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

W Rust, K Stedronsky, G Tillmann, S Morley¹, N Walther and R Ivell

Institute for Hormone and Fertility Research, University of Hamburg, Grandweg 64, 22529 Hamburg, Germany

¹Department of Clinical Biochemistry, Edinburgh Royal Infirmary, Edinburgh, UK

(Requests for offprints should be addressed to R Ivell)

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-

ing to the two proximal SF-1/Ad4BP elements at –100 and –240, though with only low relative effect. Furthermore, additional cotransfection of a construct expressing a constitutive protein kinase A catalytic subunit to mimic the effects of cAMP stimulation, led to a small SF-1/Ad4BP-dependent increase in reporter activity mediated only by the same proximal sites. Since the bovine StAR gene promoter does not appear to have a functional cAMP responsive element (CRE), either this effect is mediated in this system directly by SF-1/Ad4BP, or by other factors interacting with this transcription factor, but which do not involve CRE-mediated gene activation. Taken together, the results show that there is a discrepancy between the results of the EMSA experiments and those using transfection of promoter-reporter constructs, which needs to be resolved before a clear understanding of SF-1/Ad4BP-mediated regulation of the StAR gene is attained.

Journal of Molecular Endocrinology (1998) **21**, 189–200

INTRODUCTION

One of the major rate-limiting steps in the production of steroids in steroidogenic tissues is the transport of the substrate cholesterol into the inner compartment of the mitochondrion, where it is made available to the principal enzymes of the steroidogenic pathway. Elaborate studies by Stocco and colleagues (Clark *et al.* 1994, Stocco & Clark 1996, Liu & Stocco 1997, Stocco 1997) have convincingly shown that one factor responsible for this transport is the steroidogenic acute regulatory protein (StAR). It is the interaction of this protein, encoded by a nuclear gene, with the mitochondrion,

which, presumably by establishing transient contact points between the outer and inner mitochondrial membrane, facilitates the transfer of cholesterol from one hydrophobic compartment to another. In the MA10 Leydig cell, it is the acute upregulation of this protein by the effector luteinizing hormone (LH) which is responsible for the rapid rise in testosterone production (Clark *et al.* 1994, Stocco & Clark 1996). This role is substantiated by the discovery that a mutation in the human StAR gene causes the disease congenital lipid adrenal hyperplasia, which is characterized by a major disruption of cholesterol transport and hence steroidogenesis (Lin *et al.* 1995). The genes for the mouse and

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-Suchet *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 polyadenylation sites, thus accounting for the different 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 P450_{SCC} 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'-CAGGCACTGTCTGTGTCC; 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 λ 1-3 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).

FIGURE 1. Schematic organization of the functional bovine StAR gene, indicating the approximate positions of the bacteriophage clones from which the sequence was derived.

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 [γ^{32} P]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 [35 S]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 μ g 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 μ l 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

FIGURE 2. The 5' upstream region of the bovine StAR gene indicating the positions of the three SF-1/Ad4BP motifs and the CRE-like sequence mentioned in the text, the subfragments used as probes in the EMSA analyses, and the restriction enzyme sites employed in the construction of the promoter deletion-reporter constructs used for the transfection assays. The numbering in the right-hand margin corresponds to that of the full gene sequence deposited in the EMBL database; numbering relative to the transcription start site is indicated above the sequence.

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 (BamI/PstI, -101/+6), one (HpaII/PstI, -203/+6), two (PstI/PstI, -315/+6 and KpnI/AvaI, -1009/+22) or all

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

Probe	Forward primer (5'–3')	Reverse primer (5'–3')
1	CCTTTCTGCACCTCCCTC	TAAATACCTCCAGCTGAAGGC
2	AGAGCCCACTCTAAACAGC	GGAAGGGAGGTTGATCCTG
3	GAGCACTCTACCTGGGC	TGCAGCTGCACCGCGCC
4	CACAAATGCCTCATCC	AGGGCATCTGCTGGGAG
5	TAAGACGCTGTGCTCCC	GGGAAACTAGTCCTTCG
6	CCGAGGGCCTGGGGAGG	ACCCCAGACCACCAGG
7	GGATCCTCATCGAGTTCAGG	CCACTTCAGAGCAGTTC

three (BamHI/AvaI, –1245/+22) of these consensus 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 efficiency 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 a full-length 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 constructs 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 experiments 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 (Fig. 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 control for the quality of the nuclear extracts, a short

TABLE 2. Exon-intron structure of the bovine stAR gene

exon 1 (199 bp)	2140 v AGCATGAAGGgtgagctccc	intron 1 (965 bp)	v 3106 gtttttggcagGGCTGCAGCA
exon 2 (113 bp)	3219 v TCTCTCCTAGgtaagagctg	intron 2 (270 bp)	v 3491 ttgcatccagGTTCTCAGCT
exon 3 (126 bp)	3617 v AGAGCCGGCAgtaagggggc	intron 3 (1886 bp)	v 5504 tcaccaacagGGCCAATGGG
exon 4 (159 bp)	5664 v GGAGATCAAGgtggggagag	intron 4 (118 bp)	v 5784 ggcctggcagGTCCTGCAGA
exon 5 (184 bp)	5968 v GTGTCATCAGgtaatactgg	intron 5 (726 bp)	v 6695 tacccggcagAGCGGAGCAC
exon 6 (93 bp)	6789 v TGACCTCAAGgtaaggccga	intron 6 (730 bp)	v 7521 ttgactctagGGATGGCTGC
exon 7 (1862 bp)			

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

FIGURE 3. EMSA for the subfragments #1 to #7, as indicated, of the 5' upstream region of the bovine StAR gene, using nuclear proteins extracted from bovine corpus luteum or bovine adrenal cortex. P, probe only, with bovine serum albumin; S, probe incubated with nuclear proteins; C, probe incubated with nuclear proteins and competed with an SF-1/Ad4BP specific oligonucleotide. OT-prom, EMSA analysis using the -160 site of the bovine oxytocin gene as positive control for SF-1/Ad4BP binding. The SF-1/Ad4BP protein-DNA complex formed from adrenal and luteal nuclear extracts with fragments #2 and #7 is arrowed.

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 4. EMSA analysing subfragments #1, #2 and #7 of the 5' upstream region of the bovine StAR gene. Bovine corpus luteum (left panel), bovine adrenal cortex (middle panel) and *in vitro* translated bovine SF-1/Ad4BP (right panel) were used as sources of nuclear protein. P, probe only; S, probe plus nuclear proteins; C, probe plus nuclear proteins competed with an SF-1/Ad4BP-specific oligonucleotide; A, probe plus nuclear proteins treated with a specific anti-SF-1/Ad4BP antibody. OT-prom, EMSA analysis using the -160 site of the bovine oxytocin gene as positive control for SF-1/Ad4BP binding. Note that a supershift is evident upon addition of the antibodies, particularly for the *in vitro* translated SF-1/Ad4BP with #7 and the OT-promoter fragments as probes. The specific SF-1/Ad4BP DNA-protein complex formed from adrenal and luteal nuclear extracts with fragments #2 and #7 is arrowed.

element (fragment 2; Fig. 2) in the StAR gene promoter sequence, neither this nor any other of the seven promoter fragments used for EMSA experiments was able to bind the CREB protein (not shown).

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 adrenocorticotrophin 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_{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 *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 $\pm$ 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.

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.

ACKNOWLEDGEMENTS

We are particularly grateful to Dr Ken-Ichirou Morohashi (Kyushu University, Japan) for the generous gift of the bovine Ad4BP-encoding cDNA clone as well as the anti-Ad4BP antibodies, to Dr Richard Maurer for the gift of the protein kinase A expression construct, to Drs Birgit Gellersen and Ralph Telgmann (IHF, Hamburg) for advice in regard to the protein kinase A experiments, and to Ms Moira Nicol (Edinburgh University) for provision of the adrenal tissue. We should also like to thank the Deutsche Forschungsgemeinschaft for financial support (grant Iv7/1-4).

REFERENCES

- Bakke M & Lund J 1995 Mutually exclusive interactions of two nuclear orphan receptors determine activity of a cyclic adenosine 3',5'-monophosphate responsive sequence in the bovine *CYP17* gene. *Molecular Endocrinology* **9** 327–339.
- Caron KM, Ikeda Y, Soo SC, Stocco DM, Parker KL & Clark BJ 1997 Characterization of the promoter region of the mouse gene encoding the steroidogenic acute regulatory protein. *Molecular Endocrinology* **11** 138–147.
- Clark BJ, Wells J, King SR & Stocco DM 1994 The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. *Journal of Biological Chemistry* **269** 28314–28322.
- Clark BJ, Soo SC, Caron KM, Ikeda Y, Parker KL & Stocco DM 1995 Hormonal and developmental regulation of the steroidogenic acute regulatory protein. *Molecular Endocrinology* **9** 1346–1355.
- Deryckere F & Gannon F 1994 A one-hour miniprep preparation technique for extraction of DNA-binding proteins from animal tissue. *BioTechniques* **16** 405.
- Gellersen B, Kempf R & Telgmann R 1997 Human endometrial stromal cells express novel isoforms of the transcriptional modulator CREM and up-regulate ICER in the course of decidualization. *Molecular Endocrinology* **11** 97–113.
- Hartung S, Rust W, Balvers M & Ivell R 1995 Molecular cloning and *in vivo* expression of the bovine steroidogenic acute regulatory protein. *Biochemical and Biophysical Research Communications* **215** 646–653.
- Honda SI, Morohashi KI, Nomura M, Takeya H, Kitajama M & Omura T 1993 Ad4BP regulating steroidogenic P-450 gene is a member of steroid hormone receptor superfamily. *Journal of Biological Chemistry* **268** 7494–7502.
- Kascheike B, Ivell R & Walther N 1997 Alterations in the chromatin structure of the distal promoter region of the bovine oxytocin gene correlate with ovarian expression. *DNA and Cell Biology* **16** 1237–1248.
- Lala DS, Syka PM, Lazarchik SB, Mangelsdorf DJ & Parker KL 1997 Activation of the orphan nuclear receptor steroidogenic factor 1 by oxysterols. *Proceedings of the National Academy of Sciences of the USA* **94** 4895–4900.
- Leers-Suchet A, Morohashi KI, Mason JI & Melner MH 1997 Synergistic activation of the human type II 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase promoter by the transcription factor steroidogenic factor 1/adrenal 4-binding protein and phorbol ester. *Journal of Biological Chemistry* **272** 7960–7967.
- Lin D, Sugawara T, Strauss JF, Clark BJ, Stocco DM, Saenger P, Rogol A & Miller WL 1995 Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. *Science* **267** 1828–1831.
- Liu Z & Stocco DM 1997 Heat shock-induced inhibition of acute steroidogenesis in MA-10 cells is associated with inhibition of the synthesis of the steroidogenic acute regulatory protein. *Endocrinology* **138** 2722–2728.
- Luo X, Ikeda Y & Parker KL 1994 A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. *Cell* **77** 481–490.
- Maurer RA 1989 Both isoforms of the cAMP-dependent protein kinase catalytic subunit can activate transcription of the prolactin gene. *Journal of Biological Chemistry* **264** 6870–6873.
- Michael MD, Kilgore MW, Morohashi KI & Simpson ER 1995 Ad4BP/SF-1 regulates cyclic AMP-induced transcription from the proximal promoter (PII) of the human aromatase P450 (*CYP19*) gene in the ovary. *Journal of Biological Chemistry* **270** 13561–13566.
- Michael MD, Michael LF & Simpson ER 1997 A CRE-like sequence that binds CREB and contributes to cAMP-dependent regulation of the proximal promoter of the human aromatase P450 (*CYP19*) gene. *Molecular and Cellular Endocrinology* **134** 147–156.
- Morohashi KI, Zanger UM, Honda SI, Hara M, Waterman MR & Omura T 1993 Activation of *CYP11A* and *CYP11B* gene promoters by the steroidogenic cell-specific transcription factor, Ad4BP. *Molecular Endocrinology* **7** 1196–1204.
- Nicol MR, Morley SD, Stirling D, Ivell R, Walker SW & Mason JI 1997 The expression of steroidogenic acute regulatory protein (StAR) mRNA in bovine adrenocortical cells. *Journal of Endocrinology* **152** (Suppl) P7.
- Parker KL & Schimmer BP 1997 Steroidogenic factor 1: a key determinant of endocrine development and function. *Endocrine Reviews* **18** 361–377.
- Pescador N, Soumano K, Stocco DM, Price CA & Murphy BD 1996 Steroidogenic acute regulatory protein in bovine corpora lutea. *Biology of Reproduction* **55** 485–491.
- Rodriguez H, Hum DW, Staels B & Miller WL 1997 Transcription of the human genes for cytochrome P450 scc and P450c17 is regulated differently in human adrenal NCI-H295 cells than in mouse adrenal Y1 cells. *Journal of Clinical Endocrinology and Metabolism* **82** 365–371.
- Smith CL, Onate SA, Tsai MJ & O'Malley BW 1996 CREB binding protein acts synergistically with steroid receptor coactivator-1 to enhance steroid receptor-dependent transcription. *Proceedings of the National Academy of Sciences of the USA* **93** 8884–8888.

- Stocco DM 1997 The steroidogenic acute regulatory (StAR) protein two years later: an update. *Endocrine* **6** 99–109.
- Stocco DM & Clark BJ 1996 Role of the steroidogenic acute regulatory protein (StAR) in steroidogenesis. *Biochemical Pharmacology* **51** 197–205.
- Sugawara T, Lin D, Holt JA, Martin KO, Javitt NB, Miller WL & Strauss JF 1995 Structure of the human steroidogenic acute regulatory protein (StAR) gene: StAR stimulates mitochondrial cholesterol 27-hydroxylase activity. *Biochemistry* **34** 12506–12512.
- Sugawara T, Holt JA, Kiriakidou M & Strauss JF 1996 Steroidogenic factor 1-dependent promoter activity of the human steroidogenic acute regulatory protein (StAR) gene. *Biochemistry* **35** 9052–9059.
- Telgmann R, Maronde E, Taskén K & Gellersen B 1997 Activated protein kinase A is required for differentiation-dependent transcription of the decidual prolactin gene in human endometrial stromal cells. *Endocrinology* **138** 929–937.
- Walther N, Wehrenberg U, Brackmann B & Ivell R 1991 Mapping of the oxytocin gene control region: identification of binding sites for luteal nuclear proteins in the 5' non-coding region of the gene. *Journal of Neuroendocrinology* **3** 539–549.
- Waterman MR & Bischof LJ 1997 Diversity of ACTH (cAMP)-dependent transcription of bovine steroid hydroxylase genes. *FASEB Journal* **11** 419–427.
- Wehrenberg U, Ivell R, Jansen M, Von Goedecke S & Walther N 1994 Two orphan receptors binding to a common site are involved in the regulation of the oxytocin gene in the bovine ovary. *Proceedings of the National Academy of Sciences of the USA* **91** 1440–1444.

REVISED MANUSCRIPT RECEIVED 7 May 1998