The role of SF-1/Ad4BP in the control of the bovine gene for the steroidogenic acute regulatory (StAR) protein

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ABSTRACT

The bovine gene for the steroidogenic acute regulatory protein (StAR) was cloned and sequenced, including 2 kb of the upstream control region of the gene. The gene comprises seven exons arranged similarly to those of the human and mouse gene sequences. The sequence analysis identified three cis elements corresponding to the binding motif for the transcription factor SF-1/Ad4BP, at −100, −240 and −1190 from the transcription start site. Electrophoretic mobility shift analysis (EMSA) using nuclear proteins from bovine corpus luteum and bovine adrenal as well as in vitro transcribed/translated SF-1/Ad4BP consistently showed that only the site at −1190 bound the transcription factor significantly. Very weak binding was detectable also at the −240 site, but none at the −100 site. Heterologous transfection of StAR promoter deletion–reporter constructs into Hela cells cotransfected with an expression vector for bovine SF-1/Ad4BP, showed that this transcription factor can specifically act on the bovine StAR gene promoter, but preferentially in regions correspond-
human StAR protein have been cloned and partially analysed (Clark et al. 1995, Sugawara et al. 1995, 1996, Caron et al. 1997). Sequence analysis revealed that, within the 5' upstream region of these genes, there are consensus elements for the transcription factor steroidogenic factor 1 (SF-1/Ad4BP), and it could be shown that these elements were able to mediate an apparent SF-1-dependent transcriptional response in heterologous and homologous transfection systems (Sugawara et al. 1996, Caron et al. 1997). This transcription factor has been identified in all steroidogenic tissues, as well as in the pituitary and placenta, and appears to be involved in the regulation of a number of genes specifically expressed in these tissues (reviewed in Parker & Schimmer 1997). The manner in which regulatory signals are mediated by SF-1 is not clear. In transfected cells, SF-1 expression constructs themselves may stimulate transcription from promoter–reporter constructs (e.g. Sugawara et al. 1996), though in many cases concatamers of SF-1 response elements are needed before a significant effect is seen (e.g. Honda et al. 1993). More recently, SF-1 has been shown to mediate directly cAMP and phorbol ester upregulation of some steroidogenic enzyme genes (e.g. Bakke & Lund 1995, Leers-Suchetat et al. 1997), and to bind a putative specific ligand, 25-OH-cholesterol (Lala et al. 1997), that appears to increase its efficiency as a transcriptional activator.

We recently cloned the bovine cDNA for the ovarian StAR transcript as part of a differential cloning project to identify specific genes expressed in the corpus luteum of the late cycle and pregnancy (Hartung et al. 1995). The cDNA sequence showed considerable homology to the sequences published from other species, and indicated three alternative transcript sizes seen in Northern hybridizations. The in vivo expression pattern of the specific mRNA, with high transcript levels in corpora lutea only in the mid cycle and several days after upregulation of the P450SCC mRNA (Hartung et al. 1995, Pescador et al. 1996), appeared at odds with a role for the encoded protein as part of an acute steroidogenic response. In order to understand the molecular mechanisms involved in this regulation, and to relate expression of the StAR gene to that of other components of the steroidogenic machinery, we have isolated and characterized the gene for the bovine StAR protein, and analysed the functional elements in the promoter involved in its control. Besides DNA–protein binding studies using nuclear extracts from bovine StAR-expressing tissues, heterologous transfection studies in Hela cells were performed. The use of heterologous rather than homologous transfection was chosen in order to test specifically the effect of bovine SF-1/Ad4BP by cotransfection of the appropriate expression plasmid into cells naturally devoid of this transcription factor.

**MATERIALS AND METHODS**

**Screening and isolation of genomic clones from a bovine genomic library**

Approximately 1 million clones of a bovine genomic library in the bacteriophage vector λ Dash II (Stratagene, La Jolla, CA, USA) were screened by conventional plaque hybridization using as probe the cDNA clone 2–2 (Hartung et al. 1995), comprising approximately 1·6 kb of the 3' untranslated region of the bovine StAR cDNA. A further 1 million clones were subsequently screened using duplicate replica filters, hybridizing each replica with a 200 bp or 400 bp PstI–PstI fragment from the extreme 5’ RACE (rapid amplification of cDNA ends)–PCR product of the cDNA (see Hartung et al. 1995). These probes were chosen because they did not include the TIMP (tissue inhibitor of metalloproteinase)-1 homology region (Hartung et al. 1995), within the coding region of the StAR cDNA, which might positively hybridize to TIMP-related genes. Positive clones from the first screening were subjected to restriction digestion and mapping. Of four bacteriophage clones isolated, three proved to represent the functional StAR gene, albeit truncated in the 5’ region, and one an intronless pseudogene. In order to select only functional and full-length gene clones, 20 positive clones from the second screening were subsequently subjected to discriminatory PCR analysis, using primer pairs from intron 4 and exon 5 (for the true gene: forward primer, 5'-CAGGCCACTGTCTGTGTCC; reverse primer, 5'-AGCACACACATGGAGC; product size 374 bp) or exons 1 and 5 (for the pseudogene: forward primer, 5'-TACTGCCAGGAAAGATG; reverse primer, as above; product size 609 bp). Of the 20 independent clones thus analysed, 10 represented the functional gene, 10 the pseudogene. Altogether four clones of the functional gene were selected for further restriction analysis and mapping (see Fig. 1). Since all appeared to represent the same genomic sequence, a 14 kb HindIII restriction fragment from clone λ1-3 was subcloned into the plasmid vector pBluescript KS for detailed restriction analysis and sequencing. Altogether 8·45 kb of genomic DNA sequence information was obtained for all exonic and intronic regions of the gene, including approximately 2 kb of the promoter region 5’ to exon 1 (accession number Y17260 in the EMBL/Genbank database).
Analysis of the 5' promoter region of the StAR gene

Seven subfragments (Fig. 2) of the first 1·2 kb of the genomic region upstream of the transcription start site, as estimated by 5' RACE primer extension (Hartung et al. 1995), were prepared by PCR using the primers listed in Table 1, and the following reaction conditions: denaturing at 95 °C for 5 min, followed by 30 cycles of denaturing at 95 °C for 1 min, annealing at 60 °C for 1 min, elongation at 72 °C for 1 min. These fragments were end-labelled by T4 polynucleotidyl kinase (New England Biolabs, Beverly, MA, USA) in the presence of [32P]ATP (Amersham–Buchler, Braunschweig, Germany), and purified by electrophoresis on 4% polyacrylamide gels. After brief autoradiographic exposure the radiolabelled DNA fragments were excised from the gel and eluted into 10 mM Tris HCl, pH 8·0/0·5 mM EDTA. Approximately 10 000 c.p.m. of probe were used in each subsequent binding reaction.

Nuclear proteins were prepared from early–mid phase bovine corpora lutea, collected fresh from the local slaughterhouse, using the technique of Deryckere & Gannon (1994). Similar proteins were also prepared from bovine adrenal medulla. As a source of purified bovine SF-1/Ad4BP, a full-length cDNA clone encoding the bovine SF-1/Ad4BP transcription factor (Honda et al. 1993) was subcloned into the vector pRc-CMV (Invitrogen, Leek, The Netherlands) and used to programme in vitro transcription and translation using a commercial kit (TNT rabbit reticulocyte lysate kit; Promega, Madison, WI, USA), and T7 RNA polymerase (Promega). Test experiments were performed in the additional presence of [35S]methionine (Amersham–Buchler), and the proteins synthesized analysed by SDS-PAGE and autoradiography. The SF-1/Ad4BP construct specifically yielded protein in two bands at 46 and 52 kDa, approximately in accord with the expected molecular mass.

Electrophoretic mobility shift assays (EMSA) were performed exactly as described elsewhere (Walther et al. 1991, Kascheike et al. 1997). Briefly, binding reactions were set up containing approximately 5 µg of nuclear proteins, and 1–2 fmol (approximately 10 000 c.p.m.) of the radiolabelled probe, and the complexes analysed by electrophoresis on a non-denaturing 4% polyacrylamide gel. As control for the binding of the bovine SF-1/Ad4BP a short, 61 bp fragment of the bovine oxytocin gene promoter, including a defined SF-1/Ad4BP binding site, was subcloned into the plasmid vector pBS (Stratagene) and excised with the enzymes HindIII and EcoRI to yield a double-stranded 130 bp DNA fragment, which also includes part of the vector multiple cloning site (Wehrenberg et al. 1994). One nanogram (10-fold molar excess) of this competing oligonucleotide was added per reaction as indicated in the figure legends. Alternatively, 1 µl of the undiluted polyclonal anti-SF-1/Ad4BP antiserum was also added where indicated.

In order to determine whether there might be an effect of protein kinase A (PKA) via activation of the CREB (cAMP responsive element (CRE) binding protein) pathway and an appropriate responsive
element (CRE) in the StAR gene promoter, additional EMSA experiments were carried out using a commercial CREB-1 bZIP protein preparation (Santa Cruz Biotechnology, Santa Cruz, CA, USA) essentially as in Gellersen et al. (1997). Briefly, the same seven fragments of the bovine StAR gene promoter, as above, as well as a classic CRE motif (oligonucleotide sc-2504; Santa Cruz Biotechnology) as positive control, were labelled and incubated as above with 0·3 µg of the CREB-1 bZIP protein.

**Transfection of bovine StAR gene promoter–reporter constructs**

Sequencing of the bovine StAR gene promoter revealed the presence of three consensus motifs for the transcription factor SF-1/Ad4BP (see Fig. 2). Restriction fragments were prepared from the promoter region to include none (BanI/PstI, −101/+6), one (HpaII/PstI, −203/+6), two (PstI/PstI, −315/+6 and KpnI/AvaI, −1009/+22) or all...
three (BamHI/AvaI, -1245/+22) of these consen-
sus elements, as indicated. These promoter deletion
fragments were ligated to the SmaI site of the
pGL3-basic luciferase reporter plasmid (Promega),
and the resulting constructs used for transfection
experiments following the protocol described below.
Fifty thousand HeLa cells per well were cultured in
12-well plates using Dulbecco’s Modified Eagle’s
Medium containing 4·5 g/l glucose, 10% fetal
calf serum, 2 mM L-glutamine, 0·5% penicillin/
streptomycin (ICN, Meckenheim, Germany). A
total of 5 µg DNA per well were transfected into the
cells using calcium phosphate co-precipitation
(Profection mammalian Transfection system;
Promega). One and a half micrograms of the deleted
StAR promoter–luciferase constructs were used per
well, and as control for transfection effi-
ciency cells
were simultaneously cotransfected with 0·5 µg of a
plasmid expressing the E. coli lacZ gene from the
cytomegalovirus (CMV) immediate early promoter.
In order to determine the effects of SF-1/Ad4BP
and activated PKA on the bovine StAR gene
promoter, cells were additionally cotransfected with 1·5 µg of an expression construct for
bovine SF-1/Ad4BP (Morohashi et al. 1993) and/or
1·5 µg of an expression construct (RSV CHO PKA
C-beta) encoding a constitutively functioning PKA
catalytic subunit (Maurer 1989). As control for the
latter, this was substituted by a similar but inactive
vector with Lys72 mutated to Met (Maurer 1989).
The amount of these constructs transfected was
based on the results of similar transfection
experiments using the identical expression con-
structs to activate the human prolactin gene
promoter via the CREB/CREM (CRE modulator
protein) system (Telgmann et al. 1997). Forty-eight
hours after addition of the precipitates, cytoplasmic
extracts were prepared in 100 µl lysis buffer
(Luciferase Assay System; Promega) and extracts
stored at −20 °C until assayed. Luciferase activity
was measured using the Luciferase Assay System
(Promega) and β-galactosidase activity using the
Galacto-Light kit (Tropix, Bedford, MA, USA) in a
Berthold luminometer. All transfection exper-
iments were repeated at least three times, and
results from all experiments analysed statistically
by ANOVA.

RESULTS

Structural organization of the bovine StAR
gene

A comparison between the cDNA sequence
(Hartung et al. 1995) and the final genomic
sequence of the functional StAR gene confirmed the
preliminary analysis by restriction mapping, that
the functional bovine StAR gene is made up of
seven exons, organized as shown in Fig. 1, with the
intron splice acceptor and donor sites as listed in
Table 2. Full sequence details are deposited with
the international EMBL database (accession
number Y17260).

The sequence of the 5′ region upstream of the
transcription start site, as defined by primer
extension PCR (Hartung et al. 1995), is indicated in
Fig. 2. Consensus transcription factor binding sites,
including those for SF-1/Ad4BP are highlighted at
approximately −100, −240 and −1190 (numbered
from the transcription start site at +1), together
with the positions of the seven subfragments used
for the EMSA analysis.

EMSA

Using nuclear extracts from early–mid corpus
luteum and adrenal cortex, EMSA were performed
for seven successive fragments of the bovine StAR
gene promoter. Nuclear protein binding was evident
for most fragments (ig. 3). However, only fragments
1, 2 and 7 include consensus binding motifs for
SF-1/Ad4BP, as indicated by the loss of a shifted
band upon coincubation with an unlabelled SF-1/
Ad4BP-binding competing oligonucleotide. As con-
trol for the quality of the nuclear extracts, a short

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### Table 1. Oligonucleotide primers used for the PCR-reactions to produce the EMSA probes of the 5′ upstream region of bovine StAR gene

<table>
<thead>
<tr>
<th>Probe</th>
<th>Forward primer (5′–3′)</th>
<th>Reverse primer (5′–3′)</th>
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<tr>
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<td>4</td>
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<td>AGGGGATCTGCTGGAGG</td>
</tr>
<tr>
<td>5</td>
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<td>GGGAAAATAGTCCTTCG</td>
</tr>
<tr>
<td>6</td>
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<td>ACCCCCCAGACCAAGG</td>
</tr>
<tr>
<td>7</td>
<td>GAATCCCTCATCAGTTCCAGG</td>
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fragment from the bovine oxytocin promoter was used, which is known to bind both SF-1/Ad4BP as well as the transcription factor COUP-TF from these luteal nuclear extracts (Wehrenberg et al. 1994). In order to test for the possibility of SF-1/Ad4BP from the nuclear extracts binding to the StAR gene promoter, EMSA were repeated for fragments 1, 2 and 7, as well as the oxytocin promoter control (Fig. 4), competing for formation of the specific complexes with an SF-1-specific double-stranded oligonucleotide, as well as with a specific anti-Ad4BP antibody (Michael et al. 1995).

These experiments were repeated for the luteal nuclear extract, for a nuclear extract prepared from bovine adrenal cortex, and using bovine SF-1/Ad4BP in the form of an in vitro translation product (Fig. 4). Binding of nuclear proteins was observed for several of the promoter subfragments using both nuclear extracts. SF-1/Ad4BP, however, could only be detected by specific competition, binding significantly to subfragment 7 (arrow), and very weakly to subfragment 2 (arrow). There was no binding of an SF-1/Ad4BP-like protein to subfragment 1. It is notable that although both adrenal and luteal nuclear extracts contained a significant amount of SF-1/Ad4BP as judged by binding to the control oxytocin promoter fragment, only subfragment 7 of the StAR gene promoter indicated a similar quantitative binding of this transcription factor. The SF-1/Ad4BP-containing complexes were effectively competed not only by an excess of competing oligonucleotide, but also by the anti-SF-1/Ad4BP antibody, which additionally appeared to induce a supershift of the specific complex.

### Delimitation of SF-1/Ad4BP control of the bovine StAR gene by heterologous cotransfection in Hela cells

In order to gain insight into the functional responsiveness of the StAR gene promoter with respect to the SF-1/Ad4BP transcription factor, cotransfection experiments were performed in Hela cells, which are devoid of endogenous SF-1 (Sugawara et al. 1996), using different StAR gene promoter deletion constructs linked to a luciferase reporter gene, together with an expression construct for bovine SF-1/Ad4BP (Fig. 5). Experiments were repeated on three independent occasions, correcting for transfection efficiency by use of the additionally cotransfected β-galactosidase expression construct. The results were clear and consistent. Only when the promoter includes the SF-1/Ad4BP consensus binding motif is there reporter activity significantly above baseline, an activity which is largely dependent upon the cotransfection of the SF-1/Ad4BP expression construct (Fig. 5b). Maximal expression is obtained when the two proximal

<table>
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<th>Exon</th>
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<th>Sequence</th>
<th>Exon</th>
<th>Length (bp)</th>
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</tr>
<tr>
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<td>1862</td>
<td></td>
<td></td>
<td></td>
<td>v 3106</td>
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<td></td>
<td>v 7521</td>
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SF-1/Ad4BP consensus elements are included, with little (not statistically significant; ANOVA from three independent experiments) further increase upon inclusion of the more distal site, located within subfragment 7. It is interesting that even without the cotransfected SF-1/Ad4BP expression construct, there is a small increase in endogenous activation with the longer promoter constructs (Fig. 5b), though levels of activation are very low, by comparison with the GL3-C positive control. In two experiments, cells were additionally treated or not with the supposed ligand for mouse SF-1 (Lala et al. 1997), 25-OH-cholesterol. Particularly in non-steroidogenic host cells one would expect to see an effect of this steroidogenic metabolite. However, 25-OH-cholesterol was without effect in this heterologous assay system (not shown).

Since the mouse StAR gene appears to be regulated by activation of adenylate cyclase, additional transfection experiments were performed, wherein also a constitutive catalytic subunit of PKA was coexpressed together with the SF-1/Ad4BP transcription factor (Fig. 5c and d). A small but significant (ANOVA from three independent experiments) PKA-dependent increase in luciferase activity was detected upon cotransfection of the PKA construct, but only in the presence of SF-1/Ad4BP (Fig. 5d). Cotransfection of the mutant PKA construct had no effect. Using different deletion variants of the StAR gene promoter–reporter construct, this PKA- and SF-1/Ad4BP-dependent effect is evident most strongly following inclusion in these constructs of the first and second SF-1 response elements (Fig. 5d). The addition of the third, distal SF-1 element brought no further significant increase in reporter activity. Additional statistical analysis by ANOVA, combining all three replicate experiments, showed that the PKA effect, albeit small, was statistically significant for the constructs including the first (−203, Fig. 5; \( P<0.0001 \)) and second (−315, Fig. 5; \( P<0.001 \)) SF-1/Ad4BP consensus motifs, but not for the remaining constructs.

It has been suggested that components of the CREB/CREM pathway may interact with other transcription factors, such as nuclear steroid receptors, in the cAMP-dependent activation of genes (e.g. Morohashi et al. 1993, Smith et al. 1996). In order to determine whether there might be a possible involvement of an endogenous CRE-dependent component in the SF-1-mediated activation of the bovine StAR gene by PKA, EMSA experiments were carried out using a pure recombinant CREB protein which is specifically able to bind CRE motifs. Even though a corrupt CRE-like motif is apparent close to the second SF-1 cis

![Figure 3](image-url)
DISCUSSION

The StAR gene is actively transcribed in bovine steroidogenic tissues just as in other species, with high mRNA levels detectable in the testis, adrenal and ovarian corpus luteum (Hartung et al. 1995, Pescador et al. 1996). Also in accord with its described function in the mouse Leydig cell, there is a rapid response in StAR gene transcription in primary cultures of bovine adrenal cortical cells following adrenocorticotropic stimulation (Nicol et al. 1997). However, in vivo in the bovine ovary there appears to be a discrepancy between the upregulation of the StAR gene and other steroidogenic enzymes, mRNA for the former rising only several days after transcripts for P450\textsubscript{SCC} (Hartung et al. 1995, Pescador et al. 1996), and after the LH surge.

Like the genes for many steroidogenic enzymes, the StAR gene also appears to make use of the transcription factor SF-1/Ad4BP as part of its regulatory machinery. For the human gene, this has been shown by heterologous transfection of various StAR gene promoter constructs into BeWo choriocarcinoma cells; the two identified SF-1 \textit{cis} motifs responded to cotransfected murine SF-1 expression vectors, and SF-1 derived from transfected COS cells formed specific EMSA bands with both proximal and distal motifs (Sugawara et al. 1996). Interestingly, in this report, nuclear extracts from the murine Y1 cell line indicated SF-1-specific protein binding only with the distal element, but not or only very weakly, apparently with the proximal one. This result, however, could possibly be attributed to the use of mouse proteins with the human promoter. The mouse StAR gene promoter has also been studied (Caron et al. 1997), and in Y1 and MA10 homologous transfection studies using promoter deletion–reporter constructs, a proximal
SF-1-like binding motif at position -135 could be identified as instrumental in cAMP-dependent StAR gene upregulation. In the present study, we observed a similar discrepancy in the findings. The results of the transfection studies imply that, for the bovine StAR gene promoter, cotransfected bovine SF-1/Ad4BP is specifically acting via both of the proximal SF1-like motifs at -100 and -240, with little further stimulation resulting on addition of a further 1000 bp including the distal SF-1-like element at -1190. In contrast, assessment of specific binding of nuclear extracts from bovine corpus luteum and adrenal cortex, as well as pure bovine SF-1/Ad4BP, all indicated that the distal site showed most specific binding of SF-1, with a very weak binding also to the -240 site and none at all to the most proximal site at -100. It is also evident using the nuclear extracts from adrenal cortex and corpus luteum, that other nuclear proteins are binding close to the SF-1-like cis elements, but are evidently not related to this transcription factor.

Taking these results all together suggests that in vitro binding of a specific nuclear protein need not mean that such a protein is intimately involved in vivo. Alternatively, particularly heterologous transfection studies, or studies using abbreviated promoter regions need not imply that elements identified functionally in such tests are also involved under conditions of natural endogenous gene expression. Indeed, for other steroidogenic enzyme

FIGURE 5. Cotransfection experiments in Hela cells using deletion constructs of the bovine StAR gene 5′ upstream region linked to a luciferase reporter gene. (a) Schematic diagram of the promoter deletion–reporter constructs used. The SF-1/Ad4BP motifs are indicated by ellipses. (b) Effect of cotransfection (+Ad4BP) or not (−Ad4BP) of an expression vector encoding the full-length bovine SF-1/Ad4BP sequence. (c) Effect of cotransfection of an expression vector encoding a constitutive PKA catalytic subunit (+PKA) or an equivalent amount of a vector encoding a mutant enzyme (−PKA) in the absence of a cotransfected SF-1/Ad4BP expressing vector. (d) Effect of cotransfection of an expression vector encoding a constitutive PKA catalytic subunit (+PKA) or an equivalent amount of a vector encoding a mutant enzyme (−PKA) in the presence of a cotransfected SF-1/Ad4BP expressing vector. Units are given as relative light units detected (mean ± S.E.M.). GL3-B, the basic reporter vector. GL3-C, the same vector, but including the early immediate CMV promoter as positive control. The illustrated results are from a single experiment performed in triplicate. Similar experiments were performed altogether three times with essentially similar results, which were analysed together statistically by ANOVA.

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genes, similar discrepancies in active promoter regions have been identified using components from different species, or upon transfecting different cell types (Rodriguez et al. 1997). However, it is not known whether additional cofactors or protein modifications (e.g. phosphorylation), or some other aspect of the cellular environment used for transfection experiments, might not influence the sequence specificity of nuclear protein–DNA binding for SF-1/Ad4BP. In this context, it is important to note that very similar binding was found for pure bovine SF-1/Ad4BP as for this protein in the context of complete nuclear extracts of bovine adrenal gland and corpus luteum. This implies that the SF-1/Ad4BP in the nuclear extracts is not binding as part of a larger complex.

In order to gain more information on possible modifying factors, further cotransfection experiments were performed using, in addition to an SF-1/Ad4BP expression construct, one expressing a constitutive catalytic subunit of PKA. Since acute regulation of the StAR gene in vivo is principally via activation of adenylate cyclase, we decided to mimic this effect by using a constitutive downstream component of this pathway (PKA), as used successfully in other systems (e.g. Telgmann et al. 1997). The use of such a molecule rather than cAMP or some other more upstream component of this signal transduction pathway avoids the generation of effects by cross-talk, which are unrelated to PKA activation. This is especially important when using a heterologous cell system, such as the Hela cell. There is a small but statistically significant effect of this PKA addition on the StAR gene promoter–reporter activity, which was absolutely dependent upon the presence of expressed SF-1/Ad4BP. Since major targets of PKA in the context of gene regulation are the components of the CREB/CREM pathway, and since it has recently been shown that there may be interaction between CREB and SF-1/Ad4BP in the bovine CYP11A, CYP11B and aromatase genes (Morohashi et al. 1993, Michael et al. 1997), which may mediate specific cAMP-dependent gene activation, we additionally assessed the 1200 bp of the 5’ upstream region of the bovine StAR gene for the ability to bind CREB protein. Since these EMSA experiments proved negative, this would indicate that the effect of PKA on SF-1-mediated gene activation does not involve specific binding to a CRE-like motif, and may in fact be a direct effect on SF-1 itself, possibly involving the CREB-binding protein (Smith et al. 1996). Like the basal effects of SF-1/Ad4BP alone, also the PKA-dependent stimulation of the StAR gene promoter–reporter constructs involved the proximal, rather than distal SF-1-binding motifs, even though there was better binding of SF-1/Ad4BP protein to the distal element in vitro. In a recent discussion on cAMP-dependent activation of genes of the steroidogenic pathway in the adrenal gland, it was also concluded that such activation generally does not involve the CREB/CRE pathway (Waterman & Bischof 1997).

The mechanism of action of the transcription factor SF-1/Ad4BP is far from clear. It is expressed in all principal steroidogenic tissues, binding as a monomer to a specific consensus response element in the upstream promoter region of a number of genes. Its ablation in knock-out mice leads to a complete developmental loss of adrenal glands and gonadal tissues at an early embryonic stage (Luo et al. 1994). StAR gene expression parallels SF-1 expression in terms of both temporal and tissue specificity (Clark et al. 1995), and is absent in the SF-1 knock-out mouse (Caron et al. 1997). Although SF-1/Ad4BP has been shown to specifically mediate cAMP (Michael et al. 1995, Leers-Suchet et al. 1997) or phorbol ester (Bakke & Lund 1995) effects on several steroidogenic enzyme genes, including StAR (Sugawara et al. 1996), in vivo SF-1/Ad4BP expression appears to precede the expression of these genes, acting rather in a facilitatory manner. This would account for the observations that in luteinizing bovine follicular/luteal cells in vivo the P450_scc gene responds very acutely to activation of adenylate cyclase, whereas the oxytocin gene, which is also dependent upon SF-1/Ad4BP binding (Wehrenberg et al. 1994), requires some 16–24 h before being upregulated; and finally, the StAR gene is first apparent some days after this (Hartung et al. 1995, Pescador et al. 1996). Thus the expression of SF-1/Ad4BP-dependent genes would appear to be the result of multiple nuclear events in vivo, and we should expect there to be marked differences between the truly in vivo situation, the situation in endogenously expressing cells in culture, the heterologously transfected cell system, and the in vitro condition of protein–DNA binding. This complex situation is reflected by the observed ambiguities between the in vitro protein–DNA binding and the transfection experiments described in the present study. Some results similar to these were reported also for the human StAR gene (Sugawara et al. 1996).

Although the findings indeed appear to implicate SF-1/Ad4BP involvement in the activation of the bovine StAR gene, it could be speculated that such results are also explainable if in vivo another SF-1-like transcription factor mediates these effects at the proximal sites, and that this is mimicked by true SF-1/Ad4BP in the heterologous transfection systems. This would be supported by the generally
very weak effect of SF-1/Ad4BP on luciferase activity shown in the present experiments, well below what one would expect given the high cAMP-dependent in vivo expression. Further experimentation is essential to identify what other components besides SF-1/Ad4BP are essential for controlling the bovine StAR gene, or just what other members of the steroid receptor superfamily, which includes a number of orphan receptors like SF-1/Ad4BP, are expressed in such cells and could bind to the identified SF-1 cis motifs.

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