Transcriptional regulation of the mouse steroidogenic acute regulatory protein gene by the cAMP response-element binding protein and steroidogenic factor 1

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Abstract

Transcriptional induction by cAMP is mediated through the interaction of the cAMP response-element binding protein (CREB) with a cAMP response element (CRE) in the promoter of target genes. The steroidogenic acute regulatory (StAR) protein gene is regulated by cAMP-mediated signaling in steroidogenic cells even though its promoter lacks a consensus CRE. Previously, we have identified three highly conserved 5′-CRE half-sites within the –96/–67 bp region of the mouse StAR gene, and a member of the CREB family (CREB/CRE modulator (CREM)) was shown to be involved in its expression and regulation. Here we show that CREB and CREMτ (but not CREMα and CREMβ) have qualitatively similar effects on StAR promoter activity in response to $(Bu)_2$cAMP. Studies on the effects of the functional integrity of the CRE half-sites on CREB-dependent $(Bu)_2$cAMP-mediated StAR gene transcription demonstrated the greater importance of the CRE2 site in comparison with the CRE1 and CRE3 sites. The CRE2 sequence was also found to bind specifically to recombinant CREB protein and nuclear extract from MA-10 mouse Leydig tumor cells. The cAMP and CREB/CREM responsive region (–151/–1 bp) of the mouse StAR promoter also contains three recognition motifs for steroidogenic factor 1 (SF-1). Electrophoretic mobility shift assays and reporter gene analyses demonstrated the involvement of different SF-1 elements in StAR gene expression with the order of importance being SF-1/3>SF-1/1>SF-1/2. Specific mutations that eliminated the binding sites of CRE and SF-1 elements, either alone or in combination, resulted in an attenuation of StAR promoter activity, indicating that CREB and SF-1 can regulate StAR gene transcription in a cooperative fashion. In addition, mammalian two-hybrid assays revealed a high affinity protein–protein interaction between CREB/CREMτ and SF-1 which appeared to be dependent upon CREB protein phosphorylation. These findings further demonstrate CREB's role in StAR gene transcription and also provide evidence that the combined action of CREB/CREMτ and SF-1 results in enhanced activation of the StAR promoter.

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Introduction

The steroidogenic acute regulatory (StAR) protein mediates a key step in the regulation of steroid hormone biosynthesis in steroidogenic tissues, i.e. the delivery of cholesterol from the outer to the inner mitochondrial membrane (Privalle *et al.* 1983, Clark *et al.* 1994, Waterman 1995, Stocco & Clark 1996). The expression of StAR protein in the adrenals and gonads is induced by cAMP, and is intimately correlated with the acute steroidogenic response of these cells to tropic hormone stimulation (Epstein & Orme-Johnson 1991, Stocco & Clark 1996). It has been demonstrated that inhibition of transcription markedly affects synthesis of the StAR protein and steroid biosynthesis in mouse Leydig cells (Stocco & Clark 1996, Clark *et al.* 1997, Manna *et al.* 2001). Regulation of

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cAMP-dependent StAR expression and steroidogenesis involves transcriptional induction; however, the StAR gene promoter lacks a consensus cAMP response element (CRE) and, as such, resembles the promoter of several steroid hydroxylase genes that are regulated by cAMP (Waterman 1994).

The mechanism of cAMP signaling at the gene level is mediated through an eight-base pair (bp) palindromic sequence (5′-TGACGTCA-3′) which is referred to as the CRE (Montminy *et al.* 1986, Meyer & Habener 1993, Lalli & Sassone-Corsi 1994). However, studies have demonstrated that the 5′-TGACG part of the palindrome is highly conserved with respect to the 3′-TCA (Sassone-Corsi 1995). To date, several CRE-binding factors have been characterized, including the cAMP response-element binding protein (CREB), the CRE modulator protein (CREM) and activating transcription factor (ATF-1) (Sassone-Corsi 1995). CREB, CREM and ATF-1 are members of a basic-leucine zipper (bZIP) family of transcription factors, which contain basic DNA binding and transactivation domains both in the basal and cAMP-induced state (Yamamoto *et al.* 1990, Quinn 1993). The functions of these CRE-binding proteins are modulated after phosphorylation by the cAMP-dependent protein kinase A (PKA) and other kinases (Montminy *et al.* 1986, Quinn 1993). The cAMP-mediated increases in transcription are observed only after the phosphorylation of CREB or CREM at serine −133 or −117 respectively, and its specific binding to a nuclear protein CREB binding protein (CBP) (Gonzalez & Montminy 1989, Chrivia *et al.* 1993). We recently identified three CRE half-sites in the proximal region of the mouse StAR gene, and the involvement of CREB/CREM in increasing steroid synthesis and StAR expression was investigated using different approaches that affected CREB/CREM function (Manna *et al.* 2002*a*). It has been demonstrated that through alternative splicing CREB and CREM genes exhibit several isoforms, which act as both activators and repressors of transcription (Lee & Masson 1993, Sassone-Corsi 1995).

The StAR promoter sequences in mouse, rat, and human are highly homologous, with the region involved in regulation by cAMP possessing recognition sequences for several transcription factors, including steroidogenic factor 1 (SF-1) (Sugawara *et al.* 1996, Caron *et al.* 1997, Sandhoff *et al.* 1998, Reinhart *et al.* 1999, Wooton-Kee & Clark 2000). SF-1 is a member of the orphan nuclear receptor superfamily that has been shown to be involved in the cAMP-regulated expression of many steroidogenic genes, including the cholesterol side chain cleavage enzyme (Zhang & Mellon 1996, Liu & Simpson 1997), aromatase (Lynch *et al.* 1993, Carlone & Richards 1997), the adrenocorticotropin receptor (Cammas *et al.* 1997), and DAX-1 (dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X-chromosome) (Yu *et al.* 1998). Several PKA-dependent phosphorylation sites have been found within the SF-1 protein and are instrumental in its function (Carlone & Richards 1997, Jacob & Lund 1998, Hammer *et al.* 1999). SF-1 has also been demonstrated to play important roles in mediating StAR gene expression (Sugawara *et al.* 1997, Sandhoff *et al.* 1998, Reinhart *et al.* 1999); however, its mechanism of action remains poorly understood.

Since the StAR gene lacks consensus CREs, the mechanism of its regulation by cAMP is of considerable interest. The identification of three well conserved CRE half-sites presented the opportunity to understand this mechanism in more depth. The present investigation was undertaken to determine if CREB/CREM could interact with SF-1 to regulate StAR gene expression. Taken together, these findings provide the first evidence that cooperation between CREB/CREMτ and SF-1 is required for transactivation of the StAR gene.

Materials and methods

Cell culture, transfections, and luciferase assays

MA-10 mouse Leydig tumor cells (Ascoli 1981), a generous gift from Dr Mario Ascoli (Department of Pharmacology, University of Iowa College of Medicine, Iowa City, IA, USA) were maintained in growth medium containing antibiotics (Manna *et al.* 2002*a*). HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc., Gaithersburg, MD, USA), streptomycin (50 mg/l) and penicillin (50 000 U/l) at 37 °C with 5% CO_2.

Transfection studies were carried out using FuGENE 6-transfection reagent (Roche Diagnostics Corp., Indianapolis, IN, USA) according to the instructions of the manufacturer under optimized conditions (Manna *et al.* 1999*b*, 2001). For promoter studies, cells were transfected with CREB-pCR3·1 (wild-type (WT) CREB), CREM-pSG5 (α, β, and τ), or SF-1-pCMV expression plasmids (1 μg each), or a combination of CREB plus SF-1, in the presence of 1 μg 5′-flanking StAR plasmid (–151/–1 StAR/luc). Transfection efficiency was normalized by co-transfecting 10 ng pRL-SV40 vector (a plasmid that constitutively expresses renilla luciferase). Luciferase activity in the cell lysates was determined by the Dual-luciferase reporter assay system (Promega Corp., Madison, WI, USA), using a TD 20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA), as described previously (Manna *et al.* 2002*a*).

Site-directed mutagenesis and construction of the CRE and SF-1 mutants

The –151/–1 bp region of the mouse StAR promoter was synthesized using a PCR based cloning strategy (Manna *et al.* 2002*a*). Briefly, a XhoI and HindIII fragment containing –151/–1 bp was subcloned into the cloning sites of the pGL3 basic vector (Promega Corp.) which contains the firefly luciferase gene as a reporter. Using the –151/–1 StAR-pGL3 as template, mutations in the CRE half-sites and SF-1 recognition motifs were generated using the Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The sense strands of the oligonucleotides (corresponding positions in parentheses) used were (mutated (Mut) bases in lower case bold) the following: CRE1 Mut (–113 to –78 bp), 5′-GGCAATCATTCCATCCTT**ccgga**TCTGCACAATGAC-3′; CRE2 Mut (–99 to –60 bp), 5′-CCTTGACCCTCTGCACAAT**aga**T**ct**TGACTTTTTTATCTC-3′; CRE3 Mut (–93 to –60 bp), 5′-CCCTCTGCACAATGACTGA**gat**CTTTTTTATCTC-3′; CRE2&3 Mut (–99 to –57 bp), 5′-CCTTGACCCTCTGCACAA**gcggccgc**GACTTTTTTATCTCAAG-3′; SF-1/1 Mut (–153 to –124 bp), 5′-GAGTCTGCTCCCTC**ga**A**ttc**TGGCCAGCAC-3′; SF-1/2 Mut (–52 to –30 bp), 5′-GATGCACAGC**tg**TCCACGGGAAG-3′; SF-1/3 Mut (–111 to –84 bp), 5′-CAATCATTCCA**g**C**tg**TGACCCTCTGCAC-3′. All mutations were verified by restriction mapping using Sau3AI (CRE1 Mut), BglII (CRE2 Mut and CRE3 Mut), NotI (CRE2&3 Mut), EcoRI (SF-1/1 Mut), PvuII (SF-1/2 Mut and SF-1/3 Mut). Mutations in the CRE and SF-1 elements were also generated in combination with appropriate CRE and SF-1 single/double mutation vectors as the PCR template, and re-ligated into the pGL3 vector as a XhoI and HindIII fragment. Assessment of these mutations was confirmed by automated sequencing on a PE Biosystems 310 Genetic Analyzer (ABI PRISM 310, Perkin-Elmer) at the Texas Tech University Biotechnology Core Facility.

Preparation of recombinant CREB protein

The cDNA encoding CREB protein was cloned into the T7 expression vector pET11d, and recombinant CREB protein was produced in *Escherichia coli* as described previously (de Groot *et al.* 1994).

Electrophoretic mobility shift assays (EMSA)

The nuclear extracts (NE) from cultured MA-10 cells were prepared utilizing previously described procedures (Dignam *et al.* 1983, Manna *et al.* 2002*a*). The oligonucleotide probes were engineered and synthesized by heating sense and antisense primers to 65 °C for 5 min in annealing buffer (10 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA, pH 7·5), followed by cooling at room temperature. The following sense strands of the oligonucleotide sequences (mutated (Mut) bases in lower case bold) were used: CRE2 (–83/–67 bp), 5′-GGAATGACTGAAGTATTTT-3′; consensus CRE (Con CRE (Montminy *et al.* 1986)), 5′- GGAGAGATTGCCTGACGTCAGAGAGC TAG -3′; three CRE half-sites (–96/–67 bp), 5′-GGTGACCCTCTGCACAATGACTGATGACTTTT-3′; CRE1 Mut, 5′-GGT**ccgga**TCTGCACAATGACTGATGACTTTT-3′; CRE2 Mut, 5′-GGTGACCCTCTGCACAAT**aga**T**ct**TGACTTTT-3′; CRE3 Mut, 5′-GGTGACCCTCTGCACAATGACTGA**gat**CTTTT-3′; CRE1,2,3 Mut, 5′-GGT**ccgga**TCTGCACAAT**aga**T**ctgat**CTTTT-3′; SF-1/1 (–142/–128 bp), 5′-GGCTCCCACCTTGGCCA-3′; SF-1/1 Mut, 5′-GGCTC**ga**A**ttc**TGGCCA-3′; SF-1/2 (–48/–35 bp), 5′-GGCACAGCCTTCCACG-3′; SF-1/2 Mut, 5′-GGCACAGC**tg**TCCACG-3′; SF-1/3 (–106/–92 bp),

5′-GGATTCCATCCTTGACC-3′; SF-1/3 Mut, 5′-GGATTCCA**g**C**tg**TGACC-3′; SF-1/3-CRE2 (–103/–74 bp), 5′-GGCCATCCTTGACCCTCT GCACAATGACTGAT-3′; –103/–74 SF-1/3 Mut, 5′-GGCCA**g**C**tg**TGACCCTCTGCACAATGAC TGAT-3′; –103/–74 CRE2 Mut, 5′-GGCCA TCCTTGACCCTCTGCACAAT**aga**T**ct**T-3′; SF-1/3-CRE2 Mut (–103/–74), 5′-GGCCA**g**C**tg**TG ACCCTCTGCACAAT**aga**T**ct**T-3′. The doubled stranded oligonucleotides were end-labeled with [α^{32}P]dCTP (Easytides, 3000 Ci/mmol, PerkinElmer Life Sciences, Inc., Boston, MA, USA) using Klenow (Promega Corp.) fill-in reaction at 25 °C for 15 min and purified by spin column (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Protein–DNA binding experiments were carried out under optimized conditions as described previously (Eubank *et al.* 2001, Manna *et al.* 2002*a*). Briefly, NE (10–20 μg) and recombinant (rec) CREB protein (1–6 μg) were incubated in 15 μl reaction buffer (10 mM HEPES, 1 mM EDTA, 4% Ficoll, 10 mM dithiothreitol, 1 μg poly dIdC, 40 ng/μl BSA, and 2 μM $ZnSO_4$, pH 7·9). NE was incubated for 15 min at room temperature prior to the addition of a ^{32}P-labeled probe (∼100 000 c.p.m.) either alone, or in the presence of 50 pM (100-fold molar excess) unlabeled oligonucleotide probe for an additional 15 min. Binding reaction was carried out for 45 min on ice when SF-1 antibody (rabbit polyclonal antibody directed against full-length SF-1 protein (Morohashi *et al.* 1993)) was used (4 μg), before the addition of the labeled probe. The reaction mixture was then subjected to electrophoresis on 5% polyacrylamide gels in 0·5 × TBE buffer (90 mM Tris–borate, 2 mM EDTA, pH 8·3). The gels were dried, exposed either to phosphor screens or to Hyperfilm (Amersham Pharmacia Biotech) and quantitated using computer-assisted image analysis (Visage 2000, BioImage, Ann Arbor, MI, USA).

Mammalian two-hybrid assay

The protein–protein interaction between CREB/CREMτ and SF-1 was assessed with the mammalian two-hybrid assay, according to the instructions of the manufacturer (Promega Corp.). Briefly, CREB-pACT, CREB-M1-pACT (non-phosphorylatable mutant, Ser^{133}Ala substitution), and CREMτ-pACT plasmids were constructed using WT-CREB, CREB-M1, and CREMτ cDNAs inserted into the SalI and KpnI sites. The full-length mouse SF-1 cDNA was inserted into the BamHI and NotI sites of the pBIND vector. HeLa cells were transfected with CREB-pACT, CREB-M1-pACT, CREMτ-pACT, or SF-1-pBIND plasmids (2 μg each) or their combination, as indicated, in the presence of pG5-luciferase vector, using FuGENE 6 reagent (Roche Diagnostics Corp.). Following 48 h transfection, cells were harvested and luciferase activity in the cell lysates was determined using a TD 20/20 Luminometer (Turner Designs).

Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA) using Statview (Abacus Concepts Inc., Berkeley, CA, USA) followed by Fisher's protected least significant differences test. All experiments were repeated three to four times as indicated in the figure legends. $P<0·05$ was considered statistically significant.

Results

Role of CREB and CREM on StAR gene transcription

The role of CREB in the regulation of steroid biosynthesis and StAR protein expression has recently been demonstrated in steroidogenic cells, where the endogenous factor was determined to be predominantly the CREM protein (Manna *et al.* 2002*a*). Also, 5′-deletion analyses demonstrated the presence of a CREB/CREM responsive region(s) within the –110/–1 bp of the mouse StAR gene, the region found to be associated with a decrease in cAMP-stimulated StAR reporter activity in the absence of CREB/CREM. Thus, a relatively longer fragment (–151/–1 bp) was used in the present studies to investigate the regulatory mechanism involved in StAR gene transcription by the cAMP pathway. As illustrated in Fig. 1, MA-10 cells transfected with the –151/–1 bp StAR promoter fragment (–151 StAR) demonstrated a 3·9-fold increase in luciferase activity following 6 h of $(Bu)_2$cAMP (500 μM) stimulation. However, over-expression of WT-CREB (CREB) or CREMτ in the presence of the –151 StAR segment demonstrated a further 3·7-fold increase in promoter activity by cAMP when compared with

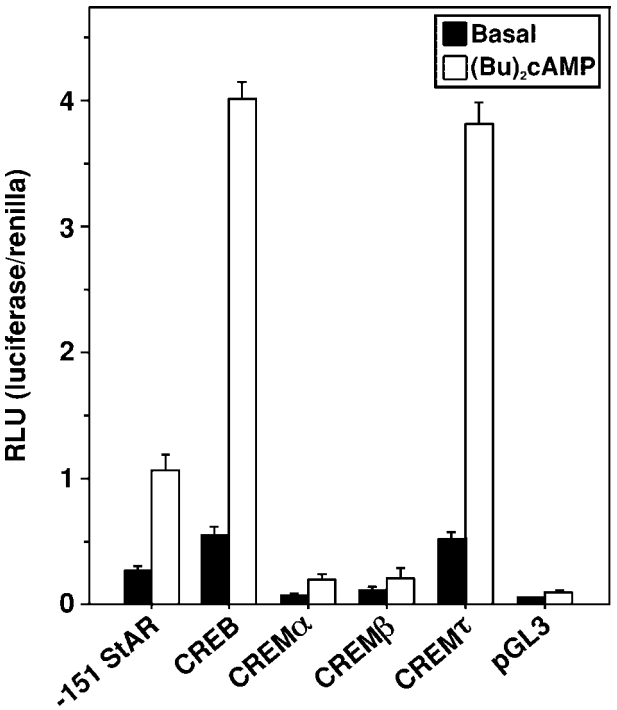


Figure 1 Over-expression of CREB and CREM on StAR promoter activity in MA-10 cells. Cells were transiently transfected with the –151/–1 bp StAR reporter plasmid (–151 StAR), without or with WT-CREB (CREB), CREMα, CREMβ, and CREMτ (2 μg each) in the presence of pRL-SV40, using FuGENE 6-transfection reagent as described under Materials and methods. Thirty-six hours after transfection, cells were stimulated without (Basal) or with $(Bu)_2$cAMP (500 μM) for 6 h. pGL3-basic (pGL3) was used as a control. Luciferase activity in the cell lysates was determined and expressed as relative light units, RLU (luciferase/renilla). Data represent the means±S.E.M. of three independent experiments.

control (–151 StAR), and the magnitude of response was qualitatively similar in both cases. In contrast, cells expressing CREMα or CREMβ markedly decreased basal and $(Bu)_2$cAMP-stimulated promoter responses when compared with controls. CREB and CREMτ also significantly increased ($P<0{\cdot}05$) basal promoter activity. CREB was used to assess the transcriptional regulation of the StAR gene in subsequent experiments.

Assessment of CRE half-sites in CREB responsiveness

Recently, we identified three canonical CRE half-sites (CRE1, CRE2 and CRE3) within the –96/–67 bp of the mouse StAR gene (Manna *et al.* 2002*a*). It was also observed that an oligonucleotide probe (–96/–67) containing these CRE half-sites demonstrated formation of three protein–DNA complexes (I, II, and III) with MA-10 nuclear extracts (NE), which were affected by the CRE mutants in EMSA. In the present findings, direct involvement of these CRE elements was assessed by generating mutations in each of the CRE half-sites using EMSA (Fig. 2). Using $(Bu)_2$cAMP-stimulated MA-10 NE, the occurrence of protein–DNA binding observed with the CRE1 mutant (lanes 1 to 3) was clearly decreased with a probe containing mutation in the CRE3 site (lanes 9 to 11). On the other hand, protein–DNA complexes were greatly diminished with the CRE2 mutant probe (lanes 5–7). Protein–DNA complexes observed with the different CRE elements were further competed with their cold competitors or with a consensus CRE sequence (Con CRE). Mutations introduced in the three CRE half-sites together markedly affected protein–DNA binding (lanes 13–15). The importance of these CRE elements on StAR promoter function using the –151 StAR segment was also studied. MA-10 cells transfected with the CRE1 (TGACCCTC to T**ccgga**TC), CRE2 (TGACTGAT to T**aga**T**ct**T), and CRE3 (TGACTTTT to **gat**CTTTT) mutants demonstrated approximately 33, 47 and 18% decreases in basal luciferase response without affecting $(Bu)_2$cAMP-mediated fold-induction when compared with the –151 StAR, an observation in agreement with the previous findings (Manna *et al.* 2002*a*). Also, mutations in the double and/or triple CRE sites demonstrated further decreases in StAR promoter activity (data not shown). These observations demonstrate the relative involvement of the three CRE half-sites in StAR gene expression.

The functional involvement of these CRE half-sites in response to CREB was then studied in MA-10 cells. Cells transfected with the –151 StAR segment demonstrated a 4·2-fold increase in $(Bu)_2$cAMP-stimulated reporter activity. Transfection of CREB in the presence of the –151 StAR segment showed a further 3·5-fold increase in luciferase activity by $(Bu)_2$cAMP when compared with the –151 StAR (Fig. 3). However, in the presence of a CRE2 mutant, CREB expression resulted in a 58% decrease in basal and $(Bu)_2$cAMP-stimulated luciferase responses when compared with controls. When expressed along

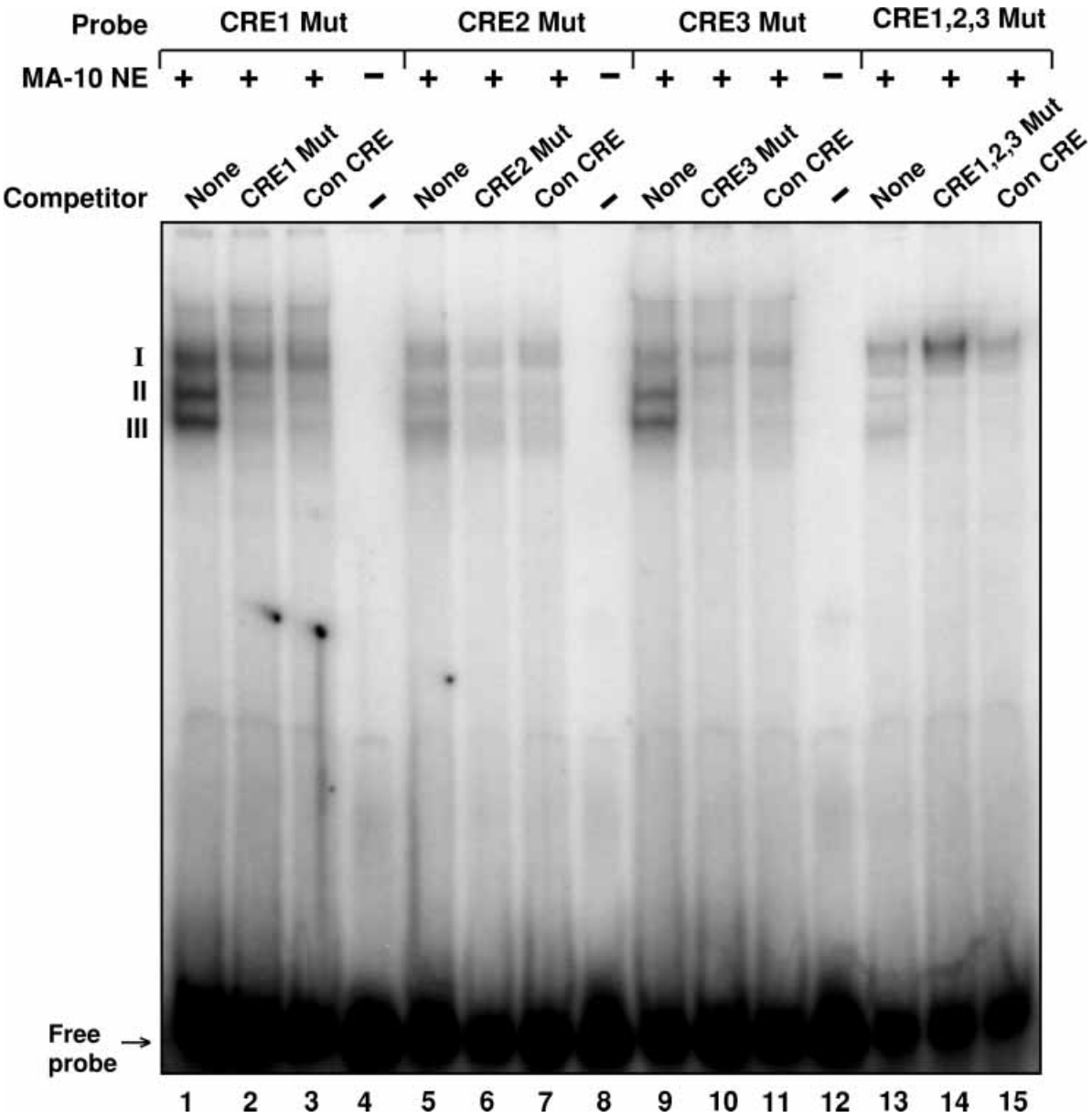


Figure 2 Binding of the different CRE mutants to MA-10 nuclear extract (NE). MA-10 cells were stimulated for 6 h with $(Bu)_2$cAMP (500 μM), and 10 μg NE were assessed in EMSA to analyze protein–DNA binding using the ^{32}P-labeled CRE elements (–96/–67 bp) containing mutations, as described in Materials and methods. Cold competitors were used at 100-fold molar excess. Protein–DNA complexes were resolved on a 5% polyacrylamide gel for about 1·5 h at 200 V, the gels were dried, and the complexes were visualized by a phosphorimaging device. A representative phosphorimage illustrates formation of protein–DNA complexes to the labeled CRE1 (lanes 1–4), CRE2 (lanes 5–8), CRE3 (lanes 9–12), and CRE1,2,3 (lanes 13–15) mutant probes. Protein–DNA complexes are marked as I, II, and III. The experiments were repeated three times. Free probes are shown in each lane. Con CRE, consensus CRE sequence.

with mutant CRE1 and CRE3 sites, CREB expression resulted in decreases in $(Bu)_2$cAMP-stimulated StAR promoter activities by 23 and 30% respectively, demonstrating that the CRE2 plays the most important role in CREB responsiveness. Notably, the $(Bu)_2$cAMP-mediated

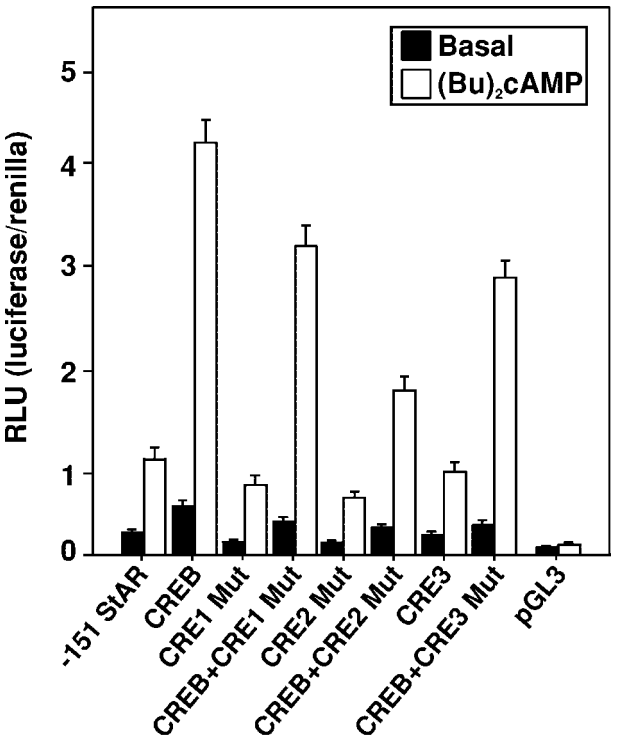


Figure 3 Influence of CRE half-sites on CREB-responsive StAR promoter activity. MA-10 cells were transfected with either the –151/–1 bp StAR segment (–151 StAR), or the –151 StAR containing mutations in each of the CRE half-sites, without or with WT-CREB (CREB), in the presence of pRL-SV40. Following 36 h of transfection, cells were incubated for a further 6 h in the absence (Basal) or presence of $(Bu)_2$cAMP (500 μM). pGL3-basic (pGL3) was used as a control. Luciferase activity in the cell lysates was determined and expressed as relative light units, RLU (luciferase/renilla). These experiments were repeated four times, and values are the means±S.E. of an experiment carried out in triplicate.

fold-induction was not affected by the CRE/CREB system in spite of decreases in basal reporter activities.

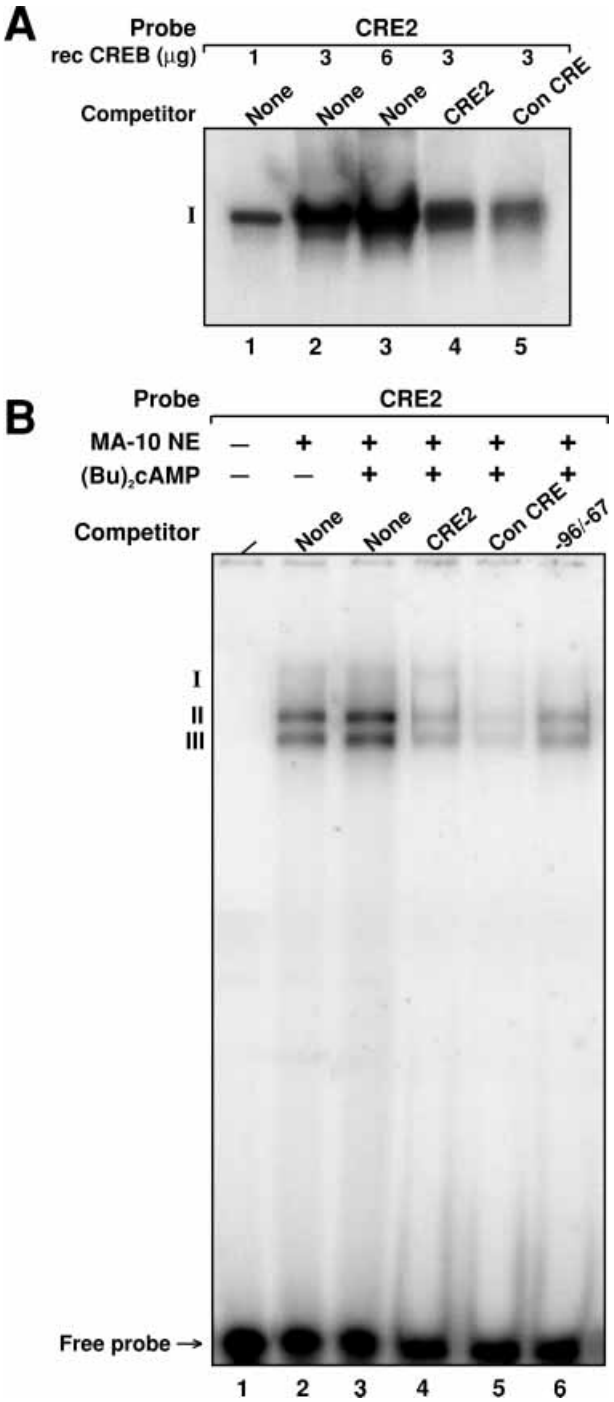
In order to determine further the importance of the CRE2 site, a wild-type CRE2 probe (–83/–67 bp) was utilized in EMSA demonstrating rec CREB protein binding. As shown in Fig. 4A, the CRE2 probe demonstrated a concentration-dependent increase in the formation of a specific protein–DNA complex (complex I) with rec CREB protein (lanes 1–3). Protein–DNA binding was strongly inhibited by cold CRE2 (lane 4) and Con CRE (lane 5) sequences. As illustrated in Fig. 4B, the CRE2 probe can also bind to MA-10 NE in a $(Bu)_2$cAMP-responsive manner (compare lanes 2 and 3), forming three protein–DNA complexes (I, II, and III). However, protein–DNA binding at complex I was nearly abolished with this probe, suggesting that CRE1 and CRE3 may have roles to play. Protein–DNA complexes were markedly decreased with cold competitors, including its unlabeled sequence (lane 4), Con CRE (lane 5), and with the –96/–67 bp (a region containing three CRE half-sites, lane 6) sequences. These data further indicate the critical involvement of CRE2 on CREB responsiveness.

CREB and SF-1 mediate maximal StAR promoter response

The cAMP and CREB/CREM responsive regions (–151/–1) of the mouse StAR gene have been shown to contain three SF-1 binding motifs which are instrumental in StAR gene expression (Caron *et al.* 1997, Wooton-Kee & Clark 2000, Manna *et al.* 2002*b*). We next studied whether CREB and SF-1 play roles in StAR gene transcription. MA-10 cells transfected with the –151/–1 bp StAR segment (–151 StAR) demonstrated a significant increase ($P<0{\cdot}01$) in $(Bu)_2$cAMP-stimulated reporter activity over untreated cells. Over-expression of either WT-CREB (CREB) or SF-1 displayed approximately 3·7- or 2-fold increases in $(Bu)_2$cAMP-mediated StAR promoter activities respectively, when compared with the –151 StAR (Fig. 5). However, co-expression of CREB and SF-1 resulted in a 5·2-fold increase in $(Bu)_2$cAMP-stimulated StAR promoter activity, demonstrating that CREB and SF-1 activate StAR promoter activity in an additive fashion.

The relative importance of different SF-1 recognition sites on StAR promoter activity was studied by mutational analysis utilizing the –151 StAR segment. When MA-10 cells were transfected with the –151 StAR/luciferase construct, the $(Bu)_2$cAMP-stimulated luciferase activity increased significantly ($P<0{\cdot}01$) over untreated cells (Fig. 6). Mutation in the SF-1/1 site (CACCTT to **a**A**ttc**T) showed a 42% decrease in basal luciferase response, but did not affect StAR promoter activity induced by $(Bu)_2$cAMP. The SF-1/2 mutant (CAGCCTT to CAGC**tg**T), on the other hand, demonstrated a modest decrease (∼20%) in reporter activity without affecting $(Bu)_2$cAMP-mediated fold activity. An approximately 54%

decrease in basal and $(Bu)_2$cAMP-mediated reporter activities was observed with the SF-1/3 mutant (CATCCTT to CA**g**C**tg**T). It is interesting to note that a different mutation in the SF-1/3 site (CATCCT**T/g**) that also involves the CRE1 site was found to decrease StAR promoter activity by ~80% (Manna *et al.* 2002*a*). Mutations carried out with double SF-1 recognition motifs, in any combination, further decreased reporter activity. These data suggest an important involvement of the SF-1/3 in StAR promoter activity in comparison with the SF-1/1 and SF-1/2 sites.



The involvement of different SF-1 binding motifs in StAR gene expression was further investigated in protein–DNA binding using EMSA. As shown in Fig. 7, oligonucleotide probes consisting of different SF-1 elements (SF-1/1, SF-1/2, and SF-1/3) demonstrated a specific protein–DNA complex with nuclear proteins isolated from $(Bu)_2$cAMP-stimulated MA-10 cells (lanes 1–12). It can be seen that formation of the protein–DNA complex was relatively higher with the SF-1/3 (lanes 9–12) in comparison with the SF-1/1 (lanes 1–4) and SF-1/2 (lanes 5–8) probes. In all cases, protein–DNA binding was decreased following addition of different unlabeled SF-1 probes or SF-1 antibody. On the other hand, similar mutations (as above) generated in different SF-1 core sequences did not affect the protein–DNA complex. These data demonstrate the relative involvement of the three SF-1 elements in MA-10 nuclear protein–DNA binding where the SF-1/3 site plays the most important role.

To assess the relationship between CRE2 and SF-1 elements, StAR promoter activity was studied utilizing the –151 StAR promoter fragment containing the CRE2 mutant in combination with

Figure 4 Binding of the CRE2 sequence to recombinant (rec) CREB protein and MA-10 NE in EMSA. (A) Increasing amounts of rec CREB protein (1–6 μg) was used for binding with a ^{32}P-labeled CRE2 (–83/–67 bp) probe (lanes 1–5). Protein–DNA reactions were performed as described in Materials and methods, and in the legend to Fig. 2. A representative autoradiogram shows rec CREB protein binding to the labeled CRE2 DNA. Cold competitors (CRE2, lane 4 and Con CRE, lane 5) were used at 100-fold molar excess. Formation of a specific protein–DNA complex is marked as I. (B) Binding of the CRE2 probe to MA-10 NE in EMSA. NE (10 μg) was used from control (lane 2) and $(Bu)_2$cAMP (500 μM; 6 h)-stimulated MA-10 cells (lanes 3–6). Formation of protein–DNA complexes is marked as I, II and III. Cold competitors (CRE2, lane 4, Con CRE, lane 5 and –96/–67 bp containing three CRE half-sites, lane 6) were used at 100-fold molar excess. These experiments were repeated 3–4 times, and a representative phosphorimage experiment is illustrated. Con CRE, consensus CRE sequence.

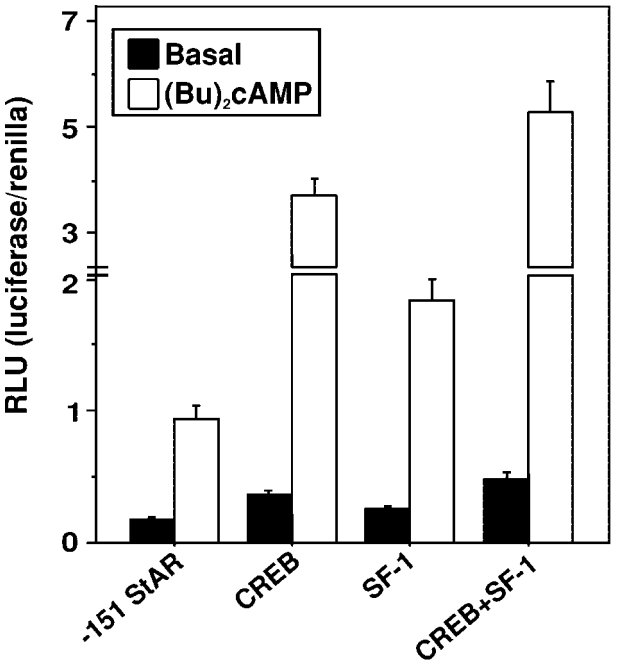


Figure 5 Over-expression of CREB and SF-1 on StAR promoter activity. MA-10 cells were transfected with the –151/–1 bp StAR segment (–151 StAR), without or with WT-CREB (CREB), SF-1 (2 μg each), or a combination of them in the presence of pRL-SV40. Thirty-six hours after transfection, cells were stimulated for a further 6 h in the absence (Basal) or presence of $(Bu)_2$cAMP (500 μM). Luciferase activity was determined in the cell lysates and expressed as relative light units, RLU (luciferase/renilla). Values represent the means±S.E.M. of four independent experiments.

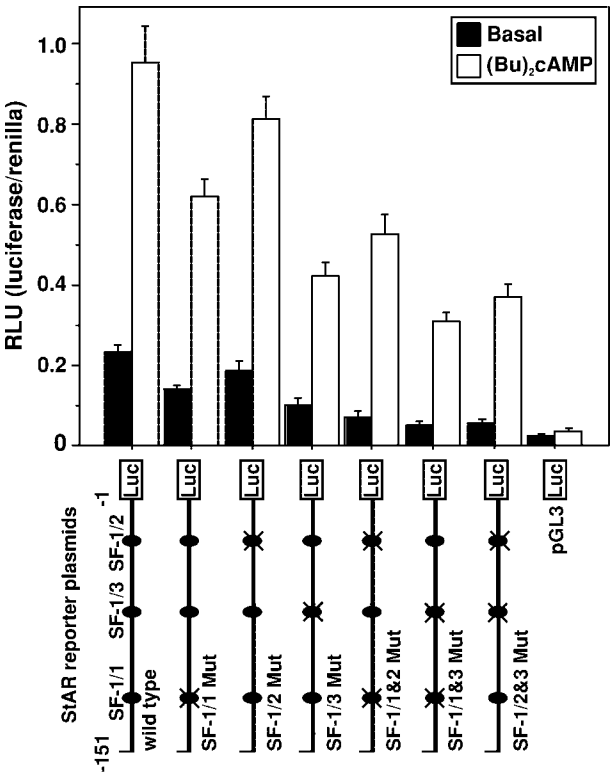


Figure 6 Assessment of different SF-1 binding sites on StAR promoter function. MA-10 cells were transfected with either the –151/–1 bp StAR reporter plasmid (–151 StAR) or the –151 StAR containing mutations in one or more SF-1 binding sites, in the presence of pRL-SV40. Following 36 h of transfection, cells were stimulated for a further 6 h without (Basal) or with 500 μM $(Bu)_2$cAMP. pGL3-basic (pGL3) was used as a control. Luciferase activity in the cell lysates was determined and expressed as relative light units, RLU (luciferase/renilla). Bottom panel shows schematic presentation of different SF-1 binding sites together with the mutations within the –151/–1 bp StAR segment. These experiments were repeated 3–4 times, and values are the means±S.E. of an experiment carried out in triplicate.

different SF-1 mutants. Transfection with the CRE2 mutant demonstrated a significant decrease ($P<0{\cdot}05$) in StAR promoter activity following $(Bu)_2$cAMP stimulation (Fig. 8). The CRE2 mutant in the presence of the SF-1/3 mutant resulted in an 85% decrease in basal promoter activity, without affecting $(Bu)_2$cAMP-mediated fold-response. Moreover, mutation in the CRE2 site in combination with the SF-1/1 or SF-1/2 mutants decreased $(Bu)_2$cAMP-stimulated luciferase responses by 60 and 47% respectively, when compared with the –151 StAR. It was also observed that transfection of CREB and SF-1 in the presence of either CRE2 or SF-1/3 mutants within the context of –151 StAR segment resulted in marked decreases in basal and $(Bu)_2$cAMP-mediated reporter activities (data not shown). In fact, mutations in the CRE2 and SF-1 binding sites demonstrated cooperation of these elements in stimulation of the StAR promoter.

To obtain more insight into these mechanisms, an oligonucleotide probe corresponding to the SF-1/3-CRE2 region (–103/–74) was studied in EMSA using NE obtained from $(Bu)_2$cAMP-stimulated MA-10 cells (Fig. 9). However, it is also necessary to take into account that this probe possesses the CRE1 site. Protein–DNA binding observed with the –103/–74 probe was strongly displaced by an excess of its unlabeled sequences

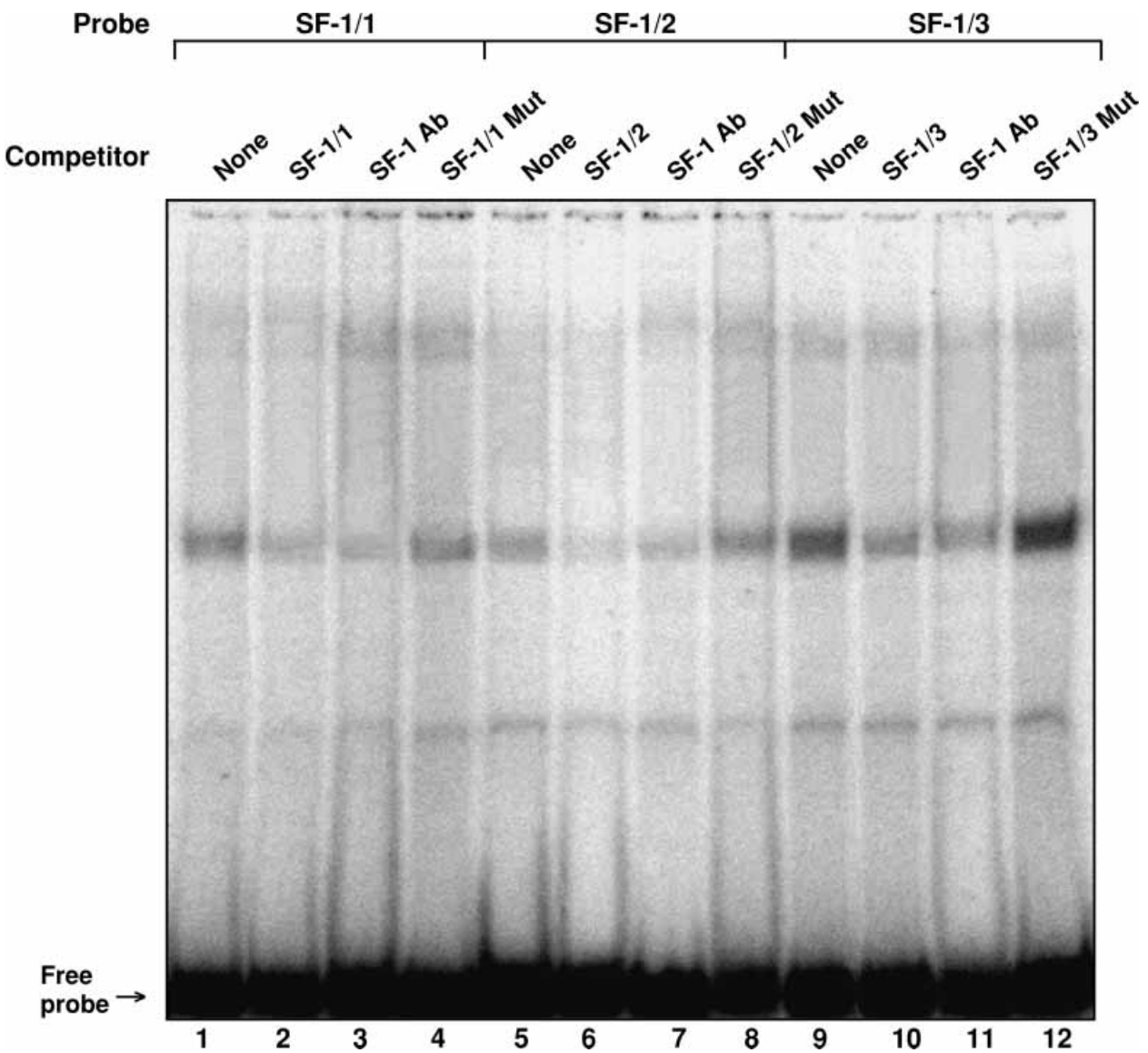


Figure 7 Relative importance of three SF-1 sites on protein–DNA binding using EMSA. NE (10 μg) from $(Bu)_2$cAMP (500 μM, 6 h)-stimulated MA-10 cells was assessed in binding with the ^{32}P-labeled SF-1/1 (–142/–128 bp), SF-1/2 (–48/–35 bp), and SF-1/3 (–106/–92 bp) probes, as described in Materials and methods and in the legend to Fig. 2. A representative phosphorimage illustrates formation of a specific protein–DNA complex with the SF-1/1 (lanes 1–4), SF-1/2 (lanes 5–8), and SF-1/3 (lanes 9–12) probes. Cold competitors were used at 100-fold molar excess. Antibody interference analysis was carried out with 4 μg polyclonal SF-1 antibody (SF-1 Ab). The experiments were repeated three times. Free probes are shown in each lane.

(compare lane 1 and 2). Mutations (as above) introduced into the SF-1/3 or CRE2 sites individually, within the context of the –103/–74 probe, showed a competition with protein–DNA complexes (lanes 3 and 4 respectively), suggesting the involvement of a non-mutated site. However, the SF-1 antibody (lane 6) and Con CRE (lane 7) also decreased protein–DNA complexes. Formation of protein–DNA complexes was not affected with a cold competitor containing mutations in the CRE2 and SF-1/3 binding sites together (lane 5). These data further demonstrate a functional cooperation between CRE2 and SF-1/3 elements in StAR gene expression.

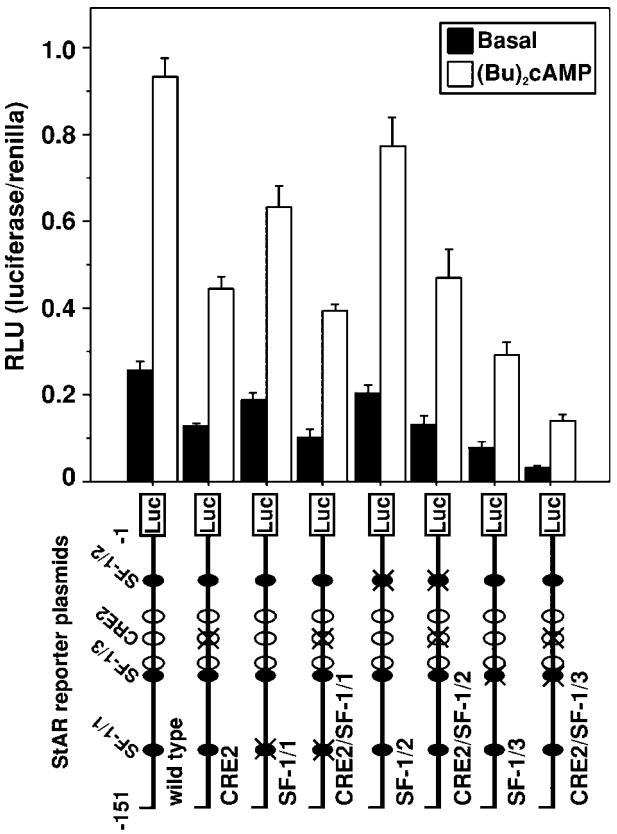


Figure 8 Functional cooperation of CRE2 and SF-1 binding sites on StAR promoter response. MA-10 cells were transfected with either the –151/–1 StAR plasmid (–151 StAR) or the –151 StAR containing mutations in the CRE2 and SF-1 elements (as indicated), in the presence of pRL-SV40. Thirty-six hours after transfection, cells were incubated for a further 6 h without (Basal) or with 500 μM $(Bu)_2$cAMP, and luciferase activity in the cell lysates was determined and expressed as relative light units, RLU (luciferase/renilla). Schematic presentation of the CRE2 and SF-1 elements along with their mutations within the –151/–1 bp StAR segment is illustrated in the bottom panel. These experiments were repeated three times, and values are the means±S.E. of an experiment carried out in triplicate.

Interaction of CREB and SF-1 in the two-hybrid assay

The protein–protein interaction between CREB/CREMτ and SF-1 was assessed using the mammalian two-hybrid assay system. HeLa cells transfected for 48 h with CREB-pACT, CREMτ-pACT, and SF-1-pBIND plasmids demonstrated limited effects on luciferase activity (Fig. 10). On the other hand, cells transfected with a non-phosphorylatable mutant ($Ser^{133}Ala$) of CREB (CREB-M1-pACT) resulted in a significant decrease ($P<0{\cdot}05$) in luciferase response as compared with CREB-pACT. Co-expression of either CREB-pACT or CREMτ-pACT with SF-1-pBIND demonstrated increases in luciferase activity, to levels 4·7-, 16-, and 4-fold higher than those observed with CREB-pACT, SF-1-pBIND, and CREMτ-pACT alone, respectively, suggesting an interaction between CREB/CREMτ and SF-1. Notably, co-transfection of both CREB-M1-pACT and SF-1-pBIND decreased luciferase activity by approximately 60% when compared with CREB-pACT and SF-1-pBIND expression together, demonstrating the importance of CREB phosphorylation on their interaction (Fig. 10). These data suggest that CREB/CREMτ and SF-1 are either capable of a direct association or interact through a common adapter protein *in vivo*.

Discussion

The combined action of multiple factors have been demonstrated to be involved in the expression and regulation of many genes involved in steroidogenesis (Roesler *et al.* 1995, Zhang & Mellon 1996, Carlone & Richards 1997, Liu & Simpson 1997, Monte *et al.* 1998). The product of one of these genes, the StAR protein, plays a critical role in steroid hormone biosynthesis by mediating intramitochondrial cholesterol transport, a process that is regulated by the cAMP pathway. In common with many cAMP-regulated steroidogenic genes, the StAR gene lacks a consensus CRE, suggesting the existence of alternative regulatory factor(s) in cAMP responsiveness. An increasing body of evidence suggests that the involvement of the nuclear receptor SF-1 is common to these pathways. Transcriptional activation of the StAR gene has also been demonstrated by multiple transcription factors including SF-1; however, the mechanism of this regulation is still not clearly understood. While it lacks a consensus CRE, three CRE half-sites located in the proximal region of the mouse StAR gene have recently been identified and the involvement of a CREB/CREM family member in StAR gene expression was demonstrated (Manna *et al.* 2002*a*). The present studies extend these observations by demonstrating that

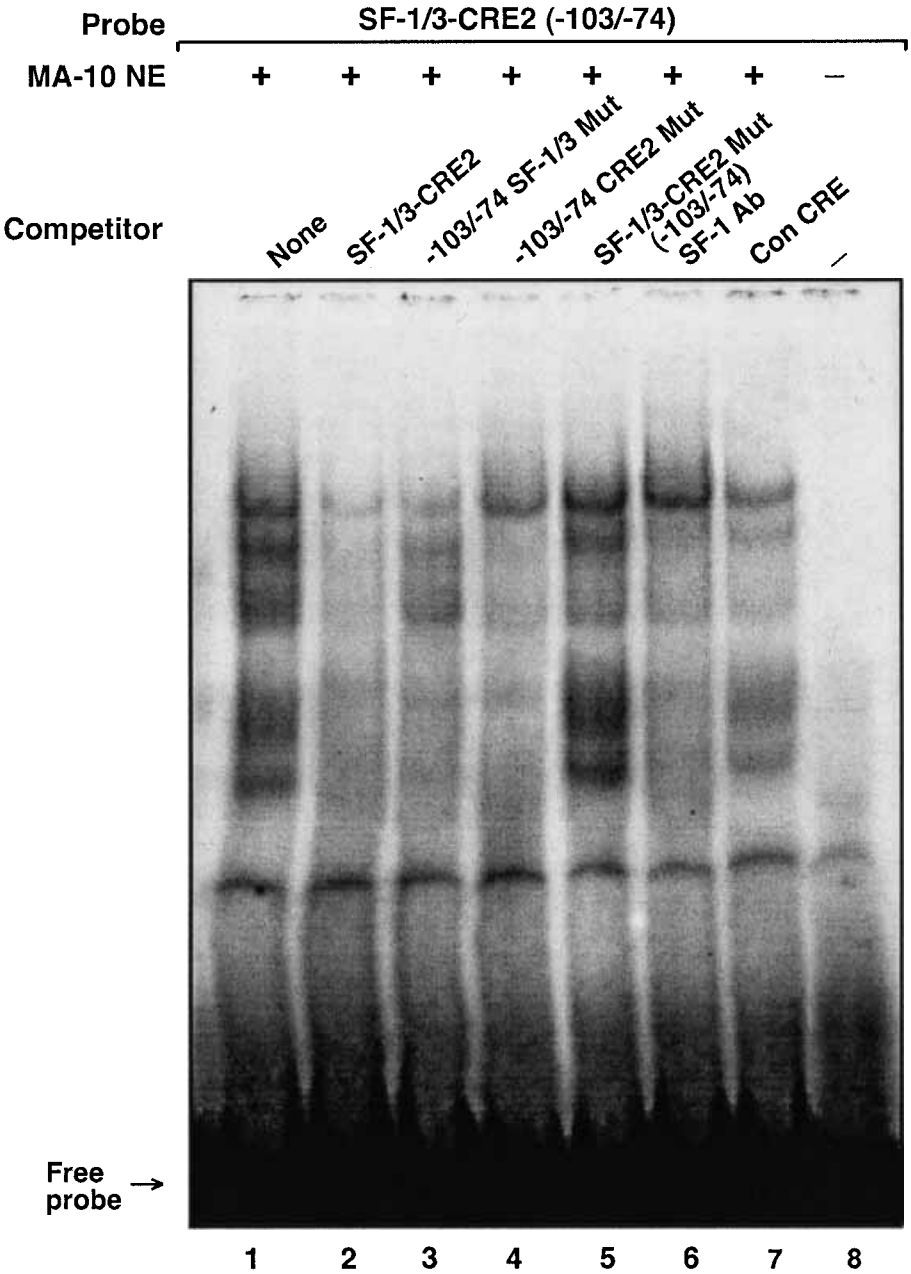


Figure 9 Binding of the SF-1/3-CRE2 region to MA-10 NE in EMSA. $(Bu)_2$cAMP (500 μM, 6 h)-stimulated MA-10 NE (10 μg) was used in binding with the ^{32}P-labeled SF-1/3-CRE2 probe (–103/–74 bp), as described in Materials and methods and in the legend to Fig. 2. A representative autoradiogram shows formation of protein–DNA complexes. The following unlabeled competitors were used at 100-fold molar excess: SF-1/3-CRE2 (lane 2), –103/–74 containing mutations in SF-1/3 (–103/–74 SF-1/3 Mut, lane 3), CRE2 (–103/–74 CRE2 Mut, lane 4), or double sites (SF-1/3-CRE2 Mut (–103/–74), lane 5). Protein–DNA binding was also assessed with 4 μg polyclonal SF-1 antibody (SF-1 Ab, lane 6) and 100-fold molar excess of consensus CRE (Con CRE, lane 7). The experiments were repeated three times. Free probes are shown in each lane.

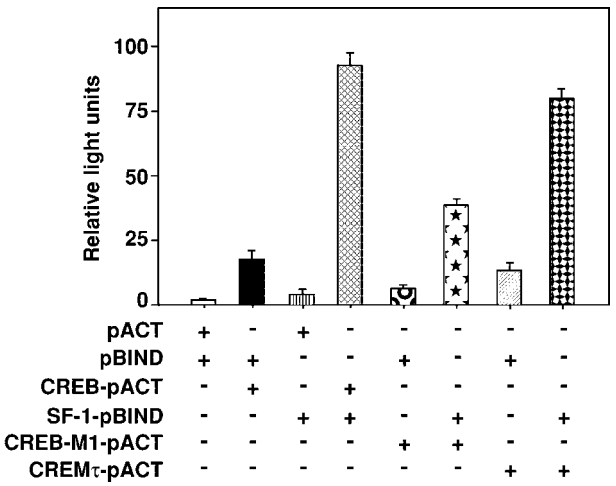


Figure 10 Interaction between CREB/CREMτ and SF-1 in the mammalian two-hybrid assay. HeLa cells were transfected with CREB-pACT, CREMτ-pACT, CREB-M1-pACT, or SF-1-pBIND (2 μg each), or a combination of them as indicated, in the presence of the pG5-luciferase vector, as described in Materials and methods. Following 48 h of transfection, cells were harvested and luciferase activity in the cell lysates was determined (relative light units). Data represent the means±S.E.M. of 3–4 independent experiments.

CREB/CREMτ and SF-1 interact and cooperate to regulate StAR gene transcription.

The CREB/CREM/ATF family of transcription factors are distinguishable by their DNA-binding bZIP domains, share many properties, and are involved in cAMP signaling (Dwarki *et al.* 1990, Gonzalez *et al.* 1991, Meyer & Habener 1993, Della Fazia *et al.* 1997). We recently demonstrated that CREB is capable of increasing cAMP-mediated steroid production and StAR expression in steroidogenic cells; however, the predominant endogenous factor was determined to be the CREM protein as assessed by EMSA and RT-PCR analyses (Manna *et al.* 2002*a*). Earlier studies have demonstrated that CREM transcripts are higher in the testis than those of CREB and specifically encode the activator form CREMτ (Delmas *et al.* 1993, Sassone-Corsi 1995). It has been demonstrated that through alternative splicing the CREM gene can give rise to several isoforms which act as activators (τ, τ1, and τ2) or repressors (α, β, and γ) of transcription (Lee & Masson 1993, Sassone-Corsi 1995). However, it is noteworthy that functional regions in the CREB protein are identical to CREMτ (Lee & Masson 1993). In addition, it was presently observed that overexpression of CREB and CREMτ resulted in qualitatively similar responses in activation of basal and $(Bu)_2$cAMP-stimulated StAR promoter activities, and these responses were markedly decreased by CREMα and CREMβ in MA-10 cells, an observation in general agreement with previous findings (Foulkes *et al.* 1992, Lee & Masson 1993, Sassone-Corsi 1995).

The identification of three CRE half-sites presented the opportunity to characterize the role of these sites on CREB function that might be involved in the acute regulation of StAR gene (Manna *et al.* 2002*a*). Our current findings clearly demonstrate the greater importance of the CRE2 site, as compared with the CRE1 and CRE3 sites, in CREB-mediated StAR gene transcription. Studies have shown that CREB's activation of a promoter can include the binding of multiple CRE binding factors to several CREs rather than the binding of a single set of heterodimers (Fisch *et al.* 1989, Liu *et al.* 1991). In the case of the phosphoenol-pyruvate carboxykinase gene promoter it has been shown that several CREs bind to multiple molecules of CREB and exhibit a response to cAMP in a synergistic fashion (Roesler *et al.* 1995). The integrity of sequence-specific binding of the CRE2 DNA was also investigated with recombinant CREB protein and MA-10 NE using EMSA, where consensus CRE or –96/–67 bp (three CRE half-sites) sequences markedly decreased protein–DNA binding. The CRE2 site is also analogous to an activator protein 1 (AP-1) sequence (TGACTGA) and, as such, a role for AP-1 in StAR gene expression cannot be eliminated. Indeed, the AP-1 family, composed of Jun and Fos oncoproteins, was found to be involved in decreasing cAMP mediated StAR gene expression in MA-10 cells (PR Manna, DW Eubank and DM Stocco unpublished data), an observation consistent with recent findings (Manna *et al.* 2002*b*, Shea-Eaton *et al.* 2002).

SF-1 is known to play a key role in adrenal and gonadal differentiation, development and function at multiple levels, including the expression of steroidogenic genes (Ikeda *et al.* 1994, Ingraham *et al.* 1994, Parker & Schimmer 1997). Overexpression of SF-1 has been demonstrated to activate StAR gene expression in steroidogenic and non-steroidogenic cells (Sugawara *et al.* 1996, Manna *et al.* 1999*a*, Reinhart *et al.* 1999). The

5′-flanking nucleotide sequences (–151/–1) of the mouse StAR gene also revealed the presence of three SF-1 binding sites at –135, –95, and –45 bp positions (Caron *et al.* 1997, Wooton-Kee & Clark 2000, Manna *et al.* 2002*b*). Our findings regarding mutations in the SF-1/1 (–135), SF-1/2 (–45), and SF-1/3 (–95) binding sites demonstrated progressive involvement of the latter on StAR promoter activity in MA-10 cells. In these findings, the $(Bu)_2$cAMP-stimulated fold responses were preserved, indicating that SF-1 elements are important for transcriptional responsiveness but dispensable for cAMP-mediated StAR gene regulation. Also, mutations in the SF-1 elements, at positions –135 and –95 in the rat and at –105/–95 in the human, have been demonstrated to be critical for basal and cAMP responsiveness (Sugawara *et al.* 1997, Sandhoff *et al.* 1998, Clark & Combs 1999). We also observed the relative importance of different SF-1 recognition motifs in MA-10 nuclear protein–DNA binding in EMSA, where the specificity of binding was verified by cold competitors, mutations in the target sites and by SF-1 antibody, an observation in agreement with the role of SF-1 elements in StAR promoter function. Collectively, using sequence- and site-specific mutational analyses, the present data demonstrate the major involvement of SF-1/3 in StAR gene expression whereas the SF-1/2 and SF-1/1 recognition sites play lesser roles.

From the results of the current findings, it is apparent that SF-1 is necessary, although not sufficient for complete cAMP responsiveness, since mutations in any of these SF-1 binding sites decreased, but did not abolish, basal and $(Bu)_2$cAMP-mediated StAR reporter activity. Furthermore, a functional cooperation between CRE and SF-1 elements was observed in mediating mouse StAR gene expression, as determined by EMSA and reporter gene analyses. Several lines of evidence suggest that transcription factors either interact and/or cooperate with other transcription factors to regulate transcription of the target genes. In the case of many cAMP-regulated steroidogenic genes that lack a consensus CRE, SF-1 can function as a transcriptional regulator alone (see Introduction) or in concert with other transcription factors. For example, interactions between SF-1 and CCAAT/enhancer binding protein β (C/EBPβ), Sp1, CBP/p300 and SOX9 have been shown to be involved in regulating the mouse StAR, bovine CYP11A, human P450scc and anti-Mullerian hormone genes respectively (Liu & Simpson 1997, Monte *et al.* 1998, de Santa Barbara *et al.* 1998, Reinhart *et al.* 1999, Sugawara *et al.* 2000).

An interesting aspect of the results obtained in these experiments is that a functional protein–protein interaction exists between CREB/CREMτ and SF-1 as demonstrated with the mammalian two-hybrid assay. This interaction appeared to be dependent, at least in part, on CREB protein phosphorylation. Studies have demonstrated that treatment of forskolin is capable of increasing SF-1 protein phosphorylation in Y-1 adrenocortical tumor cells (Gyles *et al.* 2001), and phosphorylation of SF-1 has also been shown to modulate cofactor recruitment (Hammer *et al.* 1999). Hence, a role for phosphorylation of SF-1 in CREB/CREMτ and SF-1 protein–protein interactions cannot be excluded. Since the interaction of CREB and SF-1 was impaired by the CREB-M1 ($Ser^{133}Ala$), it is possible that CBP/p300 (a factor known to interact with both CREB and SF-1 (Monte *et al.* 1998), and whose interaction with CREB is inhibited by the same mutation) may play an important role in bridging the two transcription factors. Further studies will be required to determine the nature of this interaction, which could be through a direct protein–protein interaction or through the recruitment of co-activators to the StAR promoter. It has been reported that CREB synergizes with other transcription factors for mediation of cAMP responsiveness (Roesler *et al.* 1995, Carlone & Richards 1997). Consequently, CREB and SF-1 have been demonstrated to interact synergistically in R2C rat Leydig tumor cells and additively in rat granulosa cells to confer cAMP responsiveness for the expression of the aromatase gene (Carlone & Richards 1997). Also, SF-1 and CREB acting in concert have been shown to be involved in the synergistic transactivation of the inhibin α promoter while they have relatively weak effects on transcription individually (Ito *et al.* 2000).

The 5′-flanking region (–151/–1) in the mouse StAR promoter has, in addition to several transcription factor binding sites, sites including three CRE half-sites, and appears to be the most important region in transcriptional regulation of the StAR gene. This region possesses putative binding sites for SF-1, C/EBP, GATA, and AP-1, and each has been shown to influence StAR

promoter function (Caron *et al.* 1997, Christenson *et al.* 1999, Reinhart *et al.* 1999, Silverman *et al.* 1999, Wooton-Kee & Clark 2000). Recently, the –105/–60 bp region, containing an SF-1, a C/EBPβ-AP-1-nuclear receptor half-site, and a GATA-4 binding site, has been shown to function in cAMP responsiveness (Wooton-Kee & Clark 2000). The C/EBPβ-3 (–81) and GATA-4 (–66) sites have been demonstrated to markedly affect protein–DNA binding and cAMP/follicle-stimulating hormone-mediated StAR promoter activity in granulosa cells (Silverman *et al.* 1999). As indicated above, the SF-1 elements within the region of the mouse (our present data, and Clark & Combs 1999), rat and human StAR promoters were demonstrated to play important roles in StAR gene function (Sugawara *et al.* 1997, Sandhoff *et al.* 1998). Also, the C/EBPβ elements, at positions –113 (C1) and –87 (C2) in the murine and –119 and –50 in the human StAR promoters, have been shown to be involved in regulating StAR gene transcription (Christenson *et al.* 1999, Reinhart *et al.* 1999). Work is currently underway to assess a potential role for these factors in the interaction and/or cooperation with CREB in cAMP-mediated StAR gene transcription.

The results obtained from this (Reinhart *et al.* 1999, Manna *et al.* 2002*a*,*b*, and the present work) and other (Caron *et al.* 1997, Sandhoff *et al.* 1998, Christenson *et al.* 1999, Silverman *et al.* 1999, Wooton-Kee & Clark 2000) laboratories have allowed us to speculate that the combinatorial action of these elements (CRE2/AP-1/C/EBP, SF-1, and GATA) might be involved in the transcriptional regulation of the StAR gene. However, this hypothesis does not exclude the interaction of CREB with other transcription factors or co-activators in addition to SF-1, which may be required for full promoter response. Thus, it is possible that regulation of other steroidogenic genes that lack a consensus CRE and are regulated by cAMP may also be mediated by similar mechanisms.

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