Synergistic Activation of the Inhibin α-Promoter by Steroidogenic Factor-1 and Cyclic Adenosine 3′,5′-Monophosphate

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The inhibin α-subunit gene is expressed in the ovary, testis, adrenal, and pituitary. Because this pattern of expression corresponds to that of the orphan nuclear receptor, steroidogenic factor-1 (SF-1), we hypothesized that the inhibin α promoter might be regulated by SF-1. Expression of exogenous SF-1, in an SF-1 deficient cell line, caused modest stimulation of the inhibin α promoter. However, activation of the cAMP pathway, which is known to regulate inhibin α expression, greatly enhanced the actions of SF-1. Coexpression of SF-1 with the catalytic subunit of cAMP-dependent protein kinase A caused greater than 250-fold stimulation, whereas only 4- or 7-fold stimulation was seen by the SF-1 or protein kinase A pathway alone. Synergistic stimulation by SF-1 and the cAMP pathway was also seen in GRMO2 granulosa cells, which express endogenous SF-1. Deletion and site-directed mutagenesis localized a novel SF-1 regulatory element (TCA GGGCCA; −137 to −129) adjacent to a variant cAMP-response element (CRE; −120 to −114). The synergistic property of SF-1 and cAMP stimulation was inherent within this composite inhibin α fragment (−146 and −112), as it was transferable to heterologous promoters. Mutations in either the CRE or the SF-1 regulatory element completely eliminated synergistic activation by these pathways. The binding of SF-1 and CRE binding protein (CREB) to the inhibin α regulatory elements was relatively weak in gel mobility shift assays, consistent with their deviation from consensus binding sites. However, SF-1 was found to interact with CREB using an assay in which epitope-tagged SF-1 was expressed in cells and used to pull down *in vitro* translated CREB. Expression of CREB binding protein (CBP), a coactivator that interacts with SF-1 and CREB, further enhanced transcription by these pathways. Stimulation by the SF-1 and cAMP pathways was associated with increased histone H4 acetylation, suggesting that chromatin remodeling accompanies their actions. We propose a model in which direct interactions of SF-1, CREB, and associated coactivators like CBP induce strongly cooperative transactivation by pathways that individually have relatively weak effects on transcription. (Molecular Endocrinology 14: 66–81, 2000)

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INTRODUCTION

Gonadotropins play a major role in the control of ovarian follicular development and function [see review (1)]. FSH and LH increase intracellular cAMP levels through G protein coupled membrane receptors (2, 3) and modulate the downstream expression of many ovarian genes. These include the gonadotropin receptors, steroidogenic enzymes (aromatase, side chain cleavage enzyme, and 3β-hydroxysteroid dehydrogenase), and the paracrine peptides (inhibin and activin), among others.

Inhibin was initially isolated and characterized as a factor that suppresses the synthesis and secretion of pituitary FSH (4–7). However, inhibin also acts locally in the ovary to enhance theca cell androgen synthesis, which also leads to increased estrogen production (8). Inhibin is a dimeric glycoprotein hormone consisting of a common α-subunit and either of two β-subunits (α-βA, α-βB) (9–13). In the ovary, inhibin is expressed in granulosa cells, and its expression is modulated

during the estrous cycle (14, 15). FSH stimulates the expression and secretion of inhibin from granulosa cells (16, 17). In addition, pharmacological agents that increase intracellular cAMP levels can stimulate inhibin secretion in granulosa cells (18, 19). Consistent with these findings, the inhibin common α-subunit promoter contains a functional cAMP response element (CRE) (20). Previous studies have shown that the CRE-binding protein (CREB) (21) and the inducible cAMP early repressor (ICER) (22, 23) are involved in the stimulation (20) and suppression (24), respectively, of the inhibin α gene in granulosa cells. It is probable, however, that many other transcription factors, in addition to CREB and ICER, are involved in regulation of the inhibin α gene.

The orphan nuclear receptor steroidogenic factor 1 (SF-1) (25, 26) is expressed in the adrenal cortex, testis, ovary, pituitary gonadotrope cells, and hypothalamus (27), and it plays an essential role in the development of these tissues (28). In addition, SF-1 functions as a transcriptional regulator of a variety of target genes including aromatase (29, 30), cholesterol side chain cleavage enzyme (31), steroidogenic acute regulatory protein (32), LHβ (33), and DAX-1 (dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1) (34).

SF-1 is a member of the nuclear receptor superfamily and it contains a characteristic zinc finger DNA-binding domain and putative ligand-binding/dimerization domain that are well conserved among members of this family (35). SF-1 binds to a consensus DNA recognition sequence (PyCA AGGTPyC or PuPu AGGTCA) as a monomer. Like other nuclear receptors, an AF2 transactivation domain is present at the carboxy terminus of SF-1. Recently, multiple coactivators and corepressors that mediate the transcriptional activity of steroid receptors have been identified and characterized [see review (36)]. Steroid receptor coactivator-1 (SRC-1), one of the well known coactivators, interacts with the AF2 domain of SF-1 and potentiates the activity of SF-1 (37). CREB binding protein (CBP), originally cloned as a coactivator for CREB (38), also interacts directly with SF-1 and has been shown to enhance transcription of the cholesterol side chain cleavage enzyme (CYP11A1) gene (39).

SF-1 is involved in the cAMP-regulated expression of various genes including cholesterol side chain cleavage enzyme (40), aromatase (41, 42), and StAR (steroidogenic acute regulatory protein) (43). It has been suggested that SF-1 might be phosphorylated (41, 44), and a phosphorylation site (Ser 203) in SF-1 was identified recently (45). This site, which modulates the transcriptional activity of SF-1, was shown to be phosphorylated by the mitogen-activated kinase (MAPK) pathway, but not by the cAMP-dependent pathway (45). Thus, the molecular mechanism of SF-1 action in cAMP-dependent gene regulation remains incompletely understood, despite its importance in the control of many target genes.

In addition to direct actions of phosphorylation on the transcriptional activity of SF-1, it is also possible that phosphorylation modifies other proteins that interact with SF-1, or mediate its transcriptional effects. In addition to transcriptional coactivators, SF-1 has been shown to interact functionally with a variety of other transcription factors. SF-1 enhances estrogen receptor-mediated stimulation of the salmon gonadotropin II β-subunit gene (46). SF-1 and Egr-1 synergistically stimulate promoter activity of the rat LHβ gene (47). Transcription of the anti-Müllerian hormone gene is cooperatively stimulated by SF-1 and SOX9 (48), and by SF-1 in combination with WT-1 (49). SF-1 action can also be inhibited by a direct protein interaction with DAX-1 (50).

Several lines of evidence have shown that post-translational modifications of histones, such as acetylation/deacetylation, methylation, or phosphorylation, can alter gene expression [see review (51)]. Several coactivators, including CBP/P300 (52, 53) and P/CAF (54, 55), contain histone acetyltransferase activity. Histone acetylation is thought to open chromatin structure and allow additional transcription factors to bind to DNA and activate transcription. Although CBP is recruited by SF-1 (39), little is known about the role of histone acetylation in SF-1-mediated transactivation.

In this report, we examined SF-1 regulation of the inhibin α promoter as a model of synergistic actions of these pathways. A novel SF-1 site was identified adjacent to the inhibin α CRE. We provide evidence for direct protein interactions between SF-1 and CREB, with recruitment of CBP, and increased histone acetylation as a mechanism for the strong synergism between the SF-1 and cAMP pathways.

RESULTS

Synergistic Activation of the Inhibin α-Subunit Gene by SF-1 and the cAMP Pathway

A number of promoters have both SF-1 and CRE sites, suggesting transcriptional cross-talk between these two pathways. Several promoters that are regulated by the SF-1 and cAMP pathway were analyzed using transfection assays in SF-1-deficient human embryonic kidney tsa 201 cells. Promoter-luciferase constructs included the human glycoprotein hormone α subunit, rat aromatase, and the inhibin α gene. An SF-1-expressing plasmid was cotransfected with, or without a protein kinase A (PKA) expression vector. The human common α-subunit gene contains a single SF-1 binding site (56) and two CREs (57) (Fig. 1). Despite the presence of this binding site, SF-1 did not stimulate the activity of this promoter, whereas PKA induced strong transactivation (187-fold). Cotransfection of SF-1 with PKA did not increase PKA-induced transactivation further (170-fold). The rat aromatase gene has an SF-1 binding site (29, 30) and a CRE (58), and there is functional interaction between the SF-1

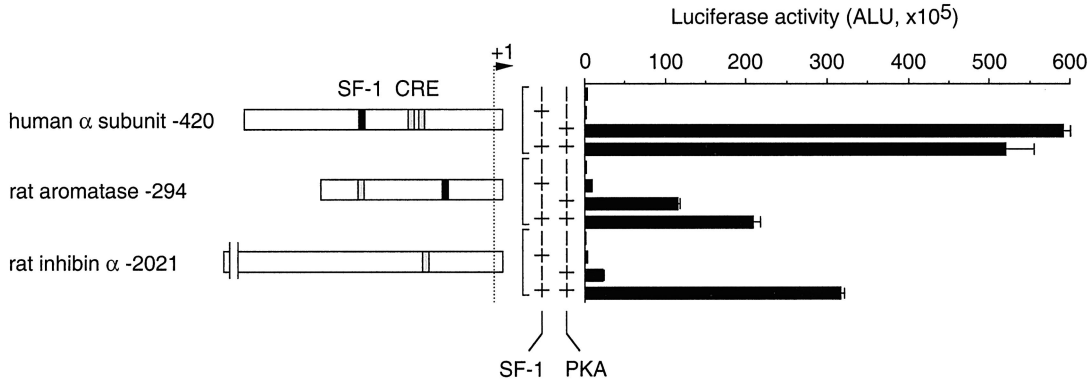

Fig. 1. Synergistic Activation of the Inhibin α Promoter by SF-1 and the cAMP Pathway

Effect of SF-1 and PKA on the activity of human gonadotropin common α-subunit, rat aromatase, and rat inhibin α promoters. Reporter plasmid (0.5 μg) was transfected into tsa cells with either a mutant or wild-type PKA expression vector (25 ng) and either an empty or SF-1 expression vector (20 ng). Results are the mean ± SEM of triplicate transfections. The locations of the SF-1 binding site and the CRE are shown as a *closed box and open box,* respectively.

and CRE-regulatory elements (41). SF-1 and PKA stimulated the promoter activity by 8- and 105-fold, respectively. SF-1 increased the PKA-induced transactivation by 2-fold. In the case of the inhibin α promoter, a single CRE site was previously identified (20), but SF-1 binding sites have not been described. SF-1 and PKA stimulated the −2021 promoter by 5- and 40-fold, respectively. When both SF-1 and PKA were cotransfected, transactivation was dramatically increased (607-fold), suggesting that a SF-1 binding site may be present in the promoter and that synergistic activation by SF-1 and the cAMP pathway plays an important role in the regulation of inhibin α gene expression. These findings illustrate that interactions between the SF-1 and PKA pathways vary markedly, depending on the promoter being studied.

The SF-1 regulatory element in the inhibin α promoter was localized further using deletion mutagenesis. Deletion of the inhibin α reporter to −769 or −547 retained strong synergistic activation by SF-1 and PKA (Fig. 2A). Further deletion to −311 or −146 also retained the synergistic activation by SF-1 and PKA (Fig. 2B), suggesting that a functional SF-1 site resides within the proximal promoter. Deletion from −146 to −134 eliminated the synergistic activation, suggesting that an SF-1 site resides within this region. Consistent with these results, SF-1 stimulation was observed with the −146 promoter (2.5-fold), but not with the −134 promoter.

Synergistic Activation Is Dose Dependent

A wild-type or mutant PKA expression vector was cotransfected with increasing amounts of the SF-1 expression vector (Fig. 3A). In addition, an empty or SF-1 expression vector was transfected with increasing amounts of wild-type PKA expression vector (Fig. 3B). In both experiments, synergistic activation was increased in proportion to the dose of the PKA or SF-1 expression constructs.

Synergistic Activation Occurs in Granulosa Cells

The GRMO2 ovarian granulosa cells express inhibin, and expression of the inhibin and activin βA-subunit genes is known to be regulated by cAMP (59). The level of inhibin α promoter activity in GRMO2 cells is similar to that in other SF-1 expressing cells, such as pituitary gonadotrope αT3 and adrenal cortical Y1 cells (data not shown). The −146 wild-type reporter was cotransfected with either an empty or SF-1 expression vector, and cells were treated with 1 mM 8-bromo-cAMP for 12 h to determine whether the synergistic activation also occurs in the GRMO2 cells (Fig. 4). SF-1 transfection and 8-bromo cAMP treatment stimulated promoter activity by 2.4- and 5.4-fold, respectively (lanes 2 and 3). When both were combined, SF-1 increased the 8-bromo-cAMP-induced transactivation by an additional 2.5-fold (lane 4). To interpret this data, it is necessary to take into account that SF-1 is expressed endogenously in granulosa cells (60), and SF-1 mRNA was detected in GRMO2 cells (data not shown). Because of the presence of endogenous SF-1, some element of synergistic activation may occur after the addition of 8-bromo cAMP alone (lane 3). Further enhancement of synergistic activation by overexpression of SF-1 was relatively small (lanes 3 and 4), presumably due to the presence of endogenous SF-1. These findings suggest that synergistic activation by SF-1 and the cAMP pathway also occurs in granulosa cells.

Identification of the SF-1 Binding Site Responsible for the Synergistic Activation

By inspection of the DNA sequence, three potential SF-1 binding sites were found (A; ATA AGGCTC, B; CTC AGGGCC, C; TCA GGGCCA) between −144 and −129 (Fig. 5). Mutations were introduced into the second and third positions of the putative nuclear receptor half-sites in the −146 reporter constructs to identify functional SF-1 regulatory elements. SF-1 and PKA stimulated the −146 wild-type reporter by 4- and

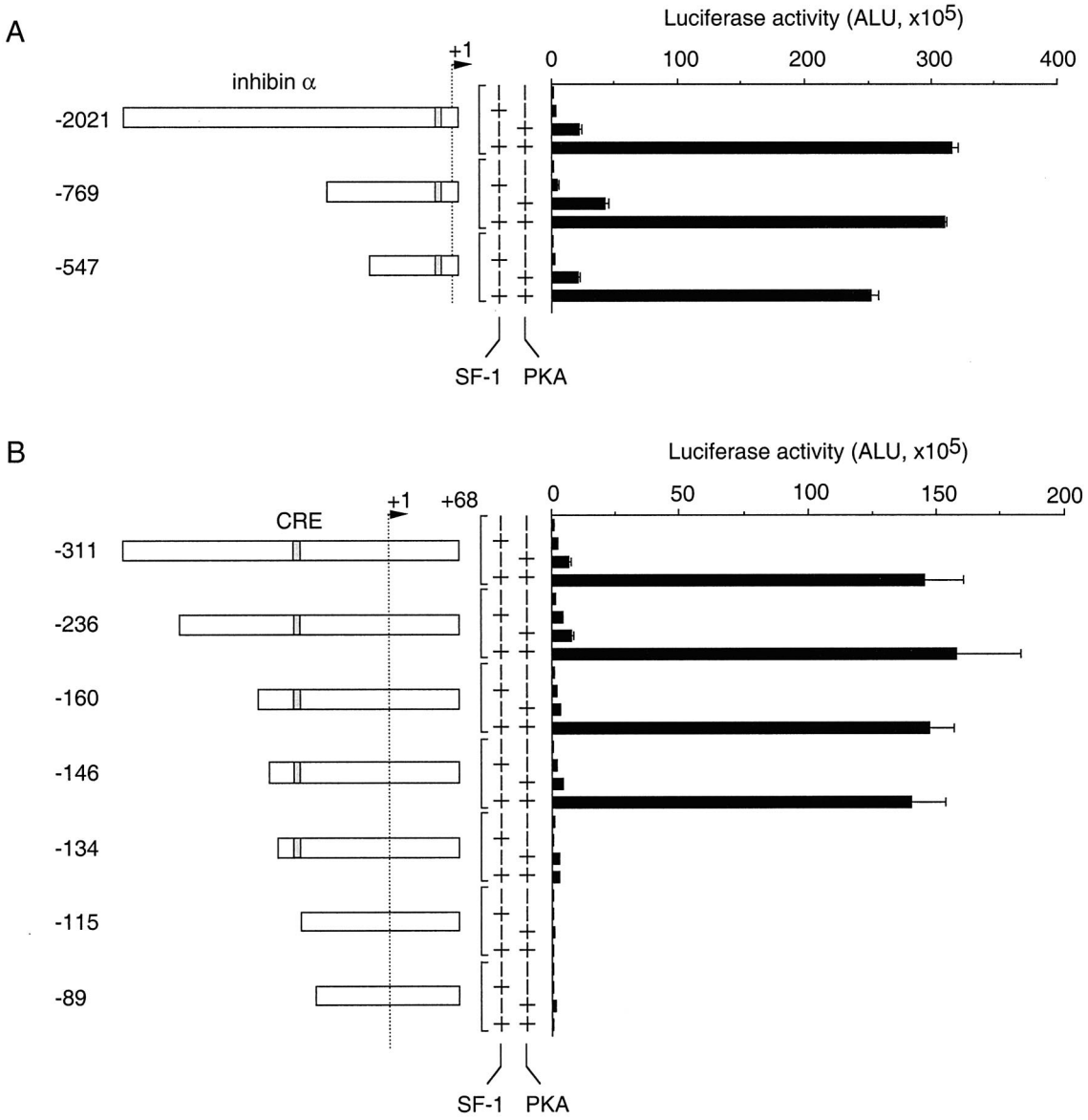

Fig. 2. Deletion Mutagenesis of Inhibin α Promoter to Localize Sequences Involved in Synergistic Activation

A deletion series of the inhibin α reporter constructs (A; -2021, -769, and -547 to $+68$, B; -311, -236, -160, -146, -134, -115, and -89 to $+68$) (0.5 μg) was transfected into tsa cells with a mutant or wild -type PKA expression vector (25 ng) and an empty or SF-1 expression vector (20 ng). Forty eight hours after transfection, cell extracts were prepared and luciferase assays were performed. Results are the mean $\pm$ SEM of triplicate transfections. The location of the CRE is shown.

7-fold, respectively. When both were transfected, synergistic activation was observed (276-fold). Introduction of mutations into the putative binding sites A (m1; AGGCTC $\rightarrow$ A*TT*CTC) and B (m3; AGGGCC $\rightarrow$ A*TT*-GCC) did not affect synergistic activation. In contrast, the m4 mutation, which disrupts the putative binding site C (GGGCCA $\rightarrow$ G*TT*CCA) eliminated synergism completely. Also, SF-1 transactivation was abolished by this mutation. These data indicate that the synergistic activation is mediated by SF-1 binding to site C. Two additional mutations introduced into site C (m2; TCA $\rightarrow$ *AA*A, m5; GGGCCA $\rightarrow$ GGG*AA*A) reduced the synergistic activation ($\sim$25% of wild type), whereas mutations adjacent to the SF-1 binding site (m1 and m6) did not alter the synergistic activation.

To confirm SF-1 binding to site C, electrophoretic mobility shift assay (EMSA) was performed using radiolabeled probes (-146 to -122) and nuclear extracts prepared from cells transfected with an empty or SF-1 expression vector (Fig. 6A). SF-1 binding was detected with extracts that contain expressed SF-1 (lane 3), but binding was not detected using extracts from cells transfected with an empty vector (lane 2). SF-1 binding was eliminated by 100-fold molar excess of competitor oligonucleotides (lane 4) or by anti-Ad4BP (anti-SF-1) antibody (lane 5). The m4 mutation

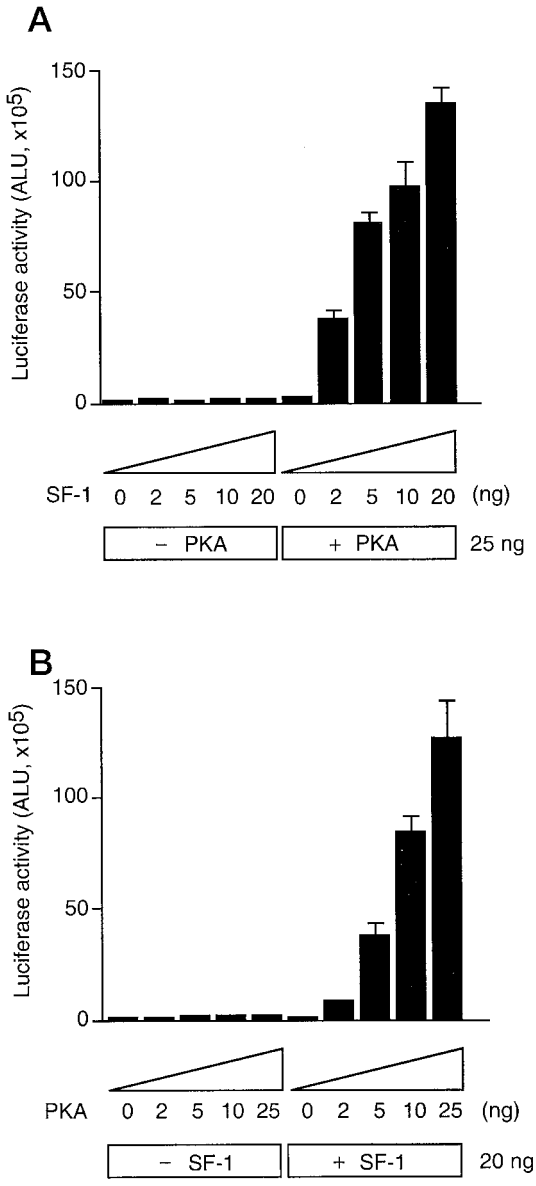

Fig. 3. Dose-Dependent Effect Of SF-1 And PKA On Synergistic Activation

A, The −146 wild-type reporter (0.5 μg) was transfected into cells with wild-type or mutant PKA expression vector (25 ng) and increasing amounts of SF-1 expression vector (0, 2, 5, 10, 20 ng). The total amount of transfected plasmid was adjusted with an empty vector. B, The −146 wild-type reporter (0.5 μg) was transfected with an empty or SF-1 expression vector (20 ng) and increasing amounts of wild-type PKA expression vector (0, 2, 5, 10, 25 ng). The total amount of transfected plasmid was adjusted with the mutant PKA expression vector. Forty eight hours after transfection, luciferase assays were performed. Results are the mean ± SEM of triplicate transfections.

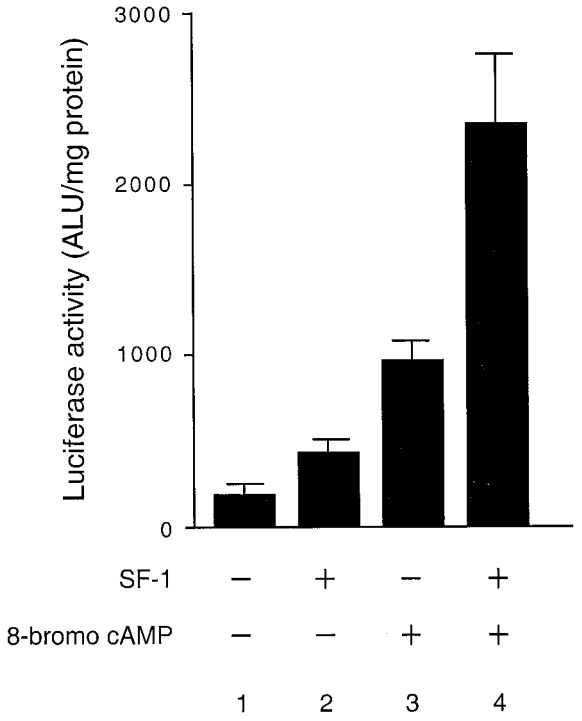

Fig. 4. Synergistic Activation In Ovarian GRMO2 Granulosa Cells

The −146 wild-type reporter (0.5 μg) was cotransfected with an empty or SF-1 expression vector (50 ng), and cells were treated with 1 mM 8-bromo-cAMP for 12 h before the luciferase assay. Luciferase activity is normalized to total cellular protein and expressed as arbitrary light units (ALU)/mg protein. Results are the mean ± SEM of triplicate transfections.

at the second and third positions of the nuclear receptor half-site completely eliminated SF-1 binding (lane 8). In contrast, SF-1 bound to the m3 probe, which contains mutations at the first and second positions of the half-site (lane 7), although its binding was reduced to 60% of the wild-type probe (lane 3). The m2 and m5 mutations significantly reduced the SF-1 binding (lanes 6 and 9), but very weak SF-1 binding was detected when the films were overexposed (data not shown). Consistent with these observations, SF-1 binding to the wild-type probe (lane 3) was not abolished by 100-fold molar excess of the m2 (lane 10), m4 (lane 12), or m5 (lane 13) competitor oligonucleotides. The m3 oligonucleotide competed for SF-1 binding to the wild-type probe, consistent with its weak binding activity (lane 11). The binding characteristics of SF-1 to the mutated probes (Fig. 6) were consistent with the promoter activities observed using mutated SF-1 binding sites (Fig. 5). Taken together, these experiments identify an SF-1 binding site (TCA GGGCCA; −137 to −129) that is responsible for the synergistic activation with PKA.

Involvement of CREB in the Synergistic Activation

It has been shown previously that CREB binds to the inhibin α CRE (TGCGTCA; −120 to −114) and that mutation of the CRE (TG*A*TCA) eliminates its binding (20). EMSA using nuclear extracts prepared from cells

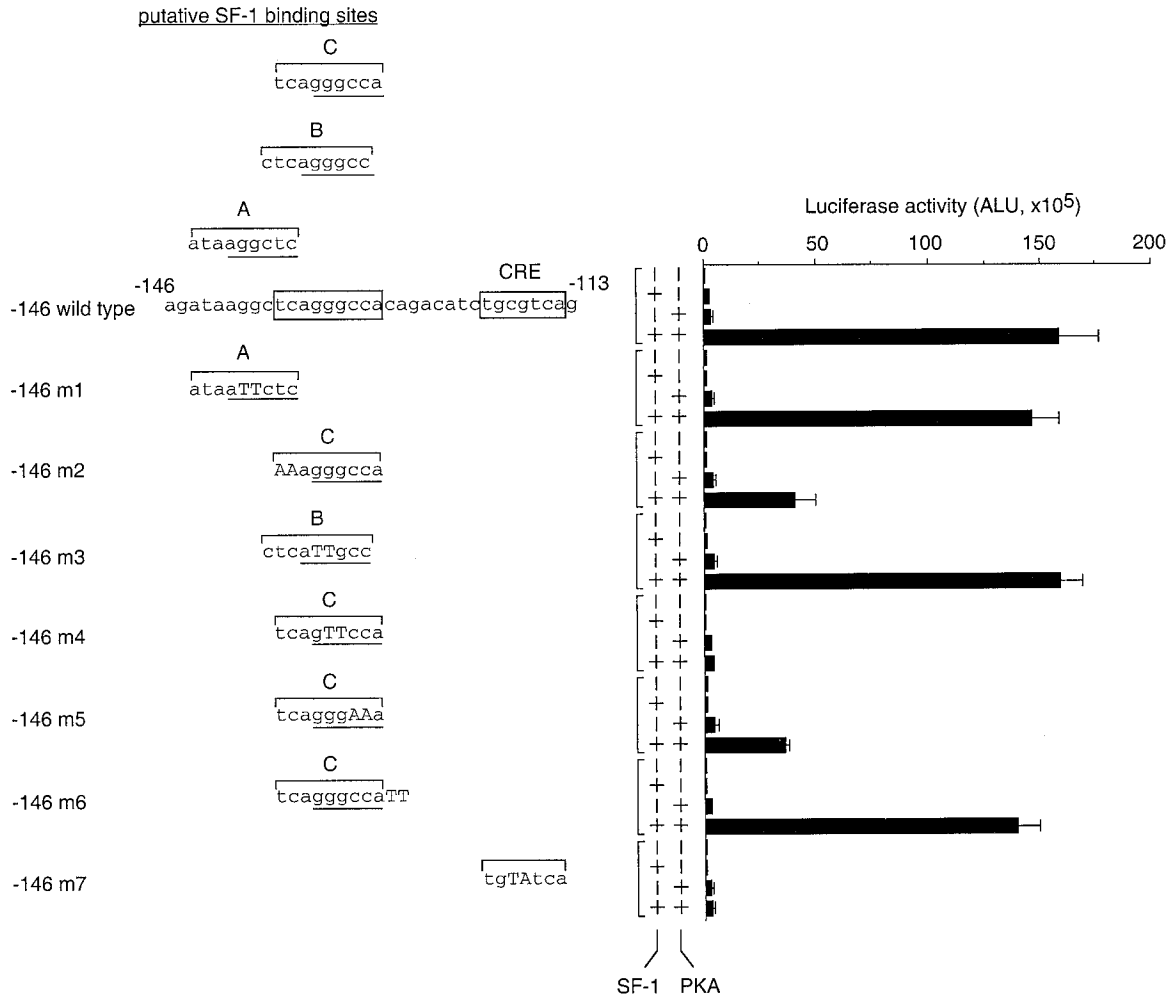

Fig. 5. Localization of the SF-1 Binding Site and CRE Responsible for the Synergistic Activation

The −146 wild-type or mutagenized reporters (0.5 μg) were transfected into tsa cells with a mutant or wild type PKA expression vector (25 ng) and an empty or SF-1 expression vector (20 ng). Forty eight hours after transfection, cells were subjected into luciferase assays. Results are the mean ± SEM of triplicate transfections. Three putative SF-1 binding sites are shown by A, B, and C.

transfected with an empty vector (Fig. 6B, *left panel*) showed binding of endogenous CREB to the CRE-containing probe (−132 to −104) (lane 15). CREB binding was eliminated by 100-fold molar excess of a consensus CRE oligonucleotide (TG*A*CGTCA) (lane 16) , and it was decreased by incubation with anti-CREB antibody (lane 17). CREB did not bind to the CRE mutant probe (m7) (lane 18), and the CREB binding was not eliminated by 100-fold molar excess of the m7 oligonucleotides (lane 19). The binding of endogenous CREB to the CRE was relatively weak compared with SF-1 binding to its element. As shown later (Fig. 7), weak CREB binding is explained by deviation of the inhibin αCRE sequence (TGCGTCA) from the consensus CRE sequence (TG*A*CGTCA). In an effort to provide additional evidence for CREB binding, EMSA was performed using nuclear extracts prepared from cells transfected with a CREB expression vector (Fig. 6B, *right panel*). CREB binding (lane 20), competition by CRE oligonucleotides (lane 21), and its supershift with anti-CREB antibody (lane 22) were clearly observed. Also, overexpressed CREB did not bind to the m7 probe (lane 23), and CREB binding was not competed by the m7 oligonucleotides (lane 24). When the m7 mutation was introduced into the CRE (Fig. 5), synergistic activation was totally eliminated. These results indicate that CREB binds to the CRE and is involved in the synergistic interaction with SF-1.

PKA Does Not Increase Binding of SF-1 or CREB to Their Response Elements

DNA binding affinities of SF-1 and CREB were examined to assess whether enhanced binding might explain synergistic interactions. Nuclear extracts were prepared in the presence of phosphatase inhibitors from cells transfected with wild-type or mutant PKA

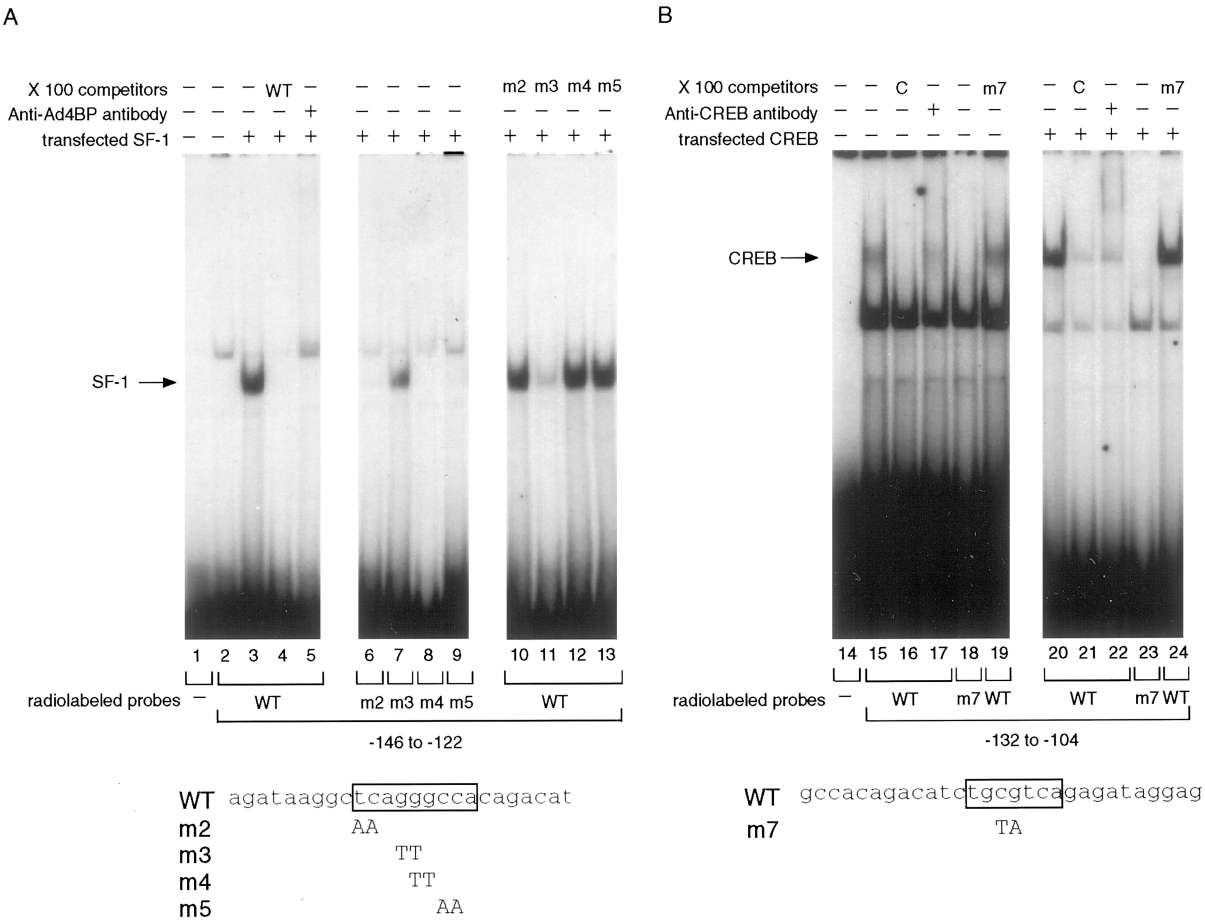

Fig. 6. DNA Binding of SF-1 and CREB

A, EMSA was performed using nuclear extracts prepared from cells transfected with an empty or SF-1 expression vector. Ten micrograms of nuclear extracts were subjected into the binding reaction along with 20 fmol of wild-type or mutagenized probes (−146 to −122). The DNA protein complexes were resolved on a 0.5× TBE polyacrylamide gel. B, EMSA was performed as described above using nuclear extracts prepared from cells transfected with an empty or CREB expression vector and the wild-type or m7 mutant probe (−132 to −104).

expression vector and empty or SF-1 expression vector. Western blot analysis using antiphosphorylated CREB antibody demonstrated increased phosphorylation of CREB and ATF-1 in cells transfected with a wild-type PKA expression vector compared with cells transfected with a mutant expression vector (data not shown). When EMSA was performed using these nuclear extracts, PKA did not increase DNA binding of SF-1 and CREB (data not shown). These results suggest that the synergistic activation is not the consequence of increased binding of SF-1 or CREB to DNA.

Increased Binding of SF-1 and CREB Diminishes Synergistic Activation.

The SF-1 binding site identified in the inhibin α promoter (GGGCCA) differs from that of the consensus binding site (AGGTCA). It was anticipated that replacement of the half-site with the consensus half-site would increase SF-1 binding. Also, introduction of the consensus CRE was expected to increase CREB binding. EMSA using nuclear extracts that express SF-1 (Fig. 7A) confirmed increased binding to the consensus SF-1 binding site (m8) compared with the inhibin α SF-1 site (lanes 1 and 2). Similarly, endogenous CREB bound to the consensus CRE (m9) much better than to the native inhibin α CRE (lanes 3 and 4). The CREB binding complexes were supershifted by anti-CREB antibody, but not by anti-ATF-1 antibody (data not shown). Introduction of the SF-1 consensus binding site did not increase the activation by SF-1 alone, whereas conversion of the CRE to a consensus sequence increased the response to PKA alone (2.5 fold). Despite increased binding of SF-1 (m8) and CREB (m9), synergistic transactivation by SF-1 and PKA was not increased further (Fig. 7B). Rather, synergistic activation was reduced (m8; 57%, m9; 50%). These results indicate that the relatively weak binding of SF-1 and CREB to the natural sites in the inhibin α promoter may facilitate synergistic activation. In addi-

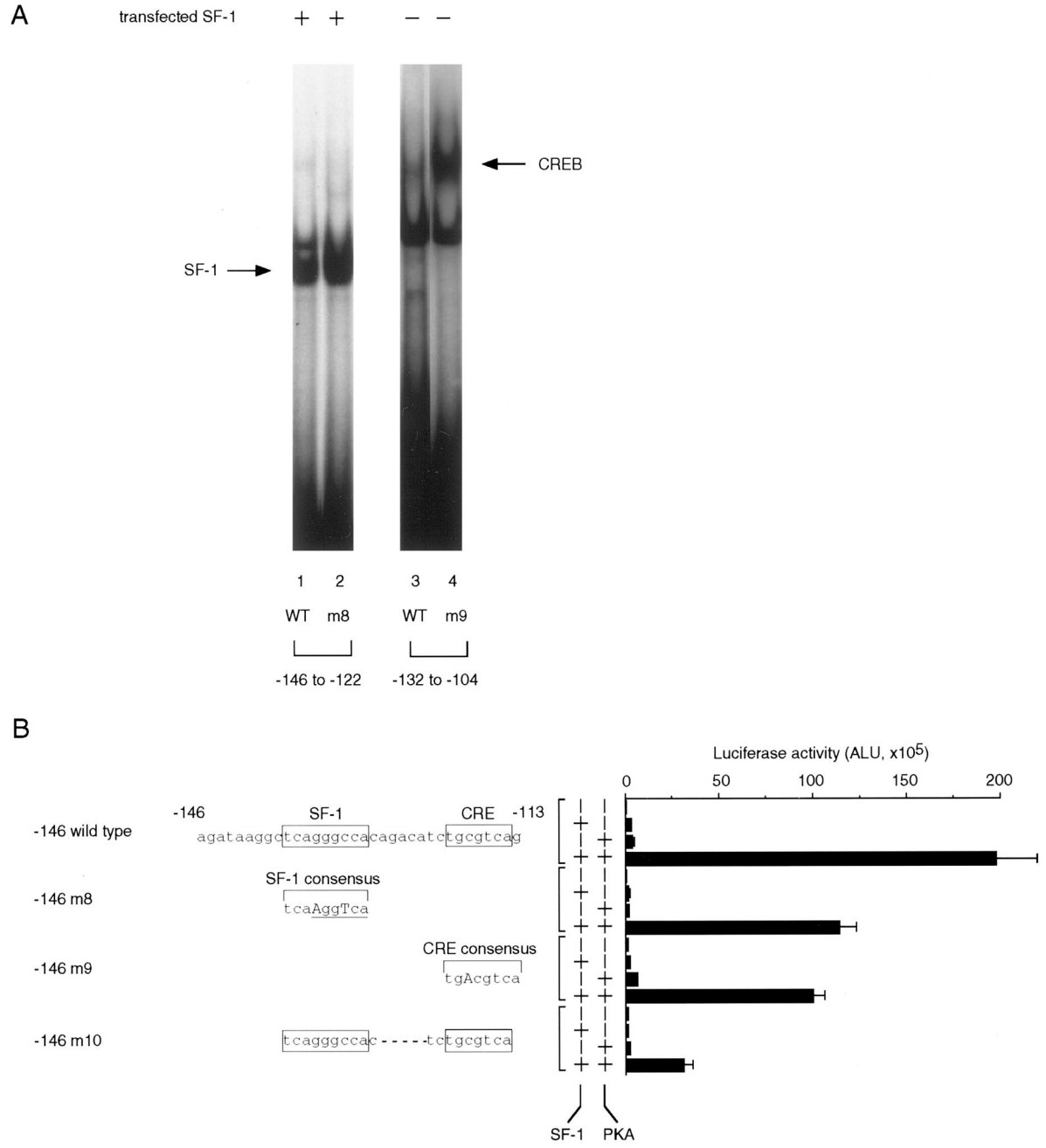

Fig. 7. Effect of Enhanced Binding of SF-1 and CREB on the Synergistic Activation

A, EMSA was performed using nuclear extracts with or without SF-1 expression. In addition to the wild-type probes (−146 to −122 and −132 to −104), mutant probes containing consensus binding sites for SF-1 (m8) and CREB (m9) were used. The consensus sequences introduced into the probes are described below. B, The −146 wild-type or mutagenized reporters (m8, m9, and m10) (0.5 μg) were transfected with a mutant or wild-type PKA expression vector (25 ng) and an empty or SF-1 expression vector (20 ng). Forty eight hours after transfection, cell extracts were subjected into luciferase assays. Results are the mean ± SEM of triplicate transfections.

tion, elimination of five bases (AGACA) from the intervening sequence between the SF-1 binding site and the CRE (m10) resulted in a marked decrease in synergistic activation (15%), suggesting that the spacing between the regulatory elements is important for optimal synergism.

Synergistic Activation Is Mediated through the Composite Enhancer Element

The promoter regions containing the responsive elements (−146 to −112, −146 to −80, and −146 to −40) were linked to the thymidine kinase minimal pro-

moter to assess whether the element containing the SF-1 binding site and CRE is sufficient to mediate synergistic activation (Fig. 8). The degree of synergism obtained with these heterologous reporters (280- to 350-fold) was similar to that obtained with the native −146 native reporter construct (276-fold). These data indicate that the minimal composite element containing the SF-1 binding site and CRE (−146 to −112) is sufficient to mediate synergistic activation. PKA activation was stronger with these reporters (35- to 49-fold) than seen with the −146 wild-type reporter (5-fold) (Fig. 5).

SF-1 Interacts Directly with CREB

Because of the proximity of the SF-1 binding site and CRE in the composite enhancer element, it is possible that SF-1 and CREB interact directly with one another. Nuclear extracts were prepared from cells transfected with either an empty or hemagglutinin (HA)-tagged SF-1 expression vector, and the expression of HA-tagged SF-1 was confirmed by Western blot analysis using anti-HA antibody (Fig. 9A). ^{35}S-labeled CREB was synthesized by *in vitro* translation and introduced into the binding reaction along with nuclear extracts. Either nonimmune IgG or anti-HA antibody was used to pull down the SF-1-CREB complexes (Fig. 9B). Labeled CREB was detected only in the presence of HA-tagged SF-1, demonstrating physical interaction between SF-1 and CREB.

CBP Enhances Synergistic Activation

CBP is a coactivator for both SF-1 (39) and CREB (38), and it is capable of directly interacting with each pro-

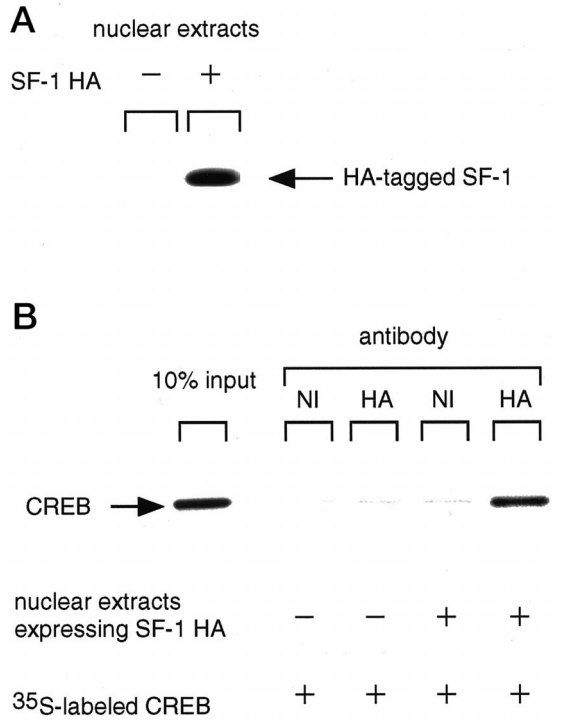

Fig. 9. Protein Interaction Assay between SF-1 and CREB

A, Nuclear extracts were prepared from cells transfected with an empty or HA-tagged SF-1 expression vector. The nuclear extracts were separated on 10% SDS-PAGE followed by Western blot analysis using anti-HA antibody. B, ^{35}S-labeled CREB proteins translated *in vitro* were introduced into the binding reaction containing nuclear extracts with or without HA-tagged SF-1 expression. The reaction was immunoprecipitated with either nonimmune IgG (NI) or anti-HA antibody (HA). Immunoprecipitates were loaded on 10% SDS-PAGE followed by autoradiography. Ten percent of the total input was also run on the gel.

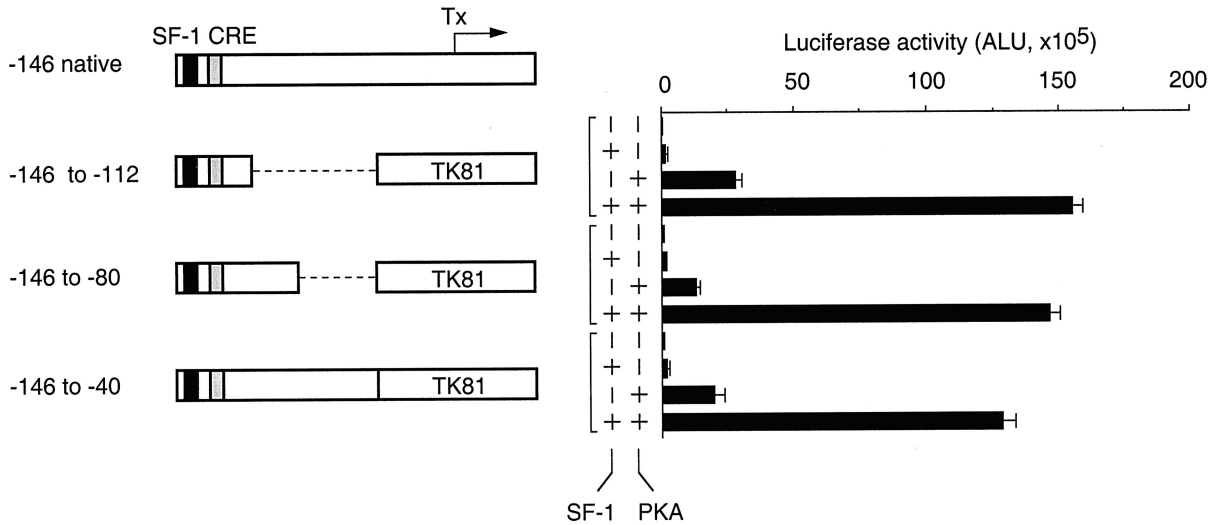

Fig. 8. Characterization of the SF-1 and CREB Composite Enhancer Element

The promoter region containing the SF-1 binding site and CRE (−146 to −112, −146 to −80, and −146 to −40) was linked to the thymidine kinase minimal promoter and luciferase gene. The reporter genes (0.5 μg) were cotransfected with a mutant or wild-type PKA expression vector (25 ng) and either an empty or SF-1 expression vector (20 ng). Forty eight hours after transfection, luciferase assays were performed. Results are the mean ± SEM of triplicate transfections.

tein. As shown above, SF-1 and CREB can interact with each other. Thus, it is possible that CBP is involved in the synergistic activation through the formation of ternary complexes with SF-1 and CREB. PKA or SF-1, or the combination of proteins, was cotransfected with increasing amounts of CBP expression vector (Fig. 10A). CBP had little or no effect on SF-1-mediated transcriptional activation, but it increased PKA-induced transactivation by 2-fold when 50 ng of CBP expression vector were cotransfected. Synergistic activation was increased (1.8- and 2.9-fold) by cotransfection with increasing amounts of CBP (20 and 50 ng). CBP lacking the carboxy-terminal region (CBP 1–460), or P/CAF, had no effect on the synergistic activation (Fig. 10B).

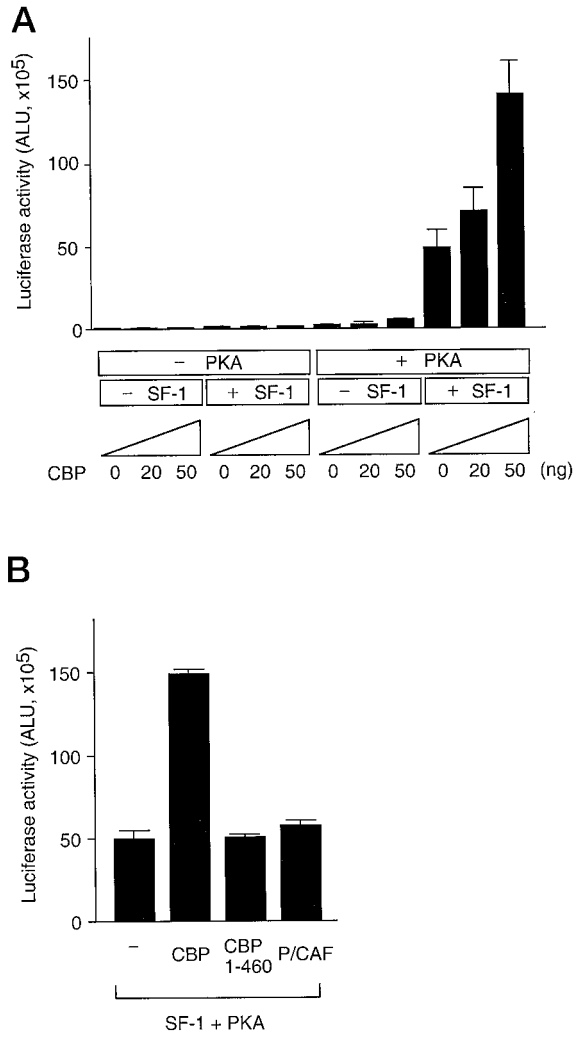

Fig. 10. Effect of CBP on the Synergistic Activation

A, The −146 wild-type reporter (0.5 μg), mutant, or wild-type PKA expression vector (25 ng) and empty or SF-1 expression vector (20 ng) were cotransfected with increasing amounts of CBP expression vector (0, 20, 50 ng). B, Empty, CBP, CBP 1–460, or P/CAF expression vectors (50 ng) were transfected into cells along with the −146 wild-type reporter (0.5 μg), and SF-1 (20 ng) and wild-type PKA expression vectors (25 ng). Forty eight hours after transfection, luciferase assays were performed. Results are the mean ± SEM of triplicate transfections.

Synergistic Activation Involves Histone Acetylation

CBP can induce histone acetylation directly (52, 53), or indirectly, through the recruitment of P/CAF (54, 55). Activation of histone acetylation may, therefore, parallel or be involved in the synergistic activation. Chromatin immunoprecipitation (CHIP) assays were performed to assess the extent of histone acetylation specific for the inhibin α promoter under various treatment conditions. The −311 inhibin α luciferase reporter construct was transfected into tsa 201 cells with mutant or wild-type PKA expression vector, and empty or SF-1 expression vector, and CHIP assays were performed 48 h after transfection (Fig. 11). PCR using immunoprecipitates with antiacetylated histone H4 antibody showed a significant increase of the 287-bp amplified products (3-fold) in cells transfected with both SF-1 and PKA expression vectors (lane 4) compared with the basal state (lane 1). SF-1 (lane 2) and PKA (lane 3) alone did not increase the band intensity. As a control, there was no difference in the PCR product using total DNA (lanes 5–8) before immunoprecipitation, and immunoprecipitates with nonimmune IgG did not amplify PCR products (data not shown).

DISCUSSION

The SF-1 and cAMP pathways are involved in the regulation of many genes in the gonads and in the adrenal gland. Because SF-1 is expressed in a highly restricted manner, it has been suggested to play a pivotal role in tissue-specific gene expression (61). On the other hand, the cAMP pathway represents one of the dominant signal transduction cascades in the gonads and adrenal, reflecting its activation by trophic hormones like FSH, LH, and ACTH. Thus, it is perhaps not surprising that these pathways converge in the control of many different target genes in these tissues. Although previous studies have shown that the inhibin α gene is regulated by cAMP (20), the potential role of SF-1 has not been studied. It is notable that the inhibin α gene is expressed primarily in the ovary, testis, adrenal gland, and pituitary gonadotropes—a pattern that largely coincides with the expression profile of SF-1. Consistent with this pattern of cell-specific expression, the inhibin α promoter is most active in SF-1 expressing cell lines (data not shown). For this reason, we were prompted to examine the role of SF-1 in control of inhibin α promoter activity. Somewhat disappointingly, initial experiments using SF-1 alone revealed modest (<5-fold) stimulation of the inhibin α promoter. However, in the presence of cAMP or PKA

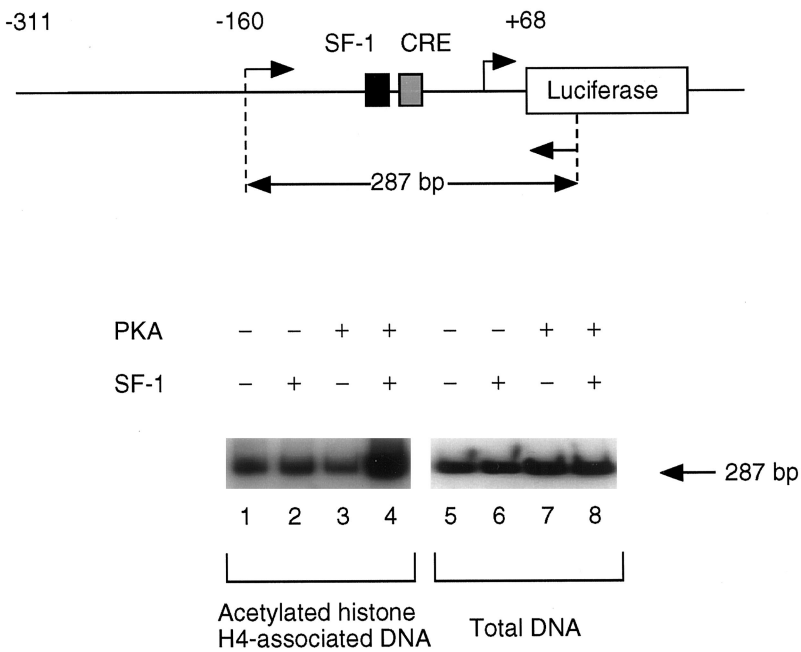

Fig. 11. Association of Synergistic Activation with Histone Acetylation

The inhibin α −311 luciferase reporter (5 μg) was transfected with either a mutant or wild-type PKA expression vector (10 μg) and either an empty or SF-1 expression vector (5 μg) into tsa 201 cells. Forty eight hours after transfection, the CHIP assay was performed. The DNA fragments (287 bp) amplified by PCR using primers corresponding to the promoter- and luciferase-coding sequences were separated on 6% nondenaturing polyacrylamide gels followed by autoradiography. The data obtained from immunoprecipitates with antiacetylated histone H4 antibody (lanes 1–4) and total DNA (lanes 5–8) are shown.

treatment, SF-1 greatly stimulated the activity of this promoter, leading to the recognition of potent synergy between these two pathways. Based on this finding, we identified a novel SF-1 binding site (TCA GGGCCA) adjacent to the imperfect CRE (TGCGTCA) in the proximal region of the inhibin α promoter.

There are several possible explanations for the synergistic transactivation of inhibin α gene by SF-1 and CREB. One possibility is that the cAMP pathway leads to the phosphorylation of SF-1 and increases its transcriptional activity. Although SF-1 is also phosphorylated by PKA *in vitro* (41, 44), recent studies suggest that phosphorylation on Ser 203 is mediated by the MAPK pathway (45). Other evidence suggests that direct phosphorylation of SF-1 by PKA is not the primary basis for synergy in the case of the inhibin α promoter. Mutation of the CRE completely eliminates synergy (and SF-1 stimulation), indicating a requirement for the CRE and its cognate transcription factors. We also found that PKA did not stimulate the activity of GAL4-SF-1 (data not shown), providing additional evidence against a direct effect of phosphorylation on the transcriptional activity of SF-1. An alternative mechanism for synergy could involve PKA-mediated alterations in the DNA-binding affinity of SF-1 or CREB. However, we found that under conditions in which PKA enhanced CREB phosphorylation on Ser 133, there was no change in the amount of DNA binding by SF-1 or CREB. Because mutations in either the SF-1 or the CRE elements are sufficient to eliminate synergy, an attractive mechanism would be for SF-1 and CREBs to act together to recruit additional transcription factors or coactivators. Although this mechanism is not assured by the current data, the inhibin α promoter appears to provide a particularly robust system for dissecting the molecular basis of transcriptional synergy.

As an initial step toward understanding the basis of synergy between the SF-1 and PKA pathways, we localized the SF-1- and cAMP-responsive regulatory elements. Deletion mutagenesis narrowed the SF-1-responsive region to the proximal −146 bp of the inhibin α promoter. Although there are no consensus SF-1 sequences in this region, several closely adjacent sequences were considered as potential SF-1 sites. A series of point mutations indicated that the sequence, TCA *G*GGCCA, which deviates somewhat from the consensus sequence (TCA AGGTCA), represents the functional SF-1 site. In addition, EMSA studies confirmed SF-1 binding to this site, and there was good correlation between the functional effects of various mutations and the effects of the mutations on SF-1 binding. We also confirmed CREB binding to the adjacent CRE (TGCGTCA), even though it deviates from the consensus octameric CRE sequence (TGACGTCA). It is probable that other members of the B-Zip transcription factor family might also bind to this variant CRE site, although CREB appears to be the major binding protein in the tsa cells used in this study, as well as in granulosa cells (20). The SF-1 and CRE

sites are closely spaced between −137 to −114 bp, but it remained possible that other sequences in the proximal inhibin promoter might be involved in the synergistic regulation by the SF-1 and PKA pathways. However, the synergistic properties of the composite SF-1/CRE element were retained after transfer to a heterologous promoter, suggesting that this feature is inherent in the SF-1/CRE element.

It is notable that there is a broad spectrum of interplay between the SF-1 and PKA pathways among the promoters that we tested. For example, although the glycoprotein hormone α-subunit promoter contains both SF-1 and CRE sites, it is controlled predominantly by the cAMP pathway, and SF-1 exerts little effect alone, or in combination with PKA. The rat aromatase promoter exhibits strong stimulation by PKA. SF-1 also activates this promoter, and it enhances PKA stimulation, although less so than seen with the inhibin α promoter. Why is synergism between the SF-1 and CRE elements so strong in the case of the inhibin α promoter? One possibility is that the inhibin α sites for both SF-1 and the CRE are imperfect binding sites. In fact, revertent mutations to optimized consensus sequences did not improve synergy, but instead reduced the ability of these two pathways to functionally interact. In addition, alteration in the spacing between the SF-1 and CRE sites diminished synergy, suggesting that DNA topology, or the physical relationship of the bound transcription factors, is critical for functional interactions to occur.

Based on these findings, we hypothesized that SF-1 might interact directly with CREB to stabilize the binding of one or both transcription factors. EMSA using the probe containing both the SF-1 binding site and the CRE (−146 to −112) did not show the formation of higher order SF-1 and CREB complexes (data not shown). However, the physical interaction between these two factors was apparent in protein interaction assays. It is not surprising that SF-1-CREB interactions are not apparent in EMSA assays, as other transcription factors that interact directly with SF-1 are not detected by gel mobility shifts. For example, DAX-1 (50), Egr-1 (early growth response protein 1) (47), WT-1 (49), and SOX9 (48) have each been shown to interact with SF-1, even though there is no evidence of heterodimer formation when studied by EMSA. It is notable that a specific, but weaker, physical interaction was detected when a glutathione *S*-transferase (GST) pull-down assay was performed using bacterially expressed GST SF-1 fusion protein and radiolabeled CREB (data not shown), suggesting that the presence of other factors in the cellular lysates may stabilize the physical interaction between SF-1 and CREB.

It is possible that the functional importance of the SF-1-CREB interaction is not so much to stabilize their binding to DNA as to facilitate the formation of an effective ternary complex with coactivators. Phosphorylation of CREB allows interaction with coactivator CBP, which also binds to components of the basal transcription machinery (62–64). In addition, CBP has been shown to interact directly with a variety of nuclear hormone receptors including the retinoic acid receptor, glucocorticoid receptor, thyroid hormone receptor, estrogen receptor, and SF-1 (39, 65–67). The ability of CBP to interact with both SF-1 and CREB raises the possibility that it may serve as a signal integrator for these two factors. Our results show that the synergistic activation of the inhibin α gene was enhanced by the addition of exogenous CBP. However, CBP 1–460, which has an interaction domain for nuclear hormone receptors (codon 1–101), but lacks an interaction domain for CREB (codon 590–669), did not increase synergism.

CBP possesses histone acetyltransferase (HAT) activity and it also recruits other proteins with HAT activity (52–55). Histone acetylation is often associated with gene activation, probably because of alterations in chromatin structure (51). Transfected plasmids generate a typical nucleosome ladder in cells (68), allowing analysis of the relationship between histone acetylation status and transcription of the transiently transfected inhibin α gene. Consistent with the transient expression studies, we found that treatment with the combination of PKA and SF-1 increased the state of histone H4 acetylation associated with the inhibin α promoter. Although both SF-1 and CREB can interact with and recruit CBP, histone acetylation was not significantly increased with each factor alone, emphasizing that the presence of both SF-1 and CREB are necessary for the effective recruitment of CBP and activation of histone acetylation associated with the inhibin α promoter. Mutations of either the SF-1 binding site or CRE also diminished histone H4 acetylation (data not shown). The extent of synergism observed with respect to histone acetylation was not as great as that seen using luciferase reporter genes. However, we find consistently that changes of reporter gene activity are much greater than changes seen in the CHIP assay, perhaps because the activated templates allow multiple rounds of transcription and amplification of the signal by the reporter enzyme. These studies of histone acetylation support the hypothesis that recruitment of CBP and other HAT enzymes may be involved in synergistic activation of the inhibin α promoter.

Based on these studies, we propose a model for the synergistic activation (Fig. 12). Because the binding of endogenous CREB to the imperfect CRE is relatively weak, CREB does not effectively mediate the signal from the cAMP pathway in SF-1 deficient cells. When SF-1 is present, it binds to the promoter, allowing interactions between CREB and SF-1. In combination, SF-1 and phosphorylated CREB recruit CBP and other cofactors, which may further stabilize their interactions. Then, CBP, in conjunction with other HATs, may induce histone acetylation and gene transactivation. In granulosa cells, endogenous SF-1 and CREB may form complexes on the composite enhancer element. Without SF-1, a robust increase of inhibin α gene expression by cAMP would not be attained. Thus,

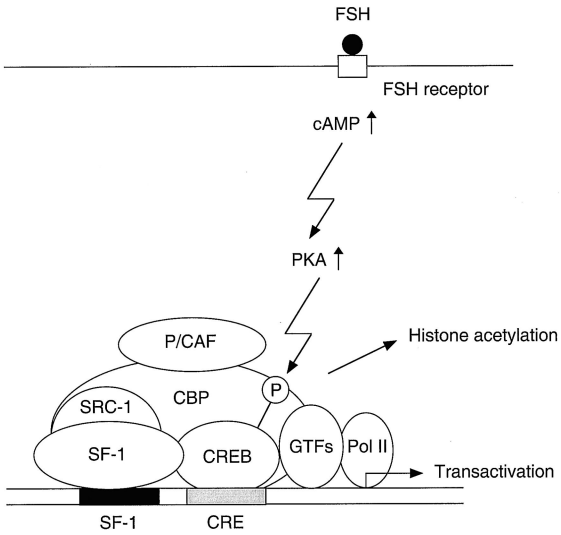

Fig. 12. Proposed Model for the Synergistic Activation

SF-1 and CREB directly interact with each other on the composite regulatory element. The formation of complexes may stabilize the weak binding of CREB to the imperfect CRE. After activation of the cAMP pathway by FSH, SF-1 and phosphorylated CREB recruit CBP, which may further stabilize their interactions. CBP may induce histone acetylation and gene transactivation. GTF, General transcription factors; Pol II, RNA polymerase II.

SF-1 may play a key role in mediating FSH signaling in granulosa cells. This model does not exclude the interaction of SF-1 with other transcription factors, or with other coactivators, aside from CBP. It is tempting to speculate that similar mechanisms involving direct protein interaction with SF-1, recruitment of cofactors, and increased histone acetylation are used in the cAMP regulation of other SF-1 responsive genes. Moreover, the functional properties of the composite regulatory element in the inhibin α promoter appear to share certain features in common with the interaction of SF-1 with Egr-1 in the context of the rat LHβ promoter. In this case, there is strong interdependence between SF-1 and Egr-1 for promoter activity (47). Thus, it is possible that a recurring feature of regulation by SF-1 will be its integrated action with other transcription factors, some of which may be regulated dynamically (*e.g.* Egr-1, WT-1) (49, 69–71), whereas others may be subject to posttranslational control (*e.g.* CREB) (62).

MATERIALS AND METHODS

Plasmid Constructions

The pA3 luciferase reporter construct for the human gonadotropin common α- subunit gene (-420 to $+44$) was previously described (72). The rat aromatase promoter region (-294 to $+20$) was amplified by PCR and cloned into the pA3 vector. The pA3 reporter constructs for the rat inhibin common α-subunit gene (-2021, -769, and -547 to $+68$) were previously described (20). The rat inhibin α promoter regions (-311, -236, -160, -146, -134, -115, and -89 to $+68$) were amplified by PCR and subcloned into the pGL3 basic luciferase reporter vector (Promega Corp., Madison, WI). The inhibin α -146 mutant reporters (m1, m2, m3, m4, m5, m6, m7, m8, m9, and m10) (see *Results* for locations of mutations) were constructed by overlapping PCR. The inhibin α enhancer elements (-146 to -112, -146 to -80, and -146 to -40) were fused to the thymidine kinase minimal promoter (TK81) and luciferase gene. Murine SF-1 cDNA (50) was cloned into the pCMX mammalian expression vector (73). In most of the experiments, the pCMX SF-1 expression vector was used along with the pCMX vector without insert (empty vector). The wild -type and mutant expression vectors for the catalytic subunit of cAMP-dependent protein kinase A (PKA) were provided by R. A. Maurer. The CREB cDNA was provided by Dr. J. Leiden. CBP, CBP 1–460 (provided by R. H. Goodman), and P/CAF (provided by Y. Nakatani) cDNAs were cloned into the pCMX expression vector. The influenza hemagglutinin (HA) epitope tag was introduced immediately after the last codon of SF-1 by PCR (74), and the HA-tagged SF-1 cDNA was subcloned into the pCMX vector. After PCR and subcloning, DNA sequence was confirmed using a dRhodamine terminator cycle sequencing kit (Perkin Elmer Corp., Norwalk, CT) and an ABI377 automated DNA sequencer (PE Applied Biosystems, Foster City, CA).

Cell Culture, Transfection, and Luciferase Assay

Human embryonic kidney tsa 201 cells (75) were grown in DMEM supplemented with 10% FBS. Cells were transfected by the calcium phosphate method (76). The immortalized granulosa cell line, GRMO2, was provided by Innogenetics N.V. (Ghent, Belgium). GRMO2 cells were maintained in Ham's F-12/DMEM containing 2% FBS, 10 μg/ml transferrin, 5 μg/ml insulin, 30 nM sodium selenite, and 3 μg/ml BSA (77). Transfection was performed using a modified lipofectin-mediated method (78). Cells were treated with 8-bromo-cAMP for 12 h before harvest. Forty eight hours after transfection, cell extracts were prepared and luciferase assays were performed (79).

Preparation of Nuclear Extracts and EMSA

Nuclear extracts were prepared from transfected cells (80). In some experiments, nuclear extracts were prepared in the presence of phosphatase inhibitors (25 mM sodium fluoride, 2 mM sodium orthovanadate). EMSA was performed as described previously (50). Briefly, nuclear extracts (10 μg) were incubated with 20 fmol of ^{32}P-labeled oligonucleotides, and the DNA protein complexes were resolved on 4% native polyacrylamide gels in 0.5$\times$ Tris-buffered EDTA (TBE) buffer. Antibodies used in the supershift assay were obtained from Dr. Morohashi (anti-Ad4BP) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) (anti-CREB).

Western Blot Analysis

Nuclear extracts were separated by 10% SDS-PAGE and were electrotransferred to nitrocellulose membranes. For detection of phosphorylated CREB, membranes were incubated with antiphosphorylated CREB antibody (Upstate Biotechnology, Inc., Lake Placid, NY) followed by incubation with horseradish peroxidase- conjugated secondary antibody. For detection of HA-tagged SF-1, membranes were probed with anti-HA antibody conjugated to horseradish peroxidase (Roche Molecular Biochemicals, Indianapolis, IN). Protein detection was performed using an enhanced chemilumines-

cence detection system (Amersham Pharmacia Biotech Inc, Piscataway, NJ).

Protein Interaction Assay

In vitro translation of CREB was performed with the TNT reticulocyte lysate system (Promega Corp.) in the presence of ^{35}S-methionine. Nuclear extracts were prepared from cells transfected with an empty vector or HA-tagged SF-1 expression vector as described above and were incubated with the labeled proteins in the presence of 2 mM dithiobis succinimidyl propionate for 15 min at room temperature. The reactions were then immunoprecipitated with either rat nonimmune IgG or rat anti-HA high affinity antibody (Roche Molecular Biochemicals) for 2 h at 4 C. Immunoprecipitates were recovered by incubation with protein G agarose. After extensive washing, bound proteins were eluted from the agarose beads and separated by 10% SDS-PAGE followed by autoradiography.

CHIP Assay

The CHIP assay was performed as described previously with minor modifications (81). Forty eight hours after transfection, tsa 201 cells were harvested and incubated in 1% formaldehyde for 15 min at room temperature. After cross-linking, cells were sonicated in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). One tenth of the total lysate was used for purification of total DNA. The rest of the lysate was incubated with either nonimmune IgG or antiacetylated histone H4 antibody (Upstate Biotechnology, Inc.) at 4 C for 18 h. Immunoprecipitates were recovered using protein A agarose pretreated with BSA and sonicated salmon sperm DNA. DNA was extracted from immunoprecipitates by phenol/chloroform extraction and ethanol precipitation. PCR was performed using either total DNA or immunoprecipitated DNA in the presence of α-P^{32}-dCTP with a temperature cycle of 1 min at 94 C, 1 min at 55 C, and 1 min at 72 C. After 30 cycles, PCR products were separated on 6% nondenaturing polyacrylamide gels followed by autoradiography. Primers used for PCR correspond to the sequence within the inhibin α promoter region (-160 to -141) (5′-TTGGCGGGAGTGGGAGATAA-3′) and luciferase coding sequence (5′-GAAATACAAAAACCGCAGAAGGTA-3′).

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