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Induction of mRNA for Tyrosine Hydroxylase by Cyclic AMP and Glucocorticoids in a Rat Pheochromocytoma Cell Line: Evidence for the Regulation of Tyrosine Hydroxylase Synthesis by Multiple Mechanisms in Cells Exposed to Elevated Levels of Both Inducing Agents

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SUMMARY

When rat pheochromocytoma PC18 cells are exposed to the cyclic AMP analog, 8-bromocyclic AMP, and/or the synthetic glucocorticoid, dexamethasone, there is a marked increase in the level of a single RNA species that hybridizes to the recombinant plasmid pTH.4, which contains sequences complementary to the RNA coding for tyrosine hydroxylase. This RNA species is 1800–1900 nucleotides in length and is presumably identical to an RNA species of similar size, isolated from rat pheochromocytoma PC8b cells and shown to code for tyrosine hydroxylase. Using RNA dot hybridization to quantitate the relative level of this tyrosine mRNA species, time course studies show that this mRNA increases relatively rapidly in PC18 cells treated with either 8-bromocyclic AMP or dexamethasone. A new steady state level of tyrosine hydroxylase mRNA is achieved after 6 hr or 12–24 hr of treatment with either 8-bromocyclic AMP or dexamethasone, respectively. The changes in the level of the mRNA slightly precede the changes in the rate of synthesis of tyrosine hydroxylase in cells treated with these inducing agents. After 24 hr of treatment with either 8-bromocyclic AMP or dexamethasone, the increases in the level of tyrosine hydroxylase mRNA are identical to the increases in the rate of synthesis of the enzyme in the cells. In cells treated simultaneously with both 8-bromocyclic AMP and dexamethasone, the increases in the enzyme level and rate of synthesis of tyrosine hydroxylase are approximately equal to the sum of the increases in these parameters observed in cells treated with either inducing agent alone. In contrast, there is not an additive increase in the level of tyrosine hydroxylase mRNA in cells treated with both inducing agents. This lack of an additive increase in mRNA for tyrosine hydroxylase is observed in total cellular RNA samples or in cytoplasmic RNA samples. Our results suggest that in cells exposed to elevated levels of either cyclic AMP or glucocorticoids, tyrosine hydroxylase is induced by a mechanism which increases the level of its mRNA, resulting in an increased rate of synthesis of the enzyme. However, in cells exposed to elevated levels of both cyclic AMP and dexamethasone, tyrosine hydroxylase enzyme levels are regulated by multiple mechanisms, one of which regulates the rate of synthesis of the enzyme without affecting the level of its mRNA.

Tyrosine hydroxylase [tyrosine 3-monooxygenase: L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating); EC 1.14.16.2] activity has been shown to increase in certain mouse neuroblastoma and rat pheochromocytoma cell lines, when they are exposed for prolonged periods of time to elevated levels of cyclic AMP or glucocorticoids (1–5). These increases in activity have been shown to be due to increases in enzyme protein (1, 4) and, in rat pheochromocytoma cells, are a result of increases in the rate of synthesis of tyrosine hydroxylase (1, 3). Furthermore, in cells exposed simultaneously to elevated levels of both cyclic AMP and glucocorticoids, the increase in enzyme level is equal to or greater than the sum of the increases in enzyme level observed in cells exposed to either inducing agent alone (1, 4). In rat pheochromocytoma PC18 cells this additive increase in enzyme level is due to an additive increase in the rate of synthesis of the enzyme (1).

It has also been shown that the level of an RNA species, 1800–1900 nucleotides in length, which codes for tyrosine hydroxylase increases in rat pheochromocytoma cells exposed to elevated levels of either cyclic AMP or glucocorticoids (5). Parallel increases in the functional activity of $mRNA^{TH}$ have also been observed in RNA samples isolated from rat pheochromocytoma cells treated with either of these inducing agents

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ABBREVIATION: $mRNA^{TH}$, the RNA species coding for tyrosine hydroxylase.

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(5). However, it has not been demonstrated that these increases in the level and functional activity of mRNATH are associated with quantitatively equal increases in the rate of synthesis of the enzyme in the intact cell. In the present report we demonstrate that the increases in the level of mRNATH observed in PC18 cells exposed to either 8-bromocyclic AMP or dexamethasone correlate well both quantitatively and temporally with the increases in the rate of synthesis of tyrosine hydroxylase in the cells. We also show that, even though there is an additive increase in enzyme level and rate of synthesis of tyrosine hydroxylase in cells exposed simultaneously to both elevated cyclic AMP and dexamethasone, there is not an additive increase in the level of either total cellular or cytoplasmic mRNATH in cells treated with both inducing agents. These results suggest that more than one mechanism is involved in the regulation of tyrosine hydroxylase synthesis in PC18 cells exposed to both cyclic AMP and glucocorticoids.

Experimental Procedures

Materials

Tissue culture dishes and flasks were obtained from Falcon Plastics Co. (Los Angeles, CA). RPMI 1640 medium was from GIBCO Laboratories (Grand Island, NY). Horse serum was purchased from KC Biologicals, Inc. (Lenexa, KS), and fetal bovine serum was from Sterile Systems, Inc. (Logan, UT). [α-^{32} P]dCTP and [α-^{32} P]TTP were purchased from New England Nuclear Corp. (Boston, MA). Vanadyl-ribonucleoside complexes were from Bethesda Research Laboratories (Bethesda, MD). 8-Bromocyclic AMP and dexamethasone were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of the highest purity available from commercial sources.

pTH.4 is a recombinant plasmid containing a 400 base-pair sequence complementary to mRNATH and was isolated as described by Lewis *et al.* (5). The PST 1-KPN 1 restriction fragment of pTH.4 contains approximately 350 base pairs complementary to mRNATH (5).

Methods

Cell culture conditions. The PC18 cells were isolated as a subclone of the rat pheochromocytoma PC12 cell line (1, 6).[1] The cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum, 50 μg/ml of streptomycin and 50 units/ml of penicillin. Cultures were maintained at 37° in a water-saturated atmosphere containing 95% air and 5% CO_2.

Induction studies. For experiments involving the induction of mRNATH, cells were subcultured in 100-mm dishes containing medium supplemented with complete serum at a cell density of 2–5 × 10^4 cells/cm^2. The cells were incubated for 12–24 hr, and then the medium was removed and replaced with fresh medium containing dialyzed serum (1). The cells were incubated in the presence of this medium for 2 days prior to the addition of the inducing agents. The cells were then treated with 8-bromocyclic AMP and/or dexamethasone.

Isolation of RNA. At the appropriate time cells were harvested by removing the medium, washing the cells once with ice-cold phosphate-buffered saline (0.15 M NaCl and 0.01 M potassium phosphate, pH 7.4), and then scraping the cells into the same ice-cold buffer using a rubber policeman. The cells were collected by centrifugation at 2500 × *g* for 5 min. When total cellular RNA was to be isolated, the cell pellets were frozen at −90°, and total cellular RNA was isolated as described by Chirgwin *et al.* (7). When cytoplasmic and nuclear RNA were to be isolated, the cells were washed once in Tris-buffered saline (0.15 M NaCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.5) and then homogenized in the same buffer, containing 0.2% Triton X-100 and 10 mM Vanadyl ribonucleoside complexes. The homogenate was layered onto a 3-ml cushion containing 1 M sucrose, 50 mM NH_4Cl, 5 mM $MgCl_2$, 0.1% Triton X-100, 0.1% deoxycholate, and 10 mM Tris-HCl, pH 7.5. The nuclei were collected by centrifugation at 2500 × *g* for 10 min in a swinging bucket rotor. This isolation procedure minimized cytoplasmic contamination of the nuclei as judged by light microscopic examination. The RNA in the nuclei was isolated as described by Chirgwin *et al.* (7). The supernatant layer above the sucrose cushion was considered to contain the cytoplasmic RNA. This layer was removed, guanidine hydrochloride added to a final concentration of 6 M, and cytoplasmic RNA isolated as described by Chirgwin *et al.* (7).

Northern blot analysis. The isolated RNA (5–10 μg) was denatured by heating at 65° for 15 min in the presence of 6.5% formaldehyde and 50% formamide. The denatured RNA was subjected to horizontal electrophoresis on 1.2% agarose gels containing formaldehyde as described by Goldberg (8). After electrophoresis, the RNA was transferred to nitrocellulose as described by Thomas (9). The immobilized RNA was then hybridized to the nick-translated PST 1-KPN 1 restriction fragment of pTH.4 as described by Lewis *et al.* (5). This restriction fragment contained only sequences complementary to the mRNATH (5). The resulting ^{32}P-labeled RNA-DNA hybrids were detected by autoradiography. Exposure times were generally 18 hr or less.

RNA dot hybridization. The isolated RNA was denatured by heating at 65° for 15 min in the presence of 7.4% formaldehyde, 1 M NaCl, and 0.1 M sodium citrate, pH 7.0. Different concentrations of the denatured RNA (0.3–10 μg) were immobilized onto nitrocellulose with the use of a Hybri-dot filtration device (Bethesda Research Laboratories). The RNA was then hybridized to the PST 1-KPN 1 restriction fragment of pTH.4 as described by Lewis *et al.* (5). The resulting radiolabeled RNA-DNA hybrids were detected by autoradiography. Exposure times were generally 12 hr or less. The intensities of the autoradiographic spots corresponding to the RNA-DNA hybrids were measured using a Hoefer GS 300 scanning densitometer. The relative levels of mRNATH were determined by calculating the slope of the linear portion of the curve relating density units to μg of RNA spotted.

Other assays and methods. Tyrosine hydroxylase enzyme activity and the rate of synthesis of tyrosine hydroxylase were determined as described in the preceding paper (1). The PST 1-KPN 1 restriction fragment of pTH.4 was radiolabeled with $^{32}P_i$ by nick-translation as described by Rigby *et al.* (10). RNA was determined by measuring the absorbance of the RNA solutions at 260 nm.

Results

Effect of 8-bromocyclic AMP and/or dexamethasone on the level of mRNATH in PC18 cells. Total cellular RNA was isolated from untreated cells or cells that were treated for 24 hr with either 1 mM 8-bromocyclic AMP, 1 μM dexamethasone, or the two inducing agents together. The isolated RNA was denatured using formaldehyde, subjected to electrophoresis on formaldehyde-agarose gels (1.2%) as described by Goldberg (8), and then transferred to nitrocellulose paper by the method of Thomas (9). The mRNATH was then detected by hybridization of the nitrocellulose-bound RNA to the nick-translated PST 1-KPN 1 restriction fragment of pTH.4 (5). In agreement with the results of a number of other studies which analyzed the RNA isolated from other rat pheochromocytoma cell lines or from rat adrenal medulla (5, 11, 12) only one major RNA species, approximately 1800–1900 nucleotides in length, hybridized to this probe (Fig. 1). This species was shown to code for tyrosine hydroxylase and thus represented the mRNATH (5). As can be clearly observed in Fig. 1, there was a dramatic increase in the level of this RNA species in cells treated with either 8-bromocyclic AMP or dexamethasone alone or with the two inducing agents together.

In order to quantitate more accurately the changes in the

[1] A. W. Tank, P. Curella, and L. Ham, submitted for publication.

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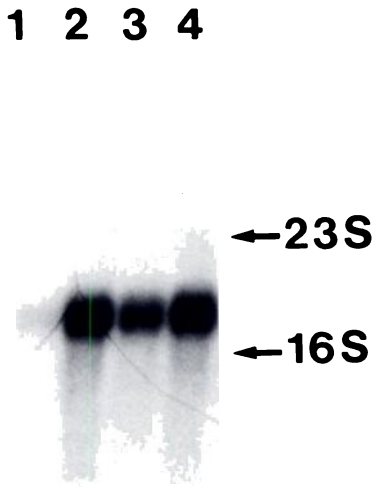

Fig. 1. Northern blot analysis of mRNATH isolated from cells treated with 8-bromocyclic AMP and/or dexamethasone. Cells were incubated in medium containing dialyzed serum for 2 days, at which time the medium was removed and replaced with fresh medium containing dialyzed serum plus the appropriate drugs. The cells were incubated for 24 hr in the absence (*lane 1*) or presence of 1 mM 8-bromocyclic AMP (*lane 2*), 1 μM dexamethasone (*lane 3*), or the two inducing agents together (*lane 4*). Total cellular RNA was then isolated from the cells, and mRNATH was analyzed by Northern blot analysis. The figure depicts an autoradiogram of a representative Northern blot in which 4.5 μg of total cellular RNA were applied to each lane of the gel. The *numbers* on the right depict the electrophoretic migration of the 16 S and 23 S *Escherichia coli* ribosomal RNA markers.

level of this RNA species, we performed RNA dot hybridization analysis (Fig. 2). Total cellular RNA was denatured and then different concentrations of the denatured RNA were spotted onto nitrocellulose paper. After hybridization of the RNA to the nick-translated PST 1-KPN 1 fragment of pTH.4, the nitrocellulose papers were exposed to X-ray film for autoradiography, and densities of the resulting spots were plotted as a function of the amount of total RNA spotted (Fig. 2). In agreement with the data in Fig. 1, there is a large increase in the relative amount of mRNATH in the RNA samples isolated from PC18 cells treated with 8-bromocyclic AMP, dexamethasone, or the two inducing agents together. Note that the relative level of mRNATH in the cells treated with both inducing agents is not greater than that observed in cells treated with either inducing agent alone.

Data obtained from numerous experiments are presented in Table 1. In these experiments cells were treated with the inducing agents for 24 hr, and then RNA was isolated and mRNATH was measured by dot hybridization. The relative level of mRNATH was increased 6–8-fold when cells were incubated for 24 hr in the presence of either 8-bromocyclic AMP, dexamethasone, or the two inducing agents simultaneously.

Time courses of the changes in the relative level of mRNATH in PC18 cells treated with 8-bromocyclic AMP and/or dexamethasone. In untreated cells the level of mRNATH remained relatively constant over the 4-day test period (Fig. 3). In cells treated with 8-bromocyclic AMP the level of mRNATH increased rapidly to a new steady state level after 6 hr of treatment and remained elevated during the 4 days of treatment with the cyclic nucleotide analog (Fig. 3). In cells treated with dexamethasone the level of mRNATH increased at a slightly slower rate, but to the same final steady state level as that observed in cells treated with 8-bromocyclic AMP. Interestingly, in cells treated with both inducing agents simultaneously, the level of mRNATH increased to the same extent as that observed in cells treated with either inducing agent alone (Fig. 3). Thus, the additive increases in enzyme activity and rate of synthesis of tyrosine hydroxylase observed in the presence of both 8-bromocyclic AMP and dexamethasone in the preceding report (1) were not paralleled by additive increases in the level of mRNATH at any time point in the PC18 cells (see Table 1 and Fig. 2).

Comparison of the changes in enzyme activity of tyrosine hydroxylase rate of synthesis of tyrosine hydroxylase, and level of mRNATH in cells treated with 8-bromocyclic AMP and/or dexamethasone. In order to verify that the changes we observed in the enzymatic activity of tyrosine hydroxylase, the rate of synthesis of tyrosine hydroxylase, and the level of mRNATH were comparable from experiment to experiment, we measured all three parameters in companion cell cultures in the same experiment (Table 2). Each parameter was measured after treatment with the inducing agents for a period of time which produced a maximal or near-maximal -fold increase. The -fold increase in the rate of synthesis of tyrosine hydroxylase in cells treated with 8-bromocyclic AMP and/or dexamethasone completely accounted for the -fold increase in enzyme levels (Table 2). Furthermore, in the presence of both inducing agents, the increases in enzyme activity and rate of synthesis were additive (Table 2). The increases in the level of mRNATH completely accounted for the increases in the rate of synthesis of the enzyme in cells treated with either 8-bromocyclic AMP or dexamethasone alone. In contrast, the increase in the level of mRNATH in cells treated simultaneously with both inducing agents was not additive and, consequently, did not account for the observed increase in the rate of synthesis of tyrosine hydroxylase in these cells (Table 2).

Effect of 8-bromocyclic AMP and/or dexamethasone on the level of total cellular, cytoplasmic, or nuclear mRNATH. In the above experiments mRNATH was measured in samples of RNA isolated from whole cells. Since only the cytoplasmic RNA was involved in protein synthesis, the discrepancy between the changes in the rate of synthesis of tyrosine hydroxylase and the level of total cellular mRNATH in cells treated with both 8-bromocyclic AMP and dexamethasone might be due to additive changes in the level of cytoplasmic mRNATH that were not measurable in total cellular RNA samples. Thus, cells were treated with the inducing agents, and mRNATH was measured in total cellular, cytoplasmic, and nuclear RNA samples. Northern blot analysis showed that the size of the cytoplasmic and nuclear RNA species complementary to the PST 1-KPN 1 fragment of pTH.4 was identical to that detected in total cellular RNA samples from the PC18 cells (Fig. 4). Even in the nuclear samples, we could not detect the presence of larger RNA species, which could represent precursors to the mature mRNATH, unless the films were exposed for very long periods of time. In cells incubated in the presence of 8-bromocyclic AMP and/or dexamethasone, there were large increases in the level of the major mRNATH species in both cytoplasmic and nuclear samples. When the mRNATH

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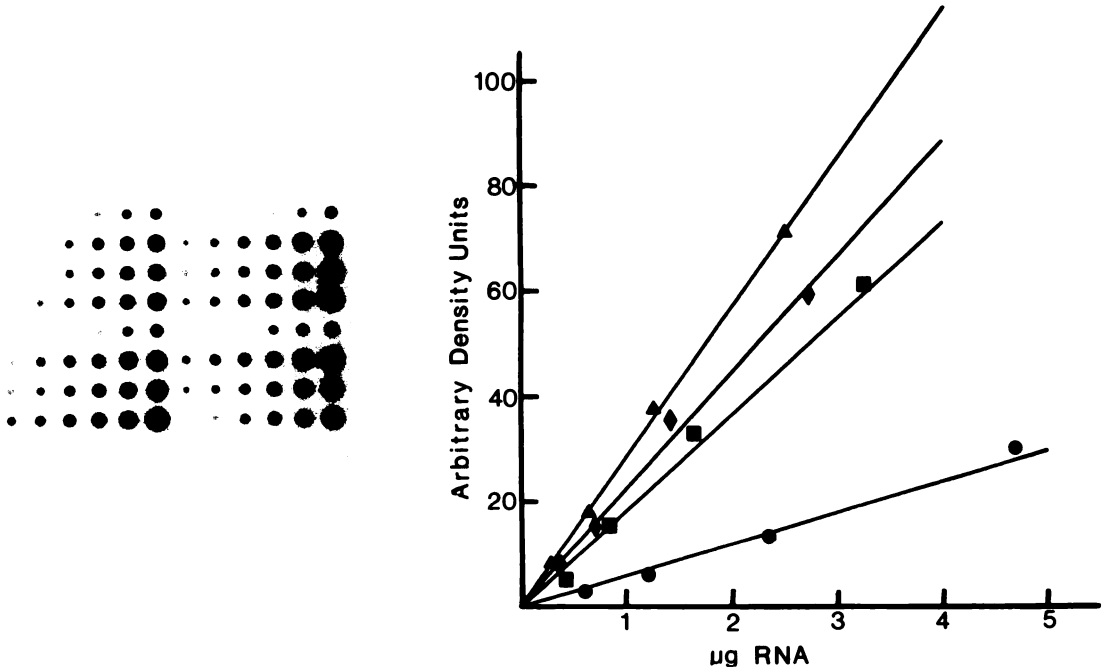

Fig. 2. RNA dot hybridization analysis of mRNATH isolated from cells treated with 8-bromocyclic AMP and/or dexamethasone. Cells were incubated in medium containing dialyzed serum for 2 days, at which time the medium was removed and replaced with fresh medium containing dialyzed serum plus the appropriate drugs. Cells were incubated for 24 hr in the absence (●) or presence of 1 mM 8-bromocyclic AMP (▲), 1 µM dexamethasone (■), or the two inducing agents together (♦). Total cellular RNA was isolated from the cells, denatured, and serially diluted five times to produce six different concentrations of RNA to be spotted onto nitrocellulose. The mRNATH was then measured by RNA dot hybridization as described in Experimental Procedures. The *photograph* on the *left* depicts a representative autoradiogram derived from such a dot hybridization analysis. The different rows of dots represent ^{32}P-labeled RNA-DNA hybrids, in which the RNA was isolated from control cells (rows 1 and 5), 8-bromocyclic AMP-treated cells (rows 2 and 6), dexamethasone-treated cells (rows 3 and 7), and cells treated with both inducing agents (rows 4 and 8). The relative levels of mRNATH were determined by measuring the relative intensities of these dots as a function of the µg of RNA spotted onto nitrocellulose, as shown on the *right*.

TABLE 1

Effects of 8-bromocyclic AMP and/or dexamethasone on the level of mRNATH in PC18 cells

Cells were incubated for 2 days in medium containing dialyzed serum, at which time the medium was removed and replaced with fresh medium containing dialyzed serum and the appropriate drugs. The cells were then incubated in the absence or presence of 1 mM 8-bromocyclic AMP, 1 µM dexamethasone, or the two inducing agents together for 24 hr. The cells were harvested, total cellular RNA was isolated, and mRNATH was measured by RNA dot hybridization. The data represent the means ± standard errors from eight dishes.

	mRNATH	
	Units/µg RNA	-Fold increase
Control	7.3 ± 0.56	
8-Bromocyclic AMP	58 ± 7.7*	7.8 ± 0.9
Dexamethasone	44 ± 4.5*	6.0 ± 0.7
8-Bromocyclic AMP + dexamethasone	52 ± 4.8*	7.4 ± 0.9

*$p < 0.01$ compared to controls.

was measured by RNA dot hybridization, the increases in the relative level of the cytoplasmic and nuclear mRNATH paralleled the increases in the relative level of the total cellular mRNATH (Table 3). Consequently, there was no additive increase in the level of cytoplasmic mRNATH in cells treated with both 8-bromocyclic AMP and dexamethasone.

Discussion

Cyclic AMP and glucocorticoids have been shown to induce tyrosine hydroxylase in a number of catecholaminergic tumor cell lines (1–5). It has also been demonstrated that these compounds increase the level of one major RNA species complementary to pTH.4 in the rat pheochromocytoma cell line, PC8b (5). This major RNA species is 1800–1900 nucleotides in length and has been shown to code for tyrosine hydroxylase (5). Higher molecular weight RNA species which hybridize with pTH.4 have also been detected, but they are present at very low concentrations relative to the major mRNATH species, and their significance is presently obscure (5, 12). In PC8b cells treated with cyclic AMP-elevating agents or glucocorticoids, the functional activity of the mRNATH increases to the same extent as the level of the mRNATH. However, in these studies it was not determined whether these increases in mRNATH result in quantitatively equal increases in the rate of synthesis of tyrosine hydroxylase in the cells.

In more recent experiments we have used a different rat pheochromocytoma cell line, PC18, which, like PC8b cells, possesses relatively high basal activity of tyrosine hydroxylase and high basal levels of mRNATH; however, the induction of tyrosine hydroxylase by cyclic AMP or glucocorticoid is much greater and less variable in the PC18 cells than in the PC8b cells. Thus, the PC18 cells represent a better model system in which to study the induction processes. In agreement with the results obtained in PC8b cells, only one major mRNATH species (1800–1900 nucleotides in length) is detectable in the PC18 cells, and this mRNATH species increases dramatically when PC18 cells are exposed to 8-bromocyclic AMP and/or dexamethasone.

In the preceding paper (1) we have shown that the induction of tyrosine hydroxylase in PC18 cells exposed to elevated levels of cyclic AMP and/or glucocorticoids is due primarily to an

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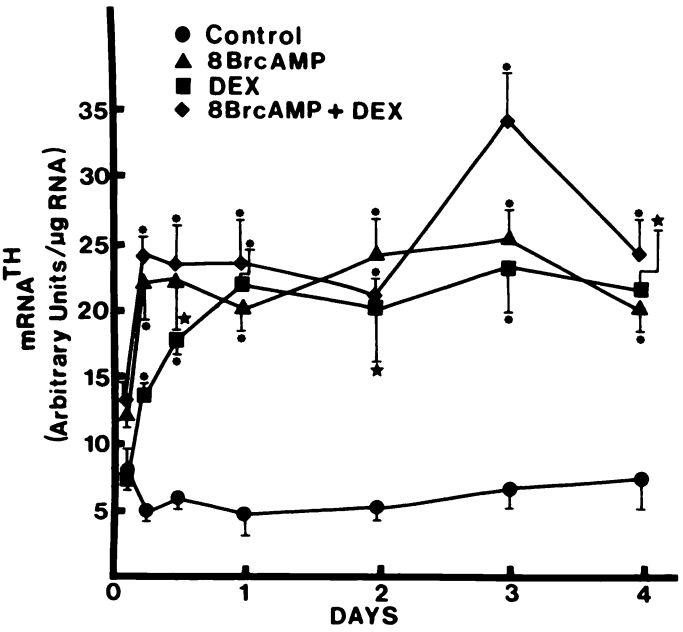

Fig. 3. Time courses of the changes in mRNATH in PC18 cells treated with 8-bromocyclic AMP and/or dexamethasone. Cells were incubated in medium containing dialyzed serum for 2 days, at which time the medium was removed and replaced with fresh medium containing dialyzed serum plus the appropriate drugs (this represents zero time in the figure). The cells were then incubated for different periods of time in the absence (*Control*) or presence of 1 mM 8-bromocyclic AMP (*8BrcAMP*), 1 μM dexamethasone (*DEX*), or the two inducing agents together. At the appropriate times the cell were harvested and total cellular RNA was isolated. The mRNATH was measured by RNA dot hybridization analysis. The data represent the means ± standard error from three or four dishes. *, $p < 0.01$ when compared to controls. ★, $p < 0.05$ when compared to controls.

TABLE 2

Effects of 8-bromocyclic AMP and/or dexamethasone on tyrosine hydroxylase (TH) activity, rate of synthesis of tyrosine hydroxylase, and mRNATH level in PC18 cells

Cells were incubated for 2 days in medium containing dialyzed serum, at which time the medium was replaced with fresh medium containing dialyzed serum and the appropriate drugs. Tyrosine hydroxylase activity, rate of synthesis of tyrosine hydroxylase, and the level of mRNATH were determined in the same experiment, using cells subcultured at the same time into companion culture dishes. These parameters were measured as described in Experimental Procedures. For the measurement of tyrosine hydroxylase activity the cells were incubated in the presence of 1 mM 8-bromocyclic AMP, 1 μM dexamethasone, or the two inducing agents together for 4 days, whereas, for the measurement of the rate of synthesis of tyrosine hydroxylase or the level of mRNATH, the cells were incubated in the presence of the inducing agents for 24 hr. The results represent the means ± standard errors from four dishes in two experiments.

	Maximal-fold increases		
	TH activity	Rate of synthesis	mRNATH
8-Bromocyclic AMP	4.1 ± 0.5	6.0 ± 1.4	5.5 ± 1.6
Dexamethasone	4.1 ± 0.2	6.9 ± 0.7	6.1 ± 2.0
8-Bromocyclic AMP + dexamethasone	7.9 ± 0.9[a]	13.5 ± 2.5[b]	8.1 ± 2.7

[a] $p < 0.01$ compared to cells treated with either inducing agent alone.
[b] $p < 0.05$ compared to cells treated with either inducing agent alone.

increase in the rate of synthesis of the enzyme. In the present report we demonstrate that, in PC18 cells treated with either 8-bromocyclic AMP or dexamethasone, the increases in the rate of synthesis of tyrosine hydroxylase are quantitatively equal to the increases in the relative level of mRNATH. The cyclic AMP-mediated or glucocorticoid-mediated changes in the level of mRNATH occur more rapidly than the corresponding

Fig. 4. Northern blot analysis of cytoplasmic and nuclear mRNATH isolated from cells treated with 8-bromocyclic AMP and/or dexamethasone. Cells were incubated in medium containing dialyzed serum for 2 days, at which time the medium was removed and replaced with fresh medium containing dialyzed serum plus the appropriate drug. Cells were then incubated for 24 hr in the absence or presence of 1 mM 8-bromocyclic AMP (*8BRCA*), 1 μM dexamethasone (*DEX*), or the two inducing agents together. RNA was isolated from the nuclear and cytoplasmic fractions of the cells. After denaturation, 5 μg of cytoplasmic RNA or 2.5 μg of nuclear RNA were loaded onto 1.2% formaldehyde agarose gels and subjected to horizontal electrophoresis. The mRNATH was measured by Northern blot analysis as described in Experimental Procedures. The *numbers* on the *right* depict the electrophoretic migration of the *E. coli* 16 S and 23 S ribosomal RNA markers.

TABLE 3

Effect of 8-bromocyclic AMP and/or dexamethasone on the relative levels of total cellular, cytoplasmic, and nuclear mRNATH

Cells were incubated for 2 days in the presence of medium containing dialyzed serum, at which time the medium was replaced with fresh medium containing dialyzed serum and the appropriate drugs. Final concentrations of 8-bromocyclic AMP and dexamethasone were 1 mM and 1 μM, respectively. The cells were treated for 24 hr with these inducing agents and then harvested. Total cellular, cytoplasmic, and nuclear RNA were isolated, and mRNATH was measured by dot hybridization. The results are expressed as arbitrary density units/μg of RNA spotted and represent the means ± standard errors of four dishes from two experiments. The differences between the mRNATH values obtained from treated cells compared to that obtained from control cells were all statistically significant with $p < 0.01$.

Treatment	mRNATH		
	Total cellular	Cytoplasmic	Nuclear
	arbitrary density units/μg RNA		
Control	3.7 ± 0.30	3.6 ± 0.25	2.6 ± 0.50
8-Bromocyclic AMP	20 ± 1.5	21 ± 2.5	21 ± 3.9
Dexamethasone	17 ± 1.7	17 ± 2.6	19 ± 2.3
8-Bromocyclic AMP + dexamethasone	25 ± 3.6	24 ± 3.7	21 ± 4.2

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changes in the rate of synthesis of the enzyme. However, both parameters approach a new steady state level after 12–24 hr of treatment with these inducing agents, and at these time points the changes in these parameters elicited by either cyclic AMP or glucocorticoids are equal in magnitude (see Table 2). These results, along with those presented in the previous paper (1), strongly support the hypothesis that cyclic AMP and glucocorticoids induce tyrosine hydroxylase by increasing the level of $mRNA^{TH}$, which results in an increase in the rate of synthesis of the enzyme.

Both glucocorticoids and cyclic AMP have been shown to regulate the level of mRNAs for specific proteins by affecting the rate of transcription of the genes for these proteins (13–19). It is tempting to speculate that cyclic AMP- and glucocorticoid-mediated increases in the rate of transcription of the tyrosine hydroxylase gene are responsible for the observed increases in the level of $mRNA^{TH}$ in the PC18 cells. This hypothesis is supported by the observed increases in the level of nuclear $mRNA^{TH}$ in PC18 cells exposed to either 8-bromocyclic AMP or dexamethasone (Fig. 4, Table 3). These increases in nuclear $mRNA^{TH}$ are quantitatively equal to those observed in cytoplasmic $mRNA^{TH}$. However, at present, other mechanisms, such as effects on the stability of the $mRNA^{TH}$, cannot be ruled out.

It has also been shown that when catecholaminergic tumor cell lines are exposed to elevated levels of both cyclic AMP and glucocorticoids simultaneously, tyrosine hydroxylase is induced to a level greater than or equal to the sum of the increases in enzyme level obtained in cells exposed to either inducing agent alone (1, 4). These additive increases in enzyme level in PC18 cells are due to additive increases in the rate of synthesis of the enzyme (1). However, additive increases in the relative level of $mRNA^{TH}$ are not observed in cells exposed to both 8-bromocyclic AMP and dexamethasone. Thus, in cells exposed to both inducing agents the rate of synthesis of the enzyme increases to a level above that observed in cells exposed to either inducing agent alone without a concomitant increase in the level of $mRNA^{TH}$.

In most of our experiments, $mRNA^{TH}$ has been measured in samples containing total cellular RNA. Since only cytoplasmic $mRNA^{TH}$ is used in the synthesis of tyrosine hydroxylase, one explanation for our results is that, in cells treated with both 8-bromocyclic AMP and dexamethasone, the transport of the $mRNA^{TH}$ from the nucleus to the cytoplasm is enhanced, resulting in a cytoplasmic level of $mRNA^{TH}$ greater than that observed in cells treated with either inducing agent alone. However, when cytoplasmic $mRNA^{TH}$ is measured in the PC18 cells, the changes in its level is paralleled by the changes in the level of total cellular $mRNA^{TH}$. Thus, there is also no additive increase in the level of cytoplasmic $mRNA^{TH}$ in cells treated with both 8-bromocyclic AMP and dexamethasone. These results suggest that, in PC18 cells exposed to elevated levels of both cyclic AMP and glucocorticoids, tyrosine hydroxylase is induced by a mechanism that regulates the level of $mRNA^{TH}$ (possibly a transcriptional mechanism) and a mechanism that regulates the translation of the $mRNA^{TH}$ (a post-transcriptional mechanism). Furthermore, our results suggest that this post-transcriptional mechanism does not involve an effect on the rate of transport of the $mRNA^{TH}$ from the nucleus to the cytoplasm. In addition, it is difficult to propose an effect of these inducing agents on the nuclear processing of the RNA precursors to $mRNA^{TH}$, because only very low levels of putative precursor RNA molecules are detected in total cellular or nuclear RNA samples derived from uninduced rat pheochromocytoma cells or from rat adrenal medulla (5, 11, 12). Thus, the evidence suggests that the post-transcriptional effect of cyclic AMP plus glucocorticoids may be to alter the functional activity of the $mRNA^{TH}$, or to affect a cellular process involved in mRNA translation, such that the synthesis of tyrosine hydroxylase is specifically increased.

Post-transcriptional effects of cyclic AMP and/or glucocorticoids on the enzyme level of tyrosine hydroxylase have not been previously reported. Thoenen and co-workers (20, 21) have shown that the induction of tyrosine hydroxylase elicited by nerve growth factor is not blocked by α-amanitan, an inhibitor of RNA polymerase II. However, this post-transcriptional effect of nerve growth factor has not been linked to cyclic AMP or glucocorticoids. There is evidence to suggest that both cyclic AMP and glucocorticoids affect post-transcriptional mechanisms involved in the induction of several other proteins (13, 22–26). Cyclic AMP has been reported by a number of workers to increase the rate of translation of the hepatic enzyme, tyrosine aminotransferase (22, 23). Noguchi *et al.* (22) have reported that, in *in vivo* experiments, multiple injections of dibutyryl cyclic AMP into rats result in an initial rise in both enzymatic activity of tyrosine aminotransferase and functional activity of the mRNA coding for tyrosine aminotransferase. However, even though the functional activity of mRNA for the enzyme decreases back to basal levels by 2.5 hr, the enzyme activity and rate of synthesis remain elevated for 4–5 hr. Furthermore, Roper and Wicks (23) have reported that cyclic AMP increases the rate of elongation of nascent tyrosine aminotransferase chains in rat hepatoma cells. Glucocorticoids or other steroid hormones have been shown in some cases to induce certain proteins or pathways, leading to subsequent effects on other cellular events unrelated to the originally induced protein (13). Examples of these indirect effects of steroid hormones are the stabilization of ovalbumin mRNA by estrogen in the chick oviduct (24), the induction of a glycoprotein-processing pathway for mouse mammary tumor viral proteins by glucocorticoids in rat hepatoma cells (25), and the induction of α_1 acid glycoprotein by glucocorticoids in rat liver (26). In all of these cases it appears that the steroid hormone activates a gene that encodes for a protein which has dramatic effects on other cellular processes.

The $mRNA^{TH}$-independent increases we observe in the rate of synthesis of tyrosine hydroxylase in PC18 cells exposed to elevated levels of both cyclic AMP and glucocorticoid may be analogous to one of the post-transcriptional mechanisms mentioned above. However, it is important to note that this effect is observed only in cells treated with both inducing agents. It is possible that a gene encoding for a protein which affects the translation of tyrosine hydroxylase requires both cyclic AMP and glucocorticoid for its activation. Alternatively, glucocorticoids may induce a protein which affects the translation of specific proteins only when it is phosphorylated by cyclic AMP-dependent protein kinase. These and other hypotheses require further investigation.

In the rat adrenal medulla and sympathetic ganglia tyrosine hydroxylase is induced after prolonged stress or treatment with reserpine (27, 28). Glucocorticoids have been shown to play a modulatory role in this transsynaptic induction of tyrosine

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hydroxylase (29). Costa *et al.* (30) have presented correlative evidence suggesting that cyclic AMP may also be involved in this stress-related induction. Thus, it is possible that, during periods of stress, catecholaminergic cells of the adrenal medulla and sympathetic ganglia may be exposed to elevated levels of both cyclic AMP and glucocorticoids. Under these conditions our results suggest that the synthesis of tyrosine hydroxylase in these organs may be regulated by multiple mechanisms.

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