Membrane depolarization of PC12 cells using 50 mM KCl leads to induction of tyrosine hydroxylase (TH) mRNA. This induction of TH mRNA is apparently due to increased TH gene promoter activity mediated by the influx of Ca\(^{2+}\). In PC12 cells transiently transfected with a chimeric gene expressing chloramphenicol acetyltransferase (CAT) driven by the proximal TH gene 5\'-flanking region, 50 mM KCl increases TH gene promoter activity 3–4 fold. Promoter analysis utilizing TH-CAT constructs containing mutagenized sequences indicates that this response to the depolarization-mediated influx of Ca\(^{2+}\) is primarily dependent on both the TH cAMP-responsive element (CRE) and TH activating protein-1 (AP1) site. Minimal promoter constructs that contain a single copy of either the TH CRE or TH AP1 site fused upstream of the TH gene basal promoter are only modestly responsive or nonresponsive, respectively, to depolarization. However, both these constructs are strongly responsive to the calcium ionophore, A23187. Gel shift assays indicate that TH AP1 complex formation is dramatically increased after treatment with either 50 mM KCl or A23187. Using antibodies to transcription factors of the Fos and Jun families, we show that the nuclear proteins comprising the inducible TH AP1 complex include c-Fos, c-Jun, JunB, and JunD. In cAMP-responsive element binding protein (CREB)-deficient cell lines that express antisense RNA complementary to CREB mRNA, the response of the TH gene promoter to cyclic AMP is dramatically inhibited, but the response to A23187 remains robust. This result indicates that transcription factors other than CREB can participate in the Ca\(^{2+}\)-mediated regulation of the TH gene. In summary, our results support the hypothesis that regulation of the TH gene by Ca\(^{2+}\) is mediated by mechanisms involving both the TH CRE and TH AP1 sites and that transcription factors other than or in addition to CREB participate in this response.

**Biosynthesis of the catecholamines is tightly regulated by**

\[ \text{TH; EC 1.14.16.2} \]

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The abbreviations used are: TH, tyrosine hydroxylase; [Ca\(^{2+}\)], intracellular calcium concentration; CAT, chloramphenicol acetyltransferase; CREB, cyclic AMP-responsive element binding protein; CRE, cyclic AMP-responsive element; S-CPT-cAMP, 8-chlorophenylthio-cyclic AMP; PBS, phosphate-buffered saline; ASCREB, antisense-CREB; bp, base pairs; RSV, Rous sarcoma virus; CREM, cyclic AMP-responsive element-modulating protein; CaRE, calcium-responsive element; AP1, activating protein-1.
in PC12 cells. Using deletion analysis, they have also reported that promoter elements upstream of the CRE do not apparently participate in the response of the TH gene promoter to Ca\(^{2+}\). Recently, these results have been extended using TH gene constructs with site-directed mutations (25). Results from these studies suggest that the TH AP1 site is not involved in Ca\(^{2+}\)-mediated TH gene regulation. In contrast, deletion analysis by Stachowiak et al. (26) has shown that the TH CRE is not required for activation of the TH gene promoter by Ca\(^{2+}\) influx in bovine adrenal chromaffin cells and that, by inference, promoter elements upstream of the CRE participate in this response. However, it is not clear from their study which promoter elements participate in this regulation.

In the present study, we demonstrate that both the CRE and AP1 sites in the proximal 5′-flanking region of the rat TH gene participate in regulating TH gene expression by Ca\(^{2+}\) influx in PC12 cells. The newly identified TH CRE2 site does not apparently play a major role in this response. To further analyze the involvement of the TH AP1 site in Ca\(^{2+}\)-mediated TH gene regulation, we have examined nuclear protein interactions at this site. Finally, we have tested the involvement of CREB in Ca\(^{2+}\)-mediated TH gene regulation using PC12 cell-derived CREB-deficient cell lines. Our results indicate that CREB is not required for Ca\(^{2+}\)-dependent regulation of the TH gene, supporting the hypothesis that multiple promoter elements are involved in regulation of the TH gene promoter in response to Ca\(^{2+}\) influx.

**EXPERIMENTAL PROCEDURES**

**Materials**—RPMI 1640 medium and penicillin/streptomycin were purchased from Life Technologies, Inc. Fetal bovine and donor horse sera were obtained from Hyclone Laboratories, Inc. (Logan, UT) and JRB Biosciences (Lenexa, KS), respectively. Cell culture flasks and dishes were Falcon or Corning brand. Primary antibodies used in the supershift assays and immunoblots were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) or Oncogene Science (Uniondale, NY). All other chemicals were purchased from Sigma. The calcium ionophore A23187 (stock solution, 10 mM) was dissolved in dimethyl sulfoxide. Liposomes for transient transfections were made by combining 1 mg of dioleoyl-L-phosphatidylethanolamine and 200 µg of dimethyldioctadecyl ammonium bromide in 1 ml of water. The lipids were suspended by sonication using a Branson Sonifier (model W140D) at 100 W for 9 min. [14C]Chloramphenicol was obtained from DuPont NEN.

TH(-372/+27)-CAT was constructed as described previously (27). The construct contains the structural gene encoding chloramphenicol acetyltransferase (CAT) fused downstream of the rat TH gene 5′-flanking region from -272 to +27. TH(-272/+27)-CAT constructs, in which the CRE (-45/-38), TGAAGCTCA, AP1 (-205/-199; TGAATTCA), or TH CRE2 (-97/0; AGGCGTGG) core sequences were mutated, were isolated using site-directed mutagenesis as described previously (27). The mutated constructs contained the following sequences (capital letters show mutated sequences; the normal sequences are given below in lowercase letters; Sequence 1).

| mCRE      | 5′-gagagggcttttcTCTAGAaacgctgctttta-3′ (bp -55 to -25) gacgtc     |
| mAP1      | 5′-gctctagagGAAATcaagagggcttgtc-3′ (bp -214 to -185) tg               |
| mTHCRE2   | 5′-gcacagccTCTAGAagagagtcgcag-3′ (bp -105 to -76) ggcgtg              |
| mTHAP1    | 5′-gagagggCTAGGCGagtgcaggtgagggc-3′ (bp -90 to -62) tggcga            |

**Sequence 1**

The numbers in parentheses indicate the positions of the designated sequences within the TH gene 5′-flanking region.

The TH gene minimal promoter constructs, TH(-44/+27)-CAT, THCRE-CAT, and THAP1-CAT, were generously provided by Dr. D. M. Chikaraishi (Duke University, NC). TH(-44/+27)-CAT (also referred to as -44PL TH CAT in Ref. 28) contains rat TH gene promoter sequences from -44 to +27 upstream of the CAT gene. THCRE-CAT and THAP1-CAT contain a single copy of either element fused upstream of the basal TH gene promoter as described by Fung et al. (28). THCRE2-CAT was constructed as described in Best et al. (27). RSVP-CAT was purchased from ATCC, and CAT-Basic was purchased from Promega Corp. (Madison, WI).

For gel shift assays, oligonucleotide sequences containing the TH CRE, TH AP1, or mutated TH AP1 element were synthesized and purified by denaturing gel electrophoresis. Mixtures of sense and antisense oligonucleotides were heated at 95°C for 2 min in a buffer containing 67 mM Tris-HCl (pH 7.8), 13 mM MgCl\(_2\), 6.7 mM diethiothreitol, 1.3 mM spermidine, and 1.3 mM EDTA. The oligonucleotides were then allowed to anneal by slowly cooling the mixture to room temperature. Double-stranded oligonucleotide containing the somatostatin SP1 site was purchased from Promega (Madison, WI). Sequences of the sense oligonucleotides are shown below (the core TH AP1 sequence is underlined and the mutated sequence is capitalized, Sequence 2):

| THAP1     | 5′-ctggggtcgtccagagggcag-3′ |
| mAP1      | 5′-ggctgagggGAAAAGgaagggcaggtcgct-3′ |
| Somatostatin SP1 | 5′-atcggcGaggggGagggcaggtcgct |
| TH CRE    | 5′-gggctttgcagtcgcacgttg-3′ |

**Sequence 2**

**Cell Culture Conditions and Treatments**—PC12 cells were cultured in RPMI 1640 medium containing 2.5% fetal bovine serum, 10% heat-inactivated donor horse serum, 50 units/ml penicillin, and 50 µg/ml streptomycin. The cells were maintained in 75-cm\(^2\) culture flasks at 37°C with 95% air and 5% CO\(_2\). For experiments, PC12 cells were plated in 100-mm dishes and treated with either control or 50 mM KCl-containing (high K\(^+\)) medium. The control medium contained 5 mM KCl. The high K\(^+\) medium contained reduced NaCl concentration (53 mM) to conserve osmotic strength. Both media were supplemented with 2.5% fetal bovine serum and 10% donor horse serum. In some experiments, PC12 cells were treated with 10 µM calcium ionophore A23187 or 100 µM 8-chlorophenylthio-CAMP (8-CPT-CAMP) in control, serum-containing medium. **Transient Transfection and Treatment of PC12 Cells**—PC12 cells were transiently transfected by the method of Rose et al. (29), as modified by Best et al. (27). Briefly, PC12 cells were cultured in 100-mm dishes at approximately 10\(^5\) cells/cm\(^2\). The cells were incubated in 2 ml of serum-free RPMI 1640 medium containing 60 µg of liposomes and 10 µg of either wild type or mutated TH-CAT construct for 4 h in an atmosphere of 93% air and 7% CO\(_2\). Serum-containing medium (4 ml) was then added to each dish, and the cells were maintained in 93% air, 7% CO\(_2\). Two to three days after transfection, the cells were treated for 8 h with either control medium, high K\(^+\) medium, and/or different drugs. The cells were harvested in ice-cold PBS and stored as cell pellets at -80°C. Most of the transfection experiment results in the figures, and tables were derived from at least three experiments using three different sets of TH-CAT plasmid preparations. Separate dishes of cells (in triplicate) were transfected with 10 µg of each RSVP-CAT to determine transfection efficiency. Transfection efficiency varied dramatically between experiments but was very reproducible between dishes within each experiment. For example, RSVP-CAT expression varied from 86,000 ± 6,000 to 377,000 ± 9,800 cpm/mg protein (n = 3) in different experiments. Separate dishes of cells were also transfected with CAT-Basic (a plasmid containing the CAT gene without a promoter to drive its expression). CAT activity in these cells was used as a blank value. In most experiments blank values were less than 20% of the values obtained from cells transfected with TH-CAT or RSVP-CAT. Depolarization did not affect these blank values.

**CAT Assay**—The cell pellets were resuspended in 60–100 µl of 0.05 M Tris-HCl (pH 7.8). The cell suspension was frozen and thawed three times and centrifuged at 12,000 × g for 5 min. The supernatant was heated at 10 min at 65°C, clarified by centrifugation at 12,000 × g for 5 min, and assayed for CAT activity. Each assay contained 0.8 to 1.0 mg of protein, 480 µM Tris-HCl (pH 7.8), 13.5 µM [\(^{14}\)C]chloramphenicol, and 670 µM acetyl-CoA in a total volume of 130 µl. The acetylation reaction was carried out at 37°C for 12–16 h. Under these conditions, the rate of acetylation was linear with respect to both protein concentration and time. Acetylated and non-acetylated forms of chloramphenicol were extracted and separated by thin layer chromatography (TLC) as described by Gorman et al. (30). The amount of acetylated chloramphenicol was quantified by scraping the TLC plate and counting the radioactivity using a liquid scintillation counter. TH-CAT activity (expressed as pmol of product formed per h) was divided by the mg of protein.
present in the original extract (prior to heating) and then normalized to RSV-CAT activity (expressed as pmol of product formed per h/mg of protein) measured in companion dishes in each experiment.

**Gel Shift Assays**—Cells were treated with either control medium, medium containing 50 mM KCl, or control medium containing 10 mM A23187 for 1 h. Nuclear proteins were isolated as described (Best et al. 27). Gel shift assays were performed as described previously (27). Briefly, approximately 0.2 pmol (50,000 cpm) of 32P-labeled THAP1 oligonucleotide probe was incubated with 10 μg of nuclear protein extract on ice for 20 min in a reaction buffer containing 25 mM HEPES (pH 7.5), 5% glycerol, 0.1 mM NaCl, 0.1% Triton X-100, 2 mM EDTA, 0.6 mM spermine, 0.2 mM spermidine, 0.1 mM phenylmethylsulfonyl fluoride, 0.25 μg/ml leupeptin, 0.25 μg/ml pepstatin, 10 μg/ml poly(dI-dC), and 0.4 mM dithiothreitol. The reaction mixture was then incubated at room temperature for 5 min. For competition assays, a 20-fold molar excess amount of unlabeled competitor oligonucleotide was added to the reaction mixture 5 min prior to the addition of the radiolabeled probe. Protein-bound DNA was then electrophoretically separated from the free DNA on a 5% polyacrylamide gel. The gel was dried onto Whatman paper, and the radiolabeled DNA was visualized by autoradiography.

In the supershift assays, 10 μg of PC12 cell nuclear proteins were first incubated on ice for 1 h with 1 μg of a rabbit IgG antibody against either c-Fos, c-Jun, Jun-B, or Jun-D. A separate sample of nuclear proteins was incubated with 1 μg of control rabbit IgG antibody against rat IgG with or without antibody (0.1% sodium azide, 2.5% gelatin in phosphate buffer (pH 7.5)) as controls. The entire antibody/protein mixture was then incubated with radiolabeled THAP1 probe and processed for the gel shift assay as described above.

**Construction of CREB-deficient Cell Lines**—Antisense CREB RNA expression vectors, CREB-AS.6 and CREB-AS.3, were constructed in pBK-RSV (Stratagene, La Jolla, CA). Expression of the antisense CREB RNAs was under control of the RSV promoter and contained base pairs 1–620 and 1–320, respectively, of rat CREB cDNA in the antisense orientation. The NIH BLAST program was used to compare CREB cDNA sequences with other transcription factor sequences. The sequences in CREB-AS.3 possess weak homology (~40%) to CREM and no significant homology to ATF1 or any other transcription factor listed in GenBank. Sequences 321–620 are 68% homologous to CREM but not significantly homologous to ATF1 or any other listed factor. PC12 cells were transfected with either CREB-AS.6 or CREB-AS.3, and stably transfected clonal cell lines (ASCR.6–2 and ASCR.3–6, respectively) were transiently transfected with CREB or phospho-CREB protein as detected using a modified protocol of that provided in the protocol provided with the enhanced chemiluminescence detection kit from Amersham Corp. Densitometric analysis of the autoradiograms obtained from these immunoblots was performed using a Hewlett-Packard ScanJet 4C scanner with a transparency adaptor and NIH Image software.

**Statistical Analyses**—Results were analyzed by one-way analysis of variance followed by Student-Newman-Keuls or Dunnett multiple comparisons tests, using the computer program INSTAT. Data are presented as means ± standard error (S.E.). A p value less than 0.05 was considered statistically significant.

**RESULTS**

**Evaluation of Promoter Elements Involved in Depolarization-mediated Activation of the TH Gene Promoter Using Mutagenized TH-CAT Constructs**—TH gene promoter activity was measured using a construct containing TH gene sequences from −272 to +27 fused upstream of the CAT reporter gene (TH-CAT construct). This region of the TH gene contains promoter elements including CRE, AP1, and TH CRE2. In order to evaluate the involvement of each of these promoter elements in response to Ca2+ influx elicited by depolarization, PC12 cells were transiently transfected with TH-CAT constructs containing either the wild type TH gene promoter or the same TH gene promoter region with a mutated CRE, AP1, or TH CRE2 site. High K+ treatment increased the expression of wild-type TH-CAT by approximately 4-fold (Table I). Mutagenesis of 6 out of the 8 bases that comprise the core CRE lowered basal TH-CAT expression by ~70% and inhibited the depolarization-mediated increase in TH-CAT expression by ~80%. However, high K+ treatment still elicited a modest, but significant, increase in TH-CAT expression when the CRE was mutated. This residual induction of TH-CAT suggested that other elements might participate in the response to Ca2+ influx. The TH AP1 and TH CRE2 are two other sites within the TH gene that are responsive to different stimuli (27, 31). Mutagenesis of both the 7 bases of the core TH AP1 site lowered basal TH-CAT expression (by ~60%) and inhibited the high K+-evoked activation of the TH gene promoter by ~80%. Mutagenesis of the TH CRE2 site was also associated with a modest but significant inhibition of the depolarization-mediated stimulation of the TH gene promoter (by ~30%). In contrast, mutagenesis of the TH CRE2 site (see Ref. 27), which is downstream and adjacent to the TH CRE2 site in the TH gene promoter, did not affect the induction of TH-CAT expression in cells treated with 50 mM K+. These results suggest that the CRE and AP1 sites within the TH gene promoter participate significantly in the response of the gene to membrane depolarization and the consequent influx of Ca2+. The TH CRE2 site plays only a minor role, if any, in this response.

**Evaluation of Different Promoter Elements in Minimal Promoter Constructs**—In order to test whether each individual promoter element is independently responsive to Ca2+, we examined the response of each element in minimal promoter constructs (Table II). PC12 cells transfected with the minimal TH gene basal promoter construct, TH(−44/+27)-CAT, did not show depolarization-mediated induction of TH-CAT expres-
Ca^{2+}-mediated Regulation of the TH Gene

TABLE II
Effect of 50 mM KCl or A23187 on TH-CAT expression in PC12 cells transiently transfected with TH minimal CAT constructs

| TH-CAT construct | Control | 50 mM KCl | 10 μM A23187 |
|------------------|---------|-----------|--------------|
| TH (-44/+27)-CAT | 0.85 ± 0.07 (11) | 1.0 ± 0.1 (13) |
| THCRE-CAT        | 0.61 ± 0.02 (5)  | 1.7 ± 0.1 (5)   |
| THAP1-CAT        | 0.75 ± 0.06 (6)  | 2.3 ± 0.6 (6)   |
| THCRE2-CAT       | 1.4 ± 0.2 (4)    | 2.1 ± 0.2 (4)   |
| THAP1-CAT        | 1.5 ± 0.2 (5)    | 11 ± 1 (5)      |
| THCRE2-CAT       | 0.77 ± 0.07 (6)  | 1.1 ± 0.1 (6)   |
| THAP1-CAT        | 0.70 ± 0.02 (5)  | 1.4 ± 0.1 (5)   |

* p < 0.001 compared with controls transfected with the same TH-CAT construct.

Depolarization of PC12 cells by 50 mM KCl induces formation of a specific TH AP1 complex. PC12 cells were treated with either control (C) or high K (K) medium for 1 h. Nuclear proteins from these cells were incubated with radiolabeled TH AP1 double-stranded oligonucleotide probe, and protein-DNA interaction was analyzed by gel shift assay in the absence or presence of 20-fold molar excess unlabeled competitor DNA. The arrow designates the TH AP1 complex that is induced by Ca^{2+} influx. mAP1, mutagenized AP1; Som SP1, somatostatin SP1.

Identification of Nuclear Proteins That Comprise the Ca^{2+}-Inducible TH AP1 Complex—Nuclear proteins that increase their interaction with the TH AP1 site upon Ca^{2+} influx were identified using specific antibodies against the AP1 family of transcription factors. These transcription factors included c-Fos, c-Jun, JunB, and JunD. The rabbit IgG antibodies raised against these factors recognized their non-DNA binding domains. The rabbit IgG antibodies raised against these factors recognized their non-DNA binding domains. The rabbit IgG antibodies raised against these factors recognized their non-DNA binding domains (Santa Cruz Biotechnology, Inc). In this set of experiments, nuclear protein extracts from A23187-treated PC12 cells were incubated with one of these antibodies prior to the gel shift assay. In some experiments a slight decrease in the intensity of the Ca^{2+}-inducible complex band was observed when nonspecific rabbit IgG was used as a control, indicating a nonspecific interaction of rabbit IgG and the nuclear proteins within the complex (experiment in Fig. 2A but not in Fig. 2B). Incubation of nuclear proteins with each specific antibody decreased the intensity and supershifted the band corresponding to the inducible TH AP1 complex (Fig. 2, A and B). No supershifted bands were observed when nuclear proteins were incubated with the control rabbit IgG antibody. As a further check on the specificity of the antibodies, the analogous supershift assays using the same nuclear protein extracts were performed using the TH CRE oligonucleotide as a probe (Fig. 2C). As expected, A23187 treatment did not increase binding to the TH CRE. The control antibody decreased TH CRE binding slightly; however, none of the antibodies targeting the Fos or Jun pro-
teins diminished TH CRE binding to a greater extent than that observed with the nonspecific control antibody nor were any supershifted bands observed when the TH CRE was used as the probe. Similar results were observed when nuclear proteins from cells treated with 50 mM KCl were used. These results demonstrate that proteins comprising the Ca\textsuperscript{2+}-inducible TH AP1 complex include c-Fos, c-Jun, JunB, and JunD and that these factors do not bind to the TH CRE.

Ca\textsuperscript{2+}-mediated TH Gene Promoter Activation in a CREB-deficient Cell Line—The role of the CRE in Ca\textsuperscript{2+}-mediated TH gene promoter activation was further evaluated by examining the participation of the CRE binding protein, CREB, in this response. The ability of Ca\textsuperscript{2+} to increase TH gene promoter activity was examined in CREB-deficient cell lines derived from PC12 cells. These CREB-deficient cell lines were generated by stably transfecting PC12 cells with one of two different antisense-CREB RNA expression vectors to diminish the expression of CREB in these cells. Both antisense CREB RNA constructs targeted the 5' region of CREB mRNA and did not include sequences encoding the basic region-leucine zipper domain. A control cell line, CN9, was generated by stably transfecting PC12 cells with the pBK-RSV plasmid without the antisense CREB RNA insert. The level of CREB protein in the CN9 cells was similar to that found in wild type PC12 cells (data not shown). Both cell lines that were stably transfected with antisense CREB constructs showed marked decreases in a 43-kDa band which presumably represents CREB protein (Fig. 3). This band was the only one recognized by the lot of CREB antiserum used in Fig. 3B. However, one lot of CREB antiserum yielded a number of nonspecific bands (seen in Fig. 3A). One of these bands migrated as a 43-kDa protein, which is the molecular size of CREB. To verify that this band represented CREB protein, we measured phospho-CREB using a different antibody in wild type PC12 cells and CN9 cells (the data from the wild type PC12 cells is shown in Fig. 3A). The phospho-CREB antibody also recognized a 43-kDa band, and the intensity of this band increased when using nuclear extracts isolated from forskolin-treated PC12 cells (Fig. 3A, lanes 3 and 4). This result indicated that phosphorylation of CREB increased after forskolin treatment, as would be expected. The phospho-CREB antiserum also recognized two other bands that we did not identify; however, the intensities of these bands did not increase with forskolin treatment. Hence, the evidence suggests that the 43-kDa band is CREB and that CREB is dramatically diminished in the ASCR.6–2 cells (Fig. 3A), as well as in the ASCR.3–6 cells (Fig. 3B). Densitometric analysis of these Western blots indicated that CREB protein was diminished by 80–90% in ASCR.6–2 cells and 70–75% in ASCR.3–6 cells.

Even though we have not yet performed exhaustive analysis of the changes that occur in other transcription factors in these CREB-deficient cell lines, we have compared a number of pertinent parameters in the ASCREB and CN9 cell lines. 1) The
basal activity of protein kinase A is essentially identical in ASCREB and control cell lines, and protein kinase A is activated by forskolin in both control and ASCREB cell lines. 2) In both ASCREB cell lines, ATF1 and CREM levels are similar to those observed in control or wild type PC12 cells. 3) c-Fos is induced by Ca\(^{2+}\) influx in both ASCREB and control cell lines. This latter result is not surprising, since Ca\(^{2+}\) induces c-Fos via phosphorylation of the serum response factor, as well as via phosphorylation of CREB (34).

Using these CREB-deficient PC12 cell lines, TH-CAT expression was examined in response to Ca\(^{2+}\) to determine whether the presence of CREB was required for Ca\(^{2+}\)-mediated TH gene promoter regulation. The CREB-deficient cells were transiently transfected with wild type TH-CAT and treated with either 0.1 mM 8-CPT-cAMP or 10 \(\mu\)M A23187 (Table III). As in the wild type PC12 cells, TH-CAT expression was increased by 8-CPT-cAMP (10-fold) or A23187 (5-fold) in the control CN9 cell line. The induction of TH-CAT expression by 8-CPT-cAMP was markedly attenuated in both CREB-deficient cell lines; in ASCR.6–2 cells, the cAMP-mediated induction of TH-CAT was completely inhibited. Most significantly, the decrease in CREB levels did not affect the ability of A23187 to increase TH-CAT expression, suggesting that CREB is not required for Ca\(^{2+}\)-mediated TH gene promoter activation. This result strongly suggests that transcription factors other than CREB can participate in the induction of TH gene promoter activity in response to Ca\(^{2+}\) influx.

**DISCUSSION**

Membrane depolarization of excitable cells and the consequent influx of Ca\(^{2+}\) leads to modulation of a number of intracellular events, including changes in transcription rates of numerous genes. Mechanisms responsible for this gene regulation have been extensively studied, particularly with respect to the c-fos gene (19, 20, 32–35). These studies have identified a Ca\(^{2+}\)-responsive element (CaRE) within the c-fos gene, which is strikingly similar to the consensus CRE. In addition, the increase in intracellular Ca\(^{2+}\) leads to activation of CREB by phosphorylation of its Ser-133 site (20, 36). Therefore, it has been postulated that Ca\(^{2+}\) influx evoked by membrane depolarization increases c-fos gene expression via the CRE/CaRE following the phosphorylation of CREB. However, the critical importance of the CRE/CaRE and CREB activation in c-fos gene regulation is unclear, because 1) CREB phosphorylation by itself is not sufficient to increase c-fos gene expression in response to Ca\(^{2+}\) influx in protein kinase A-deficient cell lines (35), and 2) c-fos gene sequences upstream of the CRE/CaRE also participate in the response to Ca\(^{2+}\) (34, 37).

Depolarizing stimuli also induce TH mRNA and stimulate TH gene promoter activity in PC12 cells (14, 24) and in cultured bovine adrenal medullary cells (26). Kilbourne et al. (24) have used deletion mutation analysis to show that the region from −60 to +27, which contains the consensus CRE core sequence, is as responsive to veratridine as the region from −773 to +27 in PC12 cells. The construct containing TH gene sequences from −41 to +27 was unresponsive. These authors have interpreted these data to support the participation of the TH CRE (at position −45 to −38) in this veratridine-mediated response. In addition, a construct, in which sequences from −209 to −193 (a region referred to as the FSE) are deleted from TH (−272/+27)-CAT, does not lose responsiveness to veratridine in PC12 cells. Since the TH AP1 site is found within this FSE region, it was concluded that this site does not participate in the response. However, other elements, such as AP2 (−220 to −213), E2A/MyoD (−194 to −189), and POU/Oct (−175 to −168), that may participate positively or negatively in regulating the TH gene are located adjacent to this region. The deletion of this FSE domain alters the normal spacing between these elements. In contrast, Stachowiak et al. (26) have used deletion analysis of the bovine TH gene promoter to show that regions of the promoter upstream of the CRE are required for the response to veratridine in bovine adrenal chromaffin cells. These regions contain numerous candidate elements, including
POU/Oct, SP1, and AP1 sites. From these previous deletion analyses, it is difficult to identify specific promoter elements involved in the response to depolarization, due to the deletion of large segments of the TH gene promoter in the TH-CAT constructs. Furthermore, these deletions alter the normal spacing of elements within the TH gene promoter and eliminate potential interactions between proximal and distal elements.

In the present study, we have used a rat TH gene promoter construct similar to that used by Kilbourne et al. (24); however, we have employed site-directed mutagenesis to knock out the function of specific sites within the promoter without altering the length of the promoter region. Using this approach, our results suggest that at least two promoter elements participate in the response to Ca\textsuperscript{2+} influx; these two elements are the CRE and AP1. This conclusion is strongly supported by the 70–80\% decrease in response to depolarization, when either the CRE or AP1 site is mutagenized within the context of the intact TH gene promoter.

The participation of the TH CRE is also supported by the results of the minimal promoter studies. Treatment of PC12 cells with 50 mM KCl leads to a significant increase in the expression of the minimal promoter construct, THCRE-CAT. This response is modest (about 3-fold) compared with that observed when the cells are treated with a cyclic AMP analog (approximately 15-fold). However, this modest response is perhaps due to the limiting amount of Ca\textsuperscript{2+} influx that occurs during depolarization with 50 mM KCl, because treatment with 10 \mu M A23187 (which raises [Ca\textsuperscript{2+}]) to levels more than 3 \mu M is associated with more than a 10-fold increase in THCRE-CAT expression.

Recently, Nankova et al. (25) have reported a similar study investigating Ca\textsuperscript{2+}-responsive elements within the TH gene promoter using TH-CAT constructs with a mutagenized CRE or AP1 site. Their results with the mutagenized CRE construct agree with our data, indicating that the CRE site plays a significant role in Ca\textsuperscript{2+}-mediated regulation of the TH gene. In contrast to our findings, these investigators do not observe a significant decrease in Ca\textsuperscript{2+}-induced TH-CAT expression when the TH AP1 site is mutagenized. One explanation for this discrepancy may be differences in the number of mutagenized nucleotides within the element. In the mutagenized AP1 construct used in our study, only the first two base pairs of the core AP1 element (TGATTCA) are modified (to GAATTCA). This modification was chosen to minimize its influence on factors binding to the dyad symmetry element that overlaps the 3' region of the core TH AP1 site (38) or other elements found within this region, such as the FSE (39) or HIE (40). The competitive displacement gel shift assays indicate that this 2-bp mutation is sufficient to inhibit the binding of Ca\textsuperscript{2+}-inducible AP1 factors to the mutagenized site (Fig. 1). Furthermore, this 2-bp mutation produces a dramatic decrease in basal promoter activity, which is in agreement with previous studies indicating that the AP1 site or adjacent, overlapping sites participate in controlling basal expression of the gene in rat pheochromocytoma cells. In contrast, the entire core TH AP1 sequence is mutagenized in the construct used by Nankova et al. (25) (mutant 2 in Ref. 38). Since a number of regulatory sequences have been mapped to this region of the gene, it is possible that these different mutations alter the response to Ca\textsuperscript{2+} due to changes in the binding of activating or repressing proteins that interact with these adjacent sites.

An alternative explanation is that the discrepancy is due to differences in the route of Ca\textsuperscript{2+} influx in the two experiments. Membrane depolarization using high K\textsuperscript{+} permits Ca\textsuperscript{2+} influx into PC12 cells via L-type voltage-sensitive calcium channels.\textsuperscript{2} Nankova et al. (25) used ionomycin to promote Ca\textsuperscript{2+} influx. Hence, the route of Ca\textsuperscript{2+} entry differs between these two studies. It is possible that CRE and AP1 transcription factors are modulated differently depending upon the route of Ca\textsuperscript{2+} influx. Support for this hypothesis has been demonstrated for the c-fos gene (41).

In order to confirm that the TH AP1 site participates in the response of the promoter to Ca\textsuperscript{2+} influx, we have performed a number of subsequent studies. Even though the minimal promoter construct, THAP1-CAT, does not respond significantly to 50 mM KCl, a slight, but statistically insignificant, response was observed in each experiment. This lack of significant response is apparently due to either the limited influx of Ca\textsuperscript{2+} elicited by high K\textsuperscript{+} treatment or to differences in the route of Ca\textsuperscript{2+} entry, because treatment of the cells with A23187 elicited a 7–8-fold increase in THAP1-CAT expression. This ability of a single copy of TH AP1 to increase CAT expression upon Ca\textsuperscript{2+} influx provides evidence that the TH AP1 functions as a Ca\textsuperscript{2+}-responsive element at least under certain stimulus conditions. However, the results using A23187 need to be interpreted with caution, because this ionophore may be producing effects that are not specific to Ca\textsuperscript{2+} influx.

The gel shift assays demonstrate that Ca\textsuperscript{2+} influx elicited by either depolarization or treatment with A23187 increases the formation of a TH AP1 complex. These results also strongly support the participation of the TH AP1 site in the response to Ca\textsuperscript{2+} influx. However, it is also possible that unidentified adjacent sites bind to PC12 cell nuclear proteins to form the observed complex. Hence, we used antibodies targeting specific transcription factors to identify the Ca\textsuperscript{2+}-inducible nuclear proteins that bind to the TH AP1 site. These studies have identified c-Fos, c-Jun, JunB, and JunD as members of the inducible TH AP1 complex. Hence, the increased formation of this complex upon Ca\textsuperscript{2+} influx is due to the increased binding of Fos and Jun family transcription factors to this site, supporting the role of the TH AP1 site in this response.

The TH CRE2 is a newly discovered site within the TH gene promoter that is homologous to the proenkephalin gene ENKCRE1 and that is responsive to cyclic AMP in PC12 cells (27). The present results suggest that this site does not play a significant role in the response to Ca\textsuperscript{2+}. This conclusion is based on the very modest decrease in response to high K\textsuperscript{+}, when the TH CRE2 is mutagenized within the normal context of the TH gene promoter and the lack of response of the THCRE2 in the minimal promoter studies to either 50 mM KCl or A23187.

A current prevailing hypothesis is that Ca\textsuperscript{2+} influx stimulates genes by activating a calcium/calmodulin-dependent protein kinase, which then phosphorylates and activates CREB, leading to the transactivation of genes downstream of CRE sites. However, our results using the CREB-deficient cell lines suggest that CREB is not necessary for Ca\textsuperscript{2+}-mediated regulation of the TH gene promoter. These results do not rule out CREB as a participant in the response to Ca\textsuperscript{2+} influx in wild type cells but suggest that other transcription factors can compensate when CREB levels are diminished. In this regard Greenberg and co-workers (42) have shown that the c-fos gene CRE is responsive to Ca\textsuperscript{2+} via phosphorylation of ATP1. It is also possible that transactivating forms of the CREM gene family may participate in this response (see Ref. 43 for review). Both of these factors are still present in the ASCREB cell lines.\textsuperscript{2} Furthermore, when CREB levels are lowered, regulation via the TH AP1 site may predominate. Further work is needed.

\textsuperscript{2} K. Nagamoto-Combs, K. M. Piech, J. A. Best, B. Sun, and A. W. Tank, unpublished observations.
to elucidate these compensatory mechanisms in the CREB-deficient cell lines.

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