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Transcriptional regulation of the tyrosine hydroxylase gene by glucocorticoid and cyclic AMP

(regulatory elements/5′ flanking sequences/neural genes)

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ABSTRACT **Glucocorticoid and cyclic AMP increase tyrosine hydroxylase (TH) activity and mRNA levels in pheochromocytoma cultures. The transcriptional activity of the TH gene, as measured by nuclear run-on assay, is also increased when cultures are treated with the synthetic glucocorticoid dexamethasone or agents that increase intracellular cyclic AMP, such as forskolin and 8-BrcAMP. Both inducers effect transcriptional changes within 10 min after treatment and are maximal after 30 min for forskolin and after 60 min for dexamethasone. The 5′ flanking sequences of the TH gene were fused to the bacterial gene chloramphenicol acetyltransferase (CAT), and the hybrid gene was transfected into pheochromocytoma cultures and GH_4 pituitary cells. In both cell lines, a region of the TH gene containing bases −272 to +27 conferred induction of CAT by cyclic AMP, but not by glucocorticoid. The same results were found when a region of the TH gene containing −773 to +27 was used. Thus, the sequences required for induction of TH by cyclic AMP are contained within 272 bases of 5′ flanking sequence, but sequences sufficient for glucocorticoid regulation are not contained within 773 bases.**

The rate of biosynthesis of specific neurotransmitters can be modulated by a variety of environmental, neuronal, and hormonal stimuli. In catecholamine biosynthesis, the rate of flow through the pathway is largely dependent on the activity of the initial enzyme, tyrosine hydroxylase (TH), which is expressed in the adrenal medulla, sympathetic ganglia, and certain defined nuclei of the brain. Activity of tyrosine hydroxylase can be modulated both by changes in the synthetic rate of new tyrosine hydroxylase polypeptide and by posttranslational modification of preexisting enzyme molecules.

The synthesis of TH is influenced by a variety of factors; *in vivo*, TH is induced in response to environmental stimuli, such as stress (1), and this induction can be mimicked pharmacologically by treatment of animals with agents that deplete cellular catecholamine stores, such as reserpine (2). In cultures of adrenal chromaffin or pheochromocytoma cells, tyrosine hydroxylase activity is induced by a number of effectors, including glucocorticoid (3, 4), cyclic AMP (5, 6), epidermal growth factor (7), and nerve growth factor (6, 8, 9).

Whether these effectors act through similar mechanisms and whether changes are modulated transcriptionally or posttranscriptionally is not known. Using a cloned cDNA probe for TH, we have previously shown increases in the mRNA for tyrosine hydroxylase following treatment of clonal rat pheochromocytoma cells from an adrenal medullary tumor with analogs of cyclic AMP and glucocorticoid (10). The TH RNA is also increased in adrenal glands and superior cervical ganglia *in vivo* when animals are subjected to cold stress or reserpine treatment (11–14). In this communication, we have extended those studies by demonstrating that both glucocorticoid and cyclic AMP stimulate the transcriptional activity of the TH gene. In addition, we report that the 5′ flanking sequences of the TH gene contain the cis information necessary for induction of TH by cyclic AMP. Sequences sufficient for glucocorticoid stimulation of transcription do not appear to be present in this 5′ flanking region.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions. The PC7e pheochromocytoma cell line is a subclone of the PC8b cell line (10), which was derived from PC12 (15). This cell line was selected for use because of its ability to induce TH RNA in response to both dexamethasone and increased cyclic AMP. PC7e cells are maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum/5% horse serum (Sterile Systems, Logan, UT). GH_4 cells were provided by Kevin Sevarino (Tufts Medical School) and are grown in F-10 medium supplemented with 15% horse serum/2.5% fetal calf serum. Cultures were maintained in a humidified atmosphere of 5% CO_2/95% air.

Analysis of Nuclear Transcription. PC7e cells at 1.5–2.0 × 10^4 per cm^2 were treated with 1 μM dexamethasone (Sigma) or 10 μM forskolin (Calbiochem) for the indicated time. Nuclei were prepared, and ^{32}P-labeled radioactive run-on RNA was synthesized and purified as described (16). Incorporation ranged from 1.5 to 3.0 dpm per cell. Between 2.2 × 10^6 and 12.8 × 10^6 dpm of nuclear run-on RNA was mixed with 3.6 × 10^4 dpm of [^{3}H]TH cRNA (5.5 × 10^7 dpm/μg) transcribed from an SP6 plasmid bearing 135 nucleotides of TH sequence and the mixture was annealed to nitrocellulose filters containing 2 μg of either linearized pBR322 DNA or pBR322 DNA bearing 6.2 kilobases (kb) of TH genomic DNA (transcribed TH sequences from approximately +2 kb to +8.2 kb downstream from the initiation site). Preliminary experiments demonstrated that run-on RNA hybridization was specific for the coding strand of this DNA segment. Filters were prepared, annealed, and washed as described by McKnight and Palmiter (17). Data are corrected for the efficiency of hybridization, which ranged from 15% to 30% using the [^{3}H]cRNA as an internal control. Each hybridization vial contained a pBR322 and a pBR322 TH filter. The radioactivity bound to the pBR322 filter was subtracted as background.

Isolation of TH Genomic Clones and Construction of Chloramphenicol Acetyltransferase (CAT) Fusion Genes. Genomic clones of TH were isolated from a library of rat liver genomic

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Abbreviations: CAT, chloramphenicol acetyltransferase; TH, tyrosine hydroxylase; RSV, Rous sarcoma virus.

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DNA constructed in λ Charon 4A. This library was a gift of Tom Sargent, R. B. Wallace, and James Bonner (California Institute of Technology). Genomic clones containing TH sequence were isolated by plaque hybridization (18) to nick-translated pTH.4, a cDNA for rat tyrosine hydroxylase (10). The transcriptional initiation site of TH RNA was mapped by S1 nuclease protection and reverse transcription methods (16). Fragments were subcloned into M13 and the DNA sequence was determined by dideoxy-chain termination (19).

A fragment of 0.8 kb from an *Hin*dIII complete *Alu* I partial digest of the TH gene, which contained the promoter region, was gel-purified and cloned into the *Hin*dIII and *Sma* I sites of pUC CAT (Fig. 2 *A* and *B*). The pUC CAT vector contains the CAT gene segment from pSV2 CAT (20) fused to pUC13 (21), such that the polylinker region of pUC13 directly precedes transcriptional initiation of the CAT gene. pUC CAT was given to us by Richard Morimoto (Northwestern University). The 0.8-kb TH fragment contains 773 bases of 5′ flanking sequence, the transcription initiation site, and 27 bases of transcribed nontranslated sequences. This construct is designated 5′TH CAT (+27/−773). A second construct, 5′TH CAT (+27/−272), was generated by digestion of the former plasmid with *Sst* I and *Hin*dIII, followed by a fill-in reaction with the Klenow fragment of DNA polymerase I (22) and religation. The junction of the TH–CAT hybrid gene was confirmed by sequence analysis (23).

All enzymes used in DNA cloning were purchased from Bethesda Research Laboratories.

DNA Transfections. PC7e cells were plated at 5×10^5 cells per 100-mm dish 2 days prior to transfection. $CaPO_4$/DNA precipitates (24) containing 5–20 μg of plasmid DNA that had been purified through CsCl were added to cultures in 0.3–0.5 ml. In experiments in which multiple cultures were transfected with the same plasmid, several individually prepared $CaPO_4$/DNA precipitates were pooled and then added to cultures. Cultures were incubated with precipitate for 4 hr at 37°C, and then medium was removed and cultures were treated with 5 ml of 15% (vol/vol) glycerol. Cultures were incubated for 4 min at room temperature, washed twice with phosphate-buffered saline, and reincubated at 37°C overnight in serum-containing medium. Inducers were added the following day.

GH_4 cultures were inoculated at 10^6 cells per 100-mm plate 2 days prior to the beginning of the experiment and were transfected by the DEAE-dextran method as described by Camper *et al.* (25).

Assay of CAT. Transfected cells were pelleted, suspended in 100 μl of 0.25 M Tris·HCl (pH 7.6), and extracted by three freeze–thaw cycles. Cytosolic protein was assayed as described by Gorman *et al.* (20). Results are expressed as the percentage of radiolabel present on the plate that was acetylated. Background activity was determined from extracts that had been transfected with the promoterless pUC CAT.

RESULTS

Analysis of Nuclear Transcription from the TH Gene. In previous studies, we had demonstrated that TH mRNA levels were increased by treatment of pheochromocytoma cultures with the synthetic glucocorticoid, dexamethasone, or with agents that increase intracellular cyclic AMP (10). In a number of different experiments using the clonal PC7e cell line, dexamethasone increased TH RNA 3.5- to 6-fold, while cyclic AMP-mediated increases in TH RNA were 2- to 3.5-fold over basal levels. These changes in message level could be due to stimulation of transcription of the TH gene or to enhanced stability of preexisting TH mRNA molecules. The relative transcriptional activity of the TH gene was evaluated by performing a nuclear "run-on" assay in nuclei of pheochromocytoma cultures treated with either dexamethasone or forskolin, an activator of adenylate cyclase. In the nuclear run-on assays, the transcriptional activity from the TH gene relative to total nuclear transcriptional activity is measured by elongating nascent RNA chains in isolated nuclei in the presence of ribonucleoside [^{32}P] triphosphate for 30 min. Treatment of PC7e cultures with either forskolin or dexamethasone produced a marked stimulation in the transcriptional activity from the TH gene (Fig. 1). In cultures treated with forskolin, a 4-fold change in TH transcription could be detected as early as 10 min after addition of inducer. The TH transcriptional activity continued to rise rapidly to an 8-fold increase over basal at 30 min, after which the activity declined to a 3-fold stimulation at 24 hr. Cultures treated with dexamethasone exhibited a slightly slower increase in TH transcription, which reached a maximum of 8-fold at 60 min, and remained near this level throughout the course of the experiment. The -fold increases in transcriptional stimulation mediated by both forskolin and dexamethasone were greater than the typical increases observed in TH RNA after 24 hr of inducer treatment.

Analysis of 5′ Flanking Sequences of TH Gene. A region of the TH gene spanning 773 bases upstream from the transcriptional initiation site and including the first 27 bases of transcribed sequences (+27/−773) was inserted in front of a "reporter" gene, bacterial CAT. The TH gene sequences were inserted directly upstream from the CAT gene in pUC CAT, a vector lacking viral promoter and enhancer elements, such that transcription of CAT is directed by TH promoter and/or enhancer elements. The 27 bases of transcribed TH sequences in this construct do not contain the AUG translational initiation site (26), and therefore translation of the CAT gene presumably begins at the bacterial translational initiation site. The 5′TH CAT vector construct and the sequence of the 5′ flanking region are illustrated in Fig. 2.

The 5′TH CAT (+27/−773) was introduced into PC7e cell cultures by calcium phosphate transfection. Preliminary experiments determined that CAT activity increased linearly

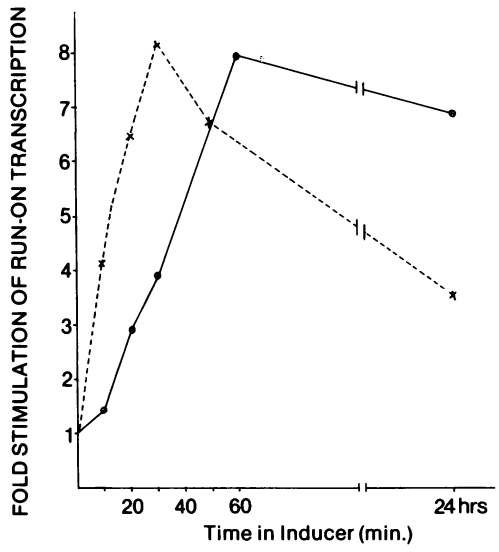

FIG. 1. Run-on RNA synthesis from PC7e cells treated with dexamethasone or forskolin. Cells were treated for the indicated time with the inducers and RNA was prepared and annealed to nitrocellulose filters containing TH DNA. ○, PC7e cells treated with 1 μM dexamethasone. Untreated cells were annealed to TH filters at the level of 44 ppm of the input ^{32}P run-on RNA. Inputs for various time points ranged from 2.2 to 9.2×10^6 dpm of [^{32}P]RNA. ×, Cells treated with 10 μM forskolin. Untreated cells were annealed at the level of 148 ppm. Input ^{32}P run-on RNA for various time points ranged from 8.2 to 12.8×10^6 dpm.

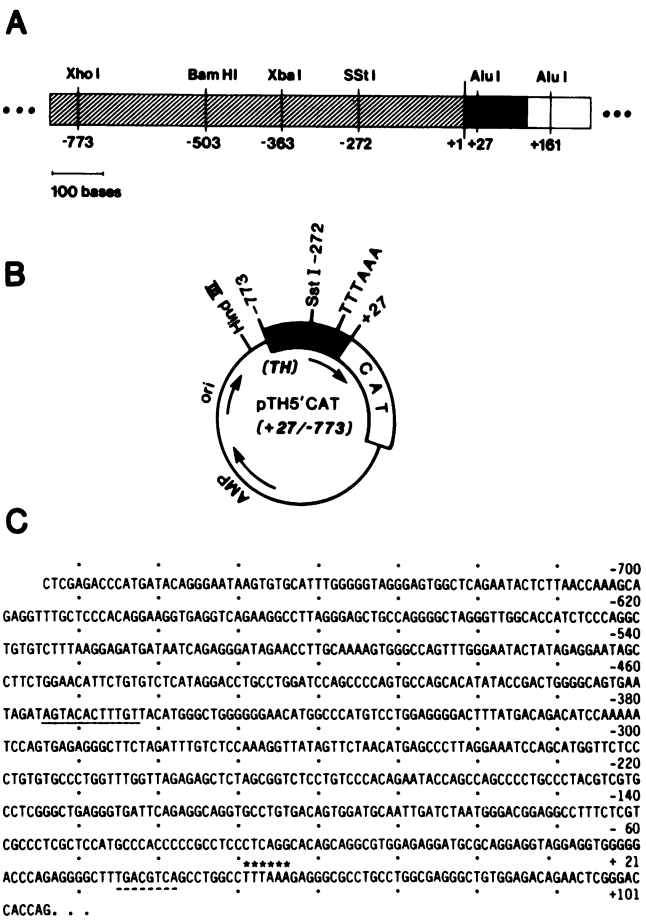

FIG. 2. (*A*) A partial restriction map of the 5′ region of the TH gene. Hatched bar, 5′ flanking region; shaded area, first exon of transcribed sequence; open area, beginning of the first intron. (*B*) The vector construct in which the 5′ flanking sequences of the TH gene are fused to the bacterial CAT gene is diagrammed. Shaded regions, TH sequences; open box, CAT gene from pSV2 CAT (20); solid lines, sequences from pUC13 (21). (*C*) The sequence of the 5′ flanking region of the TH gene. The TATA box (−24 to −29) is denoted by asterisks; the region of homology with the cyclic AMP regulatory element (−37 to −44) from the human somatostatin (27), rat PEPCK (28), and proenkephalin (29) genes is denoted by dashed underlining; and the region homologous to the glucocorticoid regulatory element of human metallothionein IIa (30) is denoted by underlining at positions −443 to −454.

with up to 20 μg of transfected 5′TH CAT DNA. One day after transfection, cultures were treated with either dexamethasone, forskolin, or 8-BrcAMP, and the following day cells were harvested and assayed for CAT activity. Transfected cultures that had been treated with forskolin or 8-BrcAMP demonstrated a 1.6- and 2.1-fold increase over basal in CAT activity, while CAT activity in cultures treated with dexamethasone was slightly lower than basal levels (Fig. 3*A*). In a series of experiments, the activity of CAT in transfected cultures treated with dexamethasone ranged from 61% to 85% of basal CAT activity. The basal level of CAT activity in cultures transfected with pSV2 CAT (20), in which simian virus 40 promoter and enhancer elements direct CAT synthesis, was substantially higher than the basal activity observed with 5′TH CAT transfections, but the CAT activity from pSV2 CAT was not altered after treatment of cultures with inducers (Fig. 3*C*). In a second experiment, RSV CAT (31), which contains enhancer elements from Rous sarcoma virus, was used as a control for the specificity of induction. In this experiment, the basal CAT activities from 5′TH CAT and RSV CAT were nearly equal (10.1 vs. 10.2), and there was a 2.1-fold induction of CAT from 5′TH CAT, but no induction from RSV CAT in cyclic AMP-treated cells (data not shown).

A second 5′TH CAT construct was developed by excision of 5′ flanking sequences from −272 to −773. When this construct, designated 5′TH CAT (+27/−272), was transfected into PC7e cultures, the basal CAT activity was lower than that observed with the flanking sequences extending to −773, although there was substantial variation in basal levels of CAT between different DNA precipitates. When cultures transfected with 5′TH CAT (+27/−272) were treated with forskolin or 8-BrcAMP, CAT activity was induced 3.3-fold (Fig. 3*B*). Although this induction was greater than that observed with the longer construct, in other experiments the -fold stimulation of cyclic AMP-induced CAT activity with 5′TH CAT (+27/−272) was the same or lower than that of 5′TH CAT (+27/−773) (data not shown). In this experiment, a 60% increase in CAT was seen when transfected cultures were treated with dexamethasone.

To determine whether the process of transfection itself altered the ability of PC7e cells to respond to inducer, the levels of endogenous TH RNA were compared between transfected and untreated cultures. Although it is likely that only a fraction of the cultured cells incorporate and express the foreign 5′TH CAT construct, while presumably all cells express endogenous TH, it was of concern that the procedure did not affect the overall capability of cultures to induce. Basal and induced TH RNA levels did not differ between transfected and nontransfected cultures (data not shown). Therefore, the inability of the 5′TH flanking sequences to confer responsiveness to dexamethasone onto the CAT gene is not due to a generalized impairment of the glucocorticoid response under conditions of transfection.

Transfection of 5′TH CAT into a Heterologous Cell Culture. To determine whether the 5′TH CAT induction by forskolin is limited to TH-expressing cells or whether the responsiveness extends to other cell types, the 5′TH CAT constructs were transfected into cell cultures that do not express TH, the GH_4 cell line, derived from a tumor of the anterior pituitary. GH_4 cells respond to forskolin by stimulation of transcription of the endogenously expressed prolactin and growth hormone genes (32, 33).

5′TH CAT (+27/−773) was introduced into GH_4 cultures by DEAE-dextran transfection. Treatment of cultures with forskolin or 8-BrcAMP resulted in a 2- and 2.2-fold increase of CAT activity, respectively, while treatment of cultures with dexamethasone produced no induction (Fig. 4*A*). pSV2 CAT is not expressed in GH_4 cells (25), but RSV CAT is expressed at high levels in these cells (25). The expression of CAT activity from RSV CAT was not altered by treatment with inducers (Fig. 4*B*).

The change in CAT activity was monitored over a 2-day period following transfection to determine the magnitude of induction over time after inducer treatment. In this experiment, forskolin was added to cultures directly after introduction of foreign DNA, while in previous experiments cells were allowed to recover from the transfection procedure for 24 hr prior to addition of inducer. Initial detection of CAT activity was observed 18 hr after transfection and rose gradually thereafter (Fig. 4*C*). In cultures treated with forskolin, CAT activity was increased over basal levels by 24 hr posttransfection, and the magnitude of increase at 43 hr was 2.4-fold.

DISCUSSION

To understand the interaction of multiple effectors on a single gene product, TH, we have investigated the mechanism by which two known inducers of TH enzyme activity, glucocorticoid and cyclic AMP, regulate synthesis of this protein in pheochromocytoma cultures. The data presented here

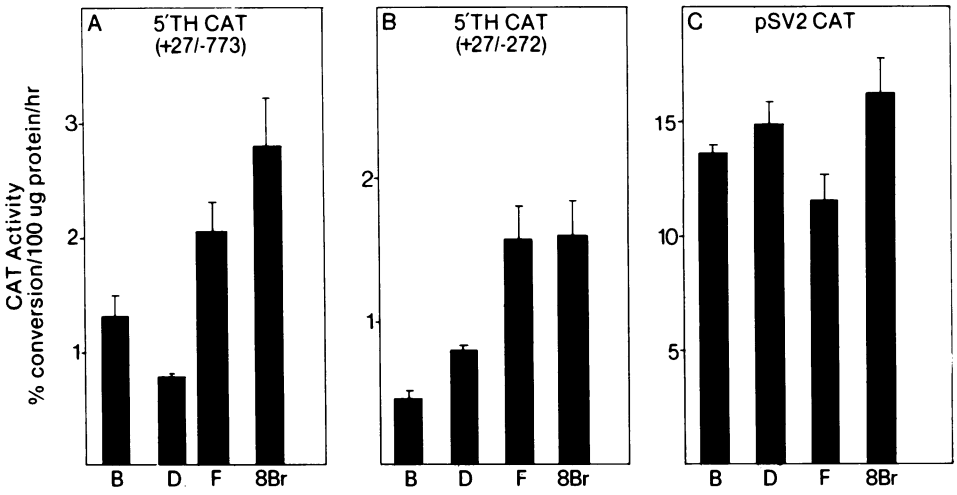

FIG. 3. Transfection of PC7e cells with recombinant CAT constructs. Cultures were transfected with 5 μg of each plasmid using the calcium phosphate method (24). The following day, transfected cultures were treated with 10 μM forskolin, 1 μM dexamethasone, or 1 mM 8-BrcAMP for 18 hr. Cells were harvested and extracted, and 100 μg of protein was assayed for CAT activity for 1 hr. Results are presented as the mean ± SEM for three individual cultures. Transfection was with 5'TH CAT (+27/−773), 5'TH CAT (+27/−272), or pSV2 CAT. Treatment was with no additives (bars B), dexamethasone (bars D), forskolin (bars F), or 8-BrcAMP (bars 8Br). By the two-tailed *t* test, the inducer-treated samples in *A* and *B* all differed significantly from basal level ($P < 0.05$). None of the treatments in *C* differed significantly from basal level.

provide evidence that both effectors rapidly stimulate transcription from the TH gene as assayed by run-on transcription from isolated nuclei. After 24 hr of treatment, the magnitude of transcriptional stimulation with inducers was reproducibly greater than the increase of TH RNA levels, suggesting that additional posttranscriptional controls may also contribute to the levels of stable TH RNA.

The initial change in TH transcription is observed within 10 min after addition of inducer to cultured cells. In contrast, changes in TH RNA levels are first observable 3–4 hr after inducer treatment (10, 34), while increases in TH enzyme activity are usually not observed until 8–16 hr after inducer treatment (10, 35). The delayed appearance of stable RNA and protein probably reflects the fact that the time required to reach new steady-state values is dependent on the degradation rate (not synthetic rate) of TH RNA and protein. The apparent half-life of TH protein is ≈30 hr (35), and, if the half-life of TH RNA were typical of other cellular RNAs (between 5 and 20 hr), the lag between transcriptional activation and the appearance of stable macromolecules would be expected. In this regard, several other mammalian genes that are transcriptionally regulated by glucocorticoids (36–39) and cyclic AMP (32, 40–43) have similar delays between rapid transcriptional events and the resulting changes in stable RNA levels.

Cyclic AMP has been recently demonstrated to increase specific mRNA levels for a number of neuropeptide precursor genes (44–46). It is of interest to determine whether there is a generalized program by which cyclic AMP can increase production of specific neurotransmitter, and whether this mechanism extends to other genes under transcriptional control by cyclic AMP. A fragment of the 5′ flanking region of the TH gene, including bases −272 to +27, is able to confer responsiveness to cyclic AMP onto a heterologous gene. It is not clear at this juncture whether this region comprises the sole cyclic AMP responsive element in the TH gene, nor is it known whether this fragment is composed of multiple elements. It has recently been noted that a number of genes regulated by cyclic AMP contain a homologous sequence, the core of which is TGACGTCA (27–29), and which is present at −44 to −37 in the TH gene (Fig. 2*C*). This core sequence alone will not confer responsiveness to cyclic AMP onto a heterologous gene, but in the somatostatin and proenkephalin genes a region of 30 bases, including and surrounding this core, can confer CAT gene induction by cyclic AMP (27, 29).

In contrast to the ability of the 5′ flanking region of the TH gene to confer induction by cyclic AMP onto the CAT gene, treatment with glucocorticoid does not generate increases in CAT activity that could account for the 3.5- to 6-fold increase in TH RNA levels. This was somewhat surprising, as sequences −454 to −443 of the TH gene bear homology in 10 of 11 consecutive bases to sequences in the human metallothionein gene that comprise a portion of the glucocorticoid regulatory element of that gene (30). These results suggest that comparison of consensus sequence homologies between hormone-responsive genes does not necessarily identify an active regulatory element. The glucocorticoid regulatory element(s) of the TH gene may be located elsewhere in the

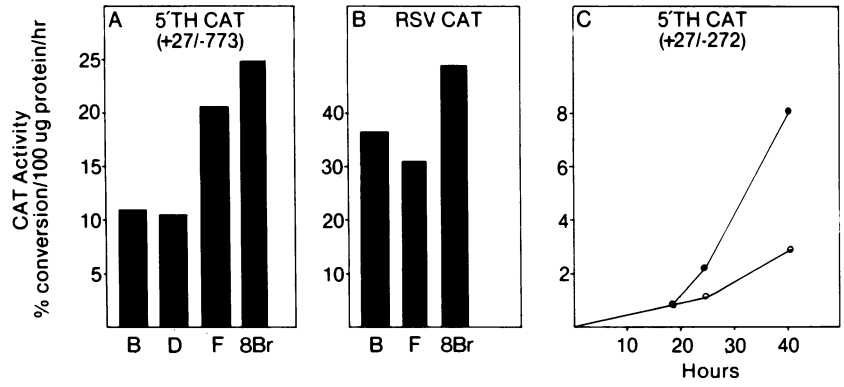

FIG. 4. Transfection of GH_4 cells with recombinant CAT constructs. (*A*) GH_4 cells were transfected with 2 μg of 5'TH CAT (+25/−773) by the DEAE-dextran method (25). The following day, inducers were added for 25 hr. Cultures were harvested and 25 μg of protein extract was assayed for 1 hr. (*B*) GH_4 cells were transfected with 0.5 μg of RSV CAT and cultures were treated as in *A*. Ten micrograms of the protein extracted was assayed. (*C*) Cultures were transfected with 2 μg of 5'TH (+27/−272) and inducers were added immediately after transfection. ○, Basal activity; ●, CAT activity in forskolin-treated cells. All values are the average of duplicate samples, which deviate from the mean by <10% (*A*), <25% (*B*), and <20% (*C*).

gene, perhaps in an intron, as has been demonstrated for the growth hormone gene (47, 48). Alternatively, there may be multiple elements on the TH gene that act cooperatively to invoke transcriptional stimulation by glucocorticoid. The decrease in CAT activity observed with the −773 construct and the small increase with the −272 construct suggest that there may be both inhibitory and stimulatory sequences involved in the TH glucocorticoid response. Primer extension and riboprobe transcription mapping analyses demonstrated that the 5′ end for the TH transcript from control and dexamethasone-treated cultures is the same (16), suggesting that both transcripts are initiated from the same promoter. Thus, it is unlikely that the lack of glucocorticoid responsiveness from the 5′ flanking sequences is due to a missing glucocorticoid-specific promoter.

The observation that the TH promoter was active in GH_4 cells was somewhat unexpected, since for many tissue-specific genes, elements encoded on the DNA restrict the expression of those genes to the appropriate cell type. The possibility that these observations provide information concerning the mechanism of expression of the TH gene, such as the presence of upstream inhibitory elements, will be of interest for future investigation.

We wish to thank Dr. Richard Morimoto for supplying us with the pUC CAT vector and Drs. Kevin Sevarino and Toshihiko Tsukada for providing us with other vectors (RSV CAT, pSV2 CAT) and cell lines, as well as helpful advice on experimental protocol. We also wish to thank Ms. Maggie Delano for technical assistance and Ms. Barbara D'Angelo and Mavis-Rose Patterson for excellent assistance in preparation of this manuscript. The studies here were supported by National Institutes of Health Grant NS22675 to D.M.C.

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