The Homeodomain Protein Arix Interacts Synergistically with Cyclic AMP to Regulate Expression of Neurotransmitter Biosynthetic Genes*

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Transcription of the neurotransmitter biosynthetic genes tyrosine hydroxylase and dopamine β-hydroxylase (DBH) is regulated by cell type-specific transcription factors, including the homeoprotein Arix, and second messengers, including cyclic AMP. The cis-acting regulatory sites of the DBH gene which respond to Arix and cAMP lie adjacent to each other, between bases −180 and −150, in a regulatory element named DB1. Neither Arix nor cyclic AMP analogs alone effectively stimulate transcription from the DBH promoter in non-neuronal cell cultures. However, when Arix is present together with cAMP, transcription is substantially activated. Synergistic transcription from the DBH promoter can also be elicited by cotransfection of Arix with an expression vector encoding the catalytic subunit of protein kinase A. Nuclear extracts from PC12 cells display a cAMP-induced complex binding to the DB1 element, and antiserum to transcription factors CREB, CREM, Fos, and Jun indicate that these proteins, or closely related family members, interact with DB1. A dominant negative construct of CREB inhibits the response of the DBH promoter to protein kinase A. These results demonstrate a synergistic interaction between a homeodomain protein and the cAMP signal transduction system and suggest that similar interactions may regulate the tissue-specific expression of neuroendocrine genes.

The phenotypic expression of genes encoding neurotransmitter biosynthetic enzymes is influenced by local environmental signals such as growth factors and neurohormones. Expression of genes encoding the catecholamine biosynthetic enzymes tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) has been shown to be modulated by extracellular signals, including glucocorticoid hormones (1–4), the neuropetide vasoactive intestinal peptide (5, 6), nicotine (7, 8), and the neurotrophic factors insulin-like growth factor I (9) and acidic fibroblast growth factor (10). In addition, physiological stimuli, such as cold or immobilization stress, can modulate expression of these genes (11–14).

These extracellular signals are transduced from the cell surface through second messenger cascades to the nucleus, where they influence transcriptional control mechanisms. The widely studied second messenger systems initiated through elevations of cyclic AMP and diacylglycerols and mediated through protein kinases A and C stimulate transcription from both the TH and DBH promoters (1, 4, 15–17, 19, 20). In the TH gene, separate regulatory elements mediate these responses: elements at −45 and −102 are essential for the response to cyclic AMP (19, 22–24), whereas an AP1 site at −205 represents the region responsive to phorbol esters (25). The cyclic AMP regulatory element (CRE) at −45 also mediates the cellular response to calcium (26) and nicotine (8), and contributes to the cell-type specificity of TH expression (19, 28). In the DBH gene, a single region, spanning from −180 to −150 of the rat gene, (−189 to −176 of the human gene), mediates the response to both cyclic AMP and phorbol ester (16, 20, 27, 29). Genetic information located within this same region, named the DB1 enhancer in the rat gene, is also essential for basal and cell type specific expression from the DBH promoter (16, 20, 28, 29). The close apposition of the basal, second messenger, and cell type specific regulatory regions has suggested an interaction between, or even identity of, the pathways leading to regulated expression of the DBH gene.

The cellular response to cAMP is often mediated by transcription factors in the CREB/ATF family. The initial factor identified, CREB, undergoes phosphorylation by protein kinase A (PKA), which subsequently leads to activation of target genes containing a CRE regulatory DNA sequence (see 30). Other members of the CREB/ATF family, including CREM and ATF-1, may also modulate transcription in response to cAMP. These factors can form heterodimers, and the composition of dimers influences the regulatory activity at the target genes. In addition to members of the CREB/ATF family, the AP-1 family of transcription factors has been implicated in the regulation of specific genes by cAMP. For example, the proenkephalin CRE-2 has been shown to interact with both Fos and Jun family proteins in chromaffin cell extracts (31), and a JunD/ATF-3 heterodimer has been implicated in mediating the response to cAMP in neuroblastoma cells (32).

For the TH and DBH genes, several factors have been reported to interact with the CREs. The CRE of the TH gene, which contains a perfect palindromic CRE consensus sequence (TGACGTCATGACGTCATA), will bind purified CREB (17) and has been demonstrated to interact with a CREM-like protein in extracts of neuronal cell lines (33). A dominant negative mutant of CREB causes partial inhibition of cAMP-mediated increase of transcription from the TH promoter (28), further strengthening the possible involvement of CREB in this response. For the DBH gene, the CRE-like sequence of the rat DB1 regulatory element

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The abbreviations used are: TH, tyrosine hydroxylase; DBH, dopamine β-hydroxylase; CRE, cAMP response element; PKA, cAMP-dependent protein kinase catalytic subunit α (protein kinase A); CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus; CPT-cAMP, 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate; 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; EMSA, electrophoretic mobility assay; HD, homeodomain; WT, wild type.
Arix and cAMP Synergistically Regulate Transcription

The CREB family of transcription factors has been shown to play a role in mediating the cellular response of the DBH gene to cAMP. In the present study, we have investigated the interaction of Arix, a homeodomain protein, with the DBH promoter. Arix exhibits limited stimulatory activity when transfected into non-neuronal cell lines (35). In any study described here, we have investigated the interaction between Arix and the second messenger systems activated through cAMP and have found that Arix augments the CAMP and PKA response of the DBH gene. An investigation of the transcription factors that may play a role in mediating the CAMP response of the DBH gene has implicated members of the CREB, CREM, Fos, and Jun families. These experiments suggest that the environmental factors that influence the catecholaminergic phenotype may act through modulation of second messenger systems, which interact with a tissue-specific homeodomain protein to specify the noradrenergic phenotype.

EXPERIMENTAL PROCEDURES

Cell Culture—The PC12 cells used in these experiments are subclone GR5, isolated by Dr. Rae Nishi at the Oregon Health Sciences University. F9 and F9 cells were cultured in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum (Hyclone). HepG2 cells were cultured in minimal essential medium plus 10% fetal bovine serum, 1% nonessential amino acids, and 110 mg/liter sodium pyruvate. All cells were cultured in an atmosphere of humidified air containing 5% CO2.

Plasmid Constructs—The construction of plasmids containing the promoter and 5′-flanking sequence of TH and DBH genes cloned adjacent to the bacterial chloramphenicol acetyltransferase (CAT) transcription unit has been described previously (1, 16). Plasmids containing deletions or point mutations of the DBH promoter region were constructed by oligonucleotide-directed mutagenesis of plasmid 5′-untranslated DBH-CAT (−232/+14) using the Sculptor in vitro mutagenesis system (Amersham), according to the recommended procedures. RSV-Arix contains Arix cDNA sequences 1–1353, which includes all 5′-untranslated and protein coding sequences and 313 bases of the Arix 3′-untranslated sequence (35). This cDNA segment is cloned into pSPRSV, where Arix transcription is under the control of the Rous sarcoma virus promoter and enhancer elements. pSPRSV also contains a poly(A) addition signal from simian virus 40.

The original RSV-Arix, described in Zellner et al. (35), contained an extensive vector sequence in the 5′-untranslated sequence, which had been transferred to the Arix cDNA from several cloning steps. This extraneous sequence was removed from RSV-Arix, and the new expression vector exhibited improved transcription regulatory activity. All experiments, except those in Fig. 4 using F9 cells, were performed using the RSV-Arix construct without the vector-derived 5′-untranslated sequence. Results with the two constructs are qualitatively similar.

The construction of RSV-PKA was described in Maurer (40) and the K-CREB expression plasmid was described in Walton et al. (41).

Transfections—DNA used for transfection was purified using the Promega Wizard kit. Following purification according to the manufacturer’s procedures, DNA was precipitated from ethanol in the presence of ammonium acetate. Cell cultures, in 100-mm culture dishes, were transfected with DNA using calcium phosphate, as described previously (16, 42). Cell cultures contained 3–6 × 10^6 cells/dish. All transfections contained 2–3 μg of pRSV-L, encoding the luciferase transcription unit under the control of promoter and enhancer elements of RSV. In any given experiment, all cultures contained the same amount of transfected DNA, which ranged from 15 to 23 μg. Cells were harvested 2 days after transfection, and all nuclear cell extracts were analyzed for protein content, CAT activity (45), and luciferase activity (44).

In experiments using inducers, these agents were added 18 h before harvesting of cells. PC12 cells were treated with 200 μM CPT-cAMP, whereas HepG2 cultures were treated with 1 mM 8-Br-cAMP. The choice of cAMP analog for each cell line was based upon previous publications demonstrating the efficacy of that compound in stimulating transcription from a cAMP responsive promoter (45, 46). CAT activity is standardized to cotransfected luciferase, except when CAMP analogs or RSV-PKA are used. Preliminary experimental results indicated a stimulatory effect of cAMP and PKA on the RSV promoter in RSV-L. For experiments using CAMP or PKA, CAT activity is standardized to total extract protein.

Preparation of Nuclear Extracts and Electrophoretic Mobility Assay (EMSA)—Crude nuclear extracts were made from PC12 cells following the method of Dignam et al. (47) modified by a high salt (0.6 M KCl) extraction of the nuclear pellet (as described by Ausbel, 48). Additionally, all buffers used contained Ficoll (0.2 mg/ml; Boehringer Mannheim), leupeptin (0.5 μg/ml), and pepstatin (0.7 μg/ml) as protease inhibitors and NaF (10 mM) as a phosphate buffer. Nuclear extracts were dialyzed against nuclear extract buffer consisting of 20 mM HEPES (pH 7.9), 20% glycerol, 0.1 mM KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 10 mM NaF. Protein concentration of each nuclear extract was determined by Bradford assay (Bio-Rad), and aliquots were frozen and stored at −70 °C.

Synthetic sense and antisense oligonucleotides were end labeled with T4 polynucleotide kinase and [γ-32P]ATP and then annealed. The EMSAs were carried out in a 20-μl final volume containing 12.5 mM HEPES (pH 7.9), 10% glycerol, 5 mM MgCl2, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 2 μg of poly(dI-dC)poly(dI-dC). Nuclear extract protein concentrations were adjusted to 1.7 μg/μl with nuclear extract buffer containing bovine serum albumin. Labeled probe (10,000–25,000 cpm Cerenkov counts) was added to the reaction buffer containing 5 μg of nuclear extracts and incubated for 30 min at room temperature. For competition EMSAs, reaction mixtures containing nuclear extracts were preincubated for 20 min in the presence of double-stranded competitor oligonucleotides (200 ng) prior to the incubation with the labeled oligonucleotide probe. Samples were loaded carefully to minimize mixing and the complexes were resolved on 6% nondenaturing polyacrylamide gels (19:1 acrylamide: bisacrylamide) using an electrophoresis buffer containing 45 mM Tris borate and 1 mM EDTA. The gels were dried, and protein-DNA complexes were visualized autoradiographically. The sequences of the wild type DB1 enhancer and mutant CRE2/HD1m oligonucleotides as well as consensus CRE, AP1, and AP2 oligonucleotide sequences used as probes and competitors are given in Fig. 5.

Antibody Supershift Assays—To identify protein constituents of EMSA complexes, antisera directed against several putative cAMP-responsive transcription factors of the CREB/CREM/ATF family, plus antisera broadly reactive against Fos and Jun family members, were tested in EMSAs. Nuclear extracts and reaction buffer components were preincubated together with 2 μg of affinity-purified IgG (or 1 μl of whole antiserum) for 20 min at room temperature prior to a 30-min incubation with labeled probe and separated by electrophoresis as described above. Several different antisera against CREB and ATP-1 were tested in these analyses with similar results. The following is a list of antisera characterized in EMSA: CREB-1 (sc-271x, Santa Cruz Biotechnology, Inc.), mouse monoclonal IgG against human CREB-1 p43; CREB (no. 9192, New England Biolabs), rabbit polyclonal IgG against amino acids 123–137 of human CREB; ATF-1 (sc-241x), mouse monoclonal IgA against human ATF-1; ATF-1 (UBI 06-325, Upstate Biotechnology, Inc.), sheep polyclonal IgG against amino acids 247–263 of mouse c-Jun p39.

RESULTS

Arix Facilitates Activation of DBH Promoter by Cyclic AMP—Previous studies have demonstrated the transcriptional responsiveness of the DBH promoter to stimulation of both PKA
FIG. 1. DBH promoter. This is a schematic representation of the rat DBH (−232/14) promoter used in this study with a detail of the sequence encompassing the DBH enhancer region, bp −189 to −150 based on Shaskus et al. (16). Numbers below and in parentheses represent the numbering from McMahon and Sabban (4). The segments that correspond to known regulatory elements are labeled. These regulatory domains are: CRE-like sites, the human DBH-CRE homolog (hCREb), and CRE/AP1 (stipped boxes); core homeodomain recognition sites, HD1 and HD2 (boxes); the binding site for RNA polymerase II, TATA. The DB1 enhancer (underlined) represents the portion of the rat DBH promoter previously found to bind the Arix homeodomain protein and also to be critical for basal and second messenger-activated transcription.

and the Ca$^{2+}$-dependent protein kinase (protein kinase C) pathways (16, 20, 27). Positive regulatory elements within the DBH proximal promoter region function to drive transcription of a reporter gene in neuronally derived cell lines, whereas lower levels of transcription are observed in non-neuronal cell lines (16, 20). A single region contains both the cell type-specific and second messenger stimulatory elements. This region falls between −189 and −150 in the rat promoter and between −189 and −176 in the human DBH promoter (16, 20). The sequence of this critical region of the rat promoter is presented in Fig. 1.

The cell type-specific responsiveness of the proximal DBH promoter to cAMP stimulation was assessed by transient transfection of the DBH-CAT (−232/14) reporter construct into either the catecholaminergic PC12 pheochromocytoma cell line or into the non-neuronal HepG2 hepatoma or C6 glialoma cells. This DBH-CAT construct encompasses 232 bases of the promoter and 5′-flanking sequences of the DBH gene containing the critical enhancer region described above (illustrated in Fig. 1). Following transfection, the cells were either left untreated (basal) or treated with a cAMP analog. In PC12 cells, CPT-cAMP stimulated DBH-CAT transcription 28-fold over basal cultures (Fig. 2A). This cAMP-dependent activation of DBH-CAT has been described previously in catecholaminergic cell lines, both with the DBH proximal promoter region and with the 30-base DB1 regulatory element, encompassing bases −150 to −180 (16, 29). However, when DBH-CAT was transfected into HepG2 cells, treatment with 8-Br-cAMP evoked little stimulation of transcription from the DBH promoter (Fig. 2A). Similarly, C6 glialoma cells showed no stimulation of DBH-CAT transcription by cAMP treatment (data not shown).

The DBH-CAT construct used in the present experiments lacks the tissue-specific negative regulatory element found between −280 and −232 (49), eliminating the possibility of interference of this element on the cAMP-evoked response. In agreement with our results, Kim et al. (27) have reported that the human DBH reporter is unresponsive to the cAMP-dependent protein kinase in the non-neuronal HeLa and C6 glialoma cell lines, whereas a substantial increase in transcription was observed in a catecholaminergic neuroblastoma cell line. In their study, the DBH-CAT construct used contained the negative regulatory element, but the experiment described in Fig. 2A demonstrates that even in the absence of this inhibitory sequence, the DBH promoter is unresponsive to cAMP treatment in a non-neuronal cell line. Because the non-neuronal cell lines used in our study have been shown to possess the intracellular machinery for second messenger-mediated transcription of other genes (45, 50), the above results suggest that catecholaminergic cells may possess a cell type-specific coactivator necessary for cAMP-mediated DBH transcription.

The tissue-restricted pattern of Arix expression and the juxtaposition of the homeodomain binding sites and second messenger response elements led to the hypothesis that Arix may be one such cell type-specific coactivator necessary for stimulated transcription of the DBH promoter. Therefore, we tested whether transfection of an Arix expression plasmid (RSV-Arix) into HepG2 cells altered the response of DBH-CAT to cAMP-mediated activation. In contrast to non-Arix-expressing HepG2 cells, cultures transfected with Arix exhibit a substantially increased transcriptional response to cAMP (Fig. 2A). Although Arix alone induced a 5-fold increase in DBH-CAT transcription when compared with non-Arix-expressing cells, the combined expression of Arix with 8-Br-cAMP treatment resulted in an 18-fold increase in DBH-CAT activity. This 18-fold increase is greater than the sum of the two effectors alone, which would be expected if Arix and 8-Br-cAMP were acting independently.

The experiments presented here demonstrate that the homeodomain protein Arix can interact with cAMP-stimulated pathways to produce a greater than additive, or synergistic, stimulation of DBH promoter activity. Thus, Arix may function as a critical factor necessary for neuroendocrine activation of the DBH promoter in response to exogenous signals.

PKA Mimics the Actions of cAMP—To evaluate further the interaction of Arix with the cAMP signaling pathway on DBH-CAT transcription, an expression construct containing the
cDNA for the catalytic subunit of cAMP-dependent protein kinase (RSV-PKA; 40) was utilized. RSV-PKA plus DBH-CAT were cotransfected along with RSV-Arix into HepG2 or F9 mouse teratocarcinoma cells and compared with similar RSV-PKA stimulation of the DBH promoter in PC12 cells. Transfection of RSV-PKA into PC12 cells elicits a strong transcriptional response from the DBH-CAT construct, such that the reporter gene activity is 160-fold greater than basal (Fig. 2B). In contrast to PC12 cells, transfection of RSV-PKA plus DBH-CAT into HepG2 and F9 cells resulted in considerably less augmentation of CAT activity, reaching values of 16-fold over basal for HepG2 cells and 25-fold increase in F9 cells (Fig. 2B). Transfection of RSV-Arix in conjunction with RSV-PKA into these cell lines resulted in a marked increase (120–170-fold) in CAT activity. This synergistic interaction of Arix with PKA is similar to and more pronounced than that observed with Arix and cAMP treatment of the HepG2 cells.

A dose-response study was conducted to establish the optimal amounts of RSV-Arix and RSV-PKA needed to elicit the maximal response. Increasing amounts of RSV-Arix in the presence of 5 μg of RSV-PKA, or increasing amounts RSV-PKA in the presence of 5 μg of RSV-Arix, along with 5’-DBH-CAT (−232), were transfected into HepG2 cells. The response to RSV-Arix was maximal with 2 μg of plasmid, after which the extent of the transcriptional response reached a plateau, with no apparent squelching of transcription at the highest Arix doses (data not shown). Transfection of HepG2 cells with maximal amounts of Arix alone typically induces a 2–5-fold increase in CAT activity over basal from DBH-CAT WT (−232). The response to RSV-PKA continues to rise with increasing amounts of plasmid, until 6 μg, the highest amount used (data not shown). All of the experiments reported in this study were performed with 5 μg of RSV-Arix plasmid, providing the plasmid in excess of that which elicited a maximal response. This indicates that the effect of PKA is not simply to increase the amount of Arix expressed from the RSV promoter plasmid.

**Response of TH Gene to Arix plus PKA**—The TH gene, encoding the initial enzyme of catecholamine biosynthesis, is also responsive to the cAMP-stimulated pathway. In PC12 cells cotransfection of the TH-CAT construct 5’-TH-CAT (−773) with PKA results in a 35-fold increase in transcriptional activity (Fig. 3A). To evaluate whether the synergistic interaction between cAMP pathway stimulation and Arix observed with the DBH promoter is also seen with the TH promoter, HepG2 cultures were cotransfected with 5’-TH-CAT (−773), 2 μg of RSV-L, ± 5 μg of RSV-PKA. Similarly, HepG2 (panel B) cell cultures were transfected with 10 μg of 5’-TH-CAT (−773), 2 μg of RSV-L, ± 5 μg of RSV-PKA, with additional groups including ± 5 μg of RSV-Arix. Cells were harvested 2 days after transfection and assayed for CAT activity and protein content. Fold basal CAT activity values were calculated as described in Fig. 2. Each bar represents the mean ± S.E. of triplicate cultures, and these analyses have been repeated with similar results.

**DB1 Regulatory Element is Found in the Sequence TTGCGTCA (Fig. 1), Identical to the CRE2 Element of the Proenkephalin Gene (50) and the CRE-3 Element of the Prodynorphin Gene (51).** This sequence in the rat DBH gene has been designated CRE/AP1 (Fig. 1). A different potential second messenger responsive site has been identified in the human DBH gene (20, 34), containing the sequence TGACGTCA, which differs by a single base from the palindromic consensus CRE TGACGTCA. In the rat DBH promoter, the homologous segment to the human DBH CRE lies adjacent to the CRE/AP1 and has been designated hCREh, for human DBH-CRE homolog. Overlapping the junction between the CRE1 and CRE2 sites is a core recognition site (CCAT) for the transcriptional regulator YY1, which has been shown to contribute to regulation of the human DBH promoter (34). Thus, based on previous analyses it would appear that this enhancer region is a complex site for transcriptional regulators acting to modulate DBH gene expression.

To define the functional cAMP and Arix response elements, we have examined DBH-CAT promoter constructs with internal and 5’-deletions that eliminate one or both CRE-like domains, or constructs containing a mutation of the sequences encompassing either HD recognition site. The deletion and mutation constructs used in this study are illustrated in Fig. 4A.

Experiments mapping the regulatory elements necessary for PKA-mediated transcriptional stimulation in a catecholaminergic cell line were performed by cotransfection of these DBH-CAT constructs into PC12 cells along with RSV-PKA. Previous experiments have demonstrated that the region of the rat DBH promoter necessary for a response to cAMP lies between −175 and −163 (16, 29). In the experiments presented in Fig. 4B, when the sequence is deleted to −169, PKA-stimulated reporter gene activity is reduced to 15% of that obtained with WT (−232), and activity is reduced further when the sequence is deleted to −162. These results confirm previous reports of the location of the rat DBH CRE and suggest that the region between −175 and −169 is critical for CRE activity. These results also demonstrate that the HD core recognition sites, beginning at −167, are not sufficient to support wild type levels of cAMP-stimulated transcription in the absence of upstream elements. The difference between the response of WT (−169)
and WT(−162) does, however, suggest that the region containing the HD1 motif may independently contribute modestly to the PKA responsiveness of the parental reporter construct.

To define further the sequence requirements for the functional CRE within the DB1 promoter, internal deletion constructs that disrupt either hCREh or CRE/AP1 (hCREhΔ and CRE/AP1Δ, respectively) were transfected into PC12 cells. After PKA cotransfection, the hCREhΔ construct exhibited a modest reduction in activity compared with the parental WT(−232) construct (Fig. 4B). In contrast, the PKA-stimulated activity from the CRE/AP1Δ construct was reduced to 14% of WT(−232). Mutation of the sequence overlapping the CRE/AP1 and HD1 elements (i.e. CRE/HDm) displayed a reduction similar to that of the construct with the deleted CRE/AP1 (Fig. 4B). These results pinpoint the CRE/AP1 site as the major PKA-responsive element in the rat DBH gene.

Regulatory Elements Involved in Arix and PKA-mediated Responses in HepG2 Cells—To ascertain whether the same elements that function in PC12 are also necessary in HepG2 cells and to define the regulatory elements involved in the synergistic interaction between Arix and PKA, the mutant DBH constructs were used to map the sites responsible for the Arix- and PKA-mediated transcription in HepG2 cells. HepG2 cells were cotransfected with DBH-CAT mutants, and reporter gene activity was compared with HepG2 cultures transfected with the DBH-CAT construct alone.

In HepG2 cells the WT(−232) DBH-CAT construct is typically stimulated 2–5-fold by transfection with RSV-Arix alone and 15–30-fold by RSV-PKA alone, but together these agents act synergistically, stimulating the promoter 170–190-fold. The results shown in Table I are representative experiments used to map the elements within the DBH enhancer region which are necessary for these individual and synergistic responses. Deletion of sequence containing the hCREh (hCREhΔ) resulted in a modest increase in stimulated activity induced by Arix or PKA alone; yet, compared with WT(−232), there was little effect of this deletion on the maximal level of transcrip-
PKA response. As observed in PC12 cells (Fig. 4B), factors.

reactivity to antisera directed against candidate transcription cific oligonucleotides (sequences given in Fig. 5) and through cAMP-treated PC12 cells in an EMSA. These complexes were

eFFmer to cAMP in neuroblastoma cultures. It is therefore likely that this site is inhibitory to induction by cAMP signaling in complex 1. In both treatment groups, all but the most rapidly migrating complex (complexes 1–5) were specifically competed by an unrelated oligonucleotide, AP2, was used as a nonspecific competitor and showed no competition of the DB1-binding complexes (Fig. 6A, lanes 5 and 10). These findings demonstrate the specificity of interaction of complexes 1–5 for the DB1 sequence.

Several distinct DNA-protein complexes (labeled 1 through 6) were formed in the EMSA when the radiolabeled DB1 oligonucleotide was incubated with untreated (basal) or CPT-cAMP (cAMP)-treated PC12 nuclear extracts (Fig. 6A, lanes 1 and 6, respectively). Although the pattern of DNA-protein complexes formed from cAMP nuclear extracts was comparable to that from basal extracts, a notable exception is the cAMP-induced increase in intensity at the position of complex 1. In both treatment groups, all but the most rapidly migrating complex (complexes 1–5) were specifically competed by an excess of unlabeled DB1 oligonucleotide (Fig. 6A, lanes 2 and 7), whereas an unrelated oligonucleotide, AP2, was used as a nonspecific competitor and showed no competition of the DB1-binding complexes (Fig. 6A, lanes 5 and 10). These findings demonstrate the specificity of interaction of complexes 1–5 for the DB1 sequence.

Because the DB1 oligonucleotide contains sequence that is similar to CRE and AP1 recognition sequences, oligonucleotides containing consensus CRE and AP1 binding sites (see Fig. 5) were also used as competitors to characterize further the sequence recognition specificity of the observed DB1-protein interactions. Preincubation of nuclear extracts with either CRE or AP1 oligonucleotides completely competed the formation of both the basal and cAMP-induced DB1 binding complexes migrating as complex 1 (Fig. 6A, lanes 3 and 8, lanes 4 and 9, respectively). The formation of complexes 2 and 3 was partially reduced by either CRE or AP1 competitor oligonucleotides, and the AP1 oligonucleotide also reduced the intensity of bands 4 and 5 in cAMP-treated cultures. These results demonstrate the presence of CRE-like and/or AP1-like binding activities that interact with the DB1 enhancer. Furthermore, these data indicate that cAMP treatment of PC12 cells results in the induction of a DNA-binding protein complex that is composed of proteins that can bind to consensus CRE and/or AP1 sites.

The contribution of the CRE/AP1-HD1 composite element to DNA-protein complex formation was examined using a DB1-based oligonucleotide containing the CRE/HDM mutation as the labeled probe in the EMSA. Fig. 6B shows the results from a representative EMSA analysis. In these assays complex 1 is completely absent, yet, two bands colocalizing with complexes 2 and 3 are present in a pattern similar to that seen with the DB1 probe (compare Fig. 6A, lanes 1 and 6 with 6B, lanes 1 and 6). Notably, cAMP-induced DNA-protein complex formation is eliminated by the mutation of the CRE/AP1 HD1 elements (lane 6). Although excess DB1 oligonucleotide completely competed the bands migrating at complexes 2 and 3 off the CRE/HDM probe (Fig. 6B, lanes 2 and 7), the CRE and AP1 oligonucleotides did not eliminate these bands (Fig. 6B, lanes 3 and 8 and lanes 4 and 9, respectively), leaving a competition band shift pattern similar to the CRE and AP1 competitions seen when the DB1 oligonucleotide was used as probe. Competition
with the unrelated AP2 oligonucleotide had no effect on the formation of these slower migrating complexes with the CRE/HDm probe (Fig. 6B, lanes 5 and 10). The intensity of complexes migrating to positions 4 and 5 is increased markedly with the CRE/HDm probe. However, the complexes are poorly competed by DB1, suggesting that they are associated with the mutant sequence of the CRE/HDm probe or are nonspecific.

These results of the EMSA analyses with the CRE/HDm probe support those of Fig. 6A and suggest that the CRE/AP1 and HD1 elements of DB1 are necessary for basal and induced DNA-protein complex formation. The same DB1-binding proteins that recognize consensus AP1 and CRE binding sites (Fig. 6A) are absent when the CRE/AP1 and HD1 segments of DB1 are mutated. Furthermore, the elimination of cAMP-induced complex formation by mutation of the CRE/HD sequence correlates well with the reduced PKA inducibility of the DBH-CAT promoter construct with the identical mutation (CRE/HDm in Fig. 4). Taken together, the transfection and EMSA results in PC12 cells demonstrate that cAMP induces a CRE/AP1 binding activity that interacts with the same site of the DB1 promoter which is necessary for PKA-induced transcription in PC12 cells.

**Immunological Characterization of Proteins Interacting with DB1**—To identify nuclear proteins that complex with DB1 in EMSA, specific antisera to candidate proteins known to interact with CRE and AP1 sites were tested in EMSAs. However, members of the Fos and the Jun families appear to be the major constituents of the DB1-binding complexes formed in EMSA with PC12 cell nuclear extracts. CREB and CREM appear to represent only a minor component of the CRE/AP1 element. Two different antisera against ATF-1 were tested in EMSA but produced no supershift or band disruption with either basal or cAMP-treated nuclear extracts on the DB1 probe, although these antisera were able to supershift recombinant ATF-1 binding to the DB1 probe (data not shown). These results suggest that CREB and CREM but not ATF-1 are present in DB1 enhancer-binding complexes from PC12 nuclear extracts. However, because these results showed weak supershifts and the lack of complex disruption with the various antisera tested, CREB and CREM do not appear to be major components of the basal or cAMP-induced complexes binding to the DB1 enhancer.

The efficacy of these antisera to recognize and interact with PC12 nuclear extract proteins was tested using a consensus CRE as probe in EMSA. Both CREB and CREM antisera produced supershifts with untreated and cAMP-treated extracts on the CRE probe (Fig. 7B, lanes 7–12). Notably, both antisera also markedly disrupted the normal band shift pattern of untreated PC12 nuclear extracts, whereas only the CREM antisera disrupted the cAMP-induced complexes formed on the CRE probe. These results confirm the presence of immunoreactive species to CREB and CREM antisera in PC12 cells and indicate the effectiveness of this amount of antisera in the disruption/supershift EMSA. These results also indicate that cAMP treatment appears to induce a CRE binding activity that may represent a CREM-reactive species. CREM proteins were previously demonstrated to increase when PC12 cells are treated with an effector that elevates cAMP (33).

The finding that classical CRE-binding proteins do not appear to be major constituents of the DB1-binding complexes prompted an initial characterization of AP1 family member proteins as candidate DB1-binding proteins. We tested antisera directed against Fos family and Jun family members of AP1-binding proteins in EMSA supershift analyses. An antiserum broadly cross-reactive for Fos family members completely disrupted the slowest migrating complexes formed on the DB1 probe, including the complex induced by cAMP treatment (Fig. 7A, lanes 9 and 10). Additionally, this antiserum produced a visible supershift with cAMP-treated nuclear extracts. The Jun family antiserum produced a marked disruption of complexes formed with basal PC12 nuclear extracts and a partial disruption of the cAMP-induced complexes, yet no supershifted bands were detectable (Fig. 7A, lanes 11 and 12). The Fos family and Jun family antisera did not alter the EMSA band shift pattern on the CRE/HDm probe, indicating the specificity of the disruption/supershift for proteins interacting with the CRE/HD region of the DB1 enhancer (data not shown). These findings indicate that Fos and Jun family members are likely constituents of the basal complex 1 as well as a major component of the cAMP-induced complex binding to the DB1 probe.

To summarize the analyses of CRE/AP1-binding proteins, CREB, CREM, Fos family, and Jun family members were all immunologically detected in DB1-associated DNA-protein complexes formed in EMSA with PC12 cell nuclear extracts. CREB and CREM appear to represent only a minor component of the DB1-binding species in either untreated or cAMP-treated cultures. However, members of the Fos and the Jun families appear to be the major constituents of the slowly migrating

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**EMSA Oligonucleotide Probes and Competitors**

- **DB1**: GAGCCTCGCTCATCCAGTTAGAGCTGCT
  - TACAGTTCCATCATCATACGGGGATCC
- **CRE/HDm**: AGTCCGACGCTGCTGAGAGCACTCCTTG
  - TACAGTTCCATCATCATACGGGGATCC
- **Consensus AP1**: GAGCCTCGCTCATCCAGTTAGAGCTGCT
  - TACAGTTCCATCATCATACGGGGATCC
- **Consensus CRE**: GAGCCTCGCTCATCCAGTTAGAGCTGCT
  - TACAGTTCCATCATCATACGGGGATCC
- **Consensus AP2**: GAGCCTCGCTCATCCAGTTAGAGCTGCT
  - TACAGTTCCATCATCATACGGGGATCC

**FIG. 5. EMSA oligonucleotide probes and competitors.** These are the oligonucleotide probes and competitors used in the EMSAs to characterize nuclear proteins that interact with the DB1 enhancer region. Putative binding sites and mutated sequences in DB1 and CRE/HDm are designated following Fig. 4A. Consensus sequences for AP1, CRE, and AP2 oligoentides are underlined.
Involvement of CREB in the Cellular PKA Response—The observations that purified CREM and CREB bind to the DB1 probe and that antiserum to CREB and CREM causes a supershift in the EMSA with the DB1 probe and PC12 cell nuclear extracts suggest the involvement of these bZip transcription factors in the cAMP response of the DBH promoter. To evaluate further the role of CREB, a dominant negative mutant of CREB, K-CREB (41), was used. This mutation, a single amino acid change in the DNA binding domain, results in a protein that is able to dimerize with normal CREB or CREM proteins but inhibits DNA binding of the normal partner. K-CREB has been shown to inhibit cAMP stimulation of transcription of several gene promoters, including somatostatin (41), tyrosine hydroxylase (28), and PEPCK (52). Fig. 8 presents experiments in which a K-CREB expression vector was cotransfected with or without PKA and the DBH-CAT or TH-CAT constructs into PC12 cells. These data show that K-CREB had no inhibitory activity on the basal activity of either DBH-CAT or TH-CAT promoters in PC12 cells. However, K-CREB inhibits the PKA response by 50% for DBH-CAT and 60% for TH-CAT. The extent to which K-CREB inhibits PKA-mediated induction of DBH-CAT is not altered by increasing the amount of K-CREB construct transfected.

These results, demonstrating an inhibitory effect of K-CREB on the PKA response of the DBH and TH promoters, support a role for CREB, or a family member capable of dimerization with K-CREB, in a portion of the cAMP response. The lack of effect on the basal activity of either TH or DBH suggests that CREB is not involved in maintenance of basal transcription of these genes.

**DISCUSSION**

The regulation of eukaryotic gene transcription involves integration of multiple nuclear signals conferred upon genetic recognition elements. The study reported here describes the interaction between two regulatory elements of the neuroendocrine-specific DBH gene. These two elements, one homeodomain and the other cAMP-responsive, together interact to influence the specificity of DBH gene expression. In so doing, these elements may influence the cellular neurotransmitter phenotype, an essential feature of neuroendocrine function.

The major finding of this study is the demonstration of a synergistic interaction between Arix and PKA in the activation of the DBH promoter. In previous studies, the DBH homeodomain binding sites were shown to interact with the transcription factor Arix, which is expressed selectively in cell lines derived from noradrenergic cells (34, 35). However, in the basal condition, Arix exerted little transcriptional stimulation of the DBH promoter. The inability of Arix to transactivate the DBH promoter in non-catecholaminergic cell lines was puzzling and

**FIG. 6.** cAMP induces a CRE/AP1-binding complex that is eliminated by the CRE/HDm mutation. Nuclear extracts (5 μg of total protein) from untreated (Basal) PC12 cells or cells treated with CPT-cAMP (cAMP) for 2 h were analyzed by EMSA using either the DB1 probe (panel A) or the CRE/HDm mutant probe (panel B). Competitor oligonucleotides (200 ng) were preincubated with extracts as indicated (lanes 2–5 and 7–10). Control lanes (1 and 6) display multiple protein-DNA complexes (complexes 1–5 appear to be specific for the DB1 probe). Note the competition of the slowest migrating complexes at position 1 (basal and induced) by CRE and AP1 oligonucleotides and the absence of these complexes with the CRE/HDm oligonucleotide probe (*).

**FIG. 7.** Characterization of CRE/AP1 binding activities from PC12 cell nuclear extracts. Nuclear extracts (5 μg of total protein) from basal (B) and cAMP-treated (C) PC12 cells were preincubated with specific antisera prior to incubation with either DB1 (panel A), CRE/HDm (panel B, lanes 1–6), or consensus CRE (panel B, lanes 7–12) oligonucleotide probes. Antisera used in these assays include anti-CREB IgG (αCREB, 2 μg of nuclear extract buffer 9192), CREM antisem (αCREM, 1 μl of serum UBI 06350), anti-Fos family IgG (αFos, 2 μg of sc-052), or anti-Jun family IgG (αJun, 2 μg of sc-044) as indicated.
suggested the need for a coregulator. One of the major enigmas in understanding homeodomain function has been the parameter of specific recognition of a homeodomain binding site. In vitro, most homeodomain proteins recognize the core sequence of TAAT (or ATTA, as written in Fig. 1), and yet in vivo, target genes are activated selectively by specific homeodomain proteins. It has been suggested that coregulatory proteins, such as Pbx or extradenticle (53–56), interact directly with certain homeodomain proteins to specify activation through the HD recognition sites. In the case of DBH promoter activation, an interaction of cAMP-dependent coactivators with Arix may be necessary for selective target sequence recognition and/or cell-type specific transcriptional activation.

To understand the mechanisms of interaction between Arix and the cAMP pathway, it is necessary to identify the sequences responsible for the cellular response to elevated cAMP and then to identify the proteins bound to this sequence. In defining the cAMP-responsive element, the experimental results are best explained by invoking the presence of a major CRE, at the position indicated as CRE/AP1 in Fig. 1. When this CRE is mutated, more than half of the PKA response is lost in PC12 cells. Our results also demonstrate the involvement of the CRE/AP1 site in the transcriptional response to PKA plus Arix in HepG2 cells. The finding that deletion of both potential CRE elements, leaving both HD elements intact, eliminates the PKA response in either cell line indicates that the CRE and HD binding sites are functionally separable and that the PKA response cannot be attributed to events occurring solely at the Arix binding sites.

As an initial step in understanding the biochemical and molecular nature of the interaction between Arix and the cAMP signaling system, we have begun to identify proteins bound at the CRE/AP1 segment within DB1. In vitro studies have identified a DNA-protein complex, induced by cAMP, which binds this CRE/AP1 site. Using antisera to known CRE-binding proteins, the presence of both CREB and CREM-related proteins, but not ATF-1, was established in DNA-protein complexes between DB1 and PC12 nuclear extracts. Consistent with a role of CREB or CREM in regulating the cAMP response of the DBH gene, the dominant negative mutant of CREB reduced the response of the DBH promoter to PKA. This mutant would be able to dimerize with either CREB or CREM and could thereby influence the transcriptional activity of either factor. The role of CREB or CREM could be to interact directly with the DBH gene, or they could induce the transcription of another factor that would then bind to and regulate transcription of DBH.

Additional experiments have shown that members of the Fos and Jun families of AP1 transcription factors also bind to the DB1 oligonucleotide and that, in fact, these proteins represent the major component of the cAMP-induced complex. The binding of Fos to DB1 agrees with experiments reported by Nankova et al. (57), who have found a slowly migrating, stress-induced complex that binds to DB1 and is recognized by Fos family antisera in extracts from rat adrenal medulla. Although Fos and Jun are not usually found to be involved in the transcriptional response to cAMP, c-fos expression is increased in PC12 cells following cAMP treatment (58, 59). In summary, the current data indicate that Fos and Jun family members, CREB, and CREM can all bind to the DB1 element in PC12 cell extracts. It is probable that the direct binding of CREM and CREB to DB1 represents only a small component of their influence on the cAMP-stimulated transcription of the DBH gene. The major role of CREM or CREB is likely to be the induction of Fos, which then, along with its partner Jun, interacts directly with the DB1 regulatory element to influence transcription.

The involvement of several distinct transcriptional regulatory proteins in signal transduction through a single site has also been described for a similar CRE element, the CRE-2 of the proenkephalin gene. CREB, ATF-3, and JunD have all been implicated in the transcriptional response of this gene to elevated cAMP (31, 32, 60). Because the CRE sequence of the ENKCRE-2 and DB1 regulatory elements is a hybrid between the consensus AP1 and CRE sites, different activators, with varied heterodimer composition, may have the potential to interact. These complexes may have a lower affinity for the hybrid site than for either consensus site, yet, the interaction of the CRE/AP1-binding proteins with other proteins, such as Arix, may stabilize their binding to the hybrid site.

The location of the rat CRE characterized in this study is different from that reported by Kim and colleagues in their study of the human DBH gene (20, 27). In those studies, researchers found that deletion of the 14-base region homologous to hCREh segment of rat DBH (Fig. 1) completely eliminated the second messenger response and also reduced basal activity. In the rat gene, the hCREh segment does not appear to play a major role in the PKA response of PC12 cells or the synergistic Arix plus PKA response of HepG2 cells, as mutation of this element caused only a modest alteration in these responses. Thus, the human and rat genes may respond to elevations of cAMP by interaction with transcriptional activator proteins at different sites. The human gene has conserved the HD binding sites, retaining the potential interaction of Arix and the cAMP signaling system.

The synergistic interaction between Arix and PKA-activated transcription factors with the DBH enhancer is not observed with the TH promoter, where each effector acts independently to stimulate transcription. The additive versus synergistic influence of these transcriptional activators may be the result of the genomic organization of the regulatory elements. In the TH gene, the likely Arix interaction site is within the octomer/ heptamer region between −168 and −188 and is separated from the promoter proximal CRE by more than 100 bases (see 28). In contrast, within the DBH promoter the Arix interaction sites are adjacent to, and even overlapping, the CRE. In other systems, it has been found that binding of transcription activator proteins to adjacent sites can result in a synergistic transcriptional response, even if the proteins are unlikely to
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interaction physically with each other. Evidence suggests that the transcription factors may recruit each other to the gene by a cooperative nucleosome-binding mechanism involving disruption of histone-DNA contacts (61). With the Arix and PKA response, the closely appositioned regulatory sites on the DBH DB1 element could lead to cooperative nucleosome binding of activating proteins, leading to synergistic gene activation, whereas the more distant spacing of the TH response elements would support independent binding and transcriptional response.

Although regulatory cross-talk is a common occurrence in the control of gene transcription, there is little precedent for the interaction of homeodomain proteins with those involved in second messenger stimulation of transcription. The paired-like homeodomain protein Phox1 has been shown to impart extracellular signal responsiveness to the serum response element of the c-fos gene (62). Similar to the DBH enhancer region, the serum response element is a composite of juxtaposed regulatory elements, including binding sites for both serum response factor and homeodomain proteins. Gruenberg et al. (62, 63) have demonstrated that Phox1 plays a role in recruiting the serum response factor to the serum response element in response to stimulation of intracellular second messenger pathways with serum or epidermal growth factor. These authors suggest that the homeodomain component of these interactions may contribute to the tissue specificity of c-fos serum response element responsiveness (62).

Because the homeodomain of Phox1 is 75% identical to Arix, a similar mechanism of action may be involved in Arix-mediated second messenger responsiveness of the DBH enhancer. Possibly the synergistic effect of Arix with the CAMP system will also involve a cooperative interaction between two closely appositioned transcription factors, Arix and the CRE/AP-1-binding protein(s). Alternatively, a coactivator protein, which does not bind DNA, may contact both Arix and the CRE/AP-1-binding protein, consequently stimulating transcription of the DBH gene. In this case, in catecholaminergic cells Arix is present and able to interact with appropriate coactivator proteins, whereas in non-catecholaminergic cells (such as HepG2 cells) the appropriate coactivator proteins may not be recruited to the DBH promoter in the absence of Arix.

The experiments in this study have described the interactions of a cell type-specific homeodomain protein with the ubiquitous cAMP signaling system on the transcriptional regulation of both the TH and DBH genes. TH and DBH are coexpressed in the peripheral nervous system and adrenal medulla and are coregulated in response to many physiological stimuli. In vivo, noradrenergic cells of the sympathetic nervous system and adrenal medulla are derived from the trunk neural crest. The cellular phenotype of neural crest-derived tissue is dependent upon extracellular signals encountered during migration (see 18, 64). The chicken and mouse homologs of Arix (Phox2) are expressed in the aggregating sympathoblasts and some cranial sensory ganglia close to the time of onset of DBH or TH expression (37–39). A recent analysis of Phox2 knockout mice suggested that this homeobox protein is necessary for development of these noradrenergic traits in peripheral cranial ganglia (21). The present study strengthens this notion and suggests a direct functional role of this homeodomain protein in the regulation of DBH expression in the cell. Perhaps the signals encountered during the migration of peripheral neuroblasts activate intracellular second messenger pathways, which then interact with cell type-specific transcription factors, such as Arix, to elicit stable expression of DBH, the consequence of which is the establishment of the noradrenergic phenotype.
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