotropin (25). The levels of C/EBPα increased with development.
and differentiation of the antral follicle into a preovulatory follicle, at which stage granulosa cells exhibit maximal levels of C/EBPα. Administration of an ovulatory dose of human chorionic gonadotropin caused a rapid loss of C/EBPα mRNA in rat granulosa cells that rebounded as the cells luteinized. In contrast to these in vivo observations in the immature rat, we observed no change in nuclear C/EBPα levels following a 24-h treatment with 8-Br-cAMP, suggesting that regulation of C/EBPα may differ in rodent and human granulosa cells.

Interestingly, Piontkewitz et al. (17) demonstrated that injection of antisense oligonucleotides that recognize C/EBPα prevented ovulation. This study failed to detect a difference in steroidogenic output (progesterone and estradiol) in mice given the antisense versus sense oligonucleotide. However, antisense treatment did not result in the complete loss of C/EBPα expression, and these observations do not rule out the possibility that other members of the C/EBP transcription factor family (e.g. C/EBPβ) could compensate for the loss of C/EBPα and permit certain promoters (i.e. StAR) to function at normal activity, although still preventing follicular differentiation and ovulation.

In the rat ovary, C/EBPβ levels were also shown to be low prior to ovulation, to increase dramatically in granulosa cells after the luteinizing hormone surge, and to then decline during the later stages of luteinization (26). We found that 8-Br-cAMP treatment of human granulosa-lutein cells increased nuclear C/EBPβ levels 3-fold, demonstrating that in vitro, the cAMP signaling pathway leads to increased C/EBPβ expression. Cyclic AMP and Ca2+/calmodulin kinase activation are also known to stimulate C/EBPβ expression in other cell lines (27). Recent observations document that cAMP causes the synthesis of C/EBPβ, -δ, and -ζ in Sertoli cells (28). C/EBPβ transactivation potential can also be augmented by extracellular signaling pathways such as inflammatory cytokines and intracellular signaling pathways such as the mitogen-activated protein kinase, AMP-dependent protein kinase A, Ca2+/calmodulin-dependent protein kinase, and protein kinase C by the differential phosphorylation of specific amino acids (see Refs. 20 and 29 and references therein). Similar to the C/EBPα antisense experiments, the targeted deletion of the mouse C/EBPβ gene did not prevent StAR gene expression in the ovary.2 It is not known whether C/EBPα could compensate for the absence of C/EBPβ in this model or whether promoter activity driven by other transcription factors is sufficient to sustain some StAR gene expression patterns of C/EBPα in other cell lines (27).

Recent observations document that C/EBPα antisense oligonucleotides caused a pronounced increase in C/EBPβ within human granulosa cells.

2 E. Sterneck and P. F. Johnson, unpublished observation.
cAMP levels can activate StAR gene transcription through a concerted mechanism that includes SF-1-dependent transactivation as well as cAMP induction of C/EBPβ, which through interactions with the distal site identified in this study raises the level of promoter activity. We have previously shown that 8-Br-cAMP-induced increases in steady state StAR mRNA levels in proliferating granulosa cells are the result of increased StAR gene transcription and that the protein synthesis inhibitor, cycloheximide, blocks the 8-Br-cAMP-provoked increase in StAR mRNA (4). Granulosa cell SF-1 expression does not dramatically change following hormone or cAMP treatment, suggesting that another factor must be involved in the increase in StAR gene transactivation (32). C/EBPβ may be the protein factor required for 8-Br-cAMP to induce StAR gene transcription.

After this paper was submitted for publication, Reinhart et al. (33) reported that C/EBP regulates mouse StAR gene transcription. These authors proposed that C/EBPβ and SF-1 interact cooperatively to enhance StAR gene expression and provided evidence for physical interactions between SF-1 and C/EBPβ. They found that the mouse promoter retained responsiveness to cAMP despite mutations in the two C/EBP response elements they identified, a finding that parallels our observations. However, in contrast to our findings, which indicate that C/EBP response elements augment the overall response to cAMP in the presence of SF-1, Reinhart et al. (33) concluded that C/EBP response elements are required for SF-1 to trans-activate the StAR promoter. This apparent discrepancy may be explained by previously described differences in the mouse and human StAR promoters. SF-1 has been reported to be important for basal but not cAMP-stimulated mouse StAR gene transcription (3), whereas SF-1 is essential for cAMP activation of the human promoter (6, 8).

The more distal site identified by Reinhart et al. (33), which is homologous to the distal site we described in the human promoter, bound C/EBPβ present in mouse Leydig cell nuclear extracts, but an antibody against C/EBPβ did not completely supershift the complex raising questions regarding the presence of other components in the complex. This differs from our findings on the human distal element. Our proximal element, which was conserved in the mouse and rat StAR promoters, is 40 bp 3′ from the proximal site identified by Reinhart et al. (33) in the mouse promoter. Reinhart et al. (33) did not identify, using immunologic probes, the factors binding to the more proximal element, which they suggested was a “low affinity C/EBP site.” We were unable to demonstrate binding of recombinant C/EBPs to two different human StAR oligonucleotides spanning the sequence of the murine proximal site. However, it is possible that the proximal site in the murine StAR promoter recognizes proteins other than C/EBPβ, as we have shown in the case of the human proximal element.

The conserved nature of the response elements within the region of the StAR promoter containing the C/EBP response element across multiple species (Fig. 1) as well as our evidence that these response elements are bona fide regulators of StAR gene promoter function suggests a regulatory role for these factors, specifically C/EBPβ, in StAR gene expression. Regula-

**References**

1. Strauss, J. F., III, Kallen, C. B., Christenson, L. K., Watari, H., Devoto, L., Arakane, F., Kiriakidou, M., and Sugawara, T. (1999) Recent Prog. Horm. Res. 54, 1–27
2. Lin, D., Sugawara, T., Strauss, J. F., III, Clark, B. J., Stocco, D. M., Saenger, P., Rogol, A., and Miller, W. L. (1995) Science 267, 1828–1831
3. Caron, K. M., Sood, S. C., Wetsel, W. C., Stocco, D. M., Clark, B. J., and Parker, K. L. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11540–11545
4. Kiriakidou, M., McAllister, J. M., Sugawara, T., and Strauss, J. F., III (1996) J. Clin. Endocrinol. Metab. 81, 4122–4128
5. Rosenn-Fuhrmann, T., Timberg, K., King, S. R., Hales, K. H., Hales, D. B., Stocco, D. M., and Orly, J. (1998) Endocrinology 139, 303–315
6. Sugawara, T., Holt, J. A., Kiriakidou, M., and Strauss, J. F., III (1996) Biochemistry 35, 9052–9059
7. Parker, K. L., and Schimmer, B. P. (1997) Endocr. Rev. 18, 361–367
8. Sugawara, T., Kiriakidou, M., McAllister, J. M., Kallen, C. B., and Strauss, J. F., III (1997) Biochemistry 36, 7249–7255
9. Caron, K. M., Ieda, Y., Sood, S. C., Stocco, D. M., Parker, K. L., and Clark, B. J. (1997) Mol. Endocrinol. 11, 138–147
10. Sandhoff, T. W., Hales, D. B., Hales, K. H., and McLean, M. P. (1998) Endocrinology 139, 4820–4831
11. Rust, W., Stedronsky, K., Tillmann, G., Morley, S., Wahiter, N., and Ivel, R. (1998) Mol. Endocrinol. 21, 189–200
12. Sugawara, T., Ieda, S., and Fujimoto, S. (1998) Endocr. Soc. Proc. 98, 118
13. Zamponi, G. L., Lalli, E., Stocco, D. M., and Sassone-Corsi, P. (1997) Nature 390, 311–315
14. Christenson, L. K., McAllister, J. M., Martin, K. O., Javitt, N. B., Osborne, T. F., and Strauss, J. F., III (1998) J. Biol. Chem. 273, 30729–30735
15. Lekstrom-Himes, J., and Xanthopoulous, K. G. (1998) J. Biol. Chem. 273, 28545–28549
16. Sterneck, E., Tessarollo, L., and Johnson, P. F. (1997) Genes Dev. 11, 2153–2162
17. Pontkeaitz, Y., Enerback, S., and Hedin, L. (1996) Dev. Biol. 179, 288–296
18. Williams, S. C., Cantwell, C. A., and Johnson, P. F. (1991) Genes Dev. 5, 1553–1567
19. Sugawara, T., Lin, D., Holt, J. A., Martin, K. O., Javitt, N. B., Miller, W. L., and Strauss, J. F., III (1995) Biochemistry 34, 12506–12512
20. Williams, S. C., Baer, M., Dillner, A. J., and Johnson, P. F. (1995) EMBO J. 14, 3170–3183
21. McAllister, J. M., Byrd, W., and Simpson, E. R. (1994) J. Clin. Endocrinol. Metab. 79, 106–112
22. Hurst, H. C., Masson, N., Jones, N. C., and Lee, K. A. (1990) Mol. Cell. Biol. 10, 6206–6209
23. Pope, R. M., Leutz, A., and Ness, S. A. (1994) J. Clin. Invest. 94, 1449–1455
24. Kageyama, R., Sasai, Y., and Nakaminishi, S. (1991) J. Biol. Chem. 266, 15523–15531
25. Pontkeaitz, Y., Eenerback, S., and Hedin, L. (1993) Endocrinology 133, 2327–2333
26. Siris, J., and Richards, J. S. (1993) J. Biol. Chem. 268, 21931–21938
27. Yukawa, K., Tanaka, T., Tsuji, S., and Akira, S. (1998) J. Biol. Chem. 273, 31345–31351
28. Greening, L. M., Duhle, M. K., Tasken, K. A., Enerback, S., Hedin, L., Tasken, K., and Knutson, K. H. (1999) Endocrinology 140, 835–843
29. Poli, V., Mancini, F. P., and Cortese, R. (1990) Cell 63, 643–653
30. Luo, X., Ieda, Y., and Parker, K. L. (1994) Cell 77, 491–496
31. Wang, H., Finogold, J. M., Bradbery, A., Du, C. N., Abdelazizy, S. V., Wilde, M. D., Taylor, L. R., Wilson, D. R., and Darlington, G. J. (1995) Science 269, 1108–1112
32. Clemea, J. W., Lala, D. S., Parker, K. L., and Richards, J. S. (1994) Endocrinology 134, 1499–1508
33. Reinhart, A., Williams, S. C., Clark, B. J., and Stocco, D. M. (1999) Mol. Endocrinol. 13, 729–741

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