Regulation of Tyrosine Hydroxylase mRNA by Glucocorticoid and Cyclic AMP in a Rat Pheochromocytoma Cell Line

ISOLATION OF A cDNA CLONE FOR TYROSINE HYDROXYLASE mRNA*

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Treatment of a subclone of the PC12 pheochromocytoma cell line, PCSb, with either dexamethasone or 8-bromo cyclic AMP resulted in increased translational activity of tyrosine hydroxylase mRNA (mRNATH). Poly(A⁺)-containing RNA from cells treated with both inducers was used to construct a cDNA library. Double-stranded cDNA was inserted into the PstI site of pBR322 using GC tailing, and plasmids were used to transform Escherichia coli HB101. Colonies containing plasmids with inserted sequences were initially screened by DNA dot hybridization, and those positive colonies were then screened by hybrid selected translation. One plasmid, pTH.4, was identified as containing a 400-base pair sequence complementary to mRNA. Nick-translated PTH.4 DNA was used to identify mRNA as containing approximately 1800 nucleotides by Northern blot analysis.

PCSb cells treated with either dexamethasone or 8-bromo cyclic AMP yielded greater mRNA hybridization on Northern blot analysis and accumulated higher molecular weight tyrosine hydroxylase RNA species. Following treatment of cells with inducers, the temporal increase in tyrosine hydroxylase enzyme activity was associated in all cases with an increase in the translational activity and relative amount of mRNA, and the fold increase in the latter two parameters was equal to or greater than the increase in enzyme activity.

The activity of tyrosine hydroxylase, the initial and rate-limiting enzyme of catecholamine biosynthesis, has been shown to be modulated by a number of effectors, including glucocorticoids (1, 2) and derivatives of cyclic AMP (3-6). Although the precise physiological role of glucocorticoids and cyclic AMP in the modulation of tyrosine hydroxylase enzyme activity in vivo has not been defined, these effectors may be important in the postnatal development of enzyme levels (7, 8), the maintenance of steady state enzyme levels in the adult animal (9), and in the transsynaptic induction of tyrosine hydroxylase following cold stress or reserpine treatment (10, 11).

Much evidence has accumulated demonstrating that glucocorticoids modulate the induction of specific proteins by increasing steady state concentrations of the mRNAs coding for those proteins (12-18), and previous studies have suggested that this may also be the mechanism by which glucocorticoids induce tyrosine hydroxylase (19). In contrast, there does not appear to be a single mechanism by which cyclic AMP mediates the induction of specific protein synthesis. In certain cases it has been demonstrated that the increase of a specific protein following treatment of tissues with derivatives of cyclic AMP can be correlated with increased RNA transcription from the gene coding for that protein (20-22). In other cases, temporal and quantitative relationships between changes in mRNA levels, mRNA translational activity, and the rate of specific protein synthesis were not observed (23-25). These studies suggest that there may be multiple sites by which cAMP may act to modify the rate of specific protein synthesis.

The isolation of a cDNA clone to rat tyrosine hydroxylase has been previously reported by Lamouroux et al. (26). In this study, we report our independent isolation of a cDNA clone containing sequences complementary to rat mRNA. We have used this cDNA clone to further characterize the mechanism of tyrosine hydroxylase induction by glucocorticoid and cyclic AMP using a cell line derived from a rat pheochromocytoma.

EXPERIMENTAL PROCEDURES AND RESULTS

Northern Blot Analysis of mRNA—In previous experiments, when poly(A⁺)-containing RNA from PCSb cells was fractionated on sucrose gradients containing methyl mercury, the peak of mRNA activity, as measured by in vitro trans-

* The abbreviations used are: mRNA, the RNA coding for tyrosine hydroxylase; 8b-cAMP, 8-bromo cyclic AMP; Bu-cAMP, dibutyryl cyclic AMP; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; NP40, Nonidet P-40.
** Portions of this paper (including "Experimental Procedures," part of "Results," Table I, and Figs. 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-1754, cite the authors, and include a check or money order for $3.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
To determine more precisely the size of the mRNA<sup>TH</sup>, total RNA from PC8b cells or rat adrenal gland was fractionated by gel electrophoresis and transferred to nitrocellulose as described under "Experimental Procedures." The 0.3-kilobase PstI-KpnI restriction fragment, containing only tyrosine hydroxylase sequences (see Fig. 2), was nick-translated and used to probe mRNA<sup>TH</sup>. This probe was used because it provided much greater sensitivity than the entire plasmid, allowing us to detect mRNA<sup>TH</sup> using total, unfraccionated RNA.

Following hybridization, a band which migrates to the position of 18 S rRNA was visualized by autoradiography (Fig. 3). Thus, the two methods of determining mRNA<sup>TH</sup> size agree well and suggest that mRNA<sup>TH</sup> consists of approximately 1,800–1,900 nucleotides. In addition, mRNA<sup>TH</sup> from RNA extracted from rat adrenal gland co-migrated with that from PC8b cells (Fig. 3). These results are in agreement with those of Lamouroux et al. (26) who estimated mRNA<sup>TH</sup> from rat and human pheochromocytomas to consist of 1,900 nucleotides. The number of amino acids residues in tyrosine hydroxylase is not known, but M<sub>r</sub> = 60,000 would correspond to approximately 550 amino acids or 1,650 nucleotides. Assuming that the poly(A) tail adds about 100 nucleotides to the length of the mRNA it would appear there are not extensive untranslated regions in mRNA<sup>TH</sup> isolated from PC8b cells or adrenal glands.

**Analysis of Relative Levels of mRNA<sup>TH</sup> in Cells Treated with Dexamethasone and Derivatives of Cyclic AMP—**RNA extracted from PC8b cells treated with dexamethasone, 8Br-cAMP, or Bt,cAMP exhibited a much stronger degree of hybridization than did RNA from untreated cultures on Northern blot analysis (Fig. 3). This indicates that these inducers act by increasing the amount of mRNA<sup>TH</sup>, as would be expected from the initial differential hybridization selection of pTH.4. There was no apparent difference in the migration of mRNA<sup>TH</sup> between RNAs from basal and induced cell cultures, suggesting that these agents act by increasing the amount of mRNA<sup>TH</sup> and do not act by inducing the appearance of a different mRNA<sup>TH</sup> species. However, because of the limits of sensitivity of Northern blot analysis, we would not be able to detect a new RNA species which differs from basal mRNA<sup>TH</sup> by 50–100 nucleotides.

Upon longer exposure of the Northern blot to x-ray film, higher molecular weight bands, corresponding to 3–6 kilobases, can be visualized in RNAs extracted from cells treated with inducers (Fig. 3B). These bands also appear in RNA from untreated cultures after even longer exposure of the blot to film (data not shown). The most likely explanation for these bands is that they are unprocessed or partially processed precursors of the mature mRNA<sup>TH</sup>. The accumulation of these high molecular weight bands would suggest that these inducers act by increasing either the rate of transcription of the tyrosine hydroxylase gene or the stability of all tyrosine hydroxylase RNA species. Thus, the induction of mRNA<sup>TH</sup> does not appear to be solely the result of an enhanced stability of mature mRNA<sup>TH</sup> molecules.

The possibility cannot be excluded that the higher molecular weight bands do not represent precursors to mRNA<sup>TH</sup>, but are instead mRNAs for other proteins induced by glucocorticoid and cAMP, which contain sequences complementary to those of tyrosine hydroxylase.

Quantitation of the relative increases in mRNA<sup>TH</sup> levels was performed by RNA dot hybridization, as described under "Experimental Procedures" (Table II). In addition to 8Br-cAMP and dexamethasone, the effects of Bt,cAMP, sodium butyrate, and forskolin (Calbiochem-Behring) on tyrosine hydroxylase activity, mRNA<sup>TH</sup> translational activity, and relative mRNA<sup>TH</sup> levels were assessed. Forskolin, an activator of adenylate cyclase (55), has been demonstrated to cause increases in intracellular cAMP concentrations, and when added to PC8b cells, a small induction of tyrosine hydroxylase was observed. Sodium butyrate, a catabolite of Bt,cAMP, has previously been demonstrated to cause an elevation of tyrosine hydroxylase activity in a mouse neuroblastoma cell line (3, 56). Treatment of PC8b cells with sodium butyrate for 2 days also resulted in a modest increase in tyrosine hydroxylase activity, although this increase was less than that observed with Bt,cAMP. Incubation of cells simultaneously with butyrate and forskolin for 2 days resulted in an increase in enzyme activity that was greater than that observed with either agent alone, and which approached the fold increase produced with Bt,cAMP. These results suggest that the action...
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Table II
Comparison of the relative mRNA\textsuperscript{Tm} concentration, translational activity, and tyrosine hydroxylase enzyme activity following treatment with various inducers

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tyrosine hydroxylase activity</th>
<th>mRNA\textsuperscript{Tm} translational activity</th>
<th>mRNA\textsuperscript{Tm} hybridized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nMol [%CO\textsubscript{2}]</td>
<td>relative units</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.16</td>
<td>0.032</td>
<td>1</td>
</tr>
<tr>
<td>Dexamethasone (1 μM)</td>
<td>1.11</td>
<td>0.355</td>
<td>7.6</td>
</tr>
<tr>
<td>8Br-cAMP (1 mM)</td>
<td>0.47</td>
<td>0.122</td>
<td>4.5</td>
</tr>
<tr>
<td>Sodium butyrate (1 mM)</td>
<td>0.35</td>
<td>0.071</td>
<td>2.7</td>
</tr>
<tr>
<td>Forskolin (2 μM)</td>
<td>0.27</td>
<td>0.051</td>
<td>2.5</td>
</tr>
<tr>
<td>Sodium butyrate (1 mM) + forskolin (2 μM)</td>
<td>0.41</td>
<td>0.133</td>
<td>3.9</td>
</tr>
</tbody>
</table>

Fig. 4. Time course of mRNA\textsuperscript{Tm} induction. Approximately 10\textsuperscript{5} cells were inoculated into 100-mm Petri dishes in serum-containing medium. Two days after inoculation, medium was changed to serum-free (for C and D) and inducers were added at various times thereafter. Cells were harvested 48 h after the beginning of inducer treatment. From three to five 100-mm Petri dishes were pooled per sample. Aliquots were taken for assay of tyrosine hydroxylase enzyme activity and the remainder was used for extraction of RNA. The relative amount of mRNA\textsuperscript{Tm} was determined as described in Table II except that the extent of hybridization was determined by liquid scintillation counting in A and B. The symbols represent mRNA\textsuperscript{Tm} levels (Δ) and tyrosine hydroxylase enzyme activity (○) following treatment of cells with 1 μM forskolin (A) or 1 μM dexamethasone (B) in serum-free medium. The absolute values are as follows: relative mRNA\textsuperscript{Tm} levels: 0.825 (0.155, 0.125), 100% = 0.14 (0.155, 0.125), 100% = 0.33; D, basal = 0.025 (0.023, 0.026), 100% = 1.03. Tyrosine hydroxylase enzyme activity: A, basal = 0.50 ± 0.09, 100% = 0.825; B, basal = 0.50 ± 0.09, 100% = 1.66. These values are in the same units as in Table II. Error estimates represent the standard deviation of four samples, while numbers in parentheses represent the range of two samples.

Discussion

It had been previously demonstrated that both glucocorticoid and derivatives of cAMP can cause an induction of tyrosine hydroxylase enzyme activity in clonal cell lines (4, 6, 57, 59), and that in the PC12 cell line the increase in enzyme activity with dexamethasone is associated with an increase in mRNA\textsuperscript{Tm} translational activity (19). Glucocorticoids have been demonstrated to alter selectively the concentration of mRNA species in a number of systems (12–18) and it is likely that the effect of glucocorticoid has been shown to be related to the rate of RNA transcription (14–15). At present we do not know whether glucocorticoids increase the rate of tyrosine
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hydroxylase RNA transcription and/or have an effect on the half-life of messenger transcripts, as is the case with metallothionein (60) and other steroid-responsive genes (61). The kinetics of induction and the accumulation of high molecular weight tyrosine hydroxylase RNA would be consistent with an effect of dexamethasone at either transcription or RNA stability. It does appear, however, that the induction of tyrosine hydroxylase activity is the result of an increase in mRNA concentration; both enzyme activity and mRNA translatibility closely followed the changes in mRNA concentration, temporally and quantitatively. The observation that the induction kinetics was similar in cells cultured in the presence or absence of serum indicates that this is a direct steroid effect on tyrosine hydroxylase, which is important in light of the well-documented permissive effect of glucocorticoids on the nerve growth factor-mediated induction of tyrosine hydroxylase (57, 58).

With cAMP-mediated induction, mRNA levels also parallel tyrosine hydroxylase enzyme activity as in glucocorticoid induction. However, in contrast to the induction with dexamethasone, induction with both forskolin and 8Br-cAMP is transient; mRNA levels rise for approximately 12 h, after which time they decline at a rate slower than that of the increase. This failure to maintain a new steady state level of enzyme activity has also been observed in the induction of tyrosine hydroxylase by 8Br-cAMP in a mouse neuroblastoma cell line (4), although the decline does not occur until 48 h after incubation with the cAMP derivative. Of more general interest, a decline following cAMP-mediated induction in enzyme activity or specific mRNA has also been reported for guanine nucleotide binding regulatory protein (21) and prolactin (20, 22). There is evidence in other systems that cAMP may have more than one mechanism of action on a single protein. 1) Induction of lactate dehydrogenase by isoproterenol is characterized by a 2-fold increase in mRNA concentration and rate of transcription but an 8-fold increase in lactate dehydrogenase translational activity (23, 25), and 2) repeated injections of BtzcAMP result in an initial increase in tyrosine aminotransferase mRNA translational activity, followed by a decline, while the rate of synthesis of the protein remained elevated (24). In our studies we find a good correlation between tyrosine hydroxylase enzyme activity, mRNA concentration, and mRNA concentration, suggesting that changes in enzyme activity result from changes in concentration of mRNA. It will be of interest to determine whether glucocorticoid and cAMP act by altering the rate of transcription of the tyrosine hydroxylase gene or the stability of mRNA, and if so, the nuclear mechanism by which this is effected.

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REFERENCES


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by C. C. W. Chua, Q. L. Kuo, and B. F. Schaper

In collaboration with Science (26 June 1982) 210:1374-1379.


In Vitro Translation and Immunoprecipitation

RNA was translated in vitro using the micrococcal nuclease-treated rabbit reticulocyte lysate as described by Feltman and Jakoby (31). Protein synthesized was tested for specific activity by dot-blot hybridization to RNA prepared from CHO cells transiently transfected with plasmid DNA containing the cDNA clone 1038. Translated proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized using Coomassie blue staining. Source of RNA is described in the legend of Figure 1. Bioactivity of the antisera was monitored by immunoprecipitation of 35S-labeled tyrosine hydroxylase synthesized in E. coli. "Anti-" indicates that the antisera was preabsorbed with boiled E. coli cell lysate. "Pre-" indicates that the antisera was preabsorbed with boiled E. coli cell lysate.

Table 1: Oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe 1</td>
<td>5'-GCTGAGTCAAGCAGATGCA-3'</td>
</tr>
<tr>
<td>Probe 2</td>
<td>5'-GGTACGTTTCATGCGAGAG-3'</td>
</tr>
</tbody>
</table>

4. Hybridization and Northern Blot

For hybridization and Northern blot analysis, total RNA was electrophoresed on agarose gel, transferred to nitrocellulose membrane, and hybridized with [32P]dCTP-labeled probe. Hybridization was performed overnight at 65°C in a solution containing 5× SSC, 5× Denhardt's solution, 1% SDS, 100 mg/ml yeast RNA, and 100 μg/ml Cot-1 DNA. Following hybridization, filters were washed at 50°C in 2× SSC containing 0.1% SDS. Autoradiograms were exposed to X-ray film at -70°C. The amount of hybridization was quantitated by densitometry of the autoradiograms. The cycle of hybridization and autoradiography was repeated three times.

5. Results

A, RNA from CHO cells transiently transfected with plasmid DNA containing the cDNA clone 1038, was electrophoresed on agarose gel, transferred to nitrocellulose membrane, and hybridized with [32P]dCTP-labeled probe. Hybridization was performed overnight at 65°C in a solution containing 5× SSC, 5× Denhardt's solution, 1% SDS, 100 mg/ml yeast RNA, and 100 μg/ml Cot-1 DNA. Following hybridization, filters were washed at 50°C in 2× SSC containing 0.1% SDS. Autoradiograms were exposed to X-ray film at -70°C. The amount of hybridization was quantitated by densitometry of the autoradiograms. The cycle of hybridization and autoradiography was repeated three times.

B, RNA from CHO cells transiently transfected with plasmid DNA containing the cDNA clone 1038, was electrophoresed on agarose gel, transferred to nitrocellulose membrane, and hybridized with [32P]dCTP-labeled probe. Hybridization was performed overnight at 65°C in a solution containing 5× SSC, 5× Denhardt's solution, 1% SDS, 100 mg/ml yeast RNA, and 100 μg/ml Cot-1 DNA. Following hybridization, filters were washed at 50°C in 2× SSC containing 0.1% SDS. Autoradiograms were exposed to X-ray film at -70°C. The amount of hybridization was quantitated by densitometry of the autoradiograms. The cycle of hybridization and autoradiography was repeated three times.
RESULTS

Cloning of tyrosine hydroxylase cDNA and screening of recombinant colonies using DNA dot hybridization

We have isolated several colonies from the P21 cell line (clone 219) and assayed for tyrosine hydroxylase activity. We determined the optimal levels of the plasmid DNA to be used in the hybridization experiments. The results of the experiment are shown in Table 1. Table 1 shows the efficiency of the DNA dot hybridization experiments. The results are expressed as the ratio of the specific activity of the cDNA to the total amount of cDNA present in the dot hybridization.

Translational Activity of mRNAs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>mRNAs 10^9 Translational Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>0.067</td>
</tr>
<tr>
<td>P1</td>
<td>0.158</td>
</tr>
<tr>
<td>P2</td>
<td>0.216</td>
</tr>
<tr>
<td>P1 + P2 + P3 + P4</td>
<td>0.822</td>
</tr>
</tbody>
</table>

Fluorescent DNA containing inserted cDNA sequences were screened for by DNA dot hybridization as described in Experimental Procedures. The cDNAs from the P1 clone were then cut with Plasmodi, the restriction enzyme, to release the cDNA sequence from the plasmid DNA. This plasmid DNA was used to transfect NIH 3T3 fibroblasts. The cDNA was then screened for by DNA dot hybridization. Table 1 shows the efficiency of the DNA dot hybridization experiments. The results are expressed as the ratio of the specific activity of the cDNA to the total amount of cDNA present in the dot hybridization.

Figure 1: Hybridization selected translation and immunoprecipitation. Poly A+ mRNA from exons 1-9 of tyrosine hydroxylase were translated in vitro with rabbit reticulocyte lysate. The translation products were then immunoprecipitated with anti-human tyrosine hydroxylase antibodies and analyzed by SDS-PAGE and fluorography. The results are shown in Figure 1. Figure 1 shows the relative efficiency of the DNA dot hybridization experiments. The results are expressed as the ratio of the specific activity of the cDNA to the total amount of cDNA present in the dot hybridization.

Figure 2: Restriction endonuclease map of the cDNA insert. The cDNA insert is shown in the figure. The relevant restriction sites are indicated. The numbers indicate the number of base pairs from the 5' end of the cDNA insert. The bars indicate the direction of transcription, as determined by hybridization of the separate strands of DNA to mRNAs

Reference:

(14637)
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