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Regulated Expression of the Tyrosine Hydroxylase Gene by Epidermal Growth Factor

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The addition of epidermal growth factor (EGF) to cultures of the rat PCG2 pheochromocytoma cell line increased the level of RNA coding for tyrosine hydroxylase (TH). A region of DNA containing 5′-flanking sequences of the TH gene was fused to a heterologous gene and transfected into a rat anterior pituitary cell line, GH_4. The TH gene sequences from +27 to −272 contained information sufficient for the induction of TH by EGF. Two regions within this TH DNA were extensively homologous to the EGF regulatory element of the rat prolactin gene.

The molecular events which modulate the synthesis of specific neurotransmitters are a primary component in the development and maintenance of a functional neuronal phenotype. The rate of synthesis of catecholamines is regulated primarily at the initial step of the biosynthetic pathway, the enzymatic conversion of tyrosine to 3,4-dihydroxyphenylalanine, which is catalyzed by tyrosine hydroxylase (TH). The major sites of synthesis of TH and catecholamines are the adrenal medulla, the sympathetic ganglia, and certain defined regions of the brain. The activity of TH is modulated both by the activation of preexisting enzyme molecules and by the induction of synthesis of new polypeptide chains, and many of the effectors which cause the activation of TH also initiate enzyme induction.

We have been investigating the molecular mechanisms underlying the induction of TH by these multiple effectors, with the goal of determining whether there are unique or convergent pathways of signal transduction. It had previously been demonstrated that the levels of TH RNA were elevated under conditions of enzyme induction during catecholamine depletion in vivo (1, 19, 31, 32) and also when cultured cells derived from a rat pheochromocytoma were treated with glucocorticoid and cyclic AMP (18). In the study reported here, we examined the mechanism of TH induction by epidermal growth factor (EGF) in a pheochromocytoma cell line, PCG2, in which TH enzyme activity has been shown to be increased by EGF (9) and found that this effector increases TH RNA levels as well. In addition, we found that the DNA sequences in the 5′-flanking region of the TH gene contain the *cis* information required for the modulation of gene activity by EGF.

Analysis of TH RNA following EGF treatment. The pheochromocytoma cell line PCG2 was provided by Roseanne Goodman. Cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum. The medium and serum were sterilized by filtration through 0.2-μm-pore filters prior to use. GH_4 cells were provided by Kevin Sevarino, Tufts University School of Medicine, and were cultured in Hams F10 supplemented with 15% horse serum and 2.5% fetal calf serum.

RNA was extracted from cultures by lysing cells in guanidine hydrochloride and centrifuging lysates through cesium chloride as previously described (3, 18). For Northern blot (RNA blot) analysis, total cellular RNA was fractionated by agarose gel electrophoresis with formaldehyde and blotted onto nitrocellulose (17, 33). For RNA dot blot analysis, RNA was serially diluted in water and applied to nitrocellulose (33). Filters were hybridized to a 0.28-kilobase *Kpn*I-*Pst*I fragment of pTH.4, a cDNA clone for rat TH (18), which had been labeled with ^{32}P by nick translation (28). The enzyme activity was measured by the method of Waymire et al (36) as modified by Meligeni et al (22). All conditions were as previously described (18).

PCG2 cell cultures were treated with EGF (Collaborative Research, Inc.) for 2 days, and samples were analyzed for TH enzyme activity and RNA levels. Both RNA levels and enzyme activity were elevated approximately twofold following treatment (Fig. 1). When RNA was analyzed by Northern blot analysis, a major band of 1,800 to 1,900 bases hybridized to the TH probe. This band corresponds in size to that of TH mRNA observed in tissues (1, 31, 32) and cell lines (16, 18), and the autoradiographic intensity was increased in RNA samples derived from EGF-treated cultures.

Transcription from the TH gene was assayed in cultures of basal and EGF-treated PCG2 cells. Nuclei were isolated from untreated cells or cells treated with EGF for 24 h and nascent RNA chains were elongated for 10 min at 37°C in the presence of [α-^{32}P] UTP. RNA was extracted, and 6×10^5 to 9×10^5 cpm was hybridized to 8.8 kilobases of transcribed TH genomic DNA sequences carried in M13 mp10. The amount of hybridization to vector sequences not containing TH sequences was subtracted, and the efficiency of hybridization was measured for each sample by determining the extent of hybridization to vector sequences of a [^3H]-cRNA which was added to each hybridization mixture. The experimental protocols are presented in greater detail elsewhere (13, 21). In one transcriptional assay, 150 ppm (number of hybridized counts per minute per million input) of radiolabeled nuclear RNA from untreated cells hybridized specifically to TH DNA, while 250 ppm of RNA from cells treated with EGF for 24 h hybridized to TH DNA. This 1.7-fold increase in TH-specific hybridizable RNA levels suggests that EGF treatment results in the stimulation of transcription from the TH gene.

Analysis of EGF regulatory sequences in the 5′-flanking region of TH DNA. The DNA sequences which modulate

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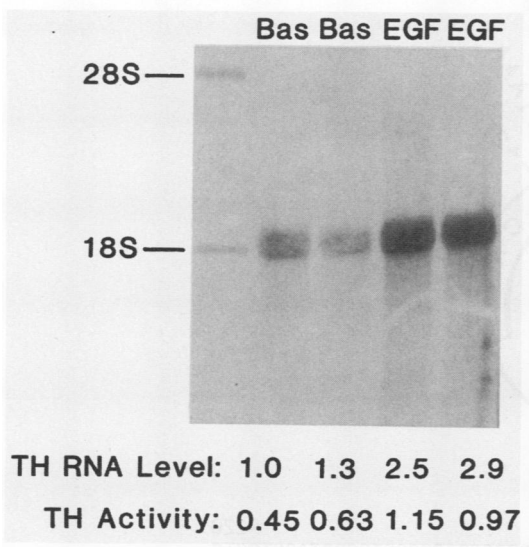

FIG. 1. Analysis of TH RNA levels and enzyme activity. PCG2 cells were plated at 5×10^5 cells per 100-mm dish, and the following day EGF was added to 10 ng/ml. Two days later cells from three dishes were pooled per sample. One-tenth of the sample was used for assay of enzyme activity, and the remainder was used for RNA extraction. Total cellular RNA (5 μg) was analyzed by Northern blot analysis by using a nick-translated 0.28-kilobase fragment of pTH.4 as a probe. The lanes represent replicate samples harvested from individual cultures, and the values below the gel represent enzyme activity and TH RNA levels, analyzed by RNA dot blot analysis, from those same samples. Enzyme activity is expressed as nanomoles of $^{14}CO_2$ hydrolyzed per minute per milligram of protein. The values for TH RNA were derived by densitometry tracing of RNA dot blots and are standardized to basal (Bas) TH RNA levels.

inducer responsiveness for several genes have been localized to the 5′-flanking regions of those genes. To initiate the localization of regulatory elements in the TH gene, we isolated a genomic clone and determined the 5′ end of the primary TH RNA transcript (13). A segment of DNA containing the transcriptional initiation site, the first 27 bases of the transcribed sequence, and 773 bases of the flanking sequence was cloned adjacent to a DNA segment coding for the bacterial enzyme chloramphenicol acetyltranferase (CAT). The recombinant DNA was carried on a plasmid derived from pSV2 CAT (11), in which the simian virus 40 enhancer and promoter elements, as well as pBR322 sequences, were removed and replaced by pUC 13 (35). This vector was designated pUC CAT. TH gene sequences were cloned into the vector by ligation of a TH *Xho*I site at −773 and an *Alu*I site at +27 with the *Sal*I and *Sma*I sites of the polylinker region of pUC CAT. A diagram of the resulting plasmid, designated 5′TH CAT (+27/−773), and the DNA sequence of a portion of the 5′-flanking region are presented in Fig. 2.

The 5′TH CAT DNA was introduced into mammalian cell cultures, and CAT activity was determined 2 days following transfection. In the 5′TH CAT constructs the synthesis of CAT was directed by the TH promoter or enhancer elements or both. Little or no CAT activity was observed when the promoterless parental CAT vector was introduced into mammalian cultures. The cultures transfected in these experiments were of the GH_4 cell line, derived from a tumor of the rat anterior pituitary. These cells do not express TH endogenously and respond to EGF treatment with a three- to six-fold increase in transcription of the prolactin gene (26). GH_4 cells were used in these experiments because both the efficiency of transfection and the magnitude of inducer responsiveness were found to be more reproducible and at higher levels than in pheochromocytoma cells.

GH_4 cell cultures were transfected by the DEAE-dextran method as described by Camper et al. (2). Cells were plated at 5×10^5 per 100-mm dish and transfected 2 days later with CsCl-purified plasmid DNA. Cultures were incubated with plasmid DNA for 1 h in 5 ml of serum-free medium containing 100 μg of DEAE-dextran per ml and buffered with 50 mM Tris (pH 7.5). Cells were then washed in serum-free medium and reincubated with fresh medium containing serum. After treatment, cells were incubated overnight, at which time EGF was added. Cultures were harvested on the following day and assayed for CAT activity. In cultures transfected with 5′TH CAT (+27/−773), CAT activity was increased 2.6-fold when cells were treated with EGF (Fig. 3A). Cultures that had been transfected with RSV CAT (10), which contains promoter and enhancer elements from Rous sarcoma virus, had no change in CAT activity following EGF treatment (Fig. 3C). A second 5′TH CAT construct was developed by excising from 5′TH CAT (+27/−773) the TH 5′-flanking sequences from −273 to −773 by digestion with restriction endonucleases *Sst*I and *Hin*dIII, followed by religation. The resulting construct, designated 5′TH CAT (+27/−272) was assayed for the presence of EGF regulatory information. GH_4 cells transfected with 5′TH CAT (+27/−272) had slightly lower basal CAT activity than did cells transfected with the parental plasmid, but CAT activity was increased 3.4-fold following treatment of cultures with EGF (Fig. 3B).

In a second experiment, EGF was added immediately after transfection with 5′TH CAT (+27/−272), and changes in CAT activity were measured over time following transfection. Twenty-four hours later, a 3.5-fold increase in CAT activity over the basal level was observed in EGF-treated cultures, and at 43 h CAT activity was increased 3.8-fold (Fig. 4). This level of induction is similar to the 3.4-fold increase observed in the experiment shown in Fig. 3B.

The data presented here demonstrate that EGF induces both TH enzyme activity and TH RNA in cultured pheochromocytoma cells. The genetic information required for regulation of the TH gene by EGF is carried within 300 bases of the 5′-flanking region, as evidenced by the ability of those TH sequences to confer responsiveness to EGF onto a heterologous gene.

Other known RNAs which respond to EGF treatment by specific elevation of those transcripts include prolactin, (26, 37), VL30 (8), transin (20), c-*fos* (12, 14, 23, 25), and the EGF receptor (5). The intracellular events which transmit the EGF-mediated signal from the cell surface receptor to the nucleus have not been fully elucidated for any of these transcripts. The binding of EGF to its cell surface receptor can result in (i) activation of the endogenous receptor tyrosine kinase (34), (ii) stimulation of phosphatidylinositol turnover (30), which may activate protein kinase C, and (iii) alterations in calcium influx and intracellular stores (24, 30). The RNA levels of prolactin (27), VL30 (29), and c-*fos* (12, 14, 23) can be elevated by treatment of cultures with phorbol esters, tumor promoters which activate protein kinase C, suggesting that protein kinase C may be involved in the induction of these RNAs. Transin RNA can also be elevated by phorbol ester, but only in the presence of serum, while serum is not a requirement for the EGF-mediated increase in transin RNA (20). Changes in intracellular calcium appear to

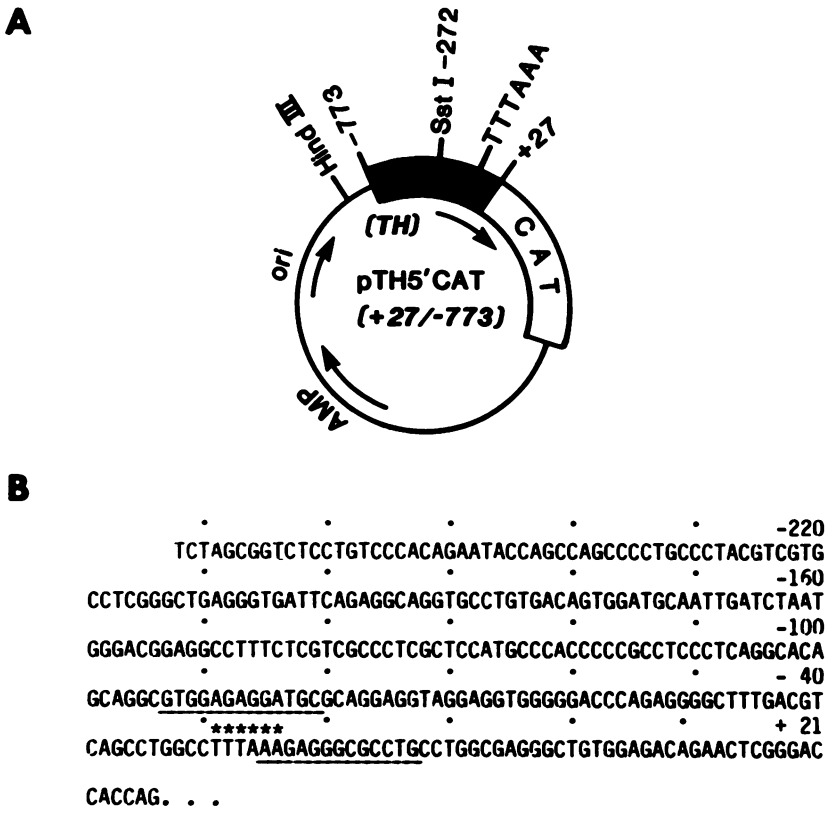

FIG. 2. (A) Vector containing the 5'-flanking region of the TH gene ligated to the promoterless CAT gene construct. The solid box represents TH sequences; the open box represents the CAT gene construct, including the small-t-antigenitron and poly(A) addition site, from pSV2 CAT (11); and the solid line represents pUC 13. pTH5'CAT = 5'TH CAT. ori, Origin of replication. AMP, β-lactamase gene. (B) Sequence of TH DNA in 5'TH CAT (+27/−272). The TATA box is represented by asterisks, and the regions of homology with the EGF regulatory element of the prolactin gene are underlined.

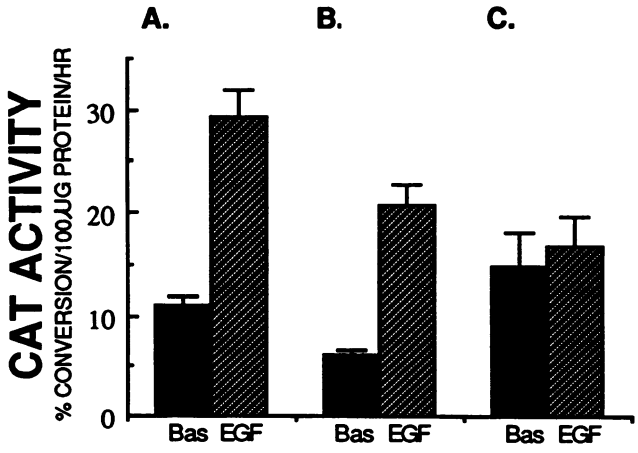

FIG. 3. Transfection of GH_4 cultures with 5'TH CAT constructs. Cells were transfected with plasmid DNA as described in the text. The following day EGF was added to 10 nM, and 24 h later cultures were harvested. Cell pellets were extracted in 100 μl of 0.25 M Tris (pH 7.6) by three rounds of a freeze-thaw cycle. Cell debris was removed by centrifugation, and 25 μg of protein was assayed for CAT activity for 1 h by using 0.1 μCi of [^{14}C]chloramphenicol and the method of Gorman et al. (11). Activity is expressed as the percentage of chloramphenicol converted to acetylated forms per 100 μg of protein per hour. Cultures were transfected with 2 μg of 5'TH CAT (+27/−773) (A), 2 μg of 5'TH CAT (+27/−272) (B), or 0.5 μg of RSV CAT (C). Values presented represent the mean of three individual cultures ± the standard error of the mean. Cultures tranfected with 2 μg of promoterless pUC CAT had a CAT activity of 0.86%. Bas, Basal activity.

alter basal levels of prolactin RNA (37), and the inhibition of calcium influx blocks the increase in prolactin transcription stimulated by either EGF or phorbol esters (27). In preliminary experiments phorbol esters had no effect on the level of TH RNA, although it is not known whether the experimental conditions were optimal for induction. At present, it is unclear whether protein kinase C or calcium or both play a role in mediating the transcriptional response to EGF.

In addition to evidence that there are differences in the second messenger(s) which mediates the EGF responses, a distinction can be made as to whether the response to EGF is a primary or secondary event with respect to protein synthesis. It has been demonstrated that de novo protein synthesis is required for transcriptional activation of the transin gene by EGF (20), but EGF-mediated increases in prolactin (27), VL30 (29), and c-*fos* (25) transcription can occur in the absence of ongoing protein synthesis. We were unable to determine whether the effect of EGF on the TH gene is a primary or secondary event, because under the current experimental conditions, the inhibition of protein synthesis decreased the basal rate of TH gene transcription. In this case, it is difficult to distinguish between events involved in the control of basal versus induced TH transcrip-

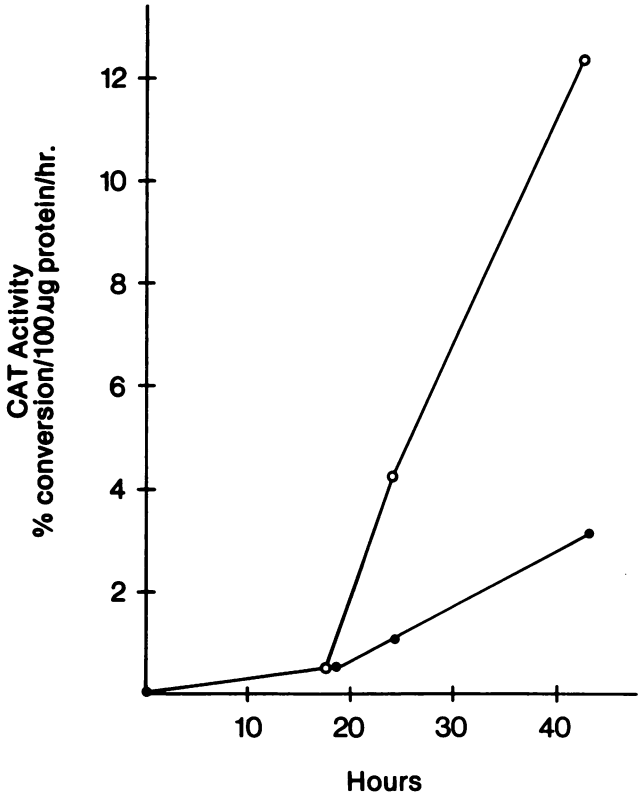

FIG. 4. Time course of CAT induction following transfection. GH_4 cultures were transfected with 2 μg of 5'TH CAT (+27/−272) by using the methods described in the text and in the legend to Fig. 3. EGF was added to 10 nM immediately after transfection, and cultures were harvested and assayed for CAT activity at various times thereafter. Symbols: ●, untreated cultures; ○, cultures treated with EGF.

tion. On the basis of current evidence it appears that there may be multiple mechanisms by which EGF regulates specific gene transcription.

In vivo, the physiological role for EGF has not been well defined. EGF-immunoreactive material has been found in the central nervous system (7, 15), and it has been suggested that EGF may function as a neurotransmitter or a neurotrophic factor. However, definitive evidence for such functions has not been demonstrated, nor is it known whether catecholaminergic cells are target tissues for EGF. The possibilities remain that EGF is not a true physiological stimulator of TH and that the induction of TH by EGF observed in pheochromocytoma cultures is due to the activation by EGF of a second messenger system which is the same as that of another physiological inducer of TH, such as stress.

To better evaluate the mechanism of TH induction by multiple effectors, we have begun to identify the sequences of the TH gene which are important for inducer responsiveness and to compare these sequences with those of other genes under similar control. As in the TH gene, the DNA sequence which denotes responsiveness to EGF is also carried in 300 bases of a 5'-flanking sequence of the rat prolactin gene (2). The DNA sequence GTGGAGAG GATGC, found at −93 to −81 relative to the start site of transcription in the TH gene, is matched in 12 of 14 bases with the DNA sequence at −78 to −65 in the prolactin gene (4). In addition, the TH sequences from −25 to −13, AAGAGGGCGCCTG, is homologous in 11 of 13 bases to the prolactin sequence from −74 to −62. These sequences are included in a 50-base-pair region (−79 to −30) of prolactin DNA which confers responsiveness to both EGF and phorbol esters onto a heterologous promoter when transfected into GH_4 cells (6). At present we do not know whether these homologous sequences are also important for EGF induction of the TH gene, nor is information yet available on the location of EGF regulatory elements in other mammalian genes. With progressive deletions of these 5'-flanking regions of responsive genes, it will be possible to identify small spans of DNA which are necessary components in induction for the individual effectors. These experiments will then begin to answer whether different inducers activate TH gene transcription by unique or common pathways and whether there are common recognition signals in genes regulated by the same inducers.

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