Hypoxia-induced Protein Binding to $O_2$-responsive Sequences on the Tyrosine Hydroxylase Gene*

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We reported recently that the gene that encodes tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of catecholamines, is regulated by hypoxia in the dopaminergic cells of the mammalian carotid body (Czyzyk-Krzeska, M. F., Bayliss, D. A., Lawson, E. E. & Millhorn, D. E. (1992) J. Neurochem. 58, 1538–1546) and in pheochromocytoma (PC12) cells (Czyzyk-Krzeska, M. F., Furnari, B. A., Lawson, E. E. & Millhorn, D. E. (1994) J. Biol. Chem. 269, 760–764). Regulation of this gene during low $O_2$ conditions occurs at both the level of transcription and RNA stability. Increased transcription during hypoxia is regulated by a region of the proximal promoter that extends from −284 to +27 bases, relative to transcription start site. The present study was undertaken to further characterize the sequences that confer $O_2$ responsiveness of the TH gene and to identify hypoxia-induced protein interactions with these sequences. Results from chloramphenicol acetyltransferase assays identified a region between bases −284 and −150 that contains the essential sequences for $O_2$ regulation. This region contains a number of regulatory elements including AP1, AP2, and HIF-1. Gel shift assays revealed enhanced protein interactions at the AP1 and HIF-1 elements of the native gene. Further investigations using supershift and shift-Western analysis showed that c-Fos and JunB bind to the AP1 element during hypoxia and that these protein levels are stimulated by hypoxia. Mutation of the AP1 sequence prevented stimulation of transcription of the TH-chloramphenicol acetyltransferase reporter gene by hypoxia.

Cells in mammalian tissues are primarily aerobic and therefore highly dependent upon a continuous supply of oxygen. A primary mechanisms by which mammals respond to reduced $O_2$ (hypoxia) is hyperventilation, which enhances the delivery of $O_2$ to cells by increasing arterial $O_2$ tension. The hyperventilation that occurs during hypoxia is mediated by the $O_2$-sensitive type I cells in the carotid body. The carotid body is located bilaterally at the bifurcation of the carotid artery and releases dopamine in response to a reduction in arterial $O_2$ tension. The activity of tyrosine hydroxylase (EC 1.14.16.2, TH), the rate-limiting enzyme in the biosynthesis of dopaminine, is enhanced in the carotid body (3) and adrenal gland (4) during hypoxia. We reported recently that hypoxia enhances TH gene expression in the rat carotid body (1) and stimulates both the rate of TH gene transcription and TH mRNA stability in pheochromocytoma (PC12) cells (2). We use the PC12 clonal cell line as a model system to study the molecular genetic mechanisms that regulate TH gene expression during hypoxia. Transcriptional studies using nested deletions of the proximal promoter region of the TH gene linked to a reporter gene revealed that the $O_2$-responsive sequences reside within a fragment that extends from −272 to +27 bases, relative to transcription start site (2).

In the present study experiments were undertaken to further characterize the sequences and trans-acting protein factors that regulate the rate of transcription of TH gene during hypoxia. We report here that the $O_2$ responsiveness of the TH gene is mediated by a short fragment of the proximal promoter that contains the AP1 and HIF-1 (hypoxia-induced factor binding site) sequences. Findings from immunological studies revealed that c-Fos and JunB bind to the AP1 element during hypoxia. In addition, we report that mutation of the AP1 element abolished the transcription response to hypoxia.

**EXPERIMENTAL PROCEDURES**

Materials

[α-32P]ATP (>3000 Ci/mmols) and [14C]chloramphenicol (50–60 mCi/mmols) were purchased from Amersham Corp. Klenow, G-25, and G-50 purification spin columns were purchased from Boehringer Mannheim. pCAT plasmids were originally obtained from Promega. Lipofectin reagent was purchased from Bio-Rad, and all cell culture reagents were obtained from Life Technologies, Inc. Antibodies for supershift assays, shift-Western analysis, and immunoblotting assays were purchased from Santa Cruz Biotechnology. Immunoblotting assays were performed using the ECL kit from Amersham.

Methods

Cell Culture—Rat pheochromocytoma (PC12) cells were maintained in Dulbecco's modified Eagle's medium/F-12 containing 15 mM Hepes buffer, 1-glutamine, 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were grown on culture dishes to 90% confluence in a strict environment (21% $O_2$, 5% $CO_2$, remainder $N_2$ at 37°C). Medium was changed twice weekly. Hypoxia exposures were carried out in an $O_2$ regulated incubator by exposing cells to 5% or 10% $O_2$, 5% $CO_2$, remainder $N_2$ and was maintained for various time courses.

TH Reporter Gene Constructs—TH plasmid constructs were made using the original −773 TH plasmid generously provided by Dr. D. M. Chikaraishi. Dr. Chikaraishi also provided the TH-AP1 mutant plasmid (5). A constant primer that contained an engineered XbaI site was made to the 3' end of the TH gene to +27 (relative to transcription start site). Different 5' primers at −284, −91, and −37 each contained an engineered HindIII site in the sense orientation. Polymerase chain reaction products were subcloned in front of the CAT gene in the pCAT Basic plasmid (a promoterless chloramphenicol acetyltransferase bacterial reporter plasmid). The −110 and −150 TH constructs were made by restriction digestion and religation of the −284 TH plasmid. All clones were confirmed by restriction digestion analysis and double-stranded.
sequencing using the Sequenase version 2.0 kit from U. S. Biochemical Corp.

Transfection and CAT Assays—PC12 cells were transfected with −284, −150, −110, −91, and −37 TH-CAT constructs, as well as a −272 construct with a mutated AP1 site. The promoterless pCAT Basic plasmid was used as a negative control for each experiment and the RSV-CAT (Rous Sarcoma Virus promoter) transfected as a positive control. PC12 cells were plated at a density of 2 × 10^5 cells/100-mm^2 dish. Plasmid DNA (5 μg of TH CAT plasmid) was mixed with 10 μg of Lipofectin reagent in a polystyrene tube in 1 ml of serum-free medium and added to the plated cells after 45 min of incubation at room temperature. Complete medium was added to the transfection 6–12 h following each transfection. Cells were exposed to either 21% O_2 (normoxia) or 5% O_2 (hypoxia) and then harvested 48 h post-transfection by washing with 1 × phosphate-buffered saline, and cell pellets and cellular debris were discarded following centrifugation. Lysates were standardized using the Bio-Rad assay. Assay incubations were for 3 h at 37°C with acetyl coenzyme A and [14C]chloramphenicol. CAT activity was measured by the method of Gorman et al. (6). Results were plotted as percent acetylation of total [14C]chloramphenicol and normalized to results from test constructs are reported as a percentage of RSV-CAT activity.

Gel Mobility Shift Assay—Mobility shift assays were performed using native DNA fragments corresponding to the −284 to −190 region of the TH gene (relative to transcription start site). Double-stranded oligonucleotide probes that contained specific cis-regulatory elements were also used in mobility shift assays. Nuclear protein extracts were prepared, as described (7), from cells that had been exposed to either 5, 10, or 21% O_2. Briefly, cells were removed from culture plates, washed, and swollen in hypotonic buffer. Intact nuclei were isolated by Dounce homogenization of the swollen cells. Nuclear proteins were extracted and dialyzed in salt buffers. Binding reactions were performed with 10 μg of nuclear protein extract, 3 μg of poly(dI: dC) (Pharmacia Biotech Inc.), and 4 ng of Klenow-labeled DNA in a previously described binding buffer (8). Binding reactions were incubated at 4°C for 12–18 h prior to electrophoresis on a 4% polyacrylamide gel with 2.5% glycerol. Running buffer was 0.5 TBE. To test the specificity of DNA-protein binding, competition experiments were performed with unlabeled DNA that was incubated with nuclear protein extracts prior to addition of radioactive probe.

Supershift Assays—Prior to the addition of radioactive DNA probe, nuclear protein extracts were incubated with antibodies (2–6 μg) against c-Fos, c-jun, or J unB for 3–5 h at 4°C. Labelled probe was then added, and DNA-protein incubation was continued for 12–18 h at 4°C. DNA-protein-antibody complexes were resolved by polyacrylamide gel electrophoresis (as described for mobility shift assays).

UV Cross-linking of DNA-Protein Complexes—DNA-protein binding reactions were performed as described above. Binding reactions were then incubated with a total of 4 × 10^5 jcm^2 UV light (UV cross-linker FB UVXL-1000; Fisher). Samples were then resuspended in an equal volume of SDS sample buffer, boiled for 10 min, and electrophoresed on an 8% SDS-polyacrylamide gel at 40 mA. Bio-Rad protein molecular weight markers were used to determine the size of resolved bands. Gels were dried and exposed to x-ray film overnight at −80°C.

Shift-Western Assays—Proteins from the gel mobility shift complex were transferred to a nitrocellulose membrane and DNA was transferred to a DEAE membrane using a semi-dry transblotter (Bio-Rad) (9). The DEAE membrane that contained the radioactively labeled DNA was transferred to x-ray film to obtain the mobility shift autoradiographic image. The nitrocellulose blot was then probed with the appropriate antibody using standard Western blotting protocols recommended in the Amersham ECL immunoblotting kit. The chemiluminescent reaction was exposed to Reflections (DuPont NEN) film for 15 to 60 s. The autoradiographic results from the two membranes were compared to determine if the DNA-protein complex (mobility shift image) contained the proteins that were identified with specific antibodies (Western blot image). Control lanes of protein alone (no DNA) were included to exclude the possibility of equivalent protein and protein-DNA migration.

RESULTS

We showed previously that a fragment of the TH 5′-flanking region that extends from −272 to +27 contains the elements necessary to regulate increased transcription during hypoxia (2). To further localize the hypoxia-responsive sequences on the TH gene, PC12 cells were transiently transfected with plasmid constructs consisting of various lengths of 5′ TH flanking se-
Hypoxia-induced binding at the AP1 and HIF-1 elements during hypoxia.

To further characterize protein interactions with the TH-AP1 and TH-HIF-1 elements during hypoxia, mobility shift assays were performed with double-stranded oligonucleotide probes that contained sequences for either the AP1 or HIF-1 element. There were no observed increases in binding activity when protein extracts from PC12 cells exposed to hypoxia were incubated with the oligonucleotide probe that contained the TH-HIF-1 binding site (data not shown). Since binding to the native fragment was partially competed by an HIF-1 oligonucleotide, we were surprised that binding to the AP1 oligonucleotide was not induced by hypoxia. The reason for this is unclear, but could be due to a requirement for additional flanking sequences or to interactions with proteins that bind to other elements. We did, however, measure hypoxia-induced binding to the TH-AP1 oligonucleotide (Fig. 3), which was blocked by addition of unlabeled TH-AP1 oligonucleotide (arrow). The figure shows the same experiment that was repeated with nuclear protein extracts from two separate experiments. Nonspecific oligonucleotides failed to block binding to the TH-AP1 oligonucleotide (data not shown), which demonstrates the specificity of hypoxia-induced binding at this site. The lower band was blocked by nonspecific oligonucleotides, which indicates that this band is due to nonspecific binding activity.

We next examined the effect of different durations of hypoxia on protein binding to the AP1 element. Fig. 4 shows a progressive increase in binding activity to the AP1 element with nuclear protein extracts from cells that were exposed to increasing durations (1–24 h) of 5% O2. A graphical representation of binding activity from optical density measurements of the DNA-protein complex at each duration of hypoxia exposure is presented in Fig. 4B. The open bar (time 0) represents binding activity to the AP1 probe in 21% O2. Maximal binding activity was observed with extracts taken from cells exposed to 5–6 h of hypoxia (Fig. 4B). A notable finding was that increased binding activity was still evident with extracts from cells exposed to reduced oxygen for 24 h.

We also performed a series of experiments (n = 3) to determine if binding activity is regulated by the level of O2 tension (Fig. 5). Cells were exposed to either 10% O2 or 5% O2 for 1, 3, or 5 h prior to extraction of nuclear proteins and performance of

**Fig. 2.** Increased protein binding to the 5′-flanking region of the TH gene during hypoxia. Gel shift assay using nuclear protein extracts (NPE) from PC12 cells exposed to either normoxia (C, 21% O2) or hypoxia (H, 5% O2). The fragment of TH DNA corresponding to sequences from −284 to −190 of the 5′ region was used as probe. DNA-protein complexes were resolved on 4% polyacrylamide gels in TBE buffer. Hypoxia-induced protein binding to this region of the TH gene (lanes 2 and 3). There was no shift in probe mobility in the absence of nuclear protein extract (lane 1). Binding to the −284/−190 fragment was competed with excess nonlabeled oligonucleotides corresponding to either the TH-AP1 (lanes 4 and 5) or the TH-HIF-1 element (lanes 6 and 7). Competition of the binding complexes differed depending on the unlabeled oligonucleotide (TH-AP1; HIF-1) added (+) to the binding reaction. Interaction of proteins at both sites was found.

**Fig. 3.** Increased binding activity to the TH-AP1 element during conditions of hypoxia. Gel shift assays using a double-stranded oligonucleotide that corresponds to the TH-AP1 element were performed with extracts from cells exposed to either 21 or 5% O2. Increased binding activity (arrow) was observed with two different sets of nuclear proteins extracted from cells exposed to hypoxia (5% O2). Unlabeled TH-AP1 oligonucleotide (50X) completely abolished activity. The lower band in the complex was competed by unlabeled nonspecific oligonucleotides, which indicates that this binding is due to nonspecific binding activity.

**Fig. 4.** Binding activity to the TH-AP1 element increases and remains elevated during prolonged exposures to hypoxia.

**A**. Nuclear protein extracts from PC12 cells exposed to increasing duration of hypoxia (5% O2) bind to the TH-AP1 oligonucleotide. Increased binding activity was observed as early as after 1 h of exposure to hypoxia; maximal activity occurred between 5 and 6 h of hypoxia. An important finding was that elevated binding was observed as long as 24 h of hypoxic exposure. B, graphical analysis of binding activity (fold change) at the AP1 element that occurred during prolonged exposure to hypoxia. Time 0 (open bar) represents control conditions (21% O2).

DNA-protein complex at each duration of hypoxia exposure is presented in Fig. 4B. The open bar (time 0) represents binding activity to the AP1 probe in 21% O2. Maximal binding activity was observed with extracts taken from cells exposed to 5–6 h of hypoxia (Fig. 4B). A notable finding was that increased binding activity was still evident with extracts from cells exposed to reduced oxygen for 24 h.

We also performed a series of experiments (n = 3) to determine if binding activity is is regulated by the level of O2 tension (Fig. 5). Cells were exposed to either 10% O2 or 5% O2 for 1, 3, or 5 h prior to extraction of nuclear proteins and performance of
gel mobility shift assays. Findings from these experiments demonstrate that even modest levels of hypoxia (10% O₂) can stimulate binding activity and that binding activity is increased more rapidly in response to more severe hypoxia (5% O₂). These data are presented graphically in Fig. 5B. Results from this experiment demonstrate that hypoxia-induced binding to the AP1 element is regulated by the severity of hypoxia and that mild hypoxia is sufficient to stimulate protein binding to the AP1 element.

To identify the protein factors that interact with the TH AP1 site, UV cross-linking experiments were first performed. Cross-linking experiments revealed that a protein complex interacts with the AP1 element, although there appeared to be no apparent qualitative differences between hypoxic and normoxic extracts. The molecular masses of the four proteins involved in the complex range from 20 kDa to about 85 kDa, as shown in Fig. 6, though the smaller molecular mass proteins might be due to degradation of protein extracts. A number of regulatory transcription factors that interact with the AP1 element fall within the molecular weight range identified by UV cross-linking. DNA-protein binding reactions were performed with DNA that show the relationship of O₂ level and binding activity to the AP1 element.

**FIG. 5.** Effect of oxygen concentration on nuclear protein binding to the TH-AP1 element. A, PC12 cells were exposed to either 10% O₂ or 5% O₂ for increasing lengths of time (h = hours) prior to extraction of nuclear proteins and performance of gel shift assays. Increased binding activity to the TH-AP1 element was observed with extracts from cells exposed to both 10 and 5% O₂. This demonstrates that a modest level of hypoxia is sufficient to induce an increase in binding activity of the TH-AP1 element and that binding activity is further increased with more severe hypoxia. B, densitometric values that show the relationship of O₂ level and binding activity to the AP1 element.

**FIG. 6.** Identification of TH-AP1/protein complexes using UV cross-linking. DNA-protein binding reactions were performed with extracts from cells exposed to normoxia (21% O₂, C) or hypoxia (5% O₂, H) and a radioactively labeled AP1 oligonucleotide. Samples were irradiated with UV light, boiled in SDS electrophoresis buffer, and electrophoresed in a 10% stacking gel. An autoradiographic exposure to the radioactive revealed a complex of proteins binding to the TH-AP1 element. The proteins ranged in size from about 20, 40, and 60 kDa to 90 kDa. Molecular sizes of the proteins were estimated by comparison of migration with known protein standard markers. Broad estimates of sizes were used only to determine possible known proteins interacting in the complex. The lower molecular weight protein signal may be due to degradation of protein extract.

**FIG. 7.** Supershift assays reveal the presence of c-Fos and J unB in the AP1 binding complex. A, an antibody against c-Fos that was incubated with the gel shift binding reaction produced a supershifted band (solid arrow) that migrated above the normal shifted band (open arrow). This supershifted band indicated the presence of c-Fos in the AP1 binding complex. c-Fos was undetectable in protein complexes from cells that were grown in normoxia (21% O₂), which indicates the induction of c-Fos during conditions of hypoxia (5% O₂). B, an antibody against c-Jun did not produce a supershifted band in the gel mobility shift assay. Higher concentrations (2–6 μg) also failed to produce a supershifted band (data not shown). This result indicated that c-Jun was not present in the TH-AP1 binding complex. Antibodies against J unB, on the other hand, resulted in a supershifted band, which indicated that J unB is present in the hypoxia-induced AP1 binding complex (panel C).

This result was confirmed by shift-Western analysis, which revealed the presence of c-Jun in the protein extract, but not in the AP1-protein binding complex (data not shown). In contrast to our finding with c-Jun, we found a very prominent supershifted band when antibodies against J unB were used (Fig. 7B). The quantity of J unB in the binding complex appeared to increase during hypoxia. The binding of J unB to the AP1 element during hypoxia was confirmed by shift-Western analysis (data not shown).
Hypoxia-induced Protein Binding to the TH Gene

**Fig. 8. Mutation of the AP1 element abolishes the hypoxic induction of TH gene expression.** CAT assays using PC12 cells transfected with the wild-type –272 TH-CAT construct (lane 1) or the AP1 mutant –272 TH-CAT construct (lane 2). This experiment was performed in triplicate. Cells transfected with the respective constructs were exposed to 5% O₂, and CAT assays were performed. Lane 2 demonstrates the abolished hypoxia-induced increase in TH gene expression with the mutated AP1 site, in comparison.

shown). These findings clearly demonstrate that binding to the AP1 complex is increased during hypoxia and that this increased binding involves both c-Fos and J unB.

To determine if DNA-protein interactions at the TH-AP1 are responsible for increased transcription of the TH gene during hypoxia, CAT assays were performed using a –272 TH-CAT plasmid with a mutated AP1 element. This mutation completely destroyed the AP1 recognition site (5). Fig. 8 shows results from a CAT assay performed with the intact TH-AP1 and AP1 mutant constructs that had been transfected into PC12 cells prior to exposure to 5% O₂. Hypoxia stimulated expression of the construct that contained the intact AP1 element (lane 1), but not in the construct with the mutant AP1 element (lane 2). These results, taken together with the protein binding data, demonstrate the importance of the AP1 element in regulation of the TH gene during hypoxia.

**DISCUSSION**

In the present study, we demonstrated that enhanced transcription of the TH gene during hypoxia is regulated by specific sequences located in the 5′-flanking region of the TH gene. Results from transient transfection experiments revealed a critical region of the TH gene that extends from –284 to –150, relative to transcription start site, that is required for transcriptional activation by hypoxia. This region of the TH gene contains a number of well characterized transcriptional regulatory elements, including an AP1, AP2, OCT/POU, and the more recently described HIF-1 element (8). The HIF-1 element has been implicated in transcriptional regulation of the erythropoietin gene during hypoxia (11). Moreover, members of the Fos and Jun proto-oncogene family interact with the AP1 sequence and are known to be induced by reduced O₂ (12). Our current findings show that hypoxia-induced proteins bind to the TH gene fragment that contains the HIF-1 and AP1 elements. Semenza and Wang (8) recently demonstrated the existence of a protein induced by hypoxia (HIF-1), which binds to its own consensus element to regulate erythropoietin gene expression during hypoxia. Others have reported changes in binding activity to the AP1-like motif of the TH gene during different environmental stimuli (13–15). We therefore focused on these two regulatory elements in our effort to further characterize the molecular mechanisms that regulate transcription of the TH gene during hypoxia.

Unlabeled oligonucleotides that corresponded to the HIF-1 and AP1 elements were used as competitors in mobility shift assays. Both the HIF-1 and AP1 oligonucleotides abolished binding to the native gene fragment that extended from –284 to –190. These results suggest that protein interactions with the TH gene during hypoxia occurred at the AP1 and HIF-1 sites. Although the HIF-1 element has been implicated in the regulation of genes during hypoxia (8), this is the first evidence for protein binding activity at an AP1 element as a step in the cascade of transcriptional regulatory events during hypoxia. Mobility shift assays were also performed with oligonucleotide probes that contained either the AP1 and HIF-1 to determine if binding to these individual elements is enhanced binding during hypoxia. Binding activity was shown to be increased at the TH AP1 motif, but not at the HIF-1 element. Since oligonucleotide probes were used in these experiments, critical flanking sequences required for HIF-1 binding might not have been included in the probe. Our finding that an HIF-1 competitor sequence successfully competes for binding to the native DNA fragment certainly supports the possibility of protein binding to this element during hypoxia. It is also possible that failure of hypoxia to enhance binding activity to the HIF-1 oligonucleotide was due to the relatively mild conditions of hypoxia (5% O₂). In contrast, binding to the AP1 element was markedly enhanced even during mild (10% O₂) and moderate (5% O₂) hypoxia. Another notable result from the current study was the sustained binding to the AP1 oligonucleotide probe when protein was extracted from cells that had been exposed to relatively long (24 h) periods of hypoxia. This is potentially a very important observation for understanding the molecular mechanisms involved in sustained TH gene expression in O₂-sensitive cells during chronic hypoxia, which is the case with most pathological and physiological conditions associated with reduced O₂ tension.

Several trans-acting protein factors are known to be induced during hypoxia, including members of the Fos and Jun families of transcription factors (12) NF-κB (16), and HIF-1 (8). It has also been shown that NF-κB and the Fos/Jun families of proteins are capable of binding to the AP1 consensus element to regulate gene expression (32). Our results show that hypoxia-inducible binding to the AP1 element is specific, and that binding to this element remain elevated during prolonged exposures to hypoxia (24 h). Findings from mobility shift experiments indicate that proteins bound to the AP1 element may play an important role in control of transcription of the TH gene during low oxygen conditions. This is supported by our finding that mutation of the AP1 element abolishes transcriptional activation of the TH-CAT transgene during hypoxia.

Regulation of gene expression involving the AP1 element has been studied extensively and involves the interactions of family members of the Fos and Jun proto-oncogenes, as well as their complex interactions with other members of the leucine zipper family of DNA-binding proteins (18–31). Immunological approaches were employed to help identify members of the Fos and Jun families that bind to the TH-AP1 element during hypoxia. Western blots showed that c-Fos, c-jun, and J unB proteins are induced in PC12 cells during hypoxia (data not shown). The same antibodies were then used in supershift assays to determine which proteins were involved in the AP1 binding complex. Results from these experiments revealed that c-Fos and J unB, but not c-jun, bind to the AP1 element during hypoxia. Our results also suggest that these proteins were more abundant extracts from cells exposed to hypoxia. Shift-Western assays confirmed the result that c-Fos and J unB bind to the AP1 element during hypoxia, with the exclusion of c-jun. This finding suggests that a selective complex formation occurs between the members of the Fos/Jun family (c-Fos/J unB) of
trans-acting protein factors that bind the TH-AP1 element during hypoxia.

Fos and Jun proteins can form dimers with other members of the bZIP class of proteins through leucine zipper interactions (18–25), and with the transcription factor NF-κB (17). It has been suggested that these proteins are expressed in a stimulus-specific and cell-specific manner with the subsequent formation of specific heterodimers (26–29). It has long been accepted that c-Jun/AP1 is capable of binding the specific heptamer known as TRE/AP1, and similar variants (30, 31). It is well documented that Jun proteins can form heterodimers with the trans-acting factor c-Fos (32). However, recent research has revealed that members of the Fos and Jun families interact in different combinations, as well as with other leucine zipper proteins to form heterodimers that interact with the AP1 element on target genes. For example, Diamond and co-workers (33) reported that the function of glucocorticoid receptor as a positive or negative regulator was determined by the ratio of Fos and Jun proteins present. Thus it appears that the functions of Fos and Jun depend on a number of factors, including the presence of other interacting trans factors, cellular milieu, cell type, and the type of environmental stimuli.

In the present study, we observed specific interactions of c-Fos and JunB at the AP1 element of the TH gene during hypoxia. Since Fos and Jun interactions are dependent on cell type and the environmental stimulus, there may be active selection of JunB over c-Jun in the TH-AP1 binding complex in PC12 cells during hypoxia. This particular choice is interesting in that JunB has been identified as a repressor of transcriptional activation by c-Jun (27). Chiu et al. (27) suggested that this type of "feed-back" regulation is extremely valuable in a system where pre generated, like Fos and Jun, are co-induced by environmental stimuli. Others have shown the involvement of c-Fos and c-Jun binding at the TH-AP1 element during various stimuli, including cold stress (14) and 12-O-tetradecanoylphorbol-13-acetate treatment (15), while nerve growth factor treatment of PC12 cells has been shown to involve the binding of FosB at the AP1 site (13). We believe that the hypoxia-induced transcription of tyrosine hydroxylase in oxygen-sensitive cells, such as PC12 and carotid body type I cells, involves a unique AP1 transcriptional complex of c-Fos and JunB. It should be noted that the TH-AP1 element is not consensus and that this may contribute to binding of specific heterodimer pairs. These data taken together suggest yet another mechanism of transcriptional regulation in response to environmental stimuli by varying the interacting trans factors and their target cis elements.

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