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The cAMP-response-element-binding protein interacts, but Fos protein does not interact, with the proenkephalin enhancer in rat striatum

(AP-1 proteins/haloperidol)

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ABSTRACT **The proenkephalin gene is a well-studied model of transcription factor–target gene interaction in the nervous system and has been proposed as a regulatory target of the protein product of the immediate-early gene c-*fos*. This regulatory mechanism has been proposed, in part, because the cAMP response element 2 (CRE-2) site, the key DNA regulatory element within the proenkephalin second-messenger-inducible enhancer, avidly binds AP-1 proteins, including Fos, *in vitro*. However, we observe a dissociation in the time course of activation of c-fos and proenkephalin mRNA in rat striatum after administration of the dopamine D_2 receptor antagonist haloperidol. This result prompted us to investigate the composition of protein complexes in striatal nuclear extracts that bind to the CRE-2 site. Even though our striatal nuclear extracts had substantial basal and haloperidol-inducible AP-1-binding activities that contained Fos, we could not detect Fos in complexes bound to the CRE-2 element. Instead, as determined by antibody supershift analysis, we detect CRE-binding protein (CREB)-like proteins binding to CRE-2 in both basal and haloperidol-stimulated conditions. Finally, we show that haloperidol induces CREB protein phosphorylation in striatum.**

The ubiquity of inducible Fos expression within the nervous system has prompted a concerted search for a biological role for this immediate-early gene product as a regulator of downstream "target genes." The proenkephalin gene, which encodes the endogenous opioid peptides [Met^5]- and [Leu^5]enkephalin, has been proposed to be such a target, putatively regulated by Fos in response to diverse stimuli in a variety of neuronal cell types. On the basis of several lines of evidence it has been suggested that proenkephalin gene activation is mediated by Fos after seizures in mouse hippocampus (1), inflammation in rat spinal cord (2), and haloperidol administration in rat striatum (3). The proposed locus of this regulation is an enhancer within the human proenkephalin gene that has been shown by mutagenesis to be required for second-messenger-regulated proenkephalin gene expression in multiple cell lines (4–7). This enhancer contains three closely spaced DNA regulatory elements, cAMP response element (CRE)-1, CRE-2, and transcription factor AP-2 (4–7). Only CRE-2 is absolutely required for induction of the gene by second messengers. Multimers of the CRE-2 sequence can confer cAMP, Ca^{2+}, and phorbol ester induction on a minimal proenkephalin promoter in a variety of cell types (8, 9), whereas multimers of the CRE-1 and AP-2 elements cannot. The CRE-2 element contains the sequence TGCGTCA, which differs by a single base from the consensus binding site for AP-1 proteins (TGACTCA) and by another single base from the consensus binding site for the cAMP-activated transcription factor CRE-binding protein (CREB) (TGACGTCA). *In vitro* the AP-1 transcription factor binds CRE-2 with high affinity (1, 5). Moreover, AP-1 proteins (9), including Fos (1), transactivate the proenkephalin gene when cotransfected into undifferentiated F9 cells. However, it has also been shown by cotransfection that CREB can activate the proenkephalin gene (10).

Blockade of D_2 dopamine receptors—e.g., by haloperidol (11–13) and lesions of the nigrostriatal dopamine pathway (14–17) have long been known to increase proenkephalin gene expression. Because acute administration of haloperidol increases c-fos mRNA (18, 19), Fos-like immunoreactivity (3, 20), and AP-1-binding activity (19) in the striatum and because haloperidol-induced Fos-like immunoreactivity occurs primarily in the D_2 receptor-expressing enkephalinergic neurons in striatum (3), it has been proposed that Fos couples the synaptically mediated effects of haloperidol to induction and maintenance of proenkephalin gene expression. Alternatively, CREB-like proteins (21) might bind the CRE-2 element and regulate proenkephalin gene expression in certain cell types or after certain stimuli. This hypothesis is supported by the observation that functional CREs, including proenkephalin CRE-2, have relatively stringent sequence requirements for an intact CGTCA motif (6), which is not necessary for AP-1 binding. Indeed, a base substitution mutation within the CRE-2 that creates a consensus AP-1 site (TGAGTCA) renders the gene unresponsive to cAMP after transfection into C6-glioma cells (5). We undertook these studies to identify which nuclear proteins actually interact with the CRE-2 site in striatum under basal and stimulated conditions.

MATERIALS AND METHODS

Drug Paradigms. Male Sprague–Dawley rats (200–250 g) were used. Drugs were administered i.p. Control rats received 0.9% saline in single daily injections for 12 days. For the acute haloperidol paradigm, saline was given for 11 days, and haloperidol was given at 2 mg/kg (Sigma) on day 12. For the chronic paradigm, haloperidol at 2 mg/kg was given in single daily injections for 12 days. Rats were sacrificed by rapid decapitation 30 min after the last injection for RNA analyses or 2 hr after the last injection for protein analyses, unless specified. All experiments were done at least four times.

Assays. RNA blot analysis was done as described (19). For gel shifts, the caudate-putamen was dissected out bilaterally

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Abbreviations: CRE, cAMP response element; CREB, CRE-binding protein; ATF, activating transcription factor; CaRE, calcium response element.

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(100 mg), and nuclear extracts were produced as described (19). Protein extracts (5 μg; ref. 22) or *in vitro*-translated proteins from reticulocyte lysates (1 μl of lysate containing Fos and/or JunD) were incubated on ice for 10 min in 10 mM Hepes, pH 7.9/10% (vol/vol) glycerol/0.1 mM EDTA/80 mM KCl/2 mM $MgCl_2$/1 mM dithiothreitol/poly(dI·dC) at 10 mg·ml^{-1} at 4°C with or without unlabeled competitor (15-μl total vol). With antiserum, preincubation was extended to 20 min. After preincubation, 1 ng of ^{32}P-labeled double-stranded oligonucleotide was added. Samples were incubated for 10 min at 23°C and then electrophoresed through a 4% nondenaturing polyacrylamide gel (acrylamide/bisacrylamide, 60:1) in 0.25× TBE (1× TBE is 89 mM Tris/89 mM boric acid/2.5 mM EDTA) and 3% glycerol.

Oligonucleotides (sequences shown in Table 1) were synthesized with partial *Bam*HI sites and hybridized in the presence of 20 mM $NaPO_4$/1 mM EDTA/100 mM KCl. The partial *Bam*HI sites were then filled in with ^{32}P-labeled dCTP and unlabeled dATP, dGTP, and dTTP. For supershifts, the Fos antiserum used was directed against the specific N terminus of the protein (Oncogene Science; Fos Ab-2). The M peptide antibody (23) was a gift of Michael Iadarola (National Institutes of Health). The CREB antiserum was a polyclonal rabbit anti-rat-CREB serum, raised against amino acids 1–205, from D. Ginty and M. Greenberg (Harvard Medical School).

Immunohistochemistry. This procedure was done as described (19), except that the initial perfusion with 0.1 M phosphate-buffered saline was omitted, and 3% bovine serum albumin was substituted for normal goat serum. The antiserum, directed against phospho-CREB (CREB phosphorylated on Ser^{133}) (24), was from D. Ginty and M. Greenberg.

RESULTS

Dissociation of the Time Courses of c-fos and Proenkephalin mRNA Induction in Response to Haloperidol. Levels of proenkephalin mRNA were increased 2- to 3-fold in all animals treated chronically with haloperidol compared with vehicle-treated controls, as previously reported. Animals treated acutely with haloperidol also exhibited a small, but reproducible, increase in proenkephalin mRNA (Fig. 1*A*). c-fos mRNA was markedly induced by an acute haloperidol injection, but after 12 daily injections the mRNA was induced to levels only slightly greater than basal (Fig. 1*A*). Expression of other immediate-early genes, *junB* and c-*jun*, also was markedly desensitized by chronic haloperidol administration (data not shown). The mRNAs encoding the constitutively expressed transcription factors CREB (Fig. 1*A*) and JunD (data not shown) were not significantly altered by acute haloperidol administration. The dissociation in time course of c-fos and proenkephalin mRNA induction makes it unlikely that Fos protein is involved in maintenance of proenkephalin gene expression with chronic haloperidol. To ensure, however, that the initial activation of c-*fos* seen with an acute haloperidol injection does not have prolonged effects on proenkephalin mRNA induction, we performed an RNA time course of proenkephalin mRNA after a single acute injection of haloperidol (2 mg/kg). Proenkephalin mRNA was induced in some animals by 15 min and in all animals by 45 min, but this mRNA declined to basal levels by 24 hr (Fig. 1*B*).

Table 1. Sequences of the oligonucleotides used in gel-shift assays

Sequence	Site
5′-*GATC*CGCG**TGACTCA**GCGC-3′	AP-1(hMT)
5′-*GATC*AGCA**TGAGTCA**CTTC-3′	AP-1(SP)
5′-*GATC*GGCC**TGCGTCA**GCTG-3′	CRE-2
5′-*GATC*GGG**CTGGCGTAGGGCCTGCGTCA**-GCTGCA-3′	CRE1&2
5′-*GATC*G**CTGACGTCA**GGG-3′	ATF
5′-*GATC*CCC**GTGACGTTTA**CA-3′	CaRE
5′-*GATC*CA**GCCCGCCGGCGA**TTG-3′	AP-2

All oligonucleotides were double stranded. The 5′ *Bam*HI site is shown in italics; core consensus sequences are in boldface type. AP-1(hMT) and AP-1(SP) have identical core consensus sequences in reverse orientation. AP-1(hMT) is derived from the human metallothionein promoter and is identical to that used by Sonnenberg *et al.* (1). ATF, activating transcription factor. CaRE, calcium response element.

CREB-Like Proteins, but Not Fos, Are Detected in Complexes Binding to CRE-2 in Striatal Nuclear Extracts. To identify proteins within striatal nuclear extracts that bind to the proenkephalin enhancer, we used gel-mobility-shift assays combined with an antibody analysis of the specific complexes. The oligonucleotide probes used for this analysis are shown in Table 1 and include both the proenkephalin CRE-2 element and a longer oligonucleotide containing proenkephalin sequences spanning both the CRE-1 and CRE-2 elements. (The rat and human CRE-1 and CRE-2 sequences are identical.) Although the CRE1&2 oligonucleotide generated a more complex pattern of bands than the CRE-2 oligonucleotide, the specific bands, determined by unlabeled competition and subsequent antibody analysis, did not differ between the two probes (see Figs. 2 and 5). For comparison we used two different oligonucleotides containing AP-1 core-binding sites, but with different flanking nucleotides, AP-1(SP) and AP-1(hMT), a palindromic consensus CREB/ATF-binding site, and the nonpalindromic cAMP and calcium-response element (CaRE) from the c-*fos* gene (Table 1). The CaRE sequence has been shown to interact with CREB *in vitro* (25).

The specific bands in the resulting gel shifts are demonstrated by effective "self" competition with unlabeled oligonucleotides (Fig. 2). As a comparison, an oligonucleotide containing the unrelated proenkephalin AP-2 sequence (7) did not compete with any of the labeled oligonucleotides (Fig. 2). The protein complexes that bind the CRE-2 oligonucleotide in striatal extracts were inhibited both by the consensus CREB/ATF sequences and by the consensus AP-1 se-

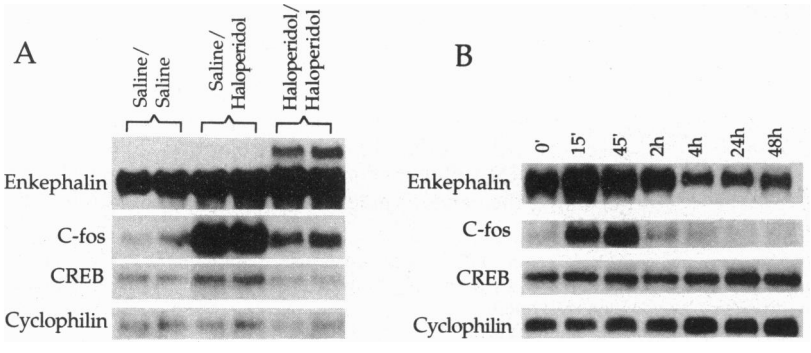

FIG. 1. Haloperidol induces proenkephalin and c-fos mRNA. (*A*) Northern analysis of striatal RNA from rats treated with the control, acute, and chronic haloperidol paradigms. Duplicate lanes represent RNA prepared from different rats. The unregulated mRNA encoding cyclophilin is used as an internal loading control. (*B*) Northern analysis showing a time course of induction of proenkephalin, c-fos, and CREB mRNA in striatum after a single haloperidol injection.

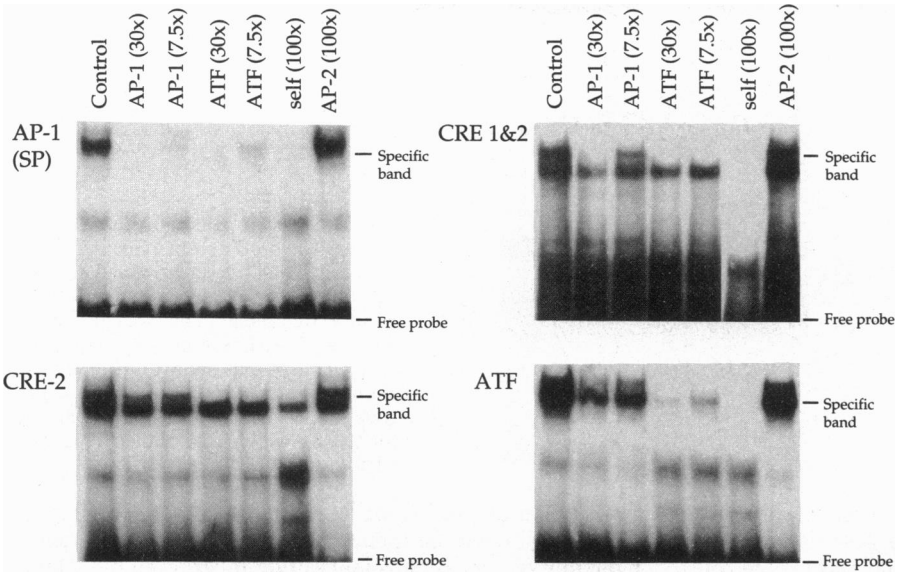

FIG. 2. Gel-shift assays with unlabeled competitor in striatal nuclear extracts. For CRE-2 and CRE1&2, two major shifted bands were generally seen. They were poorly resolved in the absence of competitor oligonucleotides, but with addition of unlabeled oligonucleotides similar in sequence to the labeled binding site, the upper band of the doublet was differentially inhibited, confirming its specificity. Fold molar excess of the competitor is shown at top. The competitor AP-1 oligonucleotide was AP-1(hMT). Experiments shown were from acutely treated rats; extracts from control or chronically treated rats did not alter either the specific bands or the relative affinities of competitor oligonucleotides.

quences; however, the CREB/ATF oligonucleotide competed for the CRE-2 binding proteins more avidly (Fig. 2).

Acute haloperidol treatment induced c-fos mRNA (Fig. 1 *A* and *B*) and is known to induce Fos protein in striatum (19). Thus, we expected acute haloperidol administration to increase protein complex formation with oligonucleotides that bind Fos. After acute haloperidol we did observe such an increase in binding to both of the AP-1 oligonucleotides tested (with chronic treatment these increases in total binding to AP-1 sites were somewhat diminished). In contrast, there was no increase in binding to the CRE-2, CRE1&2, ATF, or CaRE oligonucleotides after acute or chronic haloperidol treatment (Fig. 3). To ensure that the results were not biased by choice of a single time point, we performed a time course (Fig. 4). Again, despite a marked induction of AP-1-binding activity, there was no consistent change in binding to the CRE-2, CRE1&2, ATF, or CaRE oligonucleotides over time (Fig. 4). Given our previously observed time course of immunohistochemically detected Fos in striatum (peak at 2 hr; data not shown), the high levels of AP-1-binding activity observed at 4 hr are most likely due to binding of Fos-related antigens rather than Fos (26, 27).

To analyze the composition of the observed protein–DNA complexes we performed gel-shift experiments with specific antiserum. An affinity-purified antiserum directed against Fos partially supershifted the specific band formed with AP-1 oligonucleotides in striatal nuclear extracts from rats acutely treated with haloperidol (Fig. 5*A*). Fully supershifted bands were obtained when *in vitro*-generated Fos/JunD heterodimers (from rabbit reticulocyte lysate) were used in place of striatal extracts (Fig. 5*A*). In contrast, binding from striatal extracts to the CRE-2, CRE1&2, ATF, and CaRE oligonucleotide sequences was not affected by incubation with the Fos antiserum (Fig. 5*A*). An antibody directed against the conserved M peptide within the Fos family that detects Fos-related antigens as well as Fos (23) also almost entirely supershifted the AP-1 oligonucleotide 2 and 4 hr after haloperidol administration, but this antibody did not shift the CRE-2 or CRE1&2 oligonucleotides (data not shown). We also carried out gel-shift assays in the presence of a CREB

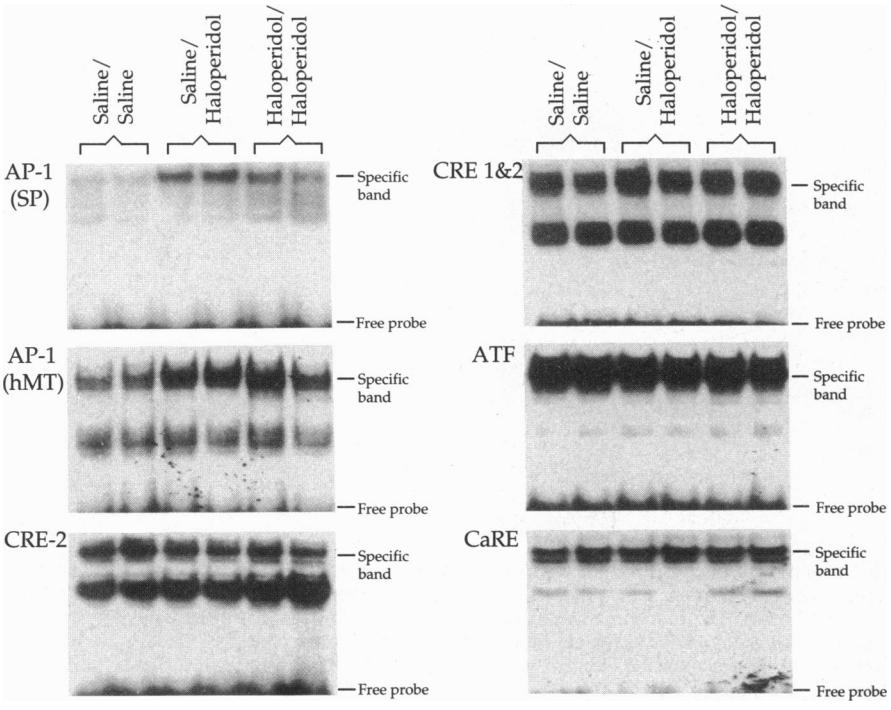

FIG. 3. Effect of acute and chronic haloperidol treatment on protein–DNA complex formation in striatal nuclear extracts. Designation of specific bands is based on competition data (see Fig. 2). Saline/Saline, control; Saline/Haloperidol, acute; and Haloperidol/Haloperidol, chronic. Each condition is shown in duplicate; each lane represents extracts prepared from a different rat.

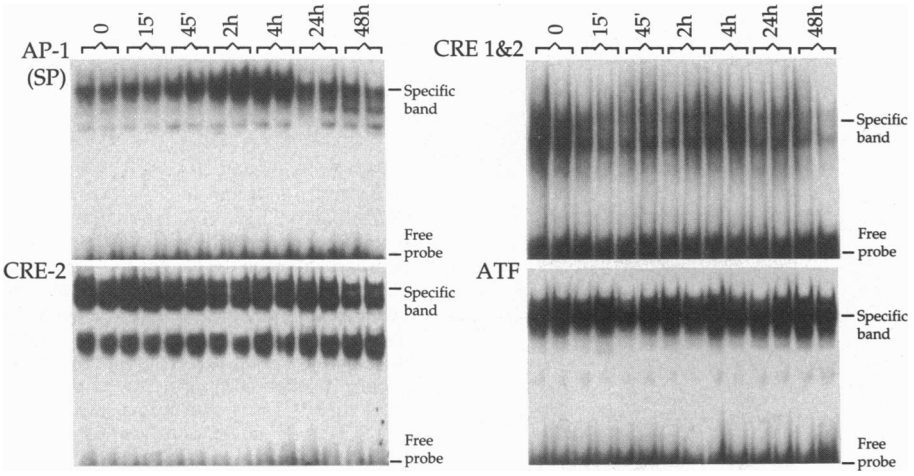

FIG. 4. Time course of induction of binding to the different oligonucleotide probes. Gel-shift assays were done with striatal nuclear extracts after acute haloperidol treatment. The specific bands designated are based on the competition data, such as that shown in Fig. 2. Not shown are the data for AP-1(hMT), which gave similar results to AP-1(SP), and data for CaRE, which gave similar results to CRE-2 and ATF. ', min; h, hr.

antiserum (Fig. 5*B*). This antiserum, which was not affinity-purified, generates a nonspecific band in gel-shift assays, which can also be observed in preimmune serum control lanes (Fig. 5*B*). Incubation with the CREB antiserum did not reduce binding at the AP-1 site (serum generally increased AP-1 binding) but blocked or supershifted the specific complexes formed with striatal extracts by the CRE-2, CRE1&2, ATF, and CaRE oligonucleotides (Fig. 5*B*).

Phosphorylation of CREB in Striatum in Response to Haloperidol. Because CREB mRNA levels are not regulated by haloperidol in striatum (see Fig. 1) and CREB protein (21) has been shown to be transcriptionally active only after phosphorylation on Ser^{133}, we examined whether CREB phosphorylation could be induced by haloperidol. Fig. 6 shows induction of CREB phosphorylation in striatum by haloperidol, compared with saline, as determined by immunohistochemistry with an antiserum that detects only the appropriately phosphorylated form of CREB (24).

DISCUSSION

In prior cotransfection experiments it has been shown that various AP-1 proteins (1, 9) and CREB (10) can trans-activate the proenkephalin promoter. Such studies, which introduce exogenous proteins, are useful for identifying candidate

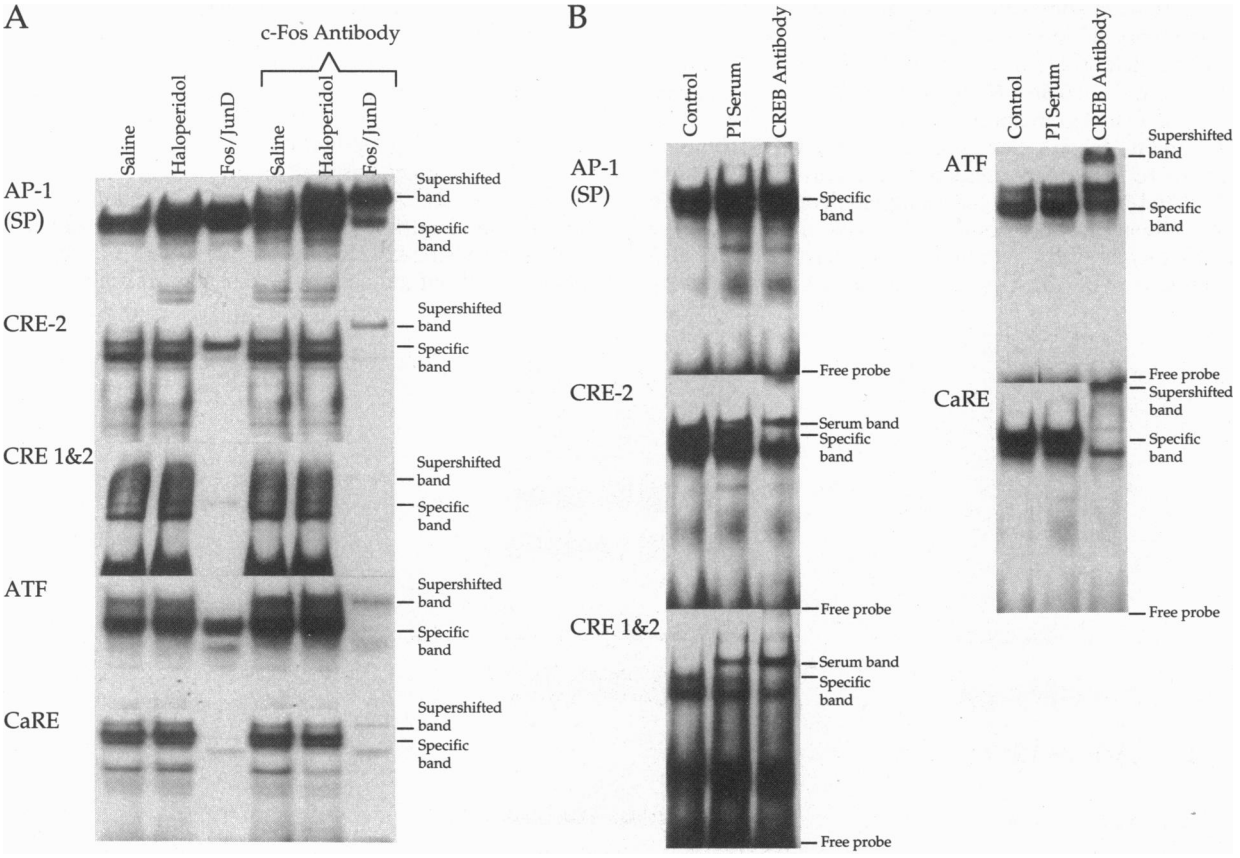

FIG. 5. Gel-shift analysis of striatal nuclear extracts in the presence of Fos or CREB antiserum. (*A*) c-Fos antiserum. The lanes show extracts from control rats (saline), acutely haloperidol-treated rats, or protein from reticulocyte lysates programmed with c-fos and junD mRNA. Unprogrammed extracts give no shifted band (data not shown). The first three lanes are without antiserum, the last three lanes are with Fos antiserum. (*B*) CREB antiserum. All lanes show shifts with striatal nuclear extracts from acutely haloperidol-treated rats. Results are identical in saline or chronic haloperidol conditions (data not shown). For each probe, control refers to a gel shift in the absence of serum or antiserum. PI Serum, preimmune serum (which produces a nonspecific band); CREB Antibody, presence of CREB antiserum.

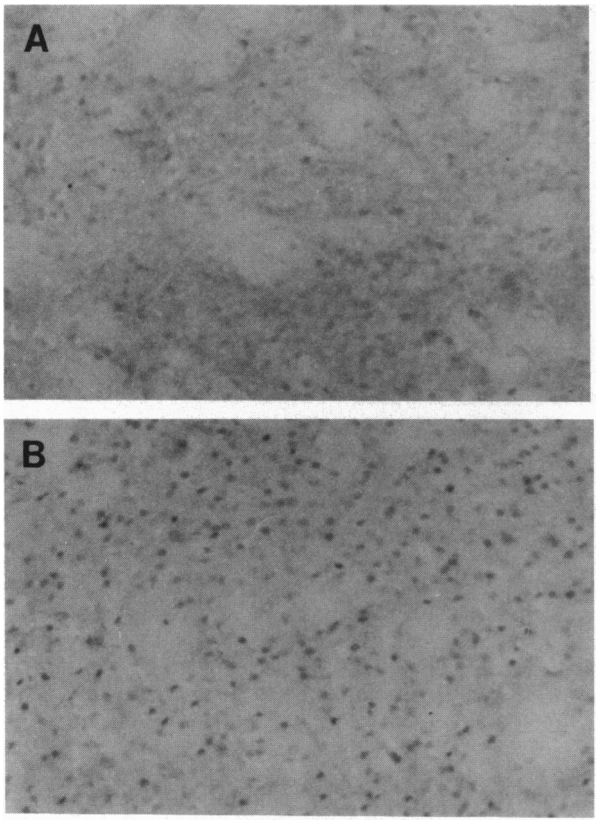

FIG. 6. Induction of phospho-CREB immunoreactivity in striatum by haloperidol. (*A*) Section from the central region of rat striatum stained with phospho-CREB antiserum 10 min after i.p. saline injection. (*B*) Section taken at an identical striatal level as shown in *A*, stained with phospho-CREB antiserum 10 min after haloperidol (2 mg/kg) injection. Staining of cell nuclei was distributed uniformly throughout the striatum. (×160.)

transcriptional mechanisms. However, to define the regulatory mechanisms actually used by particular neurons, the properties of their endogenously expressed transcription factors must be investigated.

By gel-shift analysis and consistent with our previous findings (19), haloperidol induced specific AP-1-binding activity in striatal neurons, which we now show by supershift to contain Fos. There is a partial desensitization of AP-1 binding with chronic haloperidol administration that contrasts with continued high levels of AP-1 binding after chronic cocaine (28). Despite the induction of AP-1-binding activity, haloperidol did not increase protein binding to the proenkephalin CRE-2 or CRE1&2 oligonucleotides, the palindromic CREB/ATF consensus sequence, or to the c-*fos*/CaRE oligonucleotide. Supershift analyses using antiserum revealed that the complexes binding to the AP-1 oligonucleotides contained Fos and Fos-related antigens but did not contain CREB-like proteins; in contrast, the complexes binding to the proenkephalin CRE-2 and CRE1&2, CREB/ATF, and c-*fos*/CaRE oligonucleotides contained CREB-like proteins.

A problem that characterizes attempts to study binding in extracts made from brain is that multiple cell types are included. Thus, proteins that are not in contact with the CRE-2 sequence *in vivo* may interact with it in the gel-shift assay. By immunohistochemistry CREB-like proteins are found in essentially every striatal neuron (data not shown). However, we cannot rule out the possibility that additional factors from the nonenkephalinergic cells somehow interfered with physiologic binding in our extracts.

Despite these caveats, our data suggest that in striatum, even with haloperidol stimulation, CREB-like proteins, rather than Fos, interact with the proenkephalin enhancer. Given previous observations that AP-1 proteins bind CRE-2 *in vitro* (1, 5, 9), it must be assumed that under the conditions found within striatal nuclear extracts after haloperidol stimulation, CREB-like proteins bind CRE-2 and inhibit the binding of Fos-containing AP-1 complexes. Combined with evidence that the c-*fos*/CaRE site also interacts with CREB-like proteins (Fig. 5 and ref. 25), it is likely that nonpalindromic CGTCA-containing CREs interact with CREB-like proteins rather than AP-1 proteins, at least in some cell types. The phosphorylation of CREB, observed 10 min after haloperidol injection, further supports a role for CREB in haloperidol-induced gene regulation.

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