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PRESENCE OF NUCLEAR FACTORS BOUND TO BOTH cAMP-RESPONSIVE ELEMENT AND AP1 FACTOR BINDING SITE IN THE PORCINE ANTERIOR PITUITARY

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Factors binding to consensus sequences of the cAMP-responsive element (CRE) and the AP1 factor binding site (AP1) were investigated using porcine anterior pituitary nuclear extracts. Each element showed specific gel mobility shifts. By reciprocal competition for the AP1 and CRE binding, CRE prevented AP1 binding completely. On the other hand, AP1 decreased the CRE binding considerably to 20%, suggesting that approximately 80% of the total CRE binding is due to factors which bind to a common site shared by both CRE and AP1, whereas proteins binding to AP1 alone are absent. Relative binding affinities of AP1 against CRE estimated from the reciprocal competition data were 0.17 for CRE binding and 0.56 for AP1 binding. UV cross-linking experiments showed that CRE and AP1 gave different patterns consisting of different molecular size. Inconsistency of the relative binding affinities and the multiple molecular size of binding factors, cannot be explained simply by the presence of two types of binding factor, common CRE/AP1-binding and specific CRE-binding factors. A more likely explanation is that the CRE/AP1-binding factors alter the dimer form by changing each respective partner to bind CRE and/or AP1.

The cyclic AMP-responsive element (CRE) and the AP1 factor binding site (AP1, also termed TRE), which consist of similar sequences (CRE, TGACGTCA and AP1, TGA$^{C}/_{G}$TCA), play important roles in signal transduction (1-3). cAMP-responsive element binding protein (CREB) and AP1 factor (identical with c-Jun) have been initially identified as binding proteins for CRE and AP1, respectively. It is reported that CREB binds to CRE alone, whereas AP1 factor shows a relaxed binding specificity for both CRE and AP1 (4). The structural similarity of several CRE- and AP1-binding proteins (including ATF proteins) indicates that they form a superfamily of large size. They have a common heptad leucine zipper, which forms a dimer (3). Recently, increasing evidence suggests that members of this family form a heterodimer as well as a homodimer (5,6). The heterodimer between mXBP/CRE-BP2 and c-Jun does not bind to AP1 (5). CRE-binding protein in

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brain (HB16) has a low affinity for AP1 (6). Members of c-Jun form a heterodimer with the cognate molecule and recognize both CRE and AP1 (4). In contrast, experiments on the responsiveness of the regulatory element have demonstrated that a heptanucleotide in the proenkephalin gene is a responsive element for both cAMP and phorbol ester (7). Although the types and characteristics of all the heterodimers are poorly understood, further investigation of heterodimers would provide unique information on gene regulation.

By reference to our studies of the porcine gonadotropin subunit genes (common α-, LHβ- and FSHβ-subunit) which have demonstrated the presence of variant CRE and AP1 sequences in the 5'-flanking regions (8-10), the present study focused on the basic characteristics of the binding factors for the consensus CRE and AP1 in porcine anterior pituitary nuclear extracts. It was found that a large proportion of the factors binding to both CRE and AP1 were present in the porcine pituitary extracts, and that these binding factors would alter the dimer formation to enable recognition of different sequences.

MATERIALS AND METHODS

Preparation of nuclear extracts. Nuclear extracts from porcine anterior pituitaries were prepared according to Miskimins *et al.* (11). Fifty porcine anterior pituitaries obtained from a local slaughterhouse were homogenized in 15 ml of buffer containing 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), pH 7.9, 0.5 M sucrose, 0.1 mM EDTA, 5 mM $MgCl_2$, 0.5% (v/v) Triton X-100, 1 mM dithiothreitol (DTT), 50 mM NaCl, 5 μg/ml antipain, 2 TIU/ml aprotinin, 2 μg/ml chymostatin and 5 μg/ml leupeptin. The homogenate was centrifuged at 25,000 x *g* for 10 min at 4° C. The pellet was suspended in an equal volume (5 ml) of Hepes buffer containing 0.6 M NaCl, 5 mM spermidine and 0.2 mM p-amidinophenyl methanesulfonyl fluoride hydrochloride, followed by stirring for 1 h and centrifugation at 25,000 x *g* for 30 min at 4° C. The supernatant was dialyzed against 10 mM Hepes, pH 7.9, containing 1 mM $MgCl_2$, 50% (v/v) glycerol, 50 mM NaCl and 0.5 mM EDTA. After any precipitates had been removed by centrifugation, aliquots of the extract were stored at -80° C until use.

Preparation of probes. The following oligonucleotides were synthesized on an Applied Biosystems Model 380A: CRE, 5'-GTCCAAATTGACGTCATGTCAGATCAA-3'(12); AP1, 5'-TAGTGATGAGTCAGCCGGATCAAG-3' (2); AP2 factor binding site (AP2), 5'-CACGGGCCGCGGGCGGTCAGATCAA-3' (13); primer 1, 5'-TTGATCTGAC-3'; primer 2, 5'-AGCTTGATC-3'. Double-stranded DNA was prepared by annealing each of CRE, AP1 or AP2 with a primer followed by an extension reaction with Klenow fragment in a reaction mixture containing 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 0.2 mM each of dATP, dTTP and dGTP in the presence of dCTP or [α-^{32}P]dCTP. Free nucleotides were removed by gel filtration on a Nick column (Sephadex G-50 DNA grade, Pharmacia-LKB, Uppsala, Sweden).

Gel mobility shift assay. The typical binding reaction mixture included 5-20 fmol of probe (about $1.3x10^5$ cpm) and 5-10 μg nuclear protein with 50 ng poly(dI-dC) in 10 μl of 11 mM Hepes buffer, pH 7.9, containing 0.6 mM $MgCl_2$, 14% glycerol, 58 mM NaCl and 0.6 mM DTT. The mixture was incubated at 30° C for 30 min. Then samples were subjected to electrophoresis on 6% polyacrylamide gel (acrylamide to bisacrylamide ratio, 30:1) in 50 mM Tris-0.38 M glycine, pH 8.3, containing 2 mM EDTA at 15 V/cm at room temperature, followed by autoradiography on Kodak XAR-5 film without drying the gel.

UV cross-linking. The binding mixture (10 μl) in a 1.5-ml microtube was placed under a UV illuminator (254 nm filter, 6 W) at a distance of 7 cm and irradiated for 30 min at room temperature, followed by treatment with 4 units DNaseI at 37° C for 20 min. The irradiated samples were separated on 6% nondenaturing polyacrylamide gel and 12.5% SDS-polyacrylamide gel (14).

RESULTS

Binding of nuclear factors to CRE and AP1. Binding factors for CRE and AP1 were assayed in porcine pituitary nuclear extracts by gel mobility shift assay. CRE formed two slow migrating bands (I and II) with similar intensity, as indicated in Fig. 1. In contrast, AP1 formed a predominant band (I) and a faint band (II) of slightly higher mobility, which was formed at a high dose of the extracts. Some other bands with higher mobility were formed by CRE and AP1, but these were found to consist predominantly of single-strand oligonucleotide and were not observed consistently, representing non-specific binding (data not shown). Addition of a 100-fold molar excess of unlabeled CRE or AP1 to the reaction mixture prevented the binding of the labeled probe, whereas a 100-fold molar excess of unlabeled AP2 did not decrease the binding. Treatment of nuclear extracts with alkaline phosphatase did not change these mobility shift patterns (data not shown).

Equilibrium binding studies of CRE and AP1. To estimate the concentration of the binding factors, a saturation isotherm was constructed by adding increasing amounts of labeled CRE or AP1 to a constant amount of pituitary nuclear extract (Fig.2). The concentrations of nuclear proteins bound to CRE and AP1 were estimated to be 65.1 and 51.4 pM, respectively. The ratio of AP1-binding factor to CRE-binding factor was approximately 0.8. When these saturation isotherms were analyzed by the method of Scatchard (15), the binding of CRE indicated the presence of two dissociation constants (Kd)

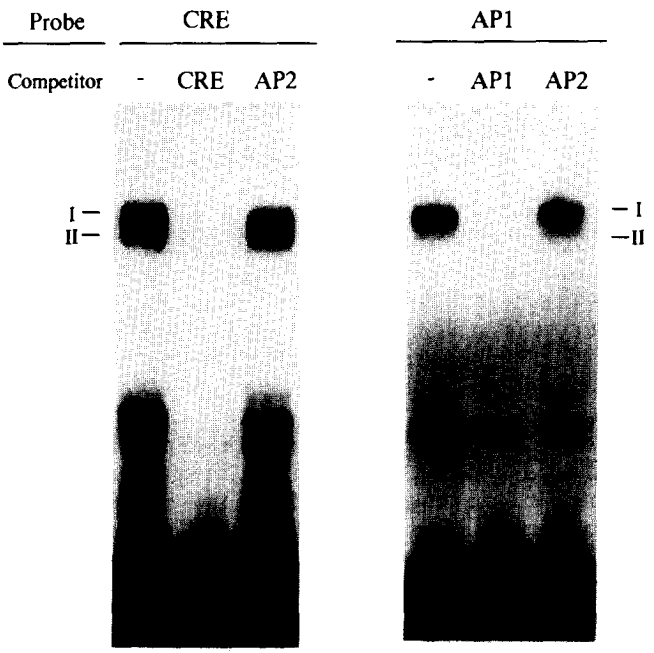

Fig. 1. Gel mobility shift assay using porcine anterior pituitary nuclear extract. In a 10-μl reaction mixture, 10 fmol of probe was mixed with 5 μg (CRE) or 10 μg (AP1) nuclear extract. A 100-fold molar excess of competitor was added to the binding reaction mixture.

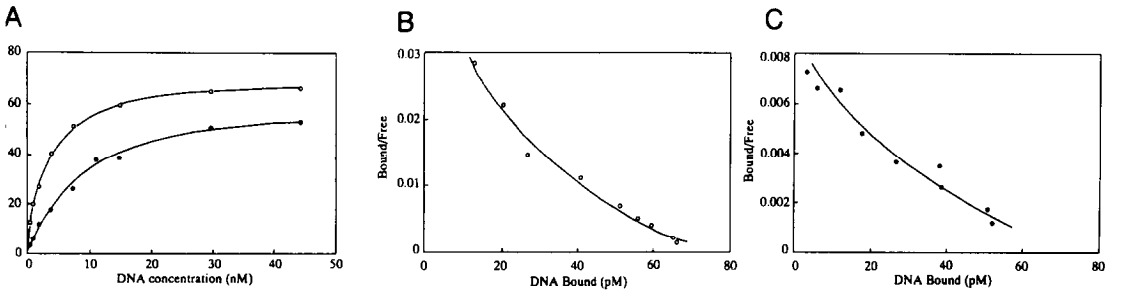

Fig. 2. Equilibrium binding studies of CRE and AP1. Increasing concentrations of labeled oligonucleotide were incubated with a constant amount (1.35 μg) of porcine anterior pituitary nuclear extract (A). The bound and free probes were separated by electrophoresis as described in Fig. 1 and quantified using a liquid scintillation counter. CRE and AP1 binding are indicated by clear and solid circles, respectively. Scatchard analyses were performed on the equilibrium binding data by plotting bound/free vs. bound CRE (B) and AP1 (C).

of 7.8×10^{-10}M and 5.9×10^{-9}M, and that of AP1 also indicated two Kds of 3.2×10^{-9}M and 2.3×10^{-8}M.

Reciprocal competitive binding of CRE and AP1. By reference to the similarity of the nucleotide sequences of CRE and AP1, the reciprocal competitive bindings between AP1 and CRE were examined. The binding of CRE was prevented by a 100-fold molar excess of unlabeled AP1, whereas the binding of AP1 was decreased to a great extent at a low molar excess (10 fold) of unlabeled CRE (Fig.3A). Subsequently, a competitive binding assay was conducted by adding increasing amounts of unlabeled AP1 and CRE. As shown in Fig. 3B, unlabeled AP1 decreased the binding of CRE. The intensities of the two CRE-binding bands decreased equally during the process of competition (data not shown). However, about 20% of the total binding remained after addition of a 3,000-fold molar excess of unlabeled AP1. This result indicated that about 20% binding is specific for CRE alone.

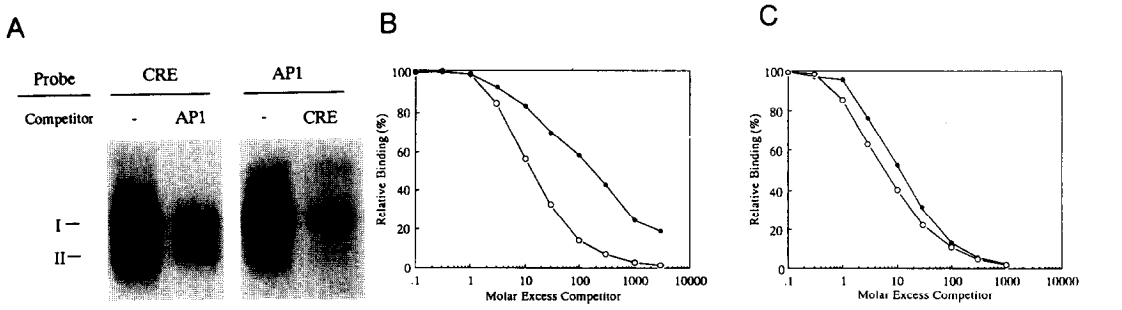

Fig. 3. Competitive binding of CRE and AP1. A. Autoradiography of competitive binding of labeled CRE with unlabeled CRE duplex. Pituitary nuclear extracts (5 μg for CRE and 10 μg for AP1) were mixed with ^{32}P labeled CRE or AP1 (each 10 fmol). The binding of labeled CRE or AP1 was competed for with unlabeled AP1 (100 fold) and CRE (10 fold). Reciprocal competition for each 20 fmol of ^{32}P labeled CRE (B) and AP1 binding (C) was achieved with the amounts indicated. Competitive binding with CRE and AP1 is indicated by clear and solid circles, respectively.

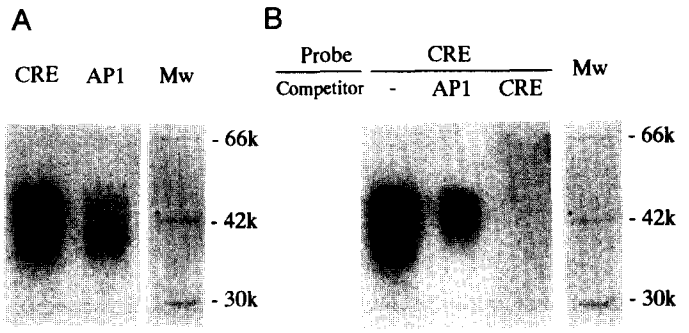

Fig. 4. UV cross-linking of CRE and AP1 with the porcine anterior pituitary nuclear extract. Nuclear extract (16 μg) and ^{32}P labeled CRE or AP1 (each 20 fmol) were mixed and irradiated with UV light. Electrophoresis on SDS gels (A and B) was performed. The irradiated samples in the presence of a 100 molar excess of competitor were prepared and analyzed independently (B). Positions of molecular size markers stained with Coomassie brilliant blue R are indicated in kDa.

However, the AP1-binding was completely prevented by CRE, indicating an absence of proteins binding to AP1 alone. The relative binding affinity of AP1 was estimated by comparing the amount of AP1 necessary to obtain 50% of the maximal displacement with that of CRE needed to obtain the same displacement. The two relative binding affinities, which were calculated to be 0.17 for CRE-binding (Fig. 3B) and 0.56 for AP1-binding (Fig. 3C),were not coincident with each other .

UV cross-linking between nuclear factors and DNA. To determine the molecular size of binding proteins CRE and AP1, UV cross-linking experiments were performed. Analysis of the UV-irradiated samples on the SDS-polyacrylamide gel showed positive bands and demonstrated the molecular distribution and the difference in size between the binding proteins, whereas the non-irradiated samples showed no positive band. The molecular sizes ranged from 37 to 50 kDa for CRE and from 35 to 47 kDa for AP1 (Fig. 4A). The diffuse bands of CRE-binding, which were apparently different from those of AP1, were decreased upon addition of AP1 to 20% and disappeared completely after addition of CRE (Fig.4B).

DISCUSSION

The present study demonstrated the definite presences of factors which bind to both AP1 and CRE in the majority of CRE-binding proteins. Competitive binding assays (Figs. 3B and C) indicated that, in the porcine anterior pituitary, two classes of CRE-binding are present with different specificities for AP1. Approximately 80% of the CRE-binding shows relaxed specificity for CRE and AP1, although the Kd values of AP1-binding were one order of magnitude lower than that of CRE-binding. On the other hand, 20% of the total CRE-binding is specific to CRE alone. In contrast to the present data, several investigators have reported that binding of AP1 competitive with CRE-binding does not occur, whereas competition is observed for the reverse binding (16,17). This inconsistency with the present results is probably due to the fact that the protein bound to both CRE and AP1 (CRE/AP1-

BP) is present at a relatively low amount as compared with the proteins in other tissues investigated, or that the properties of those binding proteins are tissue-specific.

As the reciprocal competition revealed by gel electrophoresis showed equal decreases in CRE- and AP1-binding similar to the self-competition data (Figs. 2A and 4B), the same binding factor may recognize both AP1 and CRE and the gel shift patterns are expected to be the same for CRE- and AP1-binding. However, the patterns of the gel mobility shift show that CRE-binding involves at least two components with similar affinity, in contrast to the major single band of AP1-binding. The difference between the electrophoretic patterns for CRE- and AP1-binding were observed also in the UV cross-linking experiments, suggesting that the binding forms may be rather complex. If the same binding factor recognizes both AP1 and CRE, the reciprocal competitions between CRE- and AP1-binding would show similar efficiencies. However, the relative binding affinities of AP1 calculated from the data obtained in the two competition experiments (Fig. 3B and C) were not consistent with each other, showing a lower efficiency of AP1 for the competitive binding for CRE than that for itself. This result is incompatible with recognition of both AP1 and CRE by the same binding factor. Furthermore, the UV cross-linking experiment revealed the size distribution of the CRE- and AP1-binding proteins, and suggested that multiple binding proteins are involved in CRE- and AP1-binding. An alternative explanation for these inconsistent data is that the pituitary CRE/AP1-BPs construct multiple forms of dimer to bind the respective ligands by altering each respective partner. Transition of dimer formation occurs in the presence of an appropriate ligand and forms a complex with novel binding specificity.

As reviewed (17,18), several CRE- and AP1-binding proteins form heterodimers between members of a family. The heterodimer binding to CRE has been reported, although this dimer does not bind to AP1 (5). However, based on *in vitro* data, Hirai *et al.* reported that members of the AP1 family form a heterodimer among their cognate molecules and have distinct relative binding affinities for CRE and AP1 (4). Therefore, the heterodimerization between members of the family may provide unique binding specificity and novel regulatory efficiency for each respective gene (18, 19). The present data suggest that CRE- and/or AP1-binding protein recognize their ligand by formation of alternative dimers with different partner.

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