Nerve growth factor regulates tyrosine hydroxylase gene transcription through a nucleoprotein complex that contains c-Fos

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We have studied nerve growth factor (NGF) regulation of the expression of the tyrosine hydroxylase (TH) gene in PC12 cells. The TH gene encodes the initial and rate-limiting enzyme of the catecholamine biosynthetic pathway. We show that the TH gene is transiently transcriptionally induced by a mechanism reliant on new protein synthesis during 1-2 hr of NGF stimulation, a time following the induction of the c-fos gene at 15 min post-NGF treatment. A potential regulatory sequence located within the TH gene promoter, the TH-FSE, shares homology to a known regulatory element, the fat-specific element (FSE), which is found upstream from genes activated during adipocyte differentiation and binds the Fos–Jun transcription factor complex. We show that the TH-FSE DNA sequence elevates the basal level of transcription from the rat TH promoter and is required for NGF inducibility. This DNA element binds authentic Fos–Jun products produced by in vitro translation. We demonstrate further that the TH-FSE can bind proteins present in PC12 nuclear extracts in a sequence-specific manner. The DNA/nucleoprotein complex that forms increases in abundance during NGF stimulation and reaches a maximum level at 4 hr of treatment. Antibody inhibition studies utilizing an anti-Fos antibody indicate that Fos and/or Fos-related antigen(s) associate with the TH-FSE and suggest that the Fos protein family contributes to the regulation of TH in vivo. These results support a model in which NGF-induced immediate early genes, including c-Fos, contribute to the regulation of delayed early genes such as TH and thereby control neuronal differentiation.

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During neuronal development, the peptide hormone nerve growth factor (NGF) is required for the differentiation and maintenance of sympathetic and sensory neurons of the peripheral nervous system (Levi-Montalcini 1966; Levi-Montalcini and Angeletti 1968). For example, NGF directs the differentiation of precursor cells into sympathetic adrenergic neurons following cell migration from the sympathoadrenal portion of the neural crest to specific target regions of the embryonic nervous system (Landis and Patterson 1981; Doupe et al. 1985; Anderson and Axel 1986). NGF can also serve as a mitogen, as described for primary chromaffin cell cultures in vitro (Lilloen and Claude 1985). Although the biological effects of NGF have been well established, the mechanism of its action is currently unknown. An in vitro model used extensively to study the effects and mechanism of action of NGF is the rat pheochromocytoma cell line, PC12 [Greene and Tischler 1976, 1982]. In the undifferentiated state, PC12 cells resemble their normal counterparts, neural crest-derived precursor cells. Upon NGF stimulation, PC12 cells undergo a diverse set of molecular and gross morphological changes and express many metabolic processes similar to sympathetic neurons, including extension of neurites, which are visible by 1–2 days following NGF exposure. The growth-factor-induced differentiation of PC12 cells appears to reflect developmental properties of their normal counterparts because similar morphological changes have been observed when normal neonatal rat adrenal medullary cells are exposed to NGF (Unsicker et al. 1978; Aloe and Levi-Montalcini 1979; Anderson and Axel 1986).

Previous studies in our laboratory (Leonard et al. 1987) utilizing differential screening of cDNA libraries from PC12 cells have identified several genes regulated by NGF. These studies suggest that NGF-induced differentiation is accompanied by the activation of various classes of genes whose onset of transcription occurs at different times following NGF stimulation. The first class includes the immediate early genes or early response genes. In PC12 and other cells, transcription of this class is induced rapidly (within 5–10 min) and transiently upon stimulation by growth factors when cells re-enter the cell cycle (Cochran et al. 1983; Greenberg and Ziff 1984; Lau and Nathans 1985, 1987; Lin et al. 1987; Sukhatme et al. 1987; Almendral et al. 1988) or
differentiate (Greenberg et al. 1985; Cho et al. 1989; Milbrandt 1987, 1988; Bartel et al. 1989; Tirone and Shooter 1989). Members of this class include several putative transcription factors whose products may mediate the growth factor response of the cell. In PC12 cells, these include c-fos (Curran and Morgan 1985, 1986; Greenberg et al. 1985; Kruijer et al. 1985; Milbrandt 1986), the cellular proto-oncogene counterpart of the transforming gene of the FBJ and FBR osteosarcoma viruses (Curran and Teich 1982, Curran et al. 1982, Curran and Verma 1984), c-jun (Bartel et al. 1989), the cellular proto-oncogene counterpart of the transforming gene of avian sarcoma virus 17 (Maki et al. 1987); NGFI-A and related zif/268 and egr-1 genes encoding proteins that contain a zinc-finger DNA-binding domain (Milbrandt 1987; Sukhatme et al. 1987, 1988; Christy et al. 1989), and NGFI-B (nur77), an early response gene that has sequence similarity to the glucocorticoid receptor (Hazel et al. 1988; Milbrandt 1988; Watson and Milbrandt 1989). A second class of genes, the delayed early genes, is transiently activated within 1–2 hr of NGF stimulation and includes genes whose products may be important for early events during the transition from the chromaffin to the neuronal cell state. This class includes the tyrosine hydroxylase (TH) gene (Leonard et al. 1987). A third class includes the peripherin gene, which encodes a neuron-specific intermediate filament protein (Portier et al. 1984; Leonard et al. 1987, 1988; Parysek et al. 1988, Thompson and Ziff 1989). Peripherin is representative of a class of “late” genes whose transcription is induced ~18 hr post-NGF, a time coinciding with the onset of neurite extension.

Given the order of activation of NGF-inducible genes, we hypothesized that members of the immediate early class, specifically c-fos, may control genes such as the TH gene, which are subsequently transcriptionally activated during differentiation. Recent studies have demonstrated that the products of c-fos and members of the c-jun gene family (Rauscher et al. 1988b; Ryder et al. 1988, 1989; Sassone-Corsi et al. 1988a; Hirai et al. 1989) can form heterodimeric complexes capable of binding DNA, specifically at AP-1 sites (5′-ATGACTCAT-3′; phorbol-ester-responsive sequence) with high affinity (Halazonetis et al. 1988; Kouzarides and Ziff 1988; Nakaeppu et al. 1988; Rauscher et al. 1988c; Sassone-Corsi et al. 1988c; for review, see Curran and Franza 1988). More direct evidence of the role of c-Fos as a trans-acting transcription factor has recently been demonstrated for the adipocyte gene, aP2 (Distel et al. 1987; Franza et al. 1988; Rauscher et al. 1988c), the transin gene (Kerr et al. 1988), the mouse a1[II] collagen gene (Setoyama et al. 1986), the collagenase gene (Schonthal et al. 1988), several viral promoters (Franza et al. 1988), and its own promoter (Sassone-Corsi et al. 1988b; König et al. 1989). The TH gene encodes TH, the enzyme that catalyzes the rate-limiting step in the catecholamine biosynthetic pathway, the conversion of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), a precursor to various neurotransmitters. TH is expressed in catecholaminergic neurons of the peripheral and central nervous system and in chromaffin cells of the adrenal medulla that originate from the neural crest. Both in vivo and in vitro studies have demonstrated that TH enzyme activity can be modulated by various extracellular effector molecules that include stress (Thoene 1969), glucocorticoids (Hanbauer et al. 1975, Lucas and Thoenen 1977; Edgar and Thoenen 1978; Tank et al. 1986), cAMP (Kumakara et al. 1979; Acheson et al. 1984; Tank et al. 1986), insulin (Schubert et al. 1980), epidermal growth factor (EGF; Goodman et al. 1980), and NGF (Goodman and Herschman 1978; Rohrer et al. 1978; Hatanaka 1981; Naujoks et al. 1982, Acheson et al. 1984). Several mechanisms have been proposed for regulating TH activity, including changes in the rate of enzyme synthesis (Hanbauer 1975; Goodman and Herschman 1978; Schubert et al. 1980; Tank et al. 1986), post-translational modifications of preexisting enzyme molecules (i.e., phosphorylation; Vulliet et al. 1980; Campbell et al. 1986), and other post-transcriptional events (Rohrer et al. 1978; Acheson et al. 1984). More recently, it has been demonstrated that the TH gene is transcriptionally regulated in response to EGF (Lewis and Chikaraishi 1987), glucocorticoids (Tank et al. 1986; Harrington et al. 1987; Lewis et al. 1987), and cAMP (Tank et al. 1986; Lewis et al. 1987). The exact molecular mechanism(s) by which NGF and other transmembrane signaling agents control the TH gene is currently unknown.

In this communication, we have examined the regulation of the TH gene during NGF-induced PC12 cell differentiation. Our results demonstrate that in response to NGF, the TH gene is transiently transcriptionally induced with delayed early kinetics. The 5′ regulatory region of the rat TH gene contains a potential regulatory sequence, the TH-FSE, which shows extensive homology to the fat-specific element (FSE) present in genes induced during adipocyte differentiation (Distel et al. 1987; Franza et al. 1988; Rauscher et al. 1988c), and binds inducible factors, specifically c-Fos. We present evidence that the TH-FSE forms a sequence-specific complex with proteins induced in vivo in response to NGF and contributes to the transcriptional induction of the TH gene by NGF. Changes in the DNA–protein complex that forms at the TH-FSE suggest that the element may provide both positive and negative control of TH transcriptional activity. Taken together, our evidence suggests a growth-factor-induced cascade of gene activity in which an inducible transcription factor protein, c-Fos, functions in the regulation of a gene essential to growth-factor-stimulated differentiation.

Results

NGF induction of the TH gene is transcriptionally regulated and reliant on protein synthesis

Previous studies have demonstrated that TH activity is elevated following NGF treatment in various rat clonal pheochromocytoma cell lines (Goodman and...
Herschman 1978; Hatanaka 1981), primary adrenal chromaffin cell cultures [Acheson et al. 1984], and rat superior cervical ganglia organ cultures [Rohrer et al. 1978]. Initial analysis of TH mRNA levels during NGF induction of PC12 cell differentiation revealed a slight increase (approximately twofold) in message levels by 4 hr of NGF treatment, which was followed by a decrease to initial basal levels in mRNA appearance (Leonard et al. 1987). To determine whether this control is at the transcriptional level, we utilized the nuclear run-on transcription assay [Greenberg and Ziff 1984] to measure the transcription rate of TH and other genes during the 8 hr following NGF induction of PC12 cells. Figure 1A shows that the TH gene is transcriptionally induced at a maximum rate 1 hr after NGF treatment. This response follows the transcriptional induction of the c-fos and β-actin genes at 30 min post-NGF treatment [Greenberg et al. 1985, 1986]. As expected, the late response gene, peripherin [Thompson and Ziff 1989], and other genes unresponsive to NGF during this period [Greenberg et al. 1985, 1986; Leonard et al. 1987] were not induced. The 10b gene, a second NGF-inducible delayed early gene of unknown function [Leonard et al. 1987; E. Gi-zang-Ginsberg and E.B. Ziff, unpubl.] is induced with kinetics similar to TH [Fig. 1B].

We then asked whether the induction of the TH gene by NGF relied on new protein synthesis. PC12 cells were treated with either the protein synthesis inhibitor cycloheximide or anisomycin [Lewis and Matthews 1980], with both inhibitors, or with neither inhibitor beginning 30 min prior to NGF stimulation. We then analyzed, by nuclear run-on assay, the levels of TH transcription in vitro in nuclei isolated from PC12 cells during 0–4 hr of NGF treatment. Figure 2A demonstrates that in the presence of protein synthesis inhibitors, the TH gene fails to be induced by NGF. In contrast, the c-fos gene is superinduced by a mechanism independent of protein synthesis [Fig. 2B; Greenberg et al. 1986].

Functional activity of the TH-FSE DNA sequence

To examine how NGF induces TH gene transcription transiently, we sought to identify the promoter region sequences and transcription factors that regulate the gene. Inspection of the rat TH promoter [Harrington et al. 1987] revealed a potential regulatory element between positions −205 and −193 with homology to a previously characterized regulatory sequence, the FSE, which is found in genes regulated during adipocyte differentiation [Distel et al. 1987]. Within the FSE lies a sequence homologous to the 8-bp AP-1-binding site found in both viral and animal genomes [Angel et al. 1987; Lee et al. 1987a,b]. The FSE binds the Fos–Jun complex [Rauscher et al. 1988 a,c], which is responsive to growth factors and phorbol esters. Of 13 bp, 11 bp are conserved between the aP2 FSE2 sequence and the TH-FSE [Fig. 3], with 1 bp difference (C to T) occurring within the AP-1 site and 1 bp difference (A to C) occurring outside of the AP-1 site [FSE-specific sequence].

We first asked which sequences provide NGF induction and whether the TH-FSE functions in the regulation of TH gene transcription. We transiently transfected PC12 cells with plasmids that contain various portions of the TH gene 5′-flanking sequence. We employed three constructs that differ in the length of the 5′-flanking-sequence [Fig. 3]: p5′THCaten272/+27, p5′THCATen272/+27, and p5′THCaten151/+27 [Lewis et al. 1987]. p5′THCaten151/+27 contains only 151 bp of S′ untranscribed DNA sequence and lacks the TH-FSE DNA element [positions −205 to −193], as well as sequences downstream from this element (i.e., −193 to −152). Plasmids were introduced into PC12 cells by electroporation [Potter et al. 1984; Flug et al. 1987], and cells were harvested with or without NGF stimulation for 4 hr. We used RNase protection to assay for TH–CAT [chloramphenicol acetyltransferase] hybrid RNA transcripts. Transcription from the exogenous TH promoter should yield a 192-bp protected fragment composed of a hybrid transcript consisting of 27 bases of TH plus 165 bases of CAT RNA sequence. Results typical of several trials are shown in Figure 4. A low level of the 192-nucleotide transcript is observed when a plasmid containing either 272- or 773-bp of S′-untranscribed TH sequence is present in unstimulated PC12 cells [lanes 1 and 5]. NGF induces a marked increase in the level of transcripts from the exogenous −272/+27 plasmid (cf. lanes 1 and 2). Transcripts from the −773/+27 plasmid construct are also increased in response to NGF, although to a lesser extent (cf. lanes 5 and 6 to 1 and 2).
Figure 2. Effect of protein synthesis inhibitors on the kinetics of NGF-induced TH gene transcription. Nuclei were isolated from PC12 cells treated with various protein synthesis inhibitors (concentrations noted above), beginning one-half hour prior to NGF stimulation of 0–4 hr. RNA transcripts labeled with 32P were then generated and used for nuclear run-on transcription. Transcription of the TH gene (A) and the c-fos gene, a control, (B) was analyzed by using these assay conditions.

The endogenous TH gene is also stimulated in these cells (data not shown). These results show that sequences conferring NGF responsiveness are present within the TH 5’ DNA −272 to +27 region. The result with the −773-bp plasmid construct, in which inducibility of TH transcripts is not as strong as that with the −272 plasmid construct, further suggests the presence of an element between −773 and −272, which modulates the level of NGF-induced TH gene transcription. Unstimulated PC12 cells transfected with p5’THCAT −151/+27 also yield the 192-nucleotide protected RNA transcript (lane 9) at a level slightly higher than either −272/+27 or −773/+27 plasmids (cf. lane 9 with 1 and 5). No change in the level of TH transcript is observed with this plasmid following NGF induction (cf. lanes 10 and 9), which suggests that sequences downstream of

\[
5'\text{gattctagacTGA}\text{CTCATgtoocq}3' \\
5'\text{gatcCAGTACGAGGAAAACATACq}3' \\
5'\text{gatccGTTGATTGAGGCAGTGCCTq}3' \\
\text{(-205)} \quad \text{(-193)} \\
\text{TH-FSE oligo (28mer)}
\]

\[
5'\text{TCGGGCTGAGGGTTGGTGCCTGTGAC}3' \\
\text{(-205)} \\
\text{(-193)} \\
\text{TH-FSE oligo (28mer)}
\]

\[
\Delta \text{in Mutants}
\]

\[
\text{TH-70bp} \\
\text{TH-216bp}
\]

Figure 3. Regulatory DNA regions of the rat TH promoter. The 5’-untranscribed DNA region of the rat TH promoter (Harrington et al. 1987) is shown and contains a CRE ([Hyman et al. 1988] located at positions −45 to −38 and the TH-FSE DNA element located at positions −205 to −193. The TH-FSE shares homology with the FSE2 DNA region of the adipocyte-specific gene ap2 [Distel et al. 1987]. The TH-FSE is shown in boldface type, together with adjacent flanking sequences (uppercase letters) that are present in the TH promoter. TH-FSE and ap2-FSE2 DNA elements are aligned with one another with the consensus AP-1 sequence site of the human metallothionein gene, hMTIIa [Angel et al. 1987; Lee et al. 1987a,b], shown in boldface type. Oligonucleotides corresponding to each of these sites are also displayed. DNA linker sequences are shown in lowercase letters. TH-CAT plasmid constructs are also shown. Deletion mutants of the plasmid constructs pTHCATΔFSE −272/+27 and pTHCATFSE −773/+27, described in Methods, lack the 13-bp TH-FSE DNA sequence noted above. Finally, TH DNA restriction fragments used as competitors in gel mobility-shift assays are also indicated.
-151 are not responsible for NGF regulation. Because the -151 plasmid construct is not NGF inducible and the -272 plasmid construct is, these results further suggest that sequences between -272 and -151, a region encompassing the TH-FSE, are required for the response to NGF. Sequences in this region may also affect the basal level of transcription from the TH promoter, as the basal level of transcription was elevated in the -151/-272 construct versus the -272/+27 construct.

To test the role of the TH-FSE directly, we used in vitro mutagenesis (Kunkel 1985) to remove the 13-bp TH-FSE DNA element from the -773/+27 and -272/+27 plasmids (Fig. 3) and measured the levels of RNA in transfected uninduced and NGF-induced PC12 cells. Figure 4 shows that in the absence of the TH-FSE DNA element, neither construct expresses RNA in the unstimulated cells (lanes 3 and 7). Strikingly, no transcripts are detected from these deletion DNA plasmid constructs when PC12 cells are stimulated with NGF (lanes 4 and 8). These results suggest that the 13-bp TH-FSE DNA sequence has a direct role in establishing the basal level of activity of the rat TH gene and is also required for NGF inducibility.

**TH-FSE is capable of binding Fos–Jun in vitro**

Given a role for the TH-FSE in establishing the basal activity and NGF inducibility of the TH promoter, we asked whether this sequence is capable of forming sequence-specific DNA–protein complexes in vitro with fos and jun peptide products, as shown previously for the aP2 FSE2 sequence. A 28-bp oligonucleotide corresponding to the TH-FSE DNA element plus several bases of flanking sequence from the authentic rat TH promoter (see Fig. 3) and the complementary oligonucleotide were synthesized and annealed, and the 32P-labeled-TH-FSE was incubated with fos and jun peptides made by in vitro translation in a rabbit reticulocyte system [Kouzarides and Ziff 1988] and gel electrophoresis performed for the gel mobility-shift assay [Strauss and Varshavsky 1984; Carthew et al. 1985]. As is evident in Figure 5, the TH-FSE forms a DNA–protein complex when incubated with both fos and jun proteins [lane 3], whereas no binding is detectable when the same oligonucleotide is incubated with either fos or jun proteins alone [lanes 1 and 2]. The DNA–protein complex formed between the TH-FSE and the fos and jun proteins comigrates with the complex between Fos and Jun and an oligonucleotide corresponding to the canonical FSE DNA element. The greater intensity in the signal in the FSE lane suggests that this oligonucleotide has a slightly higher affinity for the in vitro-translated fos and jun proteins than the TH-FSE (cf. lanes 3 and 10). An unrelated DNA element NF68, forms no DNA–protein complex with fos and jun proteins [lane 9]. Unlabeled TH-FSE oligonucleotide specifically decreases by competition the formation of the complex formed with fos and jun proteins in lane 4, whereas the heterologous oligonucleotide NF68 does not compete for binding [lane 5], demonstrating the specificity of complex formation. In addition, formation of the DNA–protein complex is specifically inhibited by antibodies to either Fos or Jun [lanes 6 and 7] but is unaffected by an antibody generated against an unrelated protein, peripherin [lane 8]. These data establish that the TH-FSE binds authentic Fos–Jun heterodimeric complexes.

**Sequence-specific binding of NGF-inducible PC12 protein factors**

We then asked whether protein factors present in extracts of PC12 cells could form complex(es) with the
TH-FSE and whether the time of formation of such complexes corresponded to periods of NGF induction. First, the radiolabeled 28-bp double-stranded TH-FSE was incubated in the presence of nonspecific competitor DNA with nuclear extracts obtained from PC12 cells treated for either 1 or 2 hr with NGF. Electrophoresis in a non-denaturing gel reveals one specific complex \( A \) and one nonspecific complex \( N \) with nuclear proteins from either extract [Fig. 6A,B, lanes 1]. In Figure 6, A and B, complex \( A \) is decreased as a result of competition by unlabeled TH-FSE [lane 2], the FSE sequence [lane 3], and a double-stranded oligonucleotide containing the consensus AP-1 sequence [lane 4], demonstrating that it is sequence specific. Furthermore, complex \( A \) is not decreased as a result of competition by an unrelated double-stranded oligonucleotide that contains the dyad symmetry element of the \( c-fos \) gene [Fig. 6A,B, lanes 5]. In contrast, complex \( N \) in Figure 6 is not decreased by competition effectively, except by a high molar excess of unlabeled single-stranded oligonucleotide [data not shown].

To further examine the sequence specificity of complex \( A \), unlabeled restriction fragments spanning the TH-FSE region were used as competitor [see Fig. 3]. Both the 70-bp \( Aval-StuI \) fragment and a 216-bp \( XbaI-StuI \) fragment overlapping the TH-FSE sequence effectively decreased competitively the formation of complex \( A \) by the radiolabeled TH-FSE oligonucleotide [Fig. 6A,B, lanes 6 and 7], whereas a nonhomologous DNA fragment of the \( \alpha \)-tubulin gene does not offer competition [lane 8]. The radiolabeled TH-216 bp genomic fragment, itself, also forms a sequence-specific complex with nuclear extracts from stimulated PC12 cells [data not shown]. Taken together, these results confirm that the TH-FSE can bind nuclear proteins from PC12 cells in a sequence-specific manner.

**Alteration in levels of TH-FSE–nucleoprotein complex following NGF induction**

We noted that the level of DNA–nucleoprotein complexes formed with extract from cells treated for 2 hr with NGF was greater than that for the 1-hr treated cells [cf. Fig. 6A,B]. No alteration in mobility of these complexes was apparent. This suggested that proteins interacting with the TH-FSE may change either in their level or binding capacity during the NGF induction, and that such changes may correlate with changes in the rate of transcription of the TH gene.

To test this possibility, we assayed complex formation using extracts from cells treated for varying times from 0 hr to 1 day with NGF. Complex \( A \) becomes more abundant during this period of NGF stimulation [Fig. 6C]. More specifically, a low level of complex is detected with extracts from unstimulated PC12 cells [Fig. 6C, lane 1], and the abundance of this complex gradually increases following 1 hr of NGF and continues to increase at 2 hr of NGF treatment [Fig. 6C, lanes 3 and 5]. A slight difference in mobility of the complex with unstimulated versus 1-hr NGF-stimulated PC12 extracts is observed, although its significance is unknown. The complex reaches a maximum level at 4 hr of NGF treatment and is less abundant at 1 day of NGF stimulation [Fig. 6C, lanes 7 and 9]. No variation was seen in the level of serum regulatory factor (SRF) complex with a dyad symmetry element [DSE] oligonucleotide [data not shown], arguing that the changes did not reflect differences in extraction efficiency. These kinetics are in sharp contrast to the time course of TH transcription, which reaches a maximum at 1 hr of NGF stimulation of PC12 cells and then declines. Thus, the level of TH-FSE DNA–protein complex continues to increase during a period when the transcription rate of the TH gene first increases and then declines. This result suggests that e-
c-Fos regulation of the tyrosine hydroxylase gene

Figure 6. Analysis of DNA–protein complex formation between the TH-FSE and nuclear extracts obtained from NGF-treated PC12 cells. Nuclear extracts were prepared by the method of Dignam et al. [1983] and assayed for complex formation with the 32P-labeled-TH-FSE. DNA–protein complexes were formed in A and B with nuclear extracts from 1-hr NGF- and 2-hr NGF-treated PC12 cells, respectively. Complexes were formed in the presence of either no competitor [lane 1], unlabeled TH-FSE [lane 2], FSE [lane 3], AP-1 [lane 4], DSE [lane 5], double-stranded unlabeled TH 5′ genomic restriction fragments designated TH-70 bp [lane 6], and TH-216 bp [lane 7], or an unlabeled DNA fragment of the rat α-tubulin gene [pT1-280, lane 8]. (A description of these oligonucleotides and fragments is given in Methods and in the legend to Fig. 3. [C] DNA–protein complexes formed by the 32P-labeled TH-FSE incubated with nuclear extracts obtained from either untreated [lane 1], 1-hr NGF-treated [lane 3], 2-hr NGF-treated [lane 5], 4-hr NGF-treated [lane 7], or 1-day NGF-treated [lane 9] PC12 cells. Competition with unlabeled TH-FSE is also shown [lanes 2, 4, 6, 8, 10]. [N] Nonspecific complex, which can be decreased by competition with a high molar excess of homologous or heterologous single-stranded oligomer [data not shown].

ther the level of the complex is unrelated to the rate of TH gene transcription or that the nature of the complex changes during the intervals of positive and negative control [see below].

Direct involvement of c-fos in the nucleoprotein complex at the TH-FSE-binding site

Previous studies by others have shown that the FSE2 and hMTIIα–AP-1 elements specifically bind a heterodimeric complex of Fos in association with Jun [e.g., Bohmann et al. 1987; Distel et al. 1987; Chiu et al. 1988; Rauscher et al. 1988a,c]. It has been well documented that c-fos protein [Curran and Morgan 1985; Kruijer et al. 1985] is induced in PC12 cells by NGF under conditions employed here for the transient induction of TH transcription. Because the TH-FSE can bind in vitro-translated Fos in the presence of Jun and complex A is induced by NGF, we asked whether Fos is present within this complex in vivo. In Figure 7A, we utilized the anti-Fos antibody, anti-FostrpA, generated against a Fos fusion peptide [Vosatka et al. 1989] to see whether the formation of the complex could be inhibited between proteins present in NGF-stimulated PC12 extracts and the radiolabeled TH-FSE. Preincubation of the antibody with PC12 nuclear extracts obtained after various periods of NGF treatment inhibited the complex from forming (cf. lanes 1, 2, 4, 5, 7, 8, 10, 11, 13, and 14). Whereas antibody concentrations used in Figure 7A were sufficient to completely inhibit complex formation at 0-, 1-, 2-hr, and 1-day NGF time points, 2× concentrations were required to completely inhibit the more abundant nucleoprotein complex formed with extract from 4-hr NGF-treated cells [data not shown]. Preincubation with a control anti-peripherin antibody did not inhibit the complex [Fig. 7A, lanes 3, 6, 9, 12, and 15].

As an additional control, the anti-Fos antibody was preincubated with or without excess Fos fusion peptide, and the mixture was then incubated with nuclear extracts prepared from either 1- or 4-hr NGF-treated PC12 cells. Radiolabeled TH-FSE was then added. As seen in Figure 7, B and C, preincubation with the peptide completely reversed the inhibitory effect of the anti-FostrpA antibody [cf. Fig. 7B,C, lanes 2 and 3], indicating that the effect observed is due to specific antigen recognition. These results show that anti-FostrpA antibody is capable of disrupting the protein–DNA complex detected by the mobility-shift assay by using extracts from cells at both early and late times of NGF induction. It should be noted that the FostrpA fusion peptide used to generate the antibody contains a hydrophilic amino acid sequence with antigenically related counterparts found in all members of the fos gene family [Vosatka et al. 1989]. Therefore, given the existence of multiple fos gene family members [Franza et al. 1987, 1988; Cohen and Curran 1988; Zerial et al. 1989], we asked whether a Fos-related antigen [Fra], as well as c-fos, could participate in the formation of the DNA–protein complexes in the unstimulated and NGF-stimulated cells.

To determine whether both c-Fos and Fra could contribute to TH-FSE–protein complex formation, we analyzed the PC12 nuclear extracts by Western blot analysis by using anti-FostrpA antibody. We asked whether the presence or absence of various proteins of the fos gene family correlated with changes in binding activity to the TH-FSE and changes in the transcription rate of the TH gene. PC12 cell nuclear extracts obtained after various intervals of NGF treatment were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with the anti-FostrpA antibody.

In Figure 8, virtually no Fos is detected in nuclear ex-
tracts of unstimulated PC12 cells. Only a faint band of the molecular mass range expected for Fra proteins—46–48 kD—is seen [Franza et al. 1987]. At 1 hr of NGF stimulation, a strong band of Fos is readily visible [lane 2], consistent with previous reports [Curran and Morgan 1985; Kruijer et al. 1985; Franza et al. 1987] that Fos is strongly induced by NGF. A weaker band of Fra is also detected. As the duration of NGF stimulation is extended [i.e., 2 hr, 4 hr, and 1 day], the relative levels of Fos and Fra change [lanes 3–5]. Fos protein appears to be much less abundant, most likely due to its transient expression and short protein half-life [Müller et al. 1984; Kruijer et al. 1985; Curran and Morgan 1986; Vosatka et al. 1989]. The decrease in Fos levels is most evident during the transition from 2 to 4 hr of NGF treatment. Fos is almost completely absent by 1 day of NGF stimulation. Fos is slightly slower in its migration at 4 hr of NGF treatment versus 1 hr, most likely as the result of an increase in phosphorylation [Müller et al. 1984; Franza et al. 1987; Vosatka et al. 1989]. The Fra species become the predominant fos gene family products by 4 hr of NGF stimulation of PC12 cells [lane 4], and the Fra protein levels drop slightly by 1 day of NGF treatment [lane 5]. This experiment reveals a pattern of expression in which Fos increases and gradually diminishes, whereas Fra increases and maintains its level to become the predominant product during the time interval that TH transcription is transiently induced. When taken together with the results of mutagenesis of the TH promoter demonstrating dependence on the TH-FSE for NGF inducibility, these results suggest that proteins acting at the TH-FSE have a direct role in regulating the TH gene and that the nature of this complex changes during the course of NGF-induced differentiation.

Discussion

Transcriptional regulation of the TH gene

We have analyzed the mechanism of transcriptional regulation of the TH gene by NGF. Our results demonstrate that NGF stimulation of PC12 cells induces transcription of the TH gene with delayed early kinetics. We also show that the mechanism controlling transcription of the TH gene during NGF induction is a multistep process that is reliant on new protein synthesis. During NGF induction, the expression of early response genes, including c-fos, precede the time of induction of TH transcription. We show that an element of the TH promoter, the TH-FSE, contains a Fos-Jun-binding site that is structurally related to the FSE2 element that functions in adipocyte differentiation. We show that c-Fos in complex with c-Jun can interact with the TH-FSE in vitro and that the element is required in vivo in transient transfection assays for NGF stimulation of TH transcription. These observations lead to a model in which the early response protein c-Fos in complex with c-Jun induces TH transcription by binding to the TH-FSE. We also show that during the time at which the transcription rate of TH declines, the relative proportions of fos family members in the induced cell change.
such that the Fra proteins become relatively more abundant than c-Fos protein itself. The data therefore raise the possibility that at the time TH transcription is repressed, a Fos–Jun complex at the TH-FSE site is replaced by a complex consisting of Fra proteins bound to the NGF-stimulated PC12 cell nuclear extracts. Nuclear protein extracts (15 μg/lane) were electrophoresed on SDS-PAGE, electrophotographed, and subjected to Western blot analysis using anti-FosrpA antibody, which was detected by 125I-labeled protein A, as described in Methods. The nuclear extracts were from un- treated, 1-, 2-, 4-, and 24-hr NGF-treated PC12 cells (lanes 1–5, respectively). Migration of Fos and Fra(s) are indicated by arrows. (Left) Protein standards are indicated in kilodaltons.

Figure 8. Western blot analysis of the fos family of proteins in NGF-stimulated PC12 cell nuclear extracts. Nuclear protein extracts (15 μg/lane) were electrophoresed on SDS-PAGE, electrophotographed, and subjected to Western blot analysis using anti-FosrpA antibody, which was detected by 125I-labeled protein A, as described in Methods. The nuclear extracts were from un- treated, 1-, 2-, 4-, and 24-hr NGF-treated PC12 cells (lanes 1–5, respectively). Migration of Fos and Fra(s) are indicated by arrows. (Left) Protein standards are indicated in kilodaltons.

Functional importance of the TH-FSE site

We have provided evidence that a specific DNA sequence element, TH-FSE, plays an important role in the regulation of transcription of the TH gene by NGF. The initial identification of this DNA element was based on homology with the FSE2 sequence [Distel et al. 1987] and other regulatory sequences containing Fos–Jun-binding sites [Angel et al. 1987; Lee et al. 1987a,b]. The TH-FSE is located between positions −205 to −193 in the 5′-untranscribed region of the TH gene. Our transient transfection studies support the following contentions regarding the functioning of this element.

The basal level expression of TH is dramatically decreased in the absence of TH-FSE when either 272 or 773 bp of 5′-untranscribed DNA sequence is present. Thus, the TH-FSE DNA sequence functions in the basal level expression of the TH gene. Other regulated DNA sequences, such as the cAMP response element (CRE) of the c-fos gene, have been shown to function as basal control elements and are responsive during EGF [Fisch et al. 1987] and cAMP [Berkowitz et al. 1989] stimulations. Because all TH expression is eliminated when the TH-FSE element is deleted, the TH-FSE may cooperate with other elements to modulate transcription of the TH gene by NGF. Support for this idea is suggested by our results with the p5'THCAT −151/+27 plasmid. p5'THCAT −151/+27, which contains no TH-FSE or adjacent flanking elements from −193 to −151, is active in unstimulated PC12 cells and is not induced by NGF. This is consistent with NGF control through the TH-FSE and also suggests that additional basal level elements for the TH promoter may lie in the −151/+27 region of the gene. We also find that the TH promoter with 272 bp of 5′-flanking sequence is more strongly induced than with 773 bp of 5′ DNA. This suggests the presence of a negative regulatory element located within the −272- and −773-bp region. Studies utilizing point mutations of the TH-FSE DNA may distinguish between TH-FSE DNA sequences functioning in basal versus NGF-induced regulation.

c-Fos regulation of the tyrosine hydroxylase gene

Our results indicate that the TH-FSE is a sequence-specific binding site for in vivo nuclear protein(s) inducible by NGF. Gel mobility-shift studies demonstrate that protein binding to the TH-FSE changes in abundance during NGF-stimulated differentiation, reaching a maximal level at 4 hr following treatment and subsequently decreasing in abundance to a low level by 24 hr of NGF treatment. The gel mobility assay of DNA–protein complexes did not reveal any obvious alterations in complex mobility, therefore, it is difficult to establish whether the complex undergoes any qualitative changes in response to NGF by this approach alone. Western blotting experiments suggest that changes did occur [see below]. Western blotting experiments suggest that changes did occur [see below].

Two lines of evidence suggest that the fos family of proteins can contribute to complex formation with the TH-FSE. First, we have shown that the TH-FSE binds fos and jun proteins synthesized by in vitro translation. This is in agreement with the binding potential of the canonical FSE and AP-1 DNA elements [Bohmann et al. 1987; Distel et al. 1987; Chiu et al. 1988; Rauscher et al. 1988a,c]. Second, antibody inhibition studies, using an antibody that recognizes amino acid sequences shared by all of the members of the fos protein family, effectively inhibit DNA–protein complex formation by extracts that contain c-Fos as the predominant Fos-related component. Because various investigators have demonstrated that c-Fos binds DNA elements in concert with Jun [Halazonetis et al. 1988; Kouzarides and Ziff 1988;
Nakabeppu et al. 1988, Rauscher et al. 1988a, Sassone-Corsi et al. 1988c; for review, see Curran and Franza 1988), we anticipate that the same Fos–Jun association should hold true in our system. Our results show that fos and jun proteins expressed in vitro can bind in complex with the TH-FSE. We have further attempted to analyze the TH-FSE–nucleoprotein complex for the involvement of Jun during various times of NGF stimulation in PC12 nuclear extracts by using antibody inhibition methodologies (antibody provided by D. Bohmann) similar to those used for examining Fos. These studies have not been successful to date. Hay et al. (1989) were similarly unable to demonstrate any involvement of Jun in the Fos-containing protein complex that controls the c-myc gene. It is possible that the Jun antibody used in our experiments recognizes an antigenic determinant that is not readily accessible when in vivo-synthesized Jun is in complex with Fos at the TH-FSE site. Alternatively, an inability to detect Jun may reflect a true biological feature of the DNA-binding complex. Franza et al. (1987) have shown that PC12 cells contain, in addition to Jun, various Fos–associated proteins in the low-molecular-mass range, ~40 kD. These investigators have shown further that although Jun is basic, the p40 proteins are extremely acidic, suggesting that they are structurally distinct. Therefore, it is possible that Jun may not be associated with Fos in the TH-FSE–protein complex; perhaps another protein (i.e., one of the p40 proteins) may serve an analogous function and cooperate with the fos protein gene family in binding to the TH-FSE.

Greenberg and colleagues (Bartel et al. 1989) have shown that in the PC12 cell system, induction of fos, c-jun, and jun-B has distinct programs of activation in response to NGF and EGF versus membrane-depolarizing agents. c-fos, jun-B, and c-jun are all rapidly induced by both NGF and EGF, whereas only c-fos and jun-B are transcriptionally activated by KCl. The temporal appearance of these genes and their corresponding protein products in PC12 cells implies that they may provide greater versatility of gene control by generating a diversity of combinational regulatory complexes that allow various subsequent differentiative events to be induced by distinct signal transducing pathways.

How could Fos affect TH gene activity? Our data demonstrate fos expression during the initial phase of NGF induction of PC12 cell differentiation (Curran and Moran 1985; Greenberg et al. 1985; Kruijer et al. 1985; Milbrandt 1986; fos expression is transient, and the fos protein is labile [Muller et al. 1984; Kruijer et al. 1985; Curran and Morgan 1985; Franza et al. 1987; Vosatka et al. 1989] and decays within ~1–2 hr following stimulation by growth factors. Our Western blot analysis demonstrates that this is also the case for NGF induction and further demonstrates an increase in the presence of Fra proteins, which parallels the decrease of Fos. We find that the maximal induction of the TH gene transcription occurs 1 hr following NGF treatment. The level of the TH-FSE–nucleoprotein complex increases from 0 to 1 hr and again at 2 hr, reaching a maximum at 4 hr and declining at 1 day of NGF treatment. We suggest that both Fos and Fra proteins may participate in TH gene control by binding to the TH-FSE sequentially during the course of NGF induction. This exchange of proteins at the TH-FSE site may allow for alternative functions to be provided at different times during NGF-induced differentiation. For instance, c-Fos may interact with the TH-FSE during the initial times of NGF treatment. Because transcriptional activation occurs at 1 hr of NGF treatment, Fos may serve as a positive regulator according to this model. Fra proteins are then synthesized and potentially substituted at the TH-FSE-binding site at 4 hr NGF treatment to provide a different signal and thereby a different functional role during a period of shutdown of NGF-induced TH gene transcription. Evidence for Fos acting as a trans-acting positive regulator of gene expression has been demonstrated for the α[III] collagen gene (Setoyama et al. 1986), the collagenase gene (Schonthal et al. 1988), and the transin gene (Kerr et al. 1988). In addition, other studies have shown that FSE-like or AP-1-containing elements that interact with fos proteins can function as negative regulators of gene expression such as with the adipocyte-specific gene aP2 ([Distel et al. 1987], the c-myc gene [Hay et al. 1989], and a region of the human immunodeficiency virus [HIV] long terminal repeat [Franza et al. 1988]. The concept that the TH-FSE may be a binding site that allows for alternative functions also has other precedents. In yeast mating-type determination, MCM1 control elements can serve as either positive or negative regulators of α- or α-specific genes (Passmore et al. 1989). Alternative proteins that bind adjacent to MCM1 determine the effect of the MCM1 control element on transcription. Our results show that Fos and/or Fra proteins interact with the TH-FSE-binding site of the TH gene. Antibodies directed against Fos- or Fra-specific antigenic domains should help to distinguish at which times fos family members are associated with this element. An analysis of transformed PC12 cell lines either stably transfected with an inducible c-fos gene or transiently cotransfected with c-fos- or fra-containing plasmids, along with our TH plasmid constructs, could further allow us to determine the individual roles of members of the fos gene family in TH gene control during NGF-induced neuronal differentiation.

In this communication we have provided evidence that NGF control of PC12 cell differentiation is regulated by a cascade of gene activity. This series of events includes the activation of an NGF-inducible transcription factor, c-fos, whose product contributes to generating a sequence-specific DNA–nucleoprotein complex within the TH promoter during the growth-factor-induced transcription. This binding complex thereby regulates the TH gene, whose protein product is crucial for neuronal differentiation. The involvement of additional newly synthesized fos protein family members in controlling TH gene activity during NGF induction suggests a pattern of gene regulation of even greater complexity. In the future, it will be important to determine whether this cascade of events is a general phenomenon that also
occurs in vivo in animal tissues whose differentiation and maintenance are precisely regulated by NGF.

**Methods**

**Cell culture and stimulations**

Stock cultures of PC12 cells were maintained in Dulbecco's modified Eagle medium, containing 10% defined and supplemented calf serum and 5% horse serum [DMEM, Hyclone Laboratories, Sterile Systems, Inc., Logan, UT] in a humidified 37°C incubator with a 10% CO2 atmosphere. Cells were plated on plastic culture dishes coated with collagen (Collagen Corporation, Palo Alto, CA) at a density of -3 x 10^6 cells/10-cm dish prior to all experiments, except where noted. Stimulations with NGF in the presence of fresh media [50 ng/ml; provided by Dr. Robert Stack, University of Michigan, Flint] were performed 24 hr following plating. When cultures were pretreated with anisomycin [100 µM] or cycloheximide [10 µg/ml], the protein synthesis inhibitors were added as concentrated solutions directly to the culture medium. Cells were then stimulated 30 min later by the addition of NGF.

**Nuclear run-on transcription assay**

Experimental procedures for isolation of nuclei and the in vitro nuclear run-on transcription assay have been described previously [Greenberg et al. 1983]; pODC934 (ornithine decarboxylase, ODC; Berger et al. 1984); pclone 10b, p-clone 15 (TH), pclone 2, pclone 73 (peripherin), and pclone 74 [Leonard et al. 1987].

**Plasmid constructions and mutagenesis**

Plasmids p5'THCAT -272/+27 and p5'TH CAT -773/+27 were gifts from Dona Chikaraishi [Lewis et al. 1987]. These constructs [Fig. 3] are composed of TH genomic DNA sequence that includes, respectively, 272 or 773 bp of 5'-flanking TH sequence, the transcription initiation site, and the first 27 bases of transcribed TH sequence cloned adjacent to a DNA segment coding for the bacterial enzyme chloramphenicol acetyltransferase (CAT) in a pUC-13-derived vector. p5'THCAT -151/+27, which contains only 151 bp of 5' genomic flanking sequence that excludes the TH-FSE DNA sequence region were isolated to be used for gel mobility-shift competitions. TH-70 bp was excised from p5'TH -272/+27 with Apal/Stul digestion, whereas TH-216 bp was obtained from a Xbal/Stul digestion of p5'TH CAT -773/+27. pT1, an embryonic a-tubulin cDNA-containing plasmid [Cleveland et al. 1980], was digested with PstI to obtain a 280-bp DNA fragment for competition studies.

**Synthetic DNA oligonucleotides**

Oligonucleotides used in gel retardation studies were synthesized on an Applied Biosystems automatic DNA synthesizer and purified by denaturing polyacrylamide gel electrophoresis. The 28-bp oligonucleotide, containing the TH-FSE and flanking region with BamHI linkers, and the oligonucleotide for the AP-1 consensus sequence, which was flanked from the human metallothionein IIa gene [Lee et al. 1987a], were synthesized and illustrated in Figure 3. The complementary strand was made and used for annealing. Double-stranded oligonucleotides have been described previously for the FSE [Kouzarides and Ziff 1988, Fig. 3] and the DSE of c-fos [Metz et al. 1988]. An oligonucleotide corresponding to the 5'-flanking DNA region (-136 to -105) of the NF68 gene [neurofilament protein; Lewis and Cowan 1986] was kindly provided by Nick Cowan.

**In vitro transcription and translation**

To express fos and jun proteins, a fos rat cDNA cloned into the EcoRI site of pGEM3 [Curran et al. 1987; Kouzarides and Ziff 1988] and a c-jun clone in pBSSK [Turner and Tjian 1989] were used. In vitro transcription of the constructs [1 µg] and subsequent in vitro translation of one-quarter of the product was made in a rabbit reticulocyte lysate in the presence of [35S]methionine, as described by Kouzarides and Ziff [1988].

**Nuclear extract preparation and gel retardation assays**

PC12 nuclear extracts were prepared from unstimulated and NGF-stimulated cell cultures, essentially as described by Dignam et al. [1983]. The nuclear extracts were dialyzed overnight against BC100 buffer [0.02 M HEPES at pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonylfluoride] at 4°C, aliquoted, and frozen at -70°C. Protein concentrations were determined using the Bio-Rad protein assay kit [Richmond, CA].

For DNA–protein binding reactions, 0.2 ng of kinase 32P-labeled oligonucleotide [1 µl] was mixed with 3 µg poly[dI-C] (3 µl; Pharmacia LKB Technology Inc, Piscataway, NJ), 100 ng of nonspecific single-stranded oligonucleotide [2 µl], BC100 without glycerol [15 µl], and 15 µg site of rGEF3. RNA probe generated from this construct, pTH56.CAT167, protects a 192-nucleotide THCAT hybrid mRNA in our studies.

Finally, two genomic 5'TH DNA fragments [Fig. 3] that span the TH-FSE DNA sequence region were isolated to be used for gel mobility-shift competitions. TH-70 bp was excised from p5'TH -272/+27 with Apal/Stul digestion, whereas TH-216 bp was obtained from a Xbal/Stul digestion of p5'TH CAT -773/+27. pT1, an embryonic a-tubulin cDNA-containing plasmid [Cleveland et al. 1980], was digested with PstI to obtain a 280-bp DNA fragment for competition studies.

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of unlabeled, double-stranded oligonucleotide (TH-FSE, FSE, publ.) to the nuclear extract in binding buffer for 1 hr at 4°C (TH-70, TH-216, or pTI-280) in the reaction mixture. Inhibition prior to the addition of the labeled probe. The preincubation of the Fos antibody with its corresponding fusion peptide or control peptide was conducted for 60 min at 4°C (0.5 μg of antibody, 5 μg of peptide) prior to complex formation. Binding assays with in vitro-translated proteins were carried out as described by Kouzarides and Ziff [1988]. DNA–protein complexes were resolved on 4.0% polyacrylamide gels (30:0.8 acrylamide/bis-acrylamide) in the presence of a 1 : 100 dilution of anti-Fos/anti-Jun antibody, 5 μg of peptide) prior to complex formation. Binding assays with in vitro-translated proteins were carried out as described by Kouzarides and Ziff [1988].

**Transfection cell transfections and RNA protection analysis**

Transfection of PC12 cells was performed using electroporation [Potter et al. 1984, Flug et al. 1987]. Briefly, cells were cultured in DMEM [with fresh media given to cells 24 hr prior to the day of transfection], and 6.0 × 10^7 cells were harvested as cell pellets for each DNA sample used for transfection. Supercoiled pTH–CAT constructs (20 μg) in 30 μl TE (1 mM Tris at pH 8.0, 0.2 mM EDTA) were added to 0.4 ml of Dulbecco’s phosphate-buffered saline containing 10 g/liter of glucose (PBS-g) and used to resuspend the cell pellets. The cell and DNA mixture was then added to prechilled gene pulser cuvettes (0.4 cm, electrode gap; Bio-Rad) on an ice slurry. The cells received a 1.5- to 1.6-kV pulse at 0.9 mA maximum amperage with an Isco model 3A Gen 5.000.1000 pulse generator. The cells were washed three times with PBS-g, treated with DNase for 30 min at 37°C, and washed again. RNA was hybridized with a 32P-labeled antisense RNA generated from pTH56.CAT167 in the presence of a 1 : 100 dilution of anti-Fos antibody and then incubated in 3% nonfat dry milk in 1 TST for 1 hr at room temperature with shaking, washing in 1 TST five times for 10 min each, and subsequently autoradiographed by exposure at ~ 70°C.

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