Inducible cAMP Early Repressor Can Modulate Tyrosine Hydroxylase Gene Expression after Stimulation of cAMP Synthesis*

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Members of the CREB/CREM/ATF family of transcription factors either enhance or repress transcription after binding to the cAMP response elements (CREs) of numerous genes. The rat gene for tyrosine hydroxylase (TH) bears a canonical CRE, at base pairs −38 through −45 from the transcription initiation site, that is essential for basal and cAMP-stimulated transcription (Kim, K.-S., Lee, M. K., Carroll, J., and Joh, T. H. (1993) J. Biol. Chem. 268, 15689–15695; Lazaroff, M., Patankar, S., Yoon, S. O., and Chikaraishi, D. M. (1995) J. Biol. Chem. 270, 21579–21589). The current study identifies CRE-binding proteins induced in pharmacological paradigms characterized by TH activation.

PC12- and rat adrenal gland-derived nuclear proteins retarded a TH-CRE oligonucleotide in gel mobility shift assays with virtually identical patterns. These differed substantially from patterns exhibited by extracts from locus ceruleus or from neuroblastoma (SK-N-BE(2)C) and locus ceruleus-derived (CATH.a) cell lines. Forskolin stimulation of PC12 cells and reserpine treatment of rats increased, in nuclear extracts derived from cells and adrenal glands, respectively, the amount of a fast moving CRE/protein complex that was supershifted by an anti-CREM antibody. Subsequent Western, Northern, and polymerase chain reaction analyses indicated that a specific member of the CREM family, the inducible cAMP early repressor (ICER), was strongly induced in both systems. Cotransfection of PC12 cells with TH2400CAT plasmid and the expression vector pCMV-ICER-Ib demonstrated that ICER efficiently represses the transcriptional activity of the TH gene promoter. In addition, PKA-stimulated transcriptional activity of the promoter was effectively suppressed by ICER.

These results suggest that ICER can modulate cAMP-stimulated transcription of the TH gene and provide a model accounting for rapid reversal of increased TH transcription following elevations in cAMP.

The CREB/CREM/ATF family of transcription factors (3–11)

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** The abbreviations used are: CRE, cAMP response element; PKA, cAMP-dependent protein kinase; bp, base pair(s); PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; ICER, inducible cAMP early repressor; TH, tyrosine hydroxylase.
elements play important roles in functional regulation of TH gene transcription (1, 2, 48, 49). Although conflicting data exist, probably related to cell line differences, the CRE, located from 38 to 45 bp upstream of the initiation site, appears to be crucial for both basal and cAMP-induced transcription of the TH gene (1, 2).

To identify members of the CREB/CREM/ATF family that regulate TH transcription, gel mobility shift assays were performed using an oligonucleotide representing the TH CRE (identical in sequence to bp -52 through -32 of the rat TH promoter). Nuclear proteins were obtained from TH-expressing cell lines and rat tissues under control conditions and in response to pharmacological manipulations that increase TH mRNA. We found that ICER is strongly induced in the adrenal gland by reserpine and in PC12 cells by forskolin. Cotransfection experiments confirmed that ICER is indeed capable of modulating TH promoter activity in PC12 cells. These results suggest that, in addition to its previously recognized stimulatory effects on TH transcription, the cAMP signaling cascade can also repress TH expression via an ICER-mediated mechanism.

EXPERIMENTAL PROCEDURES

Animals and Tissue Preparation—All procedures were approved by the Institutional Animal Care and Use Committee of the Cornell University Medical College. Male Sprague-Dawley rats (200–300 g), housed 2–3/cage with free access to food and water, under a 12-h light/12-h dark cycle, received subcutaneous injections of reserpine, 10 mg/kg in 20% ascorbic acid, or an equivalent volume of the vehicle, 2–3 h after lights-on. Four hours later, they were decapitated, and the adrenal glands and brains rapidly dissected, frozen in liquid N₂, and stored at −80 °C until extraction of nuclear proteins or RNA.

Tissue Culture Procedures—PC12 cells and CATH.a cells were grown in RPMI 1640 medium, pH 7.2, supplemented with 10% horse serum, 5% fetal calf serum, 50 units/ml penicillin, and 25 mg/ml streptomycin. SK-N-BE(2)C cells were maintained in Dulbecco’s modified Eagle’s medium, pH 7.5, 10% calf serum, and the above antibiotics. Cells were harvested from 30 min to 8 h after treatment with forskolin (10 μΜ) and isobutyl methylxanthine (0.5 mM), frozen in liquid nitrogen, and stored as above.

Gel Mobility Shift Assay—Micropurification of nuclear proteins from either animal tissues or cell line cultures was performed according to Roy et al. (50). Briefly, 4 ml of NE1 (250 mM sucrose, 15 mM Tris-HCl, pH 7.9, 140 mM NaCl, 2 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 25 mM KCl, and 2 mM MgCl₂) were added to tissues (0.2–1.5 mg) or cells (5–10 × 10⁶). Tissues were first homogenized in a Dounce tissue grinder and filtered through cheesecloth. To free nuclei, tissue was further homogenized with added Nonidet P-40® (0.5%), washed twice, and pelleted at 3000 × g. One “packed cell volume” of NE2 (NE1 buffer containing 350 mM KCl) was used to resuspend nuclei and, after a 5-min incubation on ice, 20 strokes in the Dounce tissue grinder were performed. The homogenate was centrifuged for 90 min at 4°C (180,000 × g), and the supernatant was dialyzed for 45 min against 50 mM KCl, 4 mM MgCl₂, 20 mM K₃PO₄ (pH 7.4), 1 mM 2-mercaptoethanol, and 20% glycerol. Extract protein concentrations were determined with a colorimetric assay (Bio-Rad).

In a 20-μl volume, nuclear proteins (4–15 μg) were incubated at room temperature for 20 min in binding buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 5 mM dithiothreitol, 5 mM EDTA, 20% glycerol) in the presence of poly(dI-dC) (1 μg) with specific double-stranded oligonucleotides end labeled with ³²P. The samples were loaded on a 6% acrylamide gel and run at 100 V in a low ionic strength buffer (0.25 × TBE) for 2 h. The gels were dried and autoradiographed at room temperature.

The following oligonucleotide sequences were used.

**CRE-TH:** 5′-GAGGGGCTTTGCAGGTACGGCTGGG-3′
3′-ACTCCCGGAACTCCGACTGTCGGAC-5′

**AP-1-TH:** 5′-GGAGGGGCTTTGCAGGTACGGCTGGG-3′
3′-ACTCCCGGAACTCCGACTGTCGGAC-5′

**Sequence 1**

**Sequence 2**

**FIG. 1.** Gel mobility shift assay: identification of TH-CRE-bind ing proteins and analysis of the effects of forskolin and reser pine. 5 μg (cell lines) or 10 μg (animal tissues) of nuclear protein derived from locus cereuleus, adrenal gland, or the cell lines PC12, SK-N-BE(2)C and CATH.a were incubated 30 min, at room temperature, with a 23-bp oligonucleotide representing rat TH promoter region −54/−32 (TH-CRE) and resolved on acrylamide gel. The oligonucleotide was labeled with T4 nucleotide kinase and [γ-³²P]ATP and 30,000 cpm used for each reaction. The retardation patterns (control (Ctl) and vehicle (Veh)) were compared with those obtained with proteins extracted 4 h after cell treatment with forskolin (Fsk) or 4 h after reserpine injection for the adrenal and locus cereuleus samples (RES). In PC12 cells and adrenal gland, the formation of a fast running complex (arrow) was enhanced by the pharmacological treatments. In the last lane, the probe was electrophoresed in the absence of nuclear proteins.

For supershift experiments, the extracts were preincubated for 20 min with appropriate antisera on ice and then with labeled oligonucleotides as above. CREB, CREM, p-CREB, ATF1, and ATF2 were purchased from Upstate Biotechnology Inc. (Lake Placid, NY).

Western Blot Analysis—Separation of nuclear proteins (50–150 μg) was carried out by SDS-polyacrylamide gel electrophoresis (8.5%) for 1 h at 20 watts in Tris-glycine buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS) at 22 °C. The proteins were electrotransferred on a supported nitrocellulose membrane (0.45 μM) as described previously (51). The membranes were soaked in phosphate-buffered saline solution and 5% nonfat dry milk for 2 h with gentle agitation. For immunological detection, the membrane was incubated with the primary antibodies, CREB (diluted 1:10,000) and anti-mouse CREM (diluted 1:1,000), for 2 h followed by three washes (10 min each in 5% nonfat dry milk, 0.02% Tween 20®, phosphate-buffered saline) under gentle agitation. Peroxidase-conjugated goat anti-rabbit immunoglobulins (DAKO, Carpenteria, CA) were used as secondary antibodies at a 1:2000 dilution (2 h incubation followed by three wash steps).

Northern Blot Analysis—The mRNA was extracted from either animal tissues or cells with the Poly(A)Tract® System1000 (Promega, Madison, WI). mRNA (1–5 μg) was electrophoresed in a 1% agarose gel, transferred to a nylon membrane (Amersham), and hybridized with specific probes labeled by random priming with [α-³²P]dCTP. Blots were exposed to Kodak XAR film for 2 days (51).

PCR—mRNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase in the presence of oligo(dT) and [α-³²P]dCTP as radioactive tracer. The cDNA obtained was amplified with specific oligonucleotides designed to prime some of the different CREM isoforms. Some of the fragments amplified by PCR reaction were purified from agarose gels, labeled, and used as probes in the Northern blot analysis. BamHI and EcoRI restriction sites were introduced in the 5′ and 3′ primers, respectively, to facilitate the following subcloning. The conditions used for amplification are the same as described by Hoeffler et al. (52). Primers are as follows: R-ICER 5′ (B5′), ACTTAGCATCCACTGTGGTAGCCAC; R-CREM 1a3 (Ia3), GAGGCTC-GAAAGCCAAATTCACATCTCAGACGAG; R-CREM 8b (Ib5′), GTTAA-TAGAATCTACATACTGTTTGGC; R-CREM 2a (A5′), GAGG-
Transfection and Chloramphenicol Acetyltransferase (CAT) Assay—
Transfections performed into PC12 cells with the reporter TH2400CAT and the expression PKAc plasmids and the following CAT assay have been already described (1). The expression vector CMV-ICER-Ib was obtained by subcloning the PCR-amplified ICER-Ib cDNA in 
BamHI and EcoRI sites of the polylinker region of the pcDNAI/AMP plasmid (Invitrogen, San Diego, CA).

RESULTS
Comparison of TH-CRE Gel Retardation Patterns in Control Conditions and after Forskolin or Reserpine Treatments—

As an initial step in defining the role of CRE-binding proteins in TH gene regulation, gel retardation analysis was performed on TH-expressing cell lines and tissues obtained from both peripheral and central nervous system of rats. Gel retardation patterns that varied among the different extracts (Fig. 1). Nuclear extracts from the adrenal gland and PC12 cells exhibited almost identical binding patterns, which differed from those

ATCCGGAAGCTGCCCGGGAGTG.

Transfection and Chloramphenicol Acetyltransferase (CAT) Assay—
Transfections performed into PC12 cells with the reporter TH2400CAT and the expression PKAc plasmids and the following CAT assay have been already described (1). The expression vector CMV-ICER-Ib was obtained by subcloning the PCR-amplified ICER-Ib cDNA in BamHI and EcoRI sites of the polylinker region of the pcDNAI/AMP plasmid (Invitrogen, San Diego, CA).

FIG. 2. Gel mobility shift competition assay: identification of the complexes formed by CRE-binding proteins. Gel shift assays, performed using unlabeled competitor oligonucleotides, demonstrated the specificity of protein/TH-CRE interactions in PC12 cells and adrenal extracts. A, competition analysis was performed against DNA/protein complexes derived from PC12 cells treated with forskolin (10 μM, 4 h). In lanes 3–5, 5 μg of nuclear proteins were incubated with γ32P-labeled TH-CRE oligonucleotide in presence of the indicated molar excess of unlabeled CRE oligonucleotide. In lanes 6–8, the same experiment was performed changing the competitor to a TH-AP1 oligonucleotide. B, the competition assay was carried out with proteins derived from adrenal gland (10 μg) of rats treated with reserpine (10 mg/kg, 4 h). Lanes 1 and 2 show adrenal nuclear extract in the absence of competitor oligonucleotide from vehicle (Veh)- and reserpine (Res)-treated rats, respectively. Lanes 3 and 4 show extracts from reserpine-treated rats in the presence of increasing concentrations of unlabeled CRE. Lane 5, free probe with no extract.

FIG. 3. Identification of CRE-binding proteins with specific antibodies in a supershift assay. A, nuclear proteins derived from forskolin-treated PC12 cells were preincubated with antibodies raised against CREB (lane 2, 0.2 μl of the antiserum), phospho-CREB (P-CREB; lane 4, 0.15 μg of IgG), CREM (lanes 5 and 6, 0.2 and 1 μl, respectively, of the antiserum), ATF-1 (lane 7, 0.5 μg of IgG), and ATF-2 (lane 8, 0.5 μg of IgG). After 30 min the nuclear homogenates were incubated with TH-CRE oligonucleotide and subjected to electrophoresis. No antibodies were added to the binding reaction in lanes 1 and 3. B, nuclear extracts were resolved in the presence (lanes 2, 4, 6, and 8) or absence (lanes 1, 3, 5, and 7) of CREM antiserum. Ctl, control; Fsk, forskolin-treated; Veh, vehicle; Res, reserpine-treated.
observed in the extracts from central nervous system or other catecholamine-producing cell lines. Extracts of human neuroblastoma-derived SK-N-BE(2)C cells (53) and rat locus ceruleus-derived CATH.a cells (54) showed unique binding patterns.

Previous studies demonstrated an increase in rat adrenal TH mRNA within 4 h of administration of reserpine (55) and within 4 h of forskolin treatment of PC12 cells (56). We therefore examined gel retardation patterns at 4 h. Nuclear extracts from both forskolin-treated PC12 cells and adrenal glands from reserpine-treated rats displayed dramatic increases in a fast moving protein-DNA complex (Fig. 1). In contrast, neither treatment substantially altered binding patterns in the CATH.a or locus ceruleus extracts (Fig. 1).

Analysis of CRE Binding Specificity—To examine the binding specificity of PC12 and adrenal gland extracts, the gel retardation assays were performed in the presence of molar excess of either TH-CRE or TH-AP1 unlabeled nucleotides (Fig. 2, A and B). In extracts from both sources, excess unlabeled CRE prevented the formation of proteins-DNA complexes (Fig. 2, A and B). In contrast, even 100-fold molar excesses of the AP-1 oligonucleotide did not efficiently interfere with the binding between the CRE probe and nuclear proteins derived from either PC12 cells (Fig. 2A) or the adrenal gland (data not shown).

Supershift and Western Blot Assays to Identify TH-CRE-binding Proteins—Using antibodies against different members of the CREB/CREM/ATF family, supershift assays were performed to make an initial identification of the proteins in the forskolin-induced fast running complex from PC12 cells. The anti-CREM antibody, even at the lowest concentration, supershifted the band in both control (Fig. 3B) and forskolin-treated (Fig. 3, A and B) PC12 preparations. Anti-CREM antibody also supershifted the fast running complex from adrenal gland (Fig. 3B).

Western blot analysis further characterized the antigen recognized by the CREM antibody. As shown in Fig. 4A, six distinct bands, representing antigens with molecular masses ranging from 12 to 43 kDa, interacted with the CREM antibody in extracts from both PC12 cells and adrenal gland. Two small proteins of similar molecular mass, 12 and 13 kDa, are strongly induced by forskolin and reserpine (Fig. 4A). The identification of these proteins as ICER is indicated by the fact that no other

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**Fig. 4. Western analysis performed to characterize the proteins recognized by CREM and CREB antisera.** 90 μg of nuclear proteins derived from untreated and forskolin-stimulated PC12 cells and 70 μg of nuclear proteins extracted from adrenal glands of vehicle and reserpine-treated animals were resolved on SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose filters, and immunolabeled with an anti-CREM antiserum (A) or with an anti-CREB antiserum (B). Abbreviations are as for Fig. 1.

**Fig. 5. Detection of CREM gene-derived products by polymerase chain reaction.** A, a schematic representation of the structure of the CREM gene and of the alternative intronic transcription initiation site from which ICER is transcribed. Q, glutamine-rich domain; P-box, phosphorylation site; γ, γ-exon; L-zip, leucine-zipper domain containing the two differentially spliced DNA-binding domains (Ia and Ib). The arrows indicate the position of the primers used for reverse transcriptase-PCR (A5’, B5’, Ia3’, Ib3’). B, cDNA, derived from mRNA extracted from PC12 cells 4 h after forskolin treatment, was amplified with different primer combinations: lane 2, A5’ and Ia3’; lane 3, A5’ and Ib3’; lane 4, B5’ and Ia3’; lane 5, B5’ and Ib3’; lane 1, DNA ladder. C, primers B5’ and Ib3’ were used to amplify cDNA derived from PC12 cells untreated (Ctl, lane 2) or forskolin-stimulated for 30 min (lane 3), 4 h (lane 4), and 8 h (lane 5). The same primers were used with cDNA derived from adrenal glands of vehicle (lane 6) or reserpine-treated (lane 7) animals. No amplification was detected in any experimental condition when cDNAs were amplified with the primers B5’ and Ia3’ (lanes 8–13). The conditions of the amplification, carried out for 30 cycles, were: 1.2 min at 94 °C, 2 min at 46 °C, and 3 min at 72 °C.
CRE-binding polypeptides of the appropriate lengths have been reported and is further supported by the Northern and PCR analysis presented below. As the CREM antiserum used cross-reacts with other members of the CREB/CREM/ATF family, it is not possible to state that all the detected polypeptides derive from the CREM gene.

CREB antiserum also produced a supershift in the protein-DNA binding pattern but did not affect the fast running complex (Fig. 3A). This finding confirms the already reported (1, 2) interaction between CREB and the TH-CRE and rules out the possibility that the protein(s) bound to DNA in the fast running complex are CREB-related. The CREB antibody in Western blot analysis (Fig. 4B) reacted with at least one protein whose molecular mass (43 kDa) matched that previously reported for CREB (21). Note also the increase in the amount of CREB following forskolin treatment (Fig. 4B). The inability of phospho-CREB to produce a supershift probably results from phosphatase activity during the nuclear protein extraction, since a supershift occurred with the CREB antibody. Additionally, CREB may already be dephosphorylated at the time analyzed, i.e. 4 h after application of the stimulus. While the ATF-2 antiserum did not affect any protein-DNA binding complex, the anti-ATF-1 antibody supershifted one of the slower moving complexes. This finding was not analyzed further in the present study.

Identification of ICER by PCR and Northern Blot—Previous reports identified a small protein, ICER, that is generated by alternative intronic promoter usage from the CREM gene and expressed in PC12 cells and adrenal gland (29, 31). Fig. 5A shows the intron-exon structure of the CREM gene (31). Four possible transcripts can be generated. All ICER transcripts contain 82 intronically derived bases not found in other CREM mRNAs. The four ICER transcripts are generated by either inclusion or exclusion of exon γ and alternative splicing of the DNA binding domains Ia and Ib (31). To confirm the presence of the ICER isoforms, we designed specific primers (Fig. 5A) that amplify from the unique 82-base site (B5') to either the Ia site (Ia3') or the Ib site (Ib3'). mRNA isolated from forskolin-treated PC12 cells and vehicle- and reserpine-treated adrenal glands were reverse transcribed and subjected to PCR. Two bands, approximately 300 bp long, were amplified from the B5' and Ib3' primers, corresponding to ICER-Ib and ICER-γ-Ib (Fig. 5C). No bands were amplified with the Ia3' primer, regardless of which 5' primer was used (Fig. 5B).

Northern blot analysis confirmed that the pharmacological treatments altered expression of ICER. A 150-bp probe recognizing the Ib domain labeled two bands of approximately 2.4 and 1.5 kb on Northern blots of mRNA obtained from untreated PC12 cells and adrenal glands from control rats (Fig. 6). The Ib domain of the CREM gene is included in all the CREM-derived transcripts that contain a DNA binding domain. The detection of only two isoforms of ICER confirms the PCR results that no full-length CREM transcripts are present either under control conditions or are inducible in PC-12 cells and adrenal glands. These data also support the possibility that 43-kDa, as well as the other, polypeptides detected by the CREM antibody can be CREB isoforms and/or ATF-related proteins. Forskolin and reserpine treatments clearly induced both mRNA bands in PC12 cells and adrenal glands, respectively. In PC12 cells, high levels of mRNA could be detected as soon as 30 min (data not shown) and even 8 h after addition of forskolin. A β-actin probe was used to normalize the amount of mRNA loaded on the gel (Fig. 6).

Transfectional Analysis of ICER: Repression of TH Promoter-driven Transcription—To test directly whether ICER can regulate TH transcription, the reporter plasmid TH2400CAT (1) and the expression plasmid pCMV-ICER-Ib were cotransfected into PC12 cells and CAT activities measured. Cotransfection of

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**Fig. 6.** Induction of ICER mRNAs in PC12 cells after forskolin stimulation and in adrenal gland after animal reserpine treatment, detected by Northern blot analysis. 5 μg of mRNA extracted from PC12 cells and rat adrenal glands were electrophoresed in a 1% agarose gel containing 2% formaldehyde, transferred to a nylon membrane, and hybridized with a probe designed to encompass the Ib DNA-binding domain. The same blot was used consecutively for hybridization with a β-actin probe. From left to right, the lanes are: PC12-derived mRNA from untreated (Ct), forskolin-stimulated 4 h (4h) and 8 h (8h), respectively, mRNA extracted from adrenal glands of rats treated with vehicle (Veh) or reserpine (Res).

**Fig. 7.** Effects of ICER on basal and PKAc-stimulated transcriptional activity of TH2400CAT reporter plasmid. Transfections were performed in PC12 cells with a total of 10 μg of DNA. In each 10-cm dish, 4 μg of pTH2400CAT were cotransfected with 3.9 μg of pCMV-ICER-Ib, 0.1 μg of PKAc, and 2 μg of RSV-β-gal. When CMV-ICER-Ib and PKAc were not cotransfected, the same amounts of pUC19 were added. CAT activities were normalized with β-gal activities. The data are expressed relative to activity in cells transfected with only pTH2400CAT set at 100 (left column). Data are expressed as mean-fold induction ± S.E. Values are from six separate samples in two replicate experiments.
the ICER expression construct substantially diminished CAT activity (Fig. 7). ICER-mediated repression of PKA-stimulated transcription of the TH construct was even more dramatic than repression of basal transcription. In control experiments, cotransfection of pUC19 or pcDNAI/AMP (data not shown) did not alter transcription of the TH reporter construct.

**DISCUSSION**

The present study investigated the role of CRE-binding proteins in TH gene regulation. Nuclear extracts from rat adrenal gland and from the adrenal medulla-derived PC12 cell line (57) produced virtually identical binding patterns in gel mobility shift assays employing a TH-CRE oligonucleotide. The binding patterns of extracts from locus ceruleus differed from patterns produced by adrenal gland, PC12 cells, the catecholamine-producing human neuroblastoma line SK-N-BE(2)C cells (53), and murine locus ceruleus-derived CATHa cells (54). Thus, relative levels of different CRE-binding transcription factors show a high degree of variability among catecholamine-producing cell types. This observation suggests that regulation of CRE-containing catecholamine genes such as TH and dopamine-β-hydroxylase (58) occurs by cell-type specific mechanisms that depend, in part, on differential expression of CRE-binding transcription factors. Evaluation of this hypothesis will require more extensive study.

Reserpine and forskolin induce rapid increases in TH levels in adrenal gland and PC12 cells, respectively (33, 37, 43, 56, 59, 60). Abundant data support a direct role for cAMP, PKA, and the TH-CRE as primary mediators of forskolin-induced increases in TH expression (61–65). In contrast, the molecular events triggering reserpine-activated TH induction are not yet fully understood. Reserpine, which inhibits monoamine uptake in storage vesicles, depletes catecholamine stores. The drop in catecholamine levels triggers a trans-synaptically mediated induction of TH transcription and protein synthesis (34, 37, 59). Although c-Fos mRNA increases after the drug treatment (55, 66) suggesting a role for the TH AP-1 site, additional regulatory mechanisms are likely involved in TH promoter transcription regulation. In fact, increased cAMP levels and induced PKA activity have been reported in rat adrenal chromaffin cells after reserpine treatment (67). Recent reports, underlining the importance of the CRE in TH gene regulation (1, 2), prompted investigation of the possible involvement of CRE-binding proteins in both the forskolin- and reserpine-induced TH gene transcriptional activation.

In gel mobility shift assays, we showed that a fast running CRE-protein complex increased substantially in nuclear extracts from adrenal glands of reserpine-treated rats and forskolin-treated PC12 cells. Supershift experiments indicated that the fast running complex contained one or more members of the CREM family of proteins. Although CREB antiserum produced a supershift of a slow moving complex, indicating that CREB family members are present in the nuclear extract, they do not contribute to the binding activity in the fast running complex. Collectively, Western blot, PCR, and Northern blot analyses demonstrated that both reserpine and forskolin treatments induced two isoforms of the CREM-related ICER protein (ICER-Ib and γ-ICER-Ib).

Our results confirm the previously reported induction of ICER by forskolin in PC12 cells (31). However, under the present experimental conditions, ICER mRNA levels peaked approximately 4 h after forskolin treatment and remained detectable at 8 h. In the earlier study, maximal levels of ICER mRNA occurred after 2 h (31). These different temporal profiles of ICER reflect either variations between the PC12 lines or differing culture conditions. Although the presence of ICER in adrenal gland has been reported (29), to our knowledge this is the first demonstration of robust induction of ICER in adrenal gland by reserpin.

To determine if ICER induction is involved with TH gene transcriptional regulation, cotransfection analyses were performed. The current data clearly demonstrate that ICER represses TH promoter-driven transcription in PC12 cells, with the strongest repression occurring after transcriptional stimulation by PKA. Since PC12 cells are immortalized adrenal medullary cells, the in vitro experiments suggest a role for ICER in TH transcriptional modulation of adrenal medullary cells. The strong ICER induction produced in the adrenal gland after reserpine treatment supported this hypothesis. Further evidence for a role of ICER in TH regulation in vivo is suggested by the dynamics of the TH mRNA response to reserpine. A relatively rapid return of TH mRNA to control levels occurs in adrenal medullary cells, as compared to the response in the locus ceruleus, following reserpine-induced up-regulation of TH mRNA levels (59, 68). Studies, now in progress, will clarify the mechanisms underlying the role of ICER in TH gene modulation in the adrenal gland.

The results of this study suggest that the transcriptional regulation of TH by cAMP-related mechanisms in PC12 cells involves both activating and repressing trans-acting proteins. Similar transcriptional regulation of TH could be simulated in cotransfection experiments performed with SK-N-BE(2)C cells, which do not normally express ICER. The data indicate that regulation of TH mRNA synthesis occurs in a tissue-specific manner that may be partially determined by a complex balance of activators and repressors. Control could occur by transcription factors competing for the same cis-acting sites. In this scheme CREB and ICER would provide a system for fine modulation of TH gene expression. In fact, CREB, which binds to the TH CRE, is rapidly activated by forskolin (21). Interactions between repressors acting at one site (e.g. ICER binding to the CRE), and activators at other sites (e.g. a Fos-Jun dimer at the AP-1 site) are additional possibilities not addressed by the present work, but represent important directions for future experiments.

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**REFERENCES**

1. Kim, K.-S., Lee, M. K., Carroll, J., and Joh, T. H. (1993) *J. Biol. Chem.* 268, 15689–15695
2. Lazaroff, M., Patankar, S., Yoon, S. O., and Chikaraisi, D. M. (1995) *J. Biol. Chem.* 270, 21579–21589
3. Yamamoto, K. K., Gonzalez, G. A., Biggs, W. H., III, and Montminy, M. R. (1988) *Nature* 334, 494–498
4. Hai, T., Liu, F., Coukos, W. J., and Green, M. R. (1989) *Genes Dev.* 3, 2083–2090
5. Macaewa, T., Sakura, H., Kanie-Ishii, C., Sudo, T., Yoshimura, T., Fujisawa, J.-I., Yoshida, M., and Ishii, S. (1989) *EMBO J.* 8, 2023–2028
6. Yoshimura, T., Fujisawa, J.-i., and Yoshida, M. (1990) *EMBO J.* 9, 2537–2542
7. Ivashkiv, L. B., Liu, H.-C., Kara, C. J., Lamm, W. W., Verma, I. M., and Glumcher, L. H. (1990) *J. Mol. Cell. Biol.* 10, 1609–1621
8. Hurst, H. C., Totty, N. F., and Jones, N. C. (1991) *Nucleic Acids Res.* 19, 4601–4609
9. Fousses, N. S., Borrelli, E., and Sassone-Corsi, P. (1991) *Cell* 64, 739–749
10. Karpiniski, B. A., Morle, G. D., Haggenvik, J., Uhler, M. D., and Leiden, J. M. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89, 4820–4824
11. Ruppert, S., Cole, T. J., Boshart, M., Schmid, E., and Schuetz, G. (1992) *EMBO J.* 11, 1505–1512
12. Comb, M., Birnberg, N. C., Seasholtz, A., Herbert, E., and Goodman, H. M. (1986) *Nature* 321, 353–356
13. Montminy, M. R., Sevarino, K. A., Wagner, J. A., Mandel, G., and Goodman, R. H. (1986) *Proc. Natl. Acad. Sci. U.S.A.* 83, 6682–6686
14. Lewis, E. J., Harrington, C. A., and Chikaraisi, D. M. (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84, 3550–3554
15. Short, J. M., Wynshaw-Boris, A., Short, H. P., and Hanson, R. W. (1986) *J. Biol. Chem.* 261, 9721–9726
16. Fisch, T. M., Frywes, R., Simon, M. C., and Roeder, R. G. (1989) *Genes Dev.* 3, 198–211
17. Bokar, J. A., Roessler, W. J., Vandenbark, G. R., Kaelter, D. M., Hanson, R. W.,

C. Tinti and T. H. Joh, unpublished observation.
