Three Zinc Finger Nuclear Proteins, Sp1, Sp3, and a ZBP-89 Homologue, Bind to the Cyclic Adenosine Monophosphate-Responsive Sequence of the Bovine Adrenodoxin Gene and Regulate Transcription†

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ABSTRACT: Adrenocorticotropin acting through cyclic adenosine monophosphate (cAMP) regulates transcription of the bovine adrenodoxin (Adx) gene in the adrenal cortex. The bovine Adx cAMP-responsive transcription sequence (CRS) has previously been found to contain two consensus GC boxes. By use of nuclear extracts from adrenocortical cells, Sp1 and Sp3 are shown here to bind to CRS. Mutations designed to enhance the identification of additional CRS binding proteins by reducing Sp protein binding showed the presence of an additional DNA-binding protein (Adx factor). Adx factor binding is inhibited by the zinc-chelating agent, 1,10-*o*-phenanthroline, suggesting it might be a zinc finger protein. By a fractionation/renaturation technique the Adx factor in mouse Y1 adrenocortical cells was found to be in the size range of 106−115 kDa by gel mobility shift assay. On the basis of size, the CRS sequence to which it binds, and its tentative identification as a zinc finger protein, Adx factor has been identified as a Krüppel-like zinc finger protein (a mouse ZBP-89 homologue). Further mutagenesis of CRS demonstrates that it can further be divided into two similar cAMP-responsive elements, and elimination of ZBP-89 binding does not affect cAMP responsiveness of either. Expression of these three nuclear proteins in *Drosophila* SL2 cells has been used to decipher the role of Adx CRS binding proteins in regulating transcription. Sp1 and Sp3 confer basal transcriptional activities, yet only Sp1 confers cAMP-responsive activity. ZBP-89 represses basal transcriptional activity.

The mammalian two-iron two-sulfur, soluble mitochondrial electron transport ferrodoxin was originally discovered in the adrenal cortex and named adrenodoxin (Adx)[1] (*1*). Electrons from NADPH are shuttled through a FAD-containing flavoprotein (Adx reductase) to Adx and subsequently to mitochondrial P450 monooxygenases (*2*). Adx is ubiquitously expressed to support the activities of mitochondrial P450s. In the adrenal cortex, mitochondrial P450s are predominantly the steroid hydroxylases P450scc (cholesterol side-chain cleavage cytochrome P450), P450aldo (aldosterone synthase cytochrome P450), and P450c11 (steroid 11β-hydroxylase cytochrome P450), with P450scc being the major form in ovary and testis. In kidney, 1α-hydroxylase of 25-hydroxyvitamin D_3 is the major mitochondrial P450, while in other tissues, including the liver, cholesterol C27 hydroxylase (P450c27) is the predominant form.

Adx is encoded by the nuclear genome, its precursor protein being proteolytically processed upon uptake into this organelle (*3*, *4*). Two distinct mRNA transcripts differing only at their 5′-sequences are derived from the bovine Adx gene (*5*). Examination of the gene has shown that exon 1′ encodes one leader peptide sequence, exon 1 encodes another, and exons 2, 3, and 4 encode mature Adx (*6*) (see Figure 1). The leader peptide sequence encoded by exon 1′ contains an in-frame stop codon and cannot be translated into a functional protein (*6*, *7*). Moreover, this transcript accounts for only 10% of the total Adx mRNA in bovine adrenocortical cells. Fully 90% of the bovine Adx mRNA begins within exon 1.

The levels of both transcripts in the adrenal cortex are enhanced transcriptionally via cAMP in response to ACTH (*8*). The major transcript is under control of a promoter within intron 1′, which contains its own unique cAMP-responsive sequence [CRS (*9*), different from the consensus CRE]. Two consensus Sp1-binding sites are found within the CRS. Sp1 has been shown to be involved in cAMP responsiveness of other bovine steroidogenic genes. For example, in CYP11A (P450scc) CRS1 consists only of a binding site for Sp1 (*10*, *11*), and CRS2 in the same gene binds Sp1 and the orphan receptor SF-1 (*12*). CRS of CYP21 (P450c21) contains overlapping binding sites for Sp1 and an uncharacterized nuclear protein named ASP (*13*). There are other examples

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[1] Abbreviations: Adx, adrenodoxin; ACTH, adrenocorticotropin; CRS, cyclic adenosine monophosphate-responsive sequence; CRE, cyclic adenosine monophosphate-responsive element; EMSA, electrophoretic mobility shift assay; PKA, cAMP-dependent protein kinase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); bp, base pair(s); kbp, kilo base pair(s); NADPH, nicotinamide adenine dinucleotide phosphate, reduced; FAD, flavin adenine dinucleotide.

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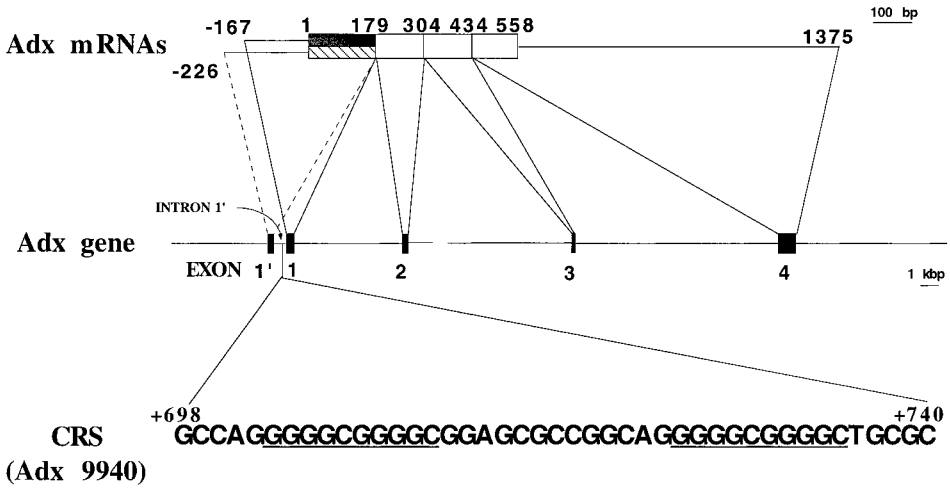

FIGURE 1: Physical map of the bovine adrenodoxin gene, its correspondence with mRNA transcripts, and the major cAMP-responsive sequence. This map is modified from Sagara et al. (*7*). Closed boxes indicate the exons of the Adx gene. Shaded and hatched regions indicate the signal peptide regions of the major and minor mRNAs, respectively. The unique cAMP-responsive sequence examined in this study is located between exon 1′ and exon 1 indicated here as Adx 9940 (*9*).

Chart 1

cctcga**GCCAGGGGGGCGGGGCGGAGCGCCGGCAGGGGGGCGGGGCTGCGC**g
tcgaggagct**CGGTCCCCCGCCCCGCCTCGCGGCCGTCCCCCGCCCCGACGCG**cagct

of genes that contain Sp1-binding sites close to or within a cAMP-responsive sequence. These include those encoding human urokinase (*14*), type IIβ cAMP-dependent protein kinase regulatory subunit (*15*), and human surfactant protein A (*16*). Sp1 is generally considered to be a ubiquitously expressed transcription factor involved in basal transcription of a wide number of genes in different tissues. The evidence for its direct role in cAMP-mediated transcription has not been clearly established. Recently, Rohlff et al. (*17*) reported that both the trans-activating and DNA binding properties of Sp1 in doxorubicin-resistant HL-60 (HL-60/AR) leukemia cells are stimulated by cAMP-dependent protein kinase (PKA). Their results strongly indicate that Sp1 can function as a cAMP-responsive transcription factor. However, in many cells, such as adrenocortical cells, it remains unclear whether Sp1-dependent genes are modulated by the ACTH-activated cAMP-dependent signaling pathway. Sp1 is a member of a multigene family of nuclear proteins (Sp1, Sp2, Sp3, and Sp4) that bind to GC- or GT-rich elements (*18, 19*). Sp1, Sp3, and Sp4 have similar structural features, the DNA binding domain of these three proteins being highly conserved. Contrary to these factors, Sp2 has different DNA binding specificities (*19*). In this study, we show that the GC-rich sequence (CRS) within intron 1′ of the bovine Adx gene binds Sp1 and Sp3 in adrenocortical cells. In addition, we demonstrate the existence of an additional CRS-binding protein, a Krüppel-like zinc finger protein [a mouse homologue of rat ZBP-89 (*20*)]. The roles that these three nuclear proteins play in regulating both the basal and cAMP-dependent transcription of the bovine Adx gene have been characterized in *Drosophila* SL2 cells and mouse Y1 adrenocortical cells.

EXPERIMENTAL PROCEDURES

Oligonucleotides and Plasmid Construction. The +698/+740-bp sequence (CRS) within intron 1′ of the bovine Adx gene called Adx 9940 (*9*) was made from synthetic oligonucleotides (Chart 1). The staggered ends were designed to enable the insertion of the oligonucleotide into *Sac*I and *Sal*I restriction enzyme sites of the OVEC reporter plasmid (*21*). We have used this plasmid, which contains the rabbit β-globin reporter gene, in several studies of CRS elements in steroidogenic genes (*22*). Mutant forms of Adx 9940 (Tables 1 and 2) were also made from synthetic oligonucleotides. The plasmids OVEC and SV40-OV were kind gifts from Drs. Thomas Gerster and Walter Schaffner (*21*). The plasmid 4xCRE-OV, which contains four tandemly repeated cAMP-responsive elements of the human chorionic gonadotropin-α gene fused to the OVEC β-globin sequence, was made by Dr. Johan Lund (*22*). The plasmids Adx 9940-OV, Adx GCM-OV, Adx V-OV, and Adx CRS-1−Adx CRS-6 were prepared by the insertion of Adx 9940, Adx GCM, Adx V, and Adx CRS-1−Adx CRS-6 oligonucleotides (Tables 1 and 2) upstream from the minimal promoter of the OVEC β-globin gene. The plasmid Adx 4V-OV, containing two copies of the Adx 2V sequence (Table 1), was made

Table 1: Wild-Type and Mutant cAMP-Responsive Sequences

Adx 9940	GCCAGGGGGCGGGGCGGAGCGCCGGCAGGGGGCGGGGCTGCGC
Adx GCM	GCCAGGGGG**TA**GGG**T**GGAGCGCCGGCAGGGGG**TA**GGGCTGCGC
Adx V	CAGGGGG**TA**GGGCTGC
Adx 2V	CAGGGGG**TA**GGGCTGCgtcgagCAGGGGG**TA**GGGCTGC
Adx L-1	GCCAGGGGGCGGGGCGGAGC
Adx M-1	GGCAGGGGGCGGGGCTGCGC
Adx M-2	GGCA**C**GGGGCGGGGCTGCGC
Adx M-3	GGCAGG**TT**CGGGGCTGCGC

Table 2: Wild-Type and Mutant cAMP-Responsive Sequences

Adx 9940	GCCAGGGGGCGGGGCGGAGCGCCGGCAGGGGGCGGGGCTGCGC
AdxCRS-1	GCCA**C**GGGGCGGGGCGGAGCGCCGGCA**C**GGGGCGGGGCTGCGC
AdxCRS-2	GCCAGGG**TT**CGGGGCGGAGCGCCGGCAGG**TT**CGGGGCTGCGC
AdxCRS-3	GCCAGG**TT**CGG**TT**CGGAGCGCCGGCAGG**TT**CGGGGCTGCGC
AdxCRS-4	GCCAGG**TT**CGGGGCGGAGCGCCGGCAGGGGGCGGGGCTGCGC
AdxCRS-5	GCCAGGGGGCGGGGCGGAGCGCCGGCAGG**TT**CGGGGCTGCGC
AdxCRS-6	GCCAGG**TT**CGG**TT**CGGAGCGCCGGCAGGGGGCGGGGCTGCGC

according to the scheme described by Westin et al. (*21*). The 9940-wt-luc contains the region between +691 and +808 of the bovine Adx gene fused to the firefly luciferase gene in the pGL3 vector (Promega Corp.). This region includes Adx 9940 and the bovine Adx promoter. The 9940-mut-luc contains the same region as 9940-wt-luc except for the mutations introduced into Adx 9940 as AdxCRS-3 (Table 2). Plasmids pPac, pPacSp1, and pPacSp3 were obtained from Dr. G. Suske.

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared according to Dignam et al. (*23*). Double-stranded oligonucleotides were labeled by polynucleotide kinase and [γ-^{32}P]ATP (6000 Ci/mmol, NEN) or by a fill-in reaction with [α-^{32}P]dCTP (3000 Ci/mmol, NEN) and DNA polymerase I, Klenow fragment. Five micrograms of nuclear extract protein was mixed with 15 μL of binding buffer (20 mM Hepes, pH 7.9, 80 mM KCl, 5 mM $MgCl_2$, 2% Ficoll, 5% glycerol, 0.1 mM EDTA, and 0.2 mM DTT), 0.5 μg of poly(dIdC), and the ^{32}P-labeled probe (5000 cpm) on ice. For competition assay, a 50- or 500-fold excess of unlabeled competitor oligonucleotide was used along with the labeled probe. In the case of antibody supershift experiments, the incubation conditions were identical except that the indicated amount of antibodies was preincubated with nuclear proteins from Y1 cells in the presence of binding buffer for 10 min prior to addition of the other components. The DNA−protein complexes were resolved by electrophoresis on a 4% polyacrylamide/0.5% Ficoll/0.5× TBE gel and visualized by autoradiography. The recombinant protein corresponding to full-length BFCOL1 (gift from Dr. T. Hasegawa) was generated by use of the TnT-coupled reticulocyte lysate system (Promega Corp.). Anti-Sp1, anti-Sp2, and anti-Sp4 were purchased from Santa Cruz Biotech (Santa Cruz, CA). Anti-Sp3 was a generous gift from Dr. G. Suske, and anti-ZBP-89 raised againt amino acids 1−521 of rat ZBP-89 (*24*) was from Dr. J. L. Merchant. This antibody does not cross-react with Sp family proteins.

Cell Culture, Transient Transfection, S1 Nuclease Protection Analysis, and Luciferase Assay. Mouse adrenocortical tumor Y1 cells were cultured in Dulbecco's modified Eagle's medium (Gibco) containing 10% bovine calf serum. The cells were transfected with 20 μg of $CsCl_2$-purified OVEC plasmid by the calcium phosphate precipitation method (*25*). The following day, cells were incubated in the presence or absence of 25 μM forskolin for 8 h and RNA was isolated and then analyzed for transcription of rabbit β-globin by S1 nuclease protection assay (*26*). The intensities of signals were quantified by cutting them from the gel and determining radioactivities by scintillation counting. *Drosophila* SL2 cells were cultured at 25 °C in Schneider medium (Sigma) supplemented with 10% fetal calf serum (Gibco). SL2 cells were also transfected by the calcium phosphate precipitation method. The luciferase assay was performed with a luciferase assay kit as described by the manufacturer (Promega).

Fractionation of Proteins from Nuclear Extract by SDS−PAGE and Elution. 200 μg of nuclear extract proteins were fractionated on a 1.5-mm thick SDS−7% polyacrylamide gel. Proteins were transferred to a poly(vinylidene difluoride) (PVDF) membrane (Immobilon-P, Millipore) with a semidry electroblotting apparatus (Gelman), according to the manufacturer's instructions. Once transferred, proteins were recovered and renatured from 2-mm slices of PVDF membrane by soaking in 100 μL of buffer (containing 25 mM Hepes, pH 7.9, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 1% Triton X-100, and 0.1 mg/mL bovine serum albumin) for 2 h at 4 °C with vigorous shaking (*27*). These fractions were then ready for analysis in EMSA experiments with 5 μL of each elution fraction.

RESULTS

cAMP-Responsive Sequence and the Adx Factor. The aim of this study is to identify and functionally characterize the factors responsible for cAMP-dependent transcription of the bovine Adx gene. Previously, we have found that the sequence between +698 and +740 (named Adx 9940, Figure 1) is sufficient to direct cAMP-dependent expression of reporter genes in mouse Y1 adrenocortical tumor cells (*9*). This region contains two consensus GC boxes (Sp1 binding sites). To determine whether other nuclear factors might bind to this sequence and participate in cAMP-responsive transcription, we designed a mutated form of Adx 9940 (Adx GCM, Table 1). The plan was to mutate vital nucleotides of the GC box and thus weaken Sp1 binding in order to more readily observe other nuclear DNA-binding proteins. Figure 2A compares the gel mobility shifts of Adx 9940 and Adx GCM. A complex binding pattern of nuclear proteins is observed with the Adx 9940 sequence (Figure 2A, lane 1). Most of these nuclear proteins are GC box binding proteins because a large portion of the protein−DNA complexes can be competed by a 500-fold excess of the consensus GC box oligonucleotide (Figure 2A, lanes 4 and 5). However, some nuclear protein binding cannot be competed even with this large excess of the GC box oligonucleotide (Figure 2A, lane 5). When the Adx GCM oligonucleotide was used as a probe, a doublet of protein−DNA complexes was observed (Figure 2A, lane 6). The upper band can be competed by the GC box oligonucleotide; however, the lower band (Adx factor) cannot (Figure 2A, lane 10). This lower band also remained when the Adx 9940 probe was competed with the GC box oligonucleotide (Figure 2A, lane 5). The Adx V and Adx 2V oligonucleotides (Table 1) were also used to detect Adx factor binding activity and clearly show its binding. When EMSA patterns were compared between Adx 9940 and mutated forms of Adx 9940 (Adx GCM, Adx V, and Adx 2V), Adx factor binding is more readily detected in mutated CRS sequences where Sp1 binding is dramatically reduced (Figure 2A).

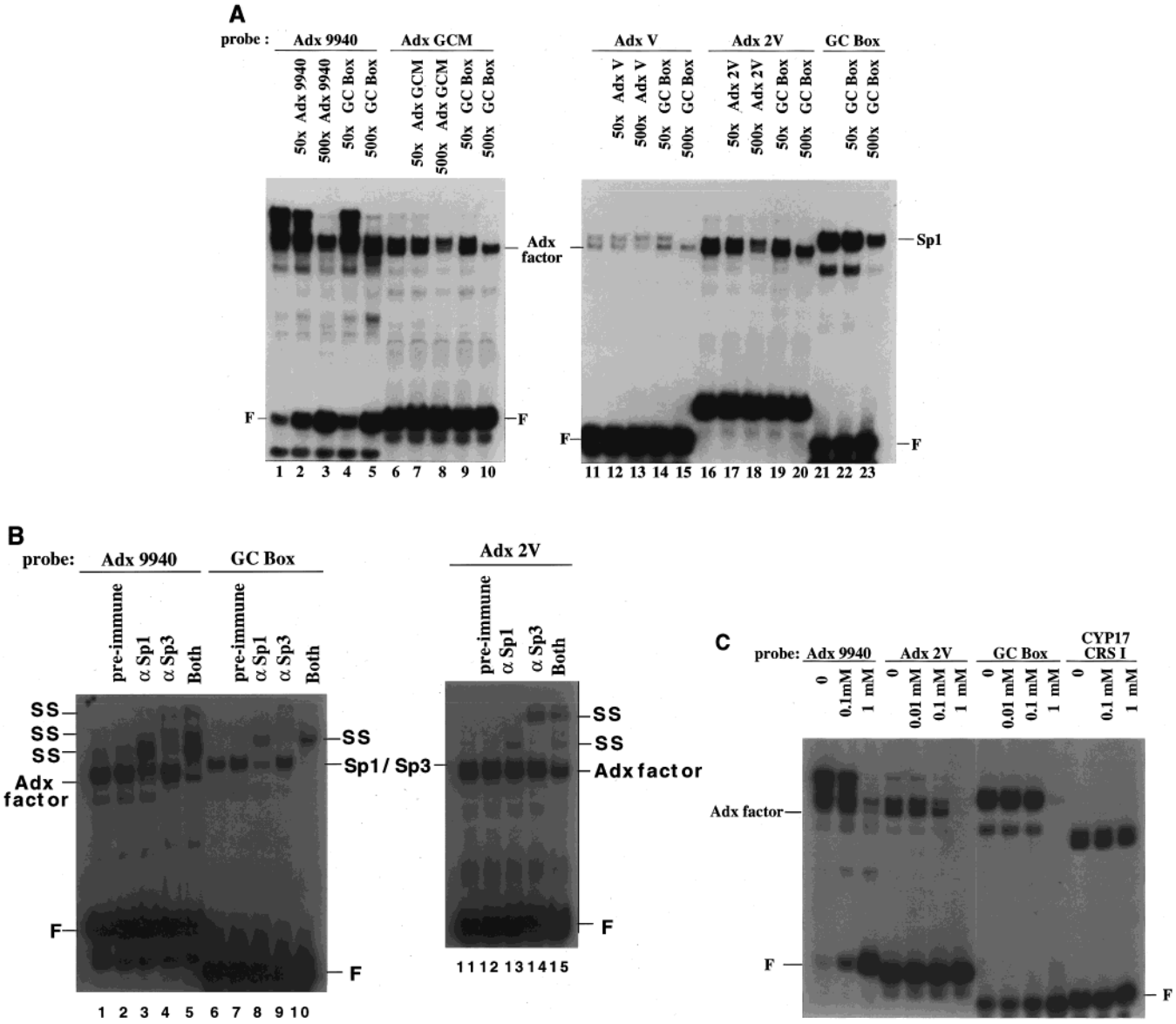

FIGURE 2: EMSA revealing that the zinc finger proteins Sp1, Sp3, and Adx factor are the Y1 nuclear proteins binding to Adx CRS. (A) ^{32}P-Labeled double-stranded oligonucleotides were incubated with 5 μg of Y1 nuclear proteins. In competition assays, a 50- or 500-fold molar excess of unlabeled oligonucleotides was added along with the probe. (B) Polyclonal Sp1 antibody (αSp1, 0.5 μg) or 1 μL of polyclonal Sp3 antibody (αSp3) was incubated with 0.5 μg of Y1 nuclear proteins (5 μg of Y1 nuclear proteins was used for Adx 2V probe). (C) ^{32}P-Labeled double-stranded oligonucleotides were incubated on ice with 5 μg of Y1 nuclear proteins and the indicated concentrations of the zinc chelator 1,10-*o*-phenanthroline for 1 min. F, free probe; SS, supershift.

It has been reported that there are several Sp nuclear proteins which bind to the consensus GC box. To investigate which Sp nuclear proteins in Y1 nuclear extracts bind to this region, we used antibodies against Sp2, Sp3, and Sp4 to test for supershifts. With Sp2 and Sp4 antibodies, no changes in binding were observed (data not shown). A weak supershift was detected with anti-Sp3 (Figure 2B, lanes 4, 9, and 14). The combination of Sp1 and Sp3 antibodies completely disrupts the Sp nuclear protein−DNA complexes on Adx 9940 or Adx 2V, leaving only the complex containing the Adx factor (Figure 2B, lanes 5 and 15). Thus Adx factor is different from the known Sp factors. To determine if Adx factor like Sp factors is a zinc finger protein, the zinc-chelating agent 1,10-*o*-phenanthroline (*28*) was used. This agent can remove zinc ions from zinc finger proteins, abolishing binding activity. Incubation of Y1 nuclear extracts with increasing concentrations of this chelator impaired the formation of protein−DNA complexes formed with Adx 9940, Adx 2V, and GC box probes (Figure 2C). In comparison, complexes formed with the CYP17 CRS I probe [a cAMP-regulatory sequence associated with the bovine CYP17 gene (*22*)] were not affected by this chelating agent. This latter result is expected because the nuclear proteins bound to CYP17 CRS I are homeodomain proteins, which do not require metal ions for DNA binding (*29*, *30*). We conclude that the Adx factor requires metal for DNA binding and is probably a zinc finger protein due to the similar effects of the zinc-chelating agent on Adx factor- and Sp1 (or Sp3) complexes with DNA.

cAMP-Dependent Transcriptional Activities of Adx 9940 and the Mutated Sequences. To study the ability of Adx factor to confer cAMP-dependent transcriptional activity, oligonucleotides in Table 1 were subcloned into the OVEC vector containing the minimal promoter and reporter gene

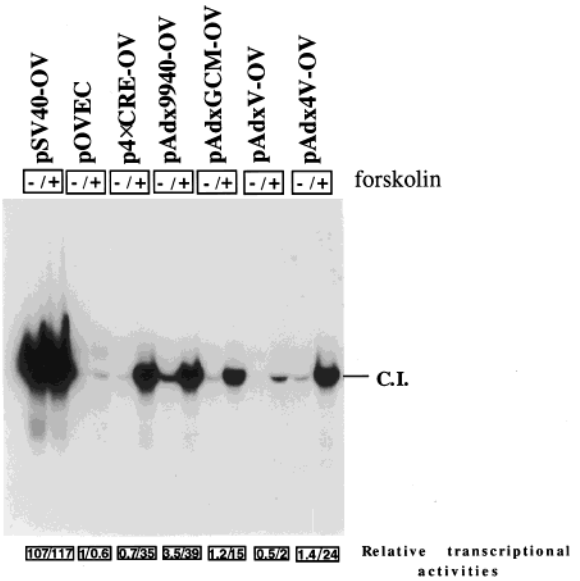

FIGURE 3: cAMP stimulates transient expression of the rabbit β-globin reporter gene in recombinant OVEC plasmids containing the Adx factor binding site. To determine the relative cAMP-stimulated transcriptional activities of sequences in Table 1, they were subcloned into the OVEC vector. Mouse Y1 cells were transfected with the recombinant OVEC plasmids and incubated in the presence and absence of 25 μM forskolin for 8 h. Cytoplasmic RNA was isolated and analyzed by S1 nuclease protection assay followed by electrophoresis on a 10% polyacrylamide−7.5 M urea sequencing gel. A radiolabeled 93-mer oligonucleotide probe was used in this assay. The protected 75-mer fragment (C. I.) represents the correctly initiated transcripts derived under the control of the β-globin promoter. This is a representative data set of three experiments.

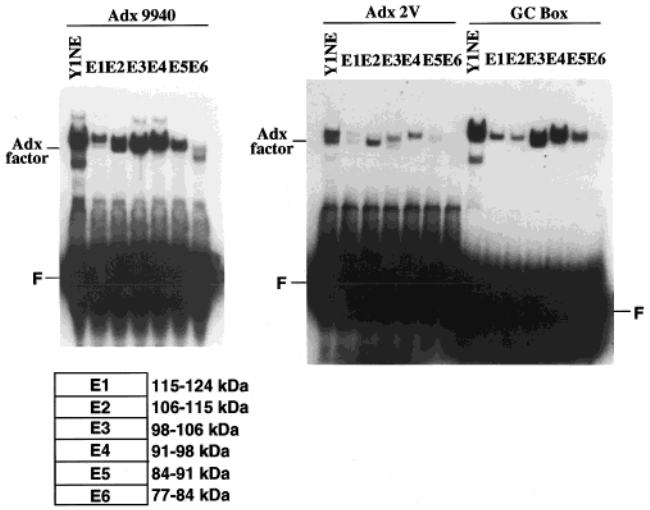

FIGURE 4: Use of a SDS fractionation and renaturation to examine the size of the Adx 9940-binding proteins in Y1 nuclear extracts. Following electrophoresis and transfer of the proteins to a PVDF membrane, the membrane was cut into six 2-mm slices (E1−E6, molecular mass 124−77 kDa) each containing proteins of distinct molecular weights. DNA-binding properties of proteins from each slice were analyzed by EMSA. F, free probe.

of rabbit β-globin. Mouse Y1 cells were transfected with the OVEC plasmids and treated with forskolin, which raises intracellular cAMP levels. When Adx 9940-OV was transfected into Y1 cells, an 11-fold increase in correctly initiated globin transcripts was observed upon forskolin treatment (Figure 3). Transfection with 4×-consensus CRE-OV leads to approximately a 50-fold increase upon forskolin treatment. Transfection with OVEC alone results in very low transcript levels, in either the presence or absence of forskolin (Figure 3). The plasmid SV40-OV in which the SV40 enhancer is inserted upstream of the minimal globin promoter was used as a positive transcription control, and this plasmid yielded high levels of globin transcripts in a cAMP-independent fashion. Transfection of Adx GCM-OV, Adx V-OV, and Adx 4V-OV into Y1 cells resulted in 12-, 4-, and 17-fold increases upon forskolin treatment, respectively, relative to untreated cells (Figure 3).

Use of SDS−PAGE Fractionation to Determine the Size of the Adx 9940-Binding Proteins. A SDS−PAGE fractionation technique (*27*) was used to separate nuclear proteins according to size and detect their DNA-binding activities by EMSA after renaturation. Y1 nuclear proteins were first denatured and subjected to SDS−PAGE. Following electrophoresis, the proteins were transferred to PVDF membrane and the membrane was cut into six 2-mm slices (E1−E6, molecular mass 124−77 kDa), each containing proteins of distinct molecular sizes. Proteins from each slice were eluted in separate microfuge tubes, and DNA-binding activity was analyzed after renaturation. When Adx 9940 was used as a probe, all fractions show DNA binding (Figure 4), although the signal with fraction E6 is very weak. Except for the lower DNA−protein complex of fractions E2 and E6, all complexes were competed by the consensus GC box oligonucleotide (data not shown). Similar but weaker gel mobility shift patterns were shown for each fraction with the GC box as a probe, except that the lower band of fraction E2 is not observed (Figure 4). By use of Adx 2V as a probe, the Adx factor was detected in fraction E2 (Figure 4). When we taped the slices of the PVDF membrane back into the original membrane and carried out Western blotting, we detected Sp1 in fractions E3 and E4 but not in E2 (data not shown). Thus, the Adx factor is localized to fraction E2 having a molecular mass range of 106−115 kDa.

Identification of Adx Factor. Examination of the literature and databases identified three candidates for Adx factor according to size (106−115 kDa), the CRS sequence to which it binds, and its tentative identification as a zinc finger protein (Figure 2C). One is rat ZBP-89 (*20*), a Krüppel-like zinc finger protein, which binds to a GC-rich element of the human gastrin promoter. The second is BERF-1 (*31*), a mouse homologue of ZBP-89 which is 99% identical to ZBP-89 at the amino acid level and which binds to a G-rich box within the muscle-specific enhancer of the β enolase gene. The third is BFCOL1 (*32*), a mouse homologue of ZBP-89 that uses a different initiator methionine and therefore is 25 amino acids shorter than ZBP-89 or BERF-1. BFCOL1 is 99% identical to the corresponding region in BERF-1 and was identified as a transcription factor binding to the promoters of the type I collagen genes. We used antiserum against ZBP-89 to perform supershift analyses and found that the gel shifts of the Adx factor with Adx 9940 or Adx 2V disappeared (Figure 5A). By contrast, this antiserum had no effect on Sp1 and Sp3 binding (Figure 5A). When a combination of antisera against Sp1, Sp3, and ZBP-89 was used, the gel shift with Adx 9940 probe completely disappeared (Figure 5B). This result clearly shows that Sp1, Sp3, and a mouse ZBP-89 homologue are the three components of Adx 9940-binding proteins.

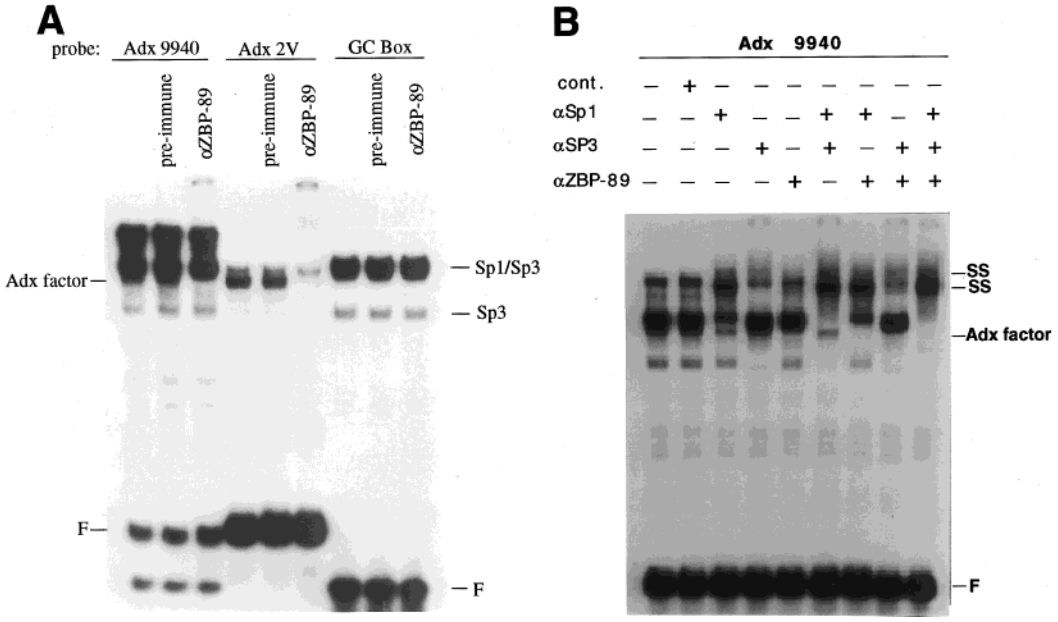

FIGURE 5: Identification of ZBP-89 as Adx factor. (A) αZBP-89 or preimmune IgG fraction (5 μg) was incubated with 5 μg of Y1 nuclear proteins, and EMSA was carried out. (B) Y1 nuclear proteins (1 μg) were used to perform EMSA. The presence of specific antibodies in the binding reaction is indicated above each lane. F, free probe.

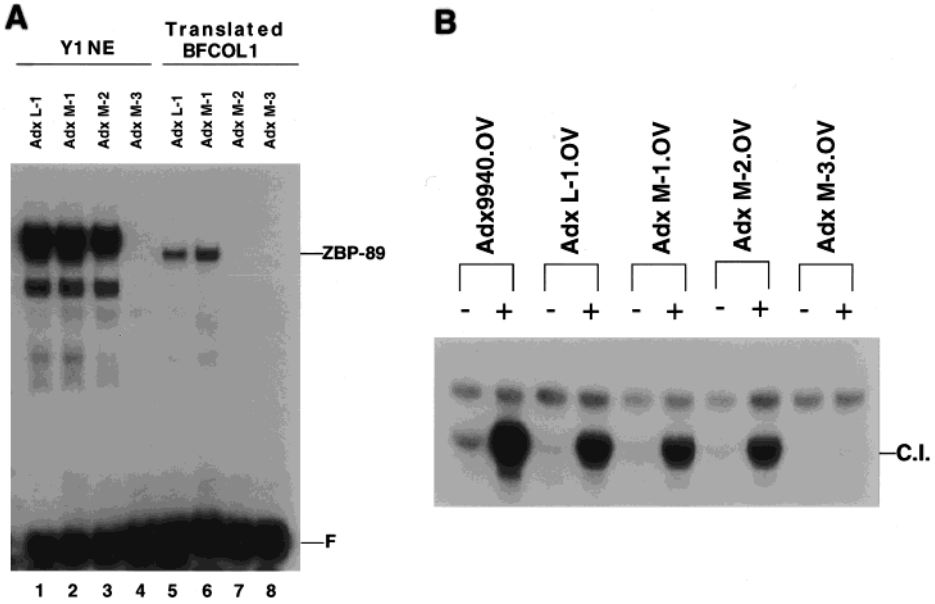

FIGURE 6: Determination of binding activities and cAMP responses of regions within mutants of Adx 9940. (A) Labeled probe was incubated with Y1 nuclear extract (5 μg) on ice or with in vitro-translated BFCOL1 at room temperature for 20 min. F, free probe. (B) cAMP-stimulated transient expression of the rabbit β-globin reporter gene in recombinant OVEC plasmids. The assay was performed as described in Figure 3. C. I., correctly initiated transcripts.

Mutagenesis of cAMP Responsive Sequence. To evaluate the role of Sp factors and ZBP-89 on the regulation of the bovine Adx gene, further mutagenesis of CRS was performed. Adx 9940 was divided into two similar GC-rich elements (Table 1, Adx L-1 and Adx M-1), and Adx M-1 was chosen for further mutation. By comparison of Adx M-1 with the consensus GC box, five consecutive G nucleotides (or C) may be important for ZBP-89 binding. On the basis of this assumption, Adx M-2 was prepared with one G changed to C. The two G nucleotides next to the central C nucleotide of a GC box are important for Sp factor binding (*33*). Mutation of these nucleotides resulted in Adx M-3. Figure 6A shows that Adx L-1 and Adx M-1 share similar gel-shift patterns with Y1 nuclear extract. Antibodies against Sp1, Sp3, and ZBP-89 show that all three can bind to both oligonucleotides (data not shown). Adx M-2 shows lower signal intensity (Figure 6A, lane 3), because ZBP-89 no longer binds (Figure 6A, lane 7). Adx M-3 eliminates all binding (Figure 6A, lane 4). Thus, the two G nucleotides next to the central C nucleotide of a GC box are essential not only for Sp factor binding but also for ZBP-89 binding. Binding assays with in vitro transcribed/translated BFCOL1 confirmed this. ZBP-89 only binds to Adx L-1 and Adx M-1 but not to Adx M-2 and Adx M-3 (Figure 6A). These oligonucleotides were also introduced into the OVEC vector to study their ability to confer cAMP-dependent transcriptional activity (Figure 6B). Adx L-1 and Adx M-1 show similar cAMP-dependent transcriptional activity, which is

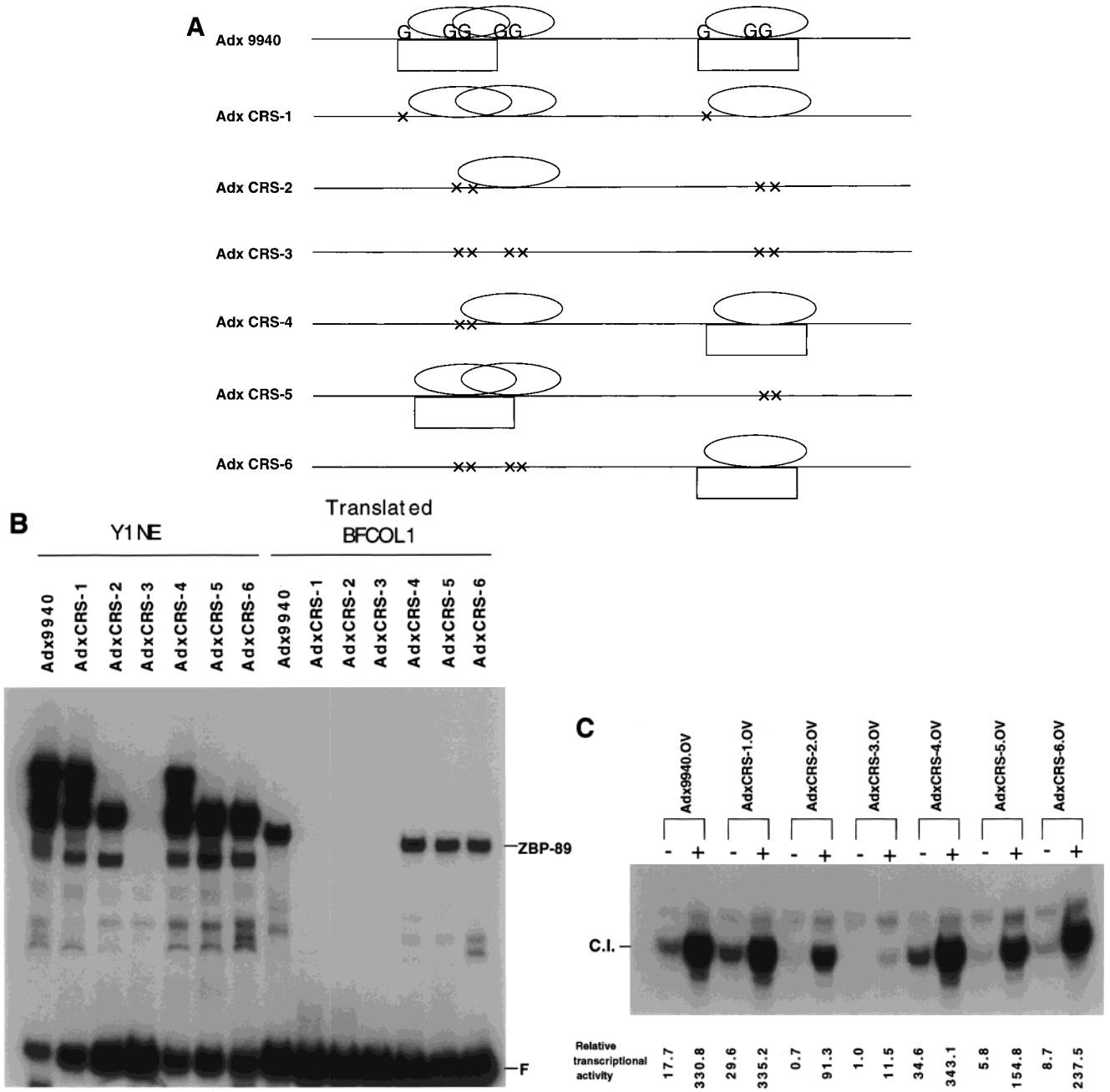

FIGURE 7: Binding activities and cAMP responses of wild-type and mutant CRS elements. (A) Putative binding activities of wild-type and mutant Adx 9940 elements. Oval indicates Sp1; rectangle, ZBP-89. In Adx 9940, G indicates residues mutated to C or T (see Table 2); ×, mutation. (B, C) Determination of binding activities and cAMP responses of wild-type and mutant Adx 9940 elements.

consistent with similar binding properties. Each one has less response than Adx 9940. Interestingly, Adx M-2 confers the same cAMP response as Adx M-1, though ZBP-89 can no longer bind to this oligonucleotide. This indicates that Sp factors can independently confer cAMP responsiveness through Adx M-2. Adx M-3 eliminates all binding and therefore no significant cAMP response was detected.

Extensive mutagenesis was applied to Adx 9940, six mutants being synthesized (AdxCRS-1−AdxCRS-6, Table 2). AdxCRS-1 was designed to eliminate ZBP-89 binding. AdxCRS-2 was designed to disrupt both functional CRS elements [Adx L-1 (L) and Adx M-1 (M) regions], but due to the two overlapping GC boxes in the 5′-CRS element of Adx 9940 (L), the two T nucleotides only block one GC box, still leaving a perfect GC box. AdxCRS-3 was designed to eliminate all binding. AdxCRS-4 was designed to block one of the two overlapping GC boxes in the 5′-CRS (L). AdxCRS-5 was designed to block binding to the 3′-CRS element of Adx 9940 (M). AdxCRS-6 was designed to disrupt all binding to the 5′-CRS element of Adx 9940 (L). Figure 7A schematically shows putative binding at each oligonucleotide. Figure 7B shows the binding activity of each oligonucleotide with Y1 nuclear extract and translated BFCOL1. The result is consistent with the prediction in Figure 7A. Transient transfection study (Figure 7C) shows that AdxCRS-1 and AdxCRS-4 confer the same cAMP response as Adx 9940. AdxCRS-5 and AdxCRS-6, both losing one CRS element of Adx 9940, confer less cAMP

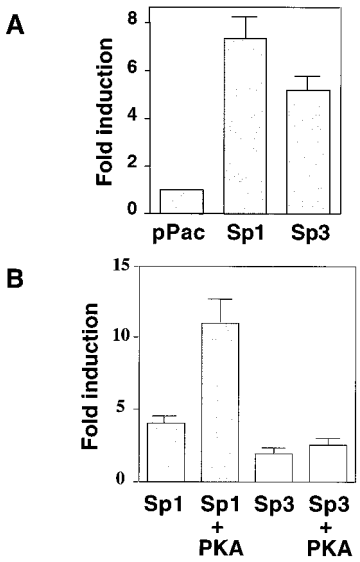

FIGURE 8: Sp1 and Sp3 confer basal transcriptional activities, but only Sp1 confers cAMP-responsive transcriptional activity. (A) SL2 cells were transiently transfected with 3 μg of 9940-wt-luc. Cells were harvested 48 h after transfection and luciferase activities were determined. Results are expressed as *x*-fold induction relative to activities obtained with the reporter gene construct transfected with pPac (empty expression vector). Values represent the mean and SE of three separate transfections. (B) SL2 cells were transfected with 9940-wt-luc (3 μg) and pPacSp1 (1 μg) or pPacSp3 (1 μg) with or without pPacPKAcα (0.1 μg) as indicated. Cells were harvested 48 h after transfection and luciferase activities were determined. Results are expressed as *x*-fold induction relative to activities obtained with reporter gene construct cotransfected with pPac and are the mean and SE of three separate transfections.

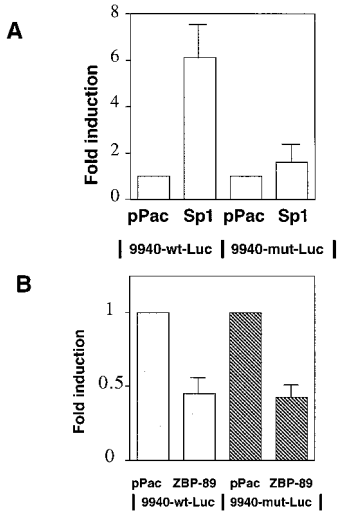

FIGURE 9: Sp1-mediated activation is based on the Adx 9940 element, but ZBP-89 can repress the activation of Adx gene construct regardless of the Adx 9940 binding element. (A) SL2 cells were transfected with 3 μg of 9940-wt-luc (or 9940-mut-luc) and pPacSp1 (1 μg) or pPac (1 μg). Cells were harvested 48 h after transfection and luciferase activities were determined. Results are expressed as *x*-fold induction relative to activities obtained with the reporter gene construct transfected with pPac. Values represent the mean and SE of three separate transfections. (B) SL2 cells were transfected with 3 μg of 9940-wt-luc (or 9940-mut-luc) and pPacZBP-89 (1 μg) or pPac (1 μg). Cells were harvested 48 h after transfection and luciferase activities were determined. Results are expressed as *x*-fold induction relative to activities obtained with reporter gene construct transfected with pPac. Values are the mean and SE of three separate transfections.

response than Adx 9940. AdxCRS-2, which contains a single Sp1 binding activity by EMSA, confers lower cAMP response. AdxCRS-3 confers minimal cAMP response, since this mutant oligonucleotide shows no binding.

Expression of Sp1, Sp3, and ZBP-89 in Drosophila SL2 Cells. To further evaluate the role of each nuclear protein, they were overexpressed in *Drosophila* SL2 cells along with reporter gene constructs. These cells were chosen because they do not contain endogeneous Sp factors (*34*, *35*) or detectable ZBP-89 (data not shown). Plasmid 9940-wt-luc was prepared by subcloning the bovine Adx promoter (including Adx 9940) upstream of the luciferase reporter gene. It is important to note that the luciferase reporter construct contains the original bovine Adx promoter, unlike the OVEC plasmid constructs used in previous experiments (Figures 3, 6B, and 7C), which contain the β-globin promoter. Expression of Sp1 and Sp3 confers basal transcriptional activity (Figure 8A). When the PKA catalytic subunit was cotransfected into SL2 cells, it further increased Sp1-mediated transcriptional activity but did not have a significant effect on Sp3-mediated transcriptional activity (Figure 8B). This Sp1-mediated activation by PKA is dependent on the Adx 9940 element, since it can be blocked by mutation (same as in Adx CRS-3) of Adx 9940 (Figure 9A). ZBP-89 represses basal transcription of both Adx 9940 wild-type and mutant luciferase constructs (Figure 9B). This result suggests that ZBP-89 represses basal transcriptional activity regardless of the binding element. Cotransfection of PKA catalytic subunit does not show any effect on ZBP-89-mediated repression of the reporter gene (data not shown).

DISCUSSION

The presence of two promoters, each containing a unique cAMP-responsive sequence, in the bovine Adx gene was known from our previous work (*9*). These unique CRS elements share no sequence homology to the traditional CRE. Since the promoter within intron 1′ is stronger and participates in production of 90% of the bovine Adx transcripts, it has been further characterized in this study. The sequence Adx 9940, identified as the CRS within this promoter, is shown here to contain two functionally similar CRS units (Adx L-1 and Adx M-1). Both confer basal and cAMP-dependent transcriptional activities to reporter genes and bind the ubiquitous zinc finger nuclear proteins Sp1, Sp3, and ZBP-89. By transient transfection in *Drosophila* SL2 cells, Sp1 and Sp3 are both shown to confer basal transcriptional activities, but only Sp1 can confer the cAMP response. ZBP-89 has been demonstrated to function as a repressor in other genes (*20*, *31*, *36*) and we find a similar role for this Adx 9940 binding protein in the bovine Adx gene. More interestingly, it represses basal transcriptional activity regardless of whether it is bound to the Adx CRS element. BFCOL1 (mouse homologue of ZBP-89) is reported to interact with the TATA-binding protein-associated factor TAF 110 (*32*), which could explain the ability of ZBP-89 to repress Adx 9940 basal transcription regardless of whether it is bound. Identification of ZBP-89-interacting protein(s) associated with the bovine Adx promoter is a study for the future.

Sp1 is generally considered to be a ubiquitous transcription factor that confers basal transcription. Several lines of evidence have shown that different hormones and growth factors can affect Sp1-mediated transcription (*37*, *38*),

suggesting an important role of Sp1 in responding to various stimuli in regulation of expression of certain genes. The involvement of Sp1 in cAMP responses has been localized at cAMP-responsive sequences from several genes (*10*, *11*, *13*−*16*). Here we show that the bovine Adx gene is one of these. By use of a Sp1-free *Drosophila* cell line it is demonstrated that Sp1 can confer cAMP-responsive transcriptional activity via Adx 9940. Whether direct PKA-dependent phosphorylation of Sp1 protein occurs is not known. However, Thr^{366} is a candidate for this modification and awaits further investigation. The Sp1 homologue, Sp3 can bind to most of the Sp1-binding sites and can function as an activator or repressor depending on the promoter and cellular context (*39*). In the bovine Adx gene, Sp3 activates Adx CRS-mediated basal transcription about as efficiently as Sp1. Sp3 has been reported to interact with basal transcription machinery (39, 40), providing a possible explanation for its role in Adx transcription. The reason that no significant cAMP response is observed in Sp3-mediated transcription of the bovine Adx gene could be due to the absence of a PKA phosphorylation site in Sp3.

Four research groups have characterized members of the ZBP-89 family of Krüppel-like zinc finger proteins. Rat ZBP-89 was identified as a transcription factor that binds specifically to the gastrin EGF response element, a GC-rich element (GGGGCGGGGTGGGGGG) (*20*). Mouse BFCOL1 was cloned and shown to bind to the proximal GC-rich promoters of the two mouse type I collagen genes (*32*). Mouse BERF-1 was identified to bind a G-rich box (AGTGGGGGAGGGGCCTGCG) within the muscle-specific enhancer of β-enolase gene (*31*). BFCOL1 and BERF-1 are both mouse homologues of ZBP-89. Due to the use of a different initiator methionine, BFCOL1 is 25 amino acids shorter than BERF-1. A human homologue of rat ZBP-89 was found to regulate the activity of the ornithine decarboxylase promoter (*36*). ZBP-89 functions as a repressor of basal and epidermal growth factor-inducible transcription of the gastrin gene (*20*, *41*). Overexpression of BFCOL1 with a pro-α2 (I) collagen promoter linked to the reporter gene does not show activation. Instead, a squelching effect was observed when highest concentrations of the BFCOL1 expression plasmid are used for cotransfection (*32*). BERF-1 functions as a repressor on basal and activated transcription mediated by the β enolase promoter (*31*). It is shown here that the bovine Adx CRS is the fifth target sequence for this Krüppel-like zinc finger protein family. Upon comparison of Adx factor binding sequences (Adx L-1 and Adx M-1) with the suggested consensus sequence for the ZBP-89 binding site (*35*; Figure 10), both are found to contain this consensus sequence. Further, we extend understanding of this consensus sequence by establishing the requirement for five consecutive G nucleotides for ZBP-89 binding, making this consensus sequence overlapping but distinct from that for Sp proteins. It is important to note that ZBP-89 alone can bind to Adx factor binding sequence (Adx 9940, Adx L, or Adx M), because supershift analyses (Figures 2B and 5B) clearly demonstrate that the combination of Sp1 and Sp3 antibodies completely disrupts the Sp nuclear protein complexes, leaving the complex containing the Adx factor (ZBP-89). In vitro transcription/translation analysis of ZBP-89 also shows that the translated product itself can bind to Adx factor binding sequence (data not shown). The approach to identify

Adx L-1*	GCTCCGCC**CC**GC**C**C**CC**TGGC
Adx M-1*	GCGCAGCC**CC**GC**C**C**CC**TGCC
ODC	GCC**CC**TC**C**C**CC**
Pro-α2(I)	GCCACGTC**CC**TC**C**C**CC**CTC
-168,Pro-α1(I)	TTCCCT**CC**TC**C**C**CC**CTCTT
-194,Pro-α(I)*	GCGCGCC**CC**CC**C**T**CC**CGCAA
TCR Vβ8.1*	CA**CC**AC**C**C**CC**AACTTC
TCR α silencer I*	CCACC**CC**AC**C**C**CC**ACC
gastrin*	CCCCCCA**C**CC**C**G**CC**CC

FIGURE 10: Known sequences for the ZBP-89 binding. Boldface and underlined letters represent nucleotides important for ZBP-89 binding suggested by the study from the ODC gene (*36*). The ZBP-89 binding sites in the promoters of the ODC gene, the type I collagen genes [Pro-α2(I); −168, Pro-α1(I); and −194, Pro-α1-(I)], the T-cell receptor genes (TCR Vβ8.1 and TCR α silencer I), and the gastrin gene are aligned with the Adx L-1 and Adx M-1 elements. The asterisk denotes sequences that are inverted in relationship to the transcriptional start site.

the ZBP-89 binding sequence was by using random PCR selection, which employs PCR amplification in conjunction with affinity purification of the specific binding oligonucleotides. The result further confirmed that five consecutive G nucleotides are important for ZBP-89 binding. Clearly, the binding sites for Sp factors and ZBP-89 are overlapping and very difficult to separate. Since Sp1 and ZBP-89 can compete for the overlapped binding sequence (*20*; our data), cotransfected ZBP-89 will be expected to antagonize Sp1-mediated transactivation in the *Drosophila* cells (*36*). ZBP-89 expression in SL2 cells represses the basal transcriptional activity of the bovine Adx gene regardless of whether it binds to the Adx CRS element. The same phenomenon was discovered upon study of regulation of β enolase gene transcription in embryonic muscle (*31*). Although ZBP-89 has been shown to function as a repressor in the gastrin gene (*20*), β-enolase gene (*31*), and ODC gene (*36*) and herein in the bovine Adx gene, this protein can also potentially stimulate transcription. By use of a GAL4 fusion polypeptide with BFCOL1 and cotransfection with a plasmid containing a GAL4-binding site upstream of an SV40 promoter linked to a reporter gene, the C-terminal segment of BFCOL1 was identified to contain a potential transcription activation domain (*37*). A similar study characterizing the functional domains of BERF-1 demonstrates that the C-terminal region can confer up to 34-fold activation of transcription of a reporter gene (*31*). Thus the ZBP-89-type Krüppel-like zinc finger protein might function either as a repressor or as an activator, depending on the specific promoter. Other transcription factors that share overlapping binding sites with ZBP-89 or bind sites close to ZBP-89 binding sites (*20*, *32*, *36*) might influence whether ZBP-89 functions as a repressor or an activator by interacting with it. While the ZBP-89 homologue seems to play a role in regulation of the bovine Adx gene, no binding of this protein is observed to the human Adx gene (P.-Y. Cheng unpublished results), indicating that it probably is important only in regulation of the bovine Adx gene.

Adx CRS in the OVEC system confers an 11-fold increase in correctly initiated globin transcripts upon forskolin treatment (Figure 3). However, in *Drosophila* SL2 cells, cotransfection of PKA catalytic subunit can only increase Sp1-mediated transcriptional activity by 2−3-fold. The discrepancy is probably due to the different promoters present in each system. In OVEC, the heterologous β-globin promoter was used, while the bovine Adx promoter was introduced into reporter gene constructs used in *Drosophila* SL2 cells. The

results in SL2 cells more accurately reflect the ACTH-mediated increase of bovine Adx mRNA in bovine adrenocortical cells (5), and apparently the β-globin promoter in OVEC vector magnifies the cAMP response. The basal transcription level of bovine Adx is quite high (Figure 3). While Adx cAMP responsiveness coordinates well with that of genes encoding mitochondrial steroid hydroxylases (8), perhaps the relatively high basal level of expression eliminates the requirement for a cAMP response for Adx transcription in nonsteroidogenic cells.

Adx is ubiquitously expressed, being most abundant in steroidogenic tissues. Since Sp1, Sp3, and ZBP-89 are ubiquitous transcription factors, it is not apparent why there should be a higher level of Adx in steroidogenic tissues than in nonsteroidogenic tissues where it is also expressed. One explanation for this difference could be that an unknown steroidogenic tissue-specific transcription factor associates with the Sp proteins on this gene to enhance transcription. Steroidogenic factor 1 (SF-1) is a candidate for this role. SF-1 is a steroidogenic tissue-specific transcription factor that regulates CYP11A (*42*), CYP11B (*42*), CYP17 (*43*), CYP19 (*44*), CYP21 (*45*), oxytocin (*46*), and Mulerian inhibitory substance (*47*) genes in a tissue-specific way. Two putative SF-1 binding sites (+34/+43 and +62/+71) are identified in exon 1′ of the bovine Adx gene. Whether or not these sites cooperate with Adx 9940 to enhance transcription remains to be investigated. Alternatively, it could be that a steroidogenic cell-specific promoter complex associated with the Adx gene leads to enhanced expression in these cells, compared to nonsteroidogenic cells. A third possibility is that a steroidogenic cell-specific coactivator is involved in Adx transcription. There is no evidence for interactions between Sp1 and known coactivators. The present results of the functional roles of Sp1, Sp3, and ZBP-89 in regulation of the bovine Adx gene provide a basis for further studies to characterize the mechanisms by which these DNA binding proteins regulate transcription of this gene.

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