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# Regulation of Tyrosine Hydroxylase Gene Transcription Rate and Tyrosine Hydroxylase mRNA Stability by Cyclic AMP and Glucocorticoid

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**SUMMARY**

Tyrosine hydroxylase mRNA is induced in rat pheochromocytoma PC18 cells by cAMP analogs and glucocorticoids. Previous studies have shown that these increases in tyrosine hydroxylase mRNA are due at least in part to stimulation of the tyrosine hydroxylase gene. However, the involvement of post-transcriptional mechanisms in the regulation of tyrosine hydroxylase mRNA by these inducing agents has not been investigated. In the present study, using nuclear run-on assays we show that the relative transcription rate of the tyrosine hydroxylase gene is stimulated 2–5-fold within 20 min after treatment of PC18 cells with cAMP analogs or dexamethasone and that the rate of transcription remains elevated 2–3-fold for at least 24 hr in the continual presence of these inducing agents. Pulse-labeling experiments using 4-thiouridine indicate that the rate of synthesis of tyrosine hydroxylase mRNA is increased approximately 3-fold or 10-fold after treatment with either a cyclic AMP analog or dexamethasone, respectively. These increases in rates of synthesis agree well with the fold increases in tyrosine hydroxylase mRNA levels after treatment with these inducers. Treatment of the cells with cycloheximide lowers the basal relative transcription rate of the tyrosine hydroxylase gene 2–3-fold; however, the relative transcription rate of the tyrosine hydroxylase gene is still elevated in cells treated with either dexamethasone or cAMP analogs in the presence of cycloheximide, compared with the transcription rate of the gene in cells treated with cycloheximide alone. These results indicate that protein synthesis is not required for the short term regulation of the gene by these inducing agents. The apparent $t_{1/2}$ for tyrosine hydroxylase mRNA has been estimated by two different procedures, approach to steady state kinetics and pulse-chase analysis. Both procedures yield an estimated apparent $t_{1/2}$ of approximately 6–9 hr for tyrosine hydroxylase mRNA under basal culture conditions. Dexamethasone does not substantially alter this apparent $t_{1/2}$ value; however, cAMP appears to lower this apparent $t_{1/2}$ value transiently. Our results suggest that cAMP and glucocorticoid regulate tyrosine hydroxylase mRNA levels primarily by stimulating the transcription rate of the tyrosine hydroxylase gene; however, cAMP may also regulate the stability of the mRNA for a short period of time, such that it is induced more rapidly in the cells.

TH (EC 1.14.16.2) in rat adrenal medulla is induced *in vivo* by prolonged stress (1, 2), by drugs that reflexively activate the sympathetic nervous system, like reserpine or 6-hydroxydopamine (3), and by cholinergic agonists, like nicotine and carbachol (4, 5). The induction of adrenal TH enzyme protein by these treatments is preceded by the induction of adrenal TH mRNA (6–8). Recently, it has been demonstrated that reserpine and cholinergic agonists elevate adrenal TH mRNA primarily by stimulating the transcription rate of the TH gene in the rat adrenal medulla (5).

The intracellular signal transduction pathways that mediate this induction of TH mRNA in rat adrenal medulla are not well understood. Two intracellular messengers that regulate gene transcription and that have been implicated in the induction of adrenal TH are cAMP and glucocorticoids. Stress or the administration of reserpine or cholinergic agonists is associated with increases in both circulating glucocorticoid levels (9, 10) and adrenal medullary cAMP levels in rats (4, 11). Furthermore, both these inducing agents elevate TH enzyme protein, TH mRNA, and TH gene transcription rate in rat pheochromocytoma cell lines (12–15). The 5′ flanking region of the TH gene possesses canonical sequences or response elements that mediate the regulation of numerous other genes by cAMP and glucocorticoid (15). The TH gene cAMP response element is functional, because deletion analysis has demonstrated that the absence of this cAMP response element in the TH gene promoter region results in the loss of response of the gene to elevated cAMP (15, 16). The functionality of the

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**ABBREVIATIONS:** TH, tyrosine hydroxylase; 8-Br-cAMP, 8-bromo-cAMP; 8-CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; bp, base pairs; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

putative TH gene glucocorticoid response element remains obscure, because TH gene promoter constructs that contain this glucocorticoid response element do not respond to elevated glucocorticoid levels in rat pheochromocytoma cells (15).

These previous studies definitively demonstrate that cAMP and glucocorticoids induce TH mRNA and TH protein at least partially by stimulating the TH gene. However, it is not clear whether post-transcriptional mechanisms also play a role in this induction. Post-transcriptional mechanisms have been postulated to be partially responsible for mediating the induction of TH protein in rat adrenal medulla after exposure to cold (17), in rat pheochromocytoma PC18 cells exposed to elevated levels of both cAMP and glucocorticoids (14), and in bovine adrenal chromaffin cells treated with the nicotinic receptor agonist (dimethylphenlpiperazinium) (18). Furthermore, recent evidence suggests that phorbol esters induce TH mRNA in rat pheochromocytoma PC12 cells by both transcriptional and post-transcriptional mechanisms (19).

In the present study we further investigate the transcriptional and post-transcriptional regulation of TH mRNA levels by cAMP and glucocorticoid using rat pheochromocytoma PC18 cells as a model system. These cells have been used to study the regulation of TH mRNA and TH enzyme protein by these inducing agents in previous investigations (13, 20). Our results suggest that the primary effect of these inducing agents is to regulate the transcription rate of the TH gene in these cells; however, the stability of TH mRNA is also influenced transiently in cells treated with cAMP analogs.

## Experimental Procedures

**Materials.** [$\alpha$-$^{32}$P]UTP and [$^3$H]uridine were purchased from ICN Biomedicals (Costa Mesa, CA). [$\alpha$-$^{32}$P]dCTP and [$^3$H]UTP were purchased from Amersham Corporation (Arlington Heights, IL). Heat-inactivated horse serum and fetal bovine serum were purchased from Hazelton Biologics Inc. (Lenexa, KS) and Hyclone Laboratories Inc. (Logan, UT), respectively. Cell culture flasks and dishes were Falcon brand. Dexamethasone, 8-Br-cAMP, 8-CPT-cAMP, cycloheximide, and 4-thiouridine were purchased from Sigma Chemical Co. (St. Louis, MO). Affi-Gel 501 (organomercuric agarose resin) was purchased from Bio-Rad Laboratories (Melville, NY). All other chemicals were of the highest purity available from commercial sources.

pTH.4 is a recombinant plasmid containing approximately 400 bp encoding the 3′ end of rat TH mRNA (12). The *Pst*I-*Kpn*I restriction fragment of pTH.4 contains 280 bp corresponding to bp 1240 to bp 1521 of the rat TH cDNA sequence. pTHg6.3 is a recombinant plasmid containing approximately 6300 bp encoding the rat TH gene (5). It encodes sequences of the TH gene extending from approximately −800 bp upstream from the transcriptional start site (at the *Kpn*I site) (21) to the *Eco*RI site in exon 11 of the coding region of the gene cloned into pGEM7Zf. pACT1.1 is a recombinant plasmid containing 1140 bp of the coding region of the mouse $\alpha$-actin cDNA [the PstI restriction fragment described by Minty *et al.* (22)] cloned into pSP64. This probe was a kind gift from Dr. Elena Ciejek (University of Rochester).

**Cell culture conditions.** The PC18 cells are a variant cell line that was isolated from rat pheochromocytoma PC12 cells and were originally described by Tank *et al.* (13). The cells were routinely cultured in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 50 $\mu$g/ml streptomycin, and 50 units/ml penicillin. Cultures were maintained at 37° in a water-saturated atmosphere containing 95% air and 5% $CO_2$. For induction experiments 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 medium supplemented with serum that had been dialyzed extensively against phosphate-buffered saline containing activated charcoal, as described by Tank *et al.* (13). The cells were then cultured in this medium for at least 24 hr before treatment with drugs. All drug treatments were performed in medium supplemented with dialyzed serum, when the cell density had reached 1–2 × $10^5$ cells/cm$^2$.

**Measurement of TH mRNA.** Total cellular RNA from PC18 cells was isolated by the method of Chomczynski and Sacchi (23). RNA concentration was estimated using absorbance at 260 nm (1 absorbance unit = 40 $\mu$g/ml). TH mRNA was measured by hybridization to the radiolabeled 280-bp *Pst*I-*Kpn*I restriction fragment of pTH.4. Previous work showed that this probe recognizes a single 1800–1900-nucleotide TH mRNA species that is present in PC18 cells (14). Northern blot analysis was performed as previously described, to detect degradation of TH mRNA in the samples. TH mRNA levels were routinely quantitated by dot hybridization analysis as described previously (5, 14). To normalize the TH mRNA signals between experiments using the dot hybridization analysis, PC18 cell TH mRNA values were compared with values obtained from a standard curve using known amounts of TH cRNA sense strand synthesized from a Gemini plasmid containing the sequences of TH mRNA present in the cDNA probe. The TH mRNA level in a sample was expressed as pg of TH cRNA sense strand equivalents/$\mu$g of total cellular RNA spotted onto the nitrocellulose. The cDNA was radiolabeled with [$^{32}$P]phosphate to a specific activity of 1–2 × $10^9$ cpm/$\mu$g of DNA, by the random priming procedure of Feinberg and Vogelstein (24).

**Nuclear run-on assays.** The nuclear run-on assays were performed by the method of McKnight and Palmiter (25), as modified by Fossom *et al.* (5). Nuclei were isolated from 10–20 × $10^6$ cells as described by McKnight and Palmiter (25). The nuclear pellet was suspended in 100 $\mu$l of 50% glycerol, 0.1 mM EDTA, 5 mM $MgCl_2$, 50 mM HEPES (pH 7.5), and mixed with 100 $\mu$l of a reaction mixture containing 0.2 M KCl, 8 mM dithiothreitol, 1 mM GTP, 1 mM CTP, 1 mM ATP, [$\alpha$-$^{32}$P]UTP (650 Ci/mmol, 0.08–0.12 mCi/assay), 0.8 mg/ml creatine kinase, 17.6 mM creatine phosphate, and 100 mM HEPES (pH 7.5). The assay was carried out at 24° for 30 min and terminated by chilling in an ice-water bath. The radiolabeled RNA was isolated as described by McKnight and Palmiter (25). An aliquot of RNA was removed for measurement of incorporation of radiolabeled UTP into total nuclear RNA synthesized during the run-on assay (see Table 1A, column 2). This measurement was made using DE81 filter paper, as described by Sambrook *et al.* (26). The remaining RNA was subjected to gel filtration using Sephadex G-50 spun columns to remove unincorporated radiolabeled UTP.

The incorporation of radiolabeled UTP into total nuclear RNA was found to be linear with respect to time (for at least 80 min of incubation at 24°) and with respect to nuclei concentration in the assay (up to at least 1.5 mg of DNA). Furthermore, this incorporation rate was inhibited by 40–45% by the RNA polymerase II inhibitor $\alpha$-amanitin (2.5 $\mu$g/ml). This proportion of total transcriptional activity catalyzed by RNA polymerase II was similar to that found in intact cultured cells (27).

The amount of radiolabeled TH RNA synthesized in the run-on assay was routinely measured by hybridization to a cloned TH genomic probe, pTHg6.3, bound to a nitrocellulose filter. pGEM7Zf bound to nitrocellulose filters was used as a control for hybridization of radiolabeled RNA to bacterial sequences. The hybridizations were carried out using 2.4 $\mu$g of plasmid DNA bound to nitrocellulose filters. Greater amounts of plasmid DNA bound to the filters did not produce an increase in signal. Furthermore, varying the input cpm of radiolabeled total nuclear RNA in the hybridization reactions from 0.2 to 3.0 × $10^6$ cpm did not significantly affect the calculated values for the relative transcription rate of the TH gene (see below for calculations). Hybridization efficiencies were routinely determined by including a known amount of [$^3$H] cRNA sense strand (synthesized from the pTHg6.3 plasmid) in the hybridization reactions. Hybridization efficiencies ranged from 60 to 80% in different experiments; however, the variabil-

ity in hybridization efficiency between hybridization reactions within each experiment was <5%. The relative transcription rates reported in Results were not corrected for these small differences in hybridization efficiency.

Hybridizations were carried out under the conditions described by McKnight and Palmiter (25). Filters were hybridized to radiolabeled RNA for 2–3 days at 45° and then washed once for 2 hr and once for 30 min at 45° with buffer containing 0.3 M NaCl, 3 mM EDTA, 10 mM Tris·HCl (pH 7.5), and 0.1% sodium dodecyl sulfate. The filters were then either dried or subjected to RNase treatment (10 μg/ml RNase A for 30 min at 37°) to remove radiolabeled sequences that were not perfectly complementary to plasmid DNA sequences. Radioactivity that remained hybridized to each filter was measured by Cerenkov counting. Filters were treated as described by McKnight and Palmiter (25) to elute radiolabeled cRNA sense strand for determination of hybridization efficiency. The radiolabeled RNA that hybridized to each filter was divided by the total amount of radiolabeled RNA added to the hybridization reactions (input cpm) and was expressed as ppm. The relative transcription rate of the TH gene was calculated as the difference in the radioactivity hybridized to filters with and without TH cDNA insert.

**Measurement of TH mRNA stability by approach to steady state analysis.** Time course studies were performed to measure the kinetics of the effects of either dexamethasone or cAMP analogs on TH mRNA levels in the PC18 cells. The data obtained from these time courses were then used to calculate the apparent $t_{1/2}$ values for TH mRNA under the different experimental conditions by the approach to steady state analysis, as described by Rodgers *et al.* (28). The apparent $t_{1/2}$ values were calculated from the data obtained from each individual experiment and are presented as the means ± standard errors averaged over these experiments (see Table 4). Alternatively, the TH mRNA values were converted to fold increases over control values, and these values were averaged together over a number of experiments to produce a composite time course from which the apparent $t_{1/2}$ values were calculated (see Figs. 3 and 4).

**Measurement of TH mRNA synthesis and degradation rates in intact PC18 cells using pulse-labeling with 4-thiouridine.** The PC18 cell RNA was thiolabeled in intact cells using 4-thiouridine, as described by Woodford *et al.* (29). The cells were incubated in the presence of 0.1 mM 4-thiouridine for 1 hr. In some experiments [$^3$H] uridine (2–3 μCi/ml) was included during this pulse-labeling period. To estimate the rate of synthesis of TH mRNA, cells were harvested after this pulse-labeling incubation with 4-thiouridine, and total cellular RNA and thiolabeled RNA were isolated. To estimate the rate of degradation of TH mRNA, pulse-chase experiments were performed by removing the medium containing 4-thiouridine and replacing it with medium containing 10 mM uridine and 10 mM cytidine. Cells were harvested at different times after the initiation of this chase, and total cellular RNA and thiolabeled RNA were isolated.

Thiolabeled RNA was isolated by subjecting total cellular RNA to organomercury affinity chromatography using Affi-gel 501, as described by Woodford *et al.* (29). The amount of thiolabeled TH mRNA was measured by dot hybridization analysis as described above and was normalized either to the μg of thiolabeled RNA applied to the nitrocellulose for the rate of synthesis estimates or to 100 μg of total cellular RNA applied to the columns for the rate of degradation estimates.

Only 5–15% of the $^3$H-labeled RNA eluted from the Affi-gel 501 column with the thiolabeled RNA when cells were incubated for 1 hr with 4-thiouridine and [$^3$H]uridine. This percentage of coelution of thiolabeled and radiolabeled RNA was much lower than that reported by Woodford *et al.* (29), who isolated RNA from cells incubated with 4-thiouridine for longer periods of time than those used in our studies. In agreement with these previous studies, we obtained a higher percentage of recovery of radiolabeled RNA with thiolabeled RNA (45–50%) when the cells were incubated with the labeled uridine compounds for 4 hr or more or with a higher concentration (500 μM) of 4-thiouridine. However, for technical (cell viability) and theoretical (minimizing the labeling period, to measure accurately the rate of synthesis of TH mRNA) reasons, our experiments were performed using a 1-hr pulse-labeling period with 0.1 mM 4-thiouridine. The lack of total recovery of newly synthesized radiolabeled RNA with newly synthesized thiolabeled RNA may be due to differences in the rates of synthesis of radiolabeled UTP and thiolabeled UTP, leading to transient differences in the proportional specific activities of the intracellular [$^3$H]UTP and thio-UTP pools, and/or to the inability of the affinity resin to bind RNA molecules that are labeled with thiouridine to only a small extent.

**Statistical analyses.** The data were statistically analyzed using one-way or two-way analysis of variance, where appropriate. Comparisons between different groups were made using the least significance difference test, unless otherwise noted. A level of $p < 0.05$ was considered statistically significant, unless otherwise noted.

## Results

**Effects of different concentrations of dexamethasone and cAMP analogs on TH mRNA levels in PC18 cells.** PC18 cells were incubated in the presence of different concentrations of dexamethasone, 8-Br-cAMP, or 8-CPT-cAMP for 24 hr, at which time the cells were harvested for measurement of TH mRNA levels. Control cells were incubated in the absence of drugs or with the addition of 0.1% ethanol, used as a vehicle for dexamethasone; this concentration of ethanol did not affect TH mRNA levels or TH gene transcription rate at any time point tested. An apparent maximal induction (8–10-fold) of TH mRNA was observed using dexamethasone concentrations of $10^{-7}$ to $10^{-6}$ M (Fig. 1). Half-maximal induction of TH mRNA was observed at approximately 3 nM dexamethasone; half-maximal induction of TH protein was also observed at 3 nM dexamethasone in a previous study (13). Concentrations of 0.1 μM or 1 μM were used in subsequent experiments.

Increasing concentrations of 8-Br-cAMP induced TH mRNA in a concentration-dependent manner, with 3 mM 8-Br-cAMP eliciting a 9-fold induction (Fig. 1). Concentrations higher than

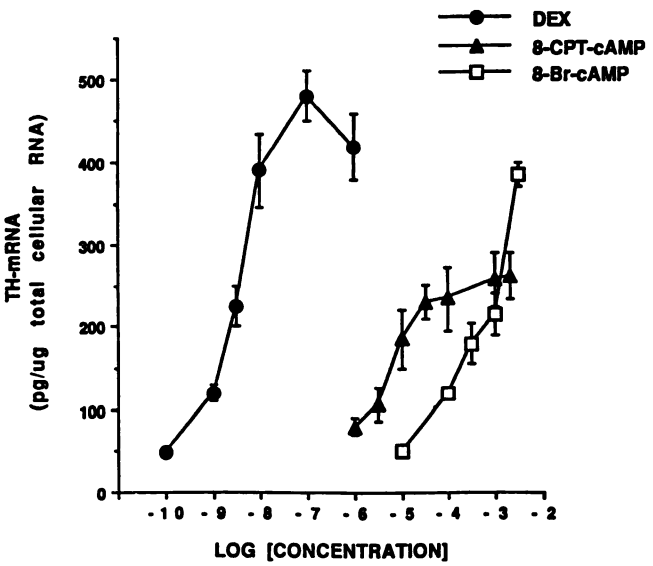


**Fig. 1.** Effects of different concentrations of dexamethasone and cAMP analogs on TH mRNA levels in PC18 cells. Cells were incubated in the presence of different concentrations of dexamethasone (*DEX*), 8-CPT-cAMP, or 8-Br-cAMP for 24 hr. Control cells were left untreated. The TH mRNA levels in the control cells were 57 ± 7 pg/μg of total cellular RNA. The results represent the means ± standard errors from five to seven dishes.

3 mM were not used due to solubility limitations. These results are similar to previously published data on the effect of different concentrations of 8-Br-cAMP on TH protein (13). 8-CPT-cAMP was a more potent cAMP analog than 8-Br-cAMP. An apparent maximal induction of approximately 4-fold was elicited using 30 μM or greater concentrations of 8-CPT-cAMP (Fig. 1). Half-maximal induction was observed at approximately 5 μM 8-CPT-cAMP. Subsequent experiments used 1 mM 8-Br-cAMP or 0.1 mM 8-CPT-cAMP.

**Effects of dexamethasone and cAMP analogs on the transcription rate of the TH gene measured using a nuclear run-on assay.** Cells were untreated or treated for 3 hr with either 0.1% ethanol, 1 μM dexamethasone, 1 mM 8-Br-cAMP, or 0.1 mM 8-CPT-cAMP, and nuclear run-on assays were performed as described in Experimental Procedures. The incorporation of radiolabeled UTP into total nuclear RNA ranged from 2 to 10 pmol/mg of DNA in different experiments. However, treatment with any of the inducing agents described above did not significantly affect this incorporation rate (Table 1A). Interestingly, this rate of incorporation into total nuclear RNA during the run-on assay was much lower than that observed in run-on assays using nuclei isolated from rat adrenal medulla (incorporation rate = 19 ± 2 pmol/mg of DNA) (5). This result suggests that the number of active RNA polymerases bound to the complete genome in adrenal medullary nuclei is significantly greater than that found in nuclei from this tumor cell line.

Nuclei isolated from 10–15 × $10^6$ PC18 cells synthesized enough radiolabeled RNA in the run-on assays (using between 80 and 120 μCi/assay [α-$^{32}$P]UTP) to add 1–3 × $10^6$ cpm of radiolabeled RNA to the hybridization reactions. In most experiments using control PC18 cell nuclei, 200–600 cpm of radiolabeled RNA hybridized to nitrocellulose filters containing pTHg6.3. When normalized to the cpm of radiolabeled total RNA added to the hybridization reactions (input cpm), approximately 200 ppm of radiolabeled RNA hybridized to the pTHg6.3 filters (Table 1B). The radioactivity that hybridized to control filters containing pGEM7Zf usually ranged from 50 to 100 ppm. Hence, the TH gene-specific signals were always at least 2–3 times greater than the nonspecific signals in run-on assays using untreated PC18 cell nuclei. When the nonspecific signals were subtracted from the TH gene-specific signals, the relative transcription rate of the TH gene in control cells was approximately 125 ppm in this set of experiments. Even though the apparent basal TH gene transcription rate (measured without RNase digestion of the filter-bound hybrids) varied significantly between experiments (from approximately 50 ppm to 200 ppm), the fold increases observed in cells treated with either dexamethasone or cAMP analogs were relatively consistent between experiments, regardless of the basal transcription rate. Both 8-Br-cAMP and 8-CPT-cAMP produced 2–3-fold increases in the relative rate of transcription of the TH gene in the PC18 cells, when measured after 3 hr of treatment (Table 1B). Ethanol (0.1%) did not affect the relative transcription rate of the TH gene. However, a 3-hr treatment with dexamethasone (1 μM) produced a 2–3-fold activation of the TH gene (Table 1B).

As an additional check on the specificity of the hybridizations, the filters were treated with RNase after hybridization, to lower background signals and to destroy hybrids that were not totally complementary to the TH gene probe. RNase treatment lowered the nonspecific hybridization signals to near-background levels (10–30 ppm in most experiments). However, this treatment also dramatically decreased the TH gene-specific hybridization signal (3–5-fold decrease in most experiments), indicating that most of the radiolabeled RNA hybridized to the pTHg6.3 filters was not totally complementary to the pTHg6.3 DNA. This result differed from that observed using nuclei isolated from rat adrenal medulla, where RNase treatment of pTHg6.3 filters lowered the TH gene-specific signals by only 30–40% (5). This decrease in TH gene-specific hybridization signal after RNase treatment was presumably partially due to degradation of TH RNA transcripts that contained radiolabeled sequences in the 3′ region of the gene (a region that is not present in pTHg6.3). However, the magnitude of this decrease in signal was rather surprising and might be due to the loss of radiolabeled transcripts that were derived from genes other than the TH gene but that shared partial homology with TH genomic sequences. This hypothesis was supported by experiments in which the nuclear run-on assay was used to measure TH gene transcription rate in cell lines that do not express TH mRNA, such as rat pituitary GH4 cells, simian kidney COS-1 cells, and rat pheochromocytoma PC16 cells. Significant rates

TABLE 1

**Effect of dexamethasone and cAMP analogs on the relative transcription rate of the TH gene**

PC18 cells were treated for 3 hr with either 1 mM 8-Br-cAMP, 0.1 mM 8-CPT-cAMP, or 0.1 μM dexamethasone. Control cells were treated with the same volume of fresh medium lacking the cAMP analog or with the same volume of ethanol (final concentration, 0.1%). Hybridizations were carried out as described in the text, using 2.4 μg of pTHg6.3 or pGEM7Zf to measure either radiolabeled RNA transcripts coding for TH or nonspecific hybridization to bacterial sequences, respectively. A, The amount of radiolabeled UTP incorporated into total nascent RNA during the run-on assay was expressed as the pmol incorporated/mg of nuclear DNA used in the assay. B, The cpm hybridized to the filter-bound plasmid DNAs were divided by the cpm of radiolabeled RNA added to the hybridizations (input cpm) and expressed as ppm. Relative transcription rates were calculated as the ppm hybridized to pTHg6.3 minus the ppm hybridized to pGEM7Zf. The filters were washed and radioactivity was measured without RNase treatment. C, Filters were washed and treated with RNase before measurement of radioactivity. The results represent the means ± standard errors from six to 13 dishes derived from two or three separate experiments.

| A. Treatment | Nuclei | Incorporation of UTP |
|---|---|---|
| | *(mg of DNA/assay)* | *pmol/mg of DNA* |
| Control | 1.0 ± 0.1 | 6.3 ± 1.2 |
| 8-Br-cAMP | 1.1 ± 0.1 | 3.8 ± 1.2 |
| 8-CPT-cAMP | 1.1 ± 0.3 | 9.3 ± 1.1 |
| Control | 1.2 ± 0.1 | 4.3 ± 0.7 |
| Dexamethasone | 1.1 ± 0.1 | 4.5 ± 0.7 |

| B. Treatment | Input radioactivity (×$10^6$) | Radioactivity hybridized to | | Relative TH gene transcription rate | Fold induction |
|---|---|---|---|---|---|
| | | pTHg6.3 | pGEM7Zf | | |
| | *cpm* | *ppm* | | *ppm* | |
| Control | 1.9 ± 0.3 | 207 ± 25 | 79 ± 12 | 128 ± 19 | |
| 8-Br-cAMP | 1.4 ± 0.4 | 453 ± 60[a] | 92 ± 18 | 362 ± 56[a] | 2.8 ± 0.5 |
| 8-CPT-cAMP | 2.5 ± 0.4 | 467 ± 43[a] | 119 ± 21 | 349 ± 39[a] | 2.7 ± 0.3 |
| Control | 2.1 ± 0.3 | 217 ± 22 | 55 ± 4 | 163 ± 20 | |
| Dexamethasone | 2.6 ± 0.5 | 568 ± 62[a] | 106 ± 17 | 462 ± 49[a] | 2.7 ± 0.2 |

| C. Treatment | Radioactivity hybridized to | | Relative TH gene transcription rate | Fold induction |
|---|---|---|---|---|
| | pTHg6.3 | pGEM7Zf | | |
| | *ppm* | | *ppm* | |
| Control | 62 ± 11 | 29 ± 9 | 33 ± 4 | |
| 8-Br-cAMP | 206 ± 45[a] | 46 ± 16 | 161 ± 32[a] | 4.4 ± 0.9 |
| 8-CPT-cAMP | 158 ± 18[a] | 24 ± 5 | 134 ± 21[a] | 4.0 ± 0.6 |
| Control | 53 ± 9 | 11 ± 2 | 42 ± 7 | |
| Dexamethasone | 136 ± 20[a] | 15 ± 2 | 119 ± 18[a] | 2.9 ± 0.2 |

[a] $p < 0.05$, compared with control values.

of TH gene transcription were measured in nuclei from these cell lines before RNase digestion of the filter-bound hybrids; however, after RNase treatment TH gene-specific signals were reduced to background levels in nuclei isolated from these cell lines (data not shown).

Even though the apparent basal TH gene transcription rate was decreased dramatically after RNase treatment of the filter-bound hybrids, TH gene-specific signals that were at least 2–3 times greater than nonspecific signals were always obtained from nuclei isolated from control PC18 cells (Table 1C). Furthermore, 8-Br-cAMP, 8-CPT-cAMP, and dexamethasone increased the relative transcription rate of the TH gene by 3–4-fold, even when the filters were treated with RNase (Table 1C). In all subsequent experiments (unless otherwise noted), RNase-resistant ppm were presented for TH gene transcription rate data, because this radioactivity represented RNA sequences that were completely complementary to the TH gene and because the variability in basal TH gene transcription rate was diminished. However, it should be noted that the fold increases in relative TH gene transcription rate observed in PC18 cells treated with inducing agents were similar whether or not the hybrids were digested with RNase.

**Time courses for the effect of dexamethasone or cAMP analogs on TH gene transcription rate.** Twenty minutes of treatment with dexamethasone produced a 2–3-fold increase in relative TH gene transcription rate (Fig. 2A). This elevation of transcription rate increased slightly to approximately 3-fold after 1 hr of drug treatment and persisted at 3–4-fold greater than controls for at least 24 hr of continuous exposure to dexamethasone (Fig. 2B).

Treatment of PC18 cells with 8-CPT-cAMP produced a rapid 2–3-fold increase in relative TH gene transcription rate after 10–20 min (Fig. 2A). The effect of 8-CPT-cAMP was maximal (4–5-fold increase) after 1 hr of treatment and then decreased to a rate 2–3-fold greater than controls after 6 hr of treatment. Relative TH gene transcription rate remained elevated (2–3-fold) for at least 24 hr in the continual presence of this drug (Fig. 2B). 8-Br-cAMP elicited a 3–5-fold elevation of relative TH gene transcription rate 20 min after its addition to the medium. Longer exposures to 8-Br-cAMP produced a persistent 2–3-fold increase in relative TH gene transcription rate (Fig. 2).

The effects of dexamethasone and the cAMP analogs on TH gene transcription rate in PC18 cells were not global, because the incorporation of radiolabeled UTP into total RNA was not increased by any of these drugs. Furthermore, the relative transcription rate of the $\alpha$-actin gene was not increased by these inducing agents after 3 hr of treatment (controls, 67 ± 7 ppm; dexamethasone-treated cells, 49 ± 10 ppm; 8-Br-cAMP-treated cells, 22 ± 3 ppm). In fact, 8-Br-cAMP significantly decreased the relative actin gene transcription rate approximately 3-fold in PC18 cells.

**Effects of dexamethasone and cAMP analogs on the rate of synthesis of thiolabeled TH mRNA in PC18 cells.** In cells treated with dexamethasone, the fold increase in TH mRNA levels (approximately 10-fold) was much greater than the fold increase in relative TH gene transcription rate (approximately 4-fold) measured by the nuclear run-on assay. Consequently, we measured the rate of synthesis of TH mRNA in PC18 cells using an alternative technique, pulse-labeling. Initial studies suggested that the radiolabeling of TH mRNA

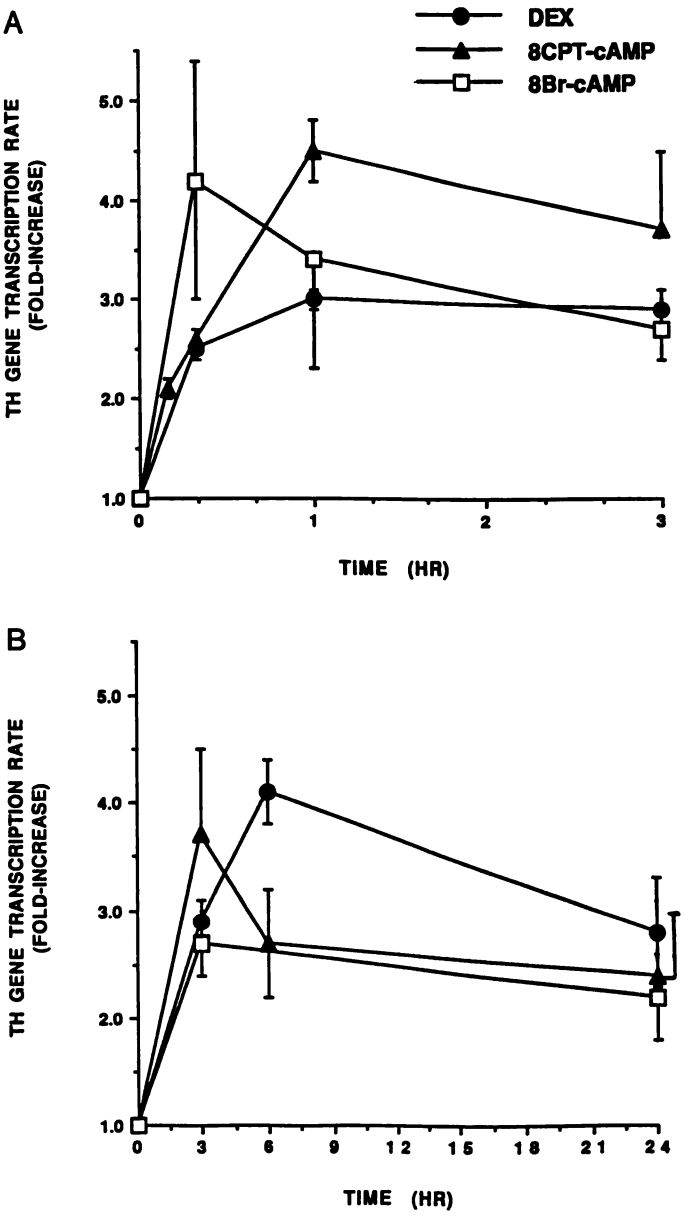


**Fig. 2.** Time courses for the effects of dexamethasone and cAMP analogs on relative TH gene transcription rate in PC18 cells. Cells were incubated in the presence of 0.1 $\mu$M dexamethasone (*DEX*), 0.1 mM 8-CPT-cAMP, or 1 mM 8-Br-cAMP for different periods of time. Control cells remained untreated (or were treated with 0.1% ethanol for the experiments using dexamethasone) and were harvested simultaneously with the treated cells. Relative TH gene transcription rate is presented as the fold increase observed in cells treated with inducing agent over the rate observed in untreated cells. Relative TH gene transcription rates in control cells in the experiments using the different inducing agents were as follows: dexamethasone treatment experiments, 42 ± 8 ppm (11 experiments); 8-CPT-cAMP treatment experiments, 36 ± 5 ppm (eight experiments); 8-Br-cAMP treatment experiments, 120 ± 26 ppm (10 experiments). All of the data in the figures represent radiolabeled hybrids that were treated with RNase, except for the data in the 8-Br-cAMP treatment experiments, for which the hybrids were not subjected to digestion with RNase. For clarity purposes, the time courses are split into two time intervals. A, Time courses from 10 min to 3 hr; B, time courses from 3 hr to 24 hr. The data representing the transcription rates at the 3-hr time points in the two panels are identical. The results represent the means ± standard errors from four to nine dishes for the dexamethasone treatment experiments, from three to five dishes for the 8-CPT-cAMP treatment experiments, and from four to eight dishes for the 8-Br-cAMP treatment experiments.

in PC18 cells using [$^3$H]uridine was not great enough to permit reliable measurement of radiolabeled TH mRNA unless very large amounts of radioactivity were used during the pulse-labeling period. In order to avoid this technical problem, the RNA pools were pulse-thiolabeled by incubating the PC18 cells with 0.1 mM 4-thiouridine for 1 hr. Thiolabeled TH mRNA was then measured by subjecting total cellular RNA to chromatography using organomercury columns, followed by dot hybridization of the thiolabeled RNA to a radiolabeled TH cDNA probe.

A 24-hr treatment with dexamethasone elicited an approximately 12-fold elevation of TH mRNA synthesis (Table 2). This fold increase in TH mRNA synthesis rate was in excellent agreement with the fold induction of TH mRNA elicited by dexamethasone but was significantly greater than the apparent effect of dexamethasone on relative TH gene transcription rate measured by the nuclear run-on assay. Twelve hours of exposure to 8-CPT-cAMP elicited a 3–4-fold increase in the rate of synthesis of TH mRNA (Table 2). This result was in good agreement with both the fold induction of TH mRNA and the fold increase in relative TH gene transcription rate measured by nuclear run-on assays in 8-CPT-cAMP-treated cells.

Neither dexamethasone nor 8-CPT-cAMP affected the incorporation of thiolabeled UTP into total cellular RNA (Table 2). This result suggests that the specific activity of the cellular pool of thio-UTP was not affected by treatment with either of these inducing agents.

**Effect of cycloheximide on the induction of TH mRNA levels and the stimulation of the TH gene by dexamethasone or cAMP analogs in PC18 cells.** In order to determine whether the immediate effects of dexamethasone or cAMP on TH gene transcription rate were direct or required the synthesis of new proteins that regulate TH gene expression, PC18 cells were treated with cycloheximide before exposure to inducing agents. A 1-hr treatment with 1 μg/ml cycloheximide inhibited total protein synthesis in PC18 cells by >90% (data not shown). Consequently, PC18 cells were treated with 1 μg/ml cycloheximide for 1 hr and then treated with inducing agents for 3 hr in the continued presence of cycloheximide. This 4-hr inhibition of protein synthesis did not affect basal TH mRNA levels in PC18 cells; however, cycloheximide dramatically inhibited the increases in TH mRNA levels elicited by either dexamethasone, 8-Br-cAMP, or 8-CPT-cAMP (Table 3). In contrast, cycloheximide lowered the basal relative transcription rate of the TH gene dramatically in PC18 cells (Table 3). Relative TH gene transcription rate was also reduced in cells treated with either dexamethasone or cAMP analogs; however, a 2–4-fold increase in the relative TH gene transcription rate was still observed in cycloheximide-treated cells exposed to these inducing agents, compared with the relative transcription rate observed in cells treated with cycloheximide alone (Table 3). In contrast, a 4-hr treatment with cycloheximide did not significantly reduce total RNA synthesis in the isolated nuclei during the run-on assay [control cells, 6.1 ± 1.4 pmol of UTP incorporated/mg of DNA (three dishes); cycloheximide-treated cells, 5.7 ± 0.6 (three dishes)] nor did this treatment significantly affect the transcription rate of the α-actin gene [actin gene transcription rate in control cells, 67 ± 7 ppm (three dishes); cycloheximide-treated cells, 58 ± 8 (three dishes)].

**Time courses for the effects of dexamethasone and cAMP analogs on TH mRNA levels in PC18 cells.** Previous work showed that in PC18 cells maximal or near-maximal induction of TH mRNA was observed after 24 hr of treatment with dexamethasone and after 6 hr of treatment with 8-Br-cAMP (14). The present results confirm and extend these findings. Dexamethasone produced a significant 2-fold elevation of TH mRNA after 3 hr of treatment (Fig. 3). TH mRNA levels then increased slowly, reaching an apparent maximal level 8–10-fold greater than that observed in control cells after 24 hr of treatment. Approach to steady state kinetic analysis was used to estimate an apparent $t_{1/2}$ for TH mRNA of approximately 8 hr in cells treated with dexamethasone (Fig. 3, *inset*; Table 4).

The time course for the effect of 8-Br-cAMP on TH mRNA levels was significantly different from that observed in dexamethasone-treated cells. TH mRNA levels were near-maximally induced by 8-Br-cAMP after 6 hr of treatment. The kinetics of the induction of TH mRNA elicited by 8-CPT-cAMP were virtually identical to those of the induction elicited

TABLE 2

**Rate of synthesis of TH mRNA in intact PC18 cells treated with dexamethasone or 8-CPT-cAMP**

PC18 cells were treated with either 0.1 μM dexamethasone for 24 hr or 0.1 mM 8-CPT-cAMP for 12 hr. The cells were then treated with 0.1 mM 4-thiouridine for 1 hr in the continued presence of the inducing agents. Control cells were exposed to 4-thiouridine in the absence of inducing agents. The rate of synthesis of total cellular RNA (incorporation of 4-thiouridine into total cellular RNA) was estimated by measuring the μg of thiolabeled RNA isolated from 100 μg of total cellular RNA applied to the Affigel 501 columns. The rate of synthesis of TH mRNA (incorporation of 4-thiouridine into TH mRNA) was estimated by measuring the pg of thiolabeled TH mRNA/μg of thiolabeled RNA used in the hybridization analysis. The results represent the means ± standard errors from the number of dishes designated in parentheses. Data were analyzed by one-way analysis of variance of logarithmically transformed data, followed by two-tailed *F* test of planned comparisons.

| | Incorporation of 4-thiouridine into | |
|---|---|---|
| | Total cellular RNA | TH mRNA |
| | *μg thio-RNA/100 μg RNA* | *pg thio-TH mRNA/μg thio-RNA* |
| Control ($n$ = 5) | 2.0 ± 0.4 | 14 ± 3 |
| Dexamethasone ($n$ = 8) | 2.8 ± 0.2 | 172 ± 17[a] |
| 8-CPT-cAMP ($n$ = 3) | 2.0 ± 0.3 | 54 ± 12[a] |

[a] $p < 0.005$, compared with control values.

TABLE 3

**Effect of cycloheximide on TH mRNA levels and TH gene transcription rate in PC18 cells treated with dexamethasone or 8-CPT-cAMP**

PC18 cells were treated with 1 μg/ml cycloheximide for 1 hr and then treated with either 0.1 μM dexamethasone, 1 mM 8-Br-cAMP, or 0.1 mM 8-CPT-cAMP for 3 hr in the continued presence of cycloheximide. Control cells remained untreated. The data represent the means ± standard errors from three dishes.

| Treatment | TH mRNA levels | Relative TH gene transcription rate |
|---|---|---|
| | *pg/μg of total cellular RNA* | *ppm* |
| Experiment 1 | | |
| None | 38 ± 6 | 170 ± 31 |
| Cycloheximide | 42 ± 5 | 48 ± 5[a] |
| 8-Br-cAMP | 87 ± 7[a] | 470 ± 30[a] |
| 8-Br-cAMP + cycloheximide | 50 ± 3 | 110 ± 12[b] |
| Dexamethasone | 72 ± 6[a] | 420 ± 57[a] |
| Dexamethasone + cycloheximide | 39 ± 4 | 150 ± 4[b] |
| Experiment 2 | | |
| None | 47 ± 10 | 180 ± 24 |
| Cycloheximide | 43 ± 6 | 98 ± 9[a] |
| 8-CPT-cAMP | 110 ± 4[a] | 520 ± 64[a] |
| 8-CPT-cAMP + cycloheximide | 66 ± 7 | 350 ± 12[a,b] |
| Dexamethasone | 130 ± 31[a] | 480 ± 31[a] |
| Dexamethasone + cycloheximide | 84 ± 19 | 340 ± 23[a,b] |

[a] $p < 0.05$, compared with control cells.
[b] $p < 0.05$, compared with cells treated only with cycloheximide.

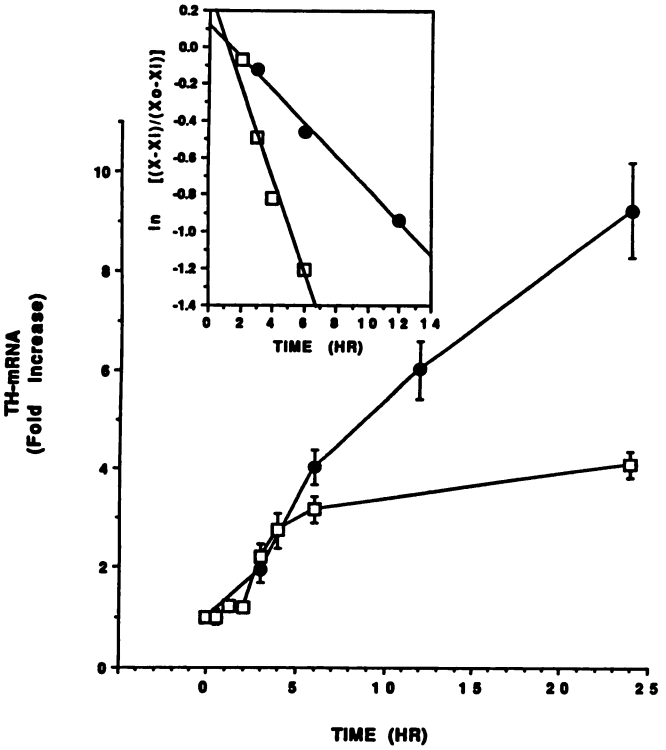


**Fig. 3.** Time courses for the effects of dexamethasone (●) and 8-Br-cAMP on TH mRNA levels in PC18 cells. Cells were treated with 0.1 μM dexamethasone (○) or 1 mM 8-Br-cAMP and harvested at the appropriate times after treatment. Control cells remained untreated but were cultured for the same periods of time as were cells treated with inducing agents. TH mRNA levels are expressed as the fold increases over control levels measured in untreated cells. Control levels of TH mRNA were 16 ± 2 pg/μg of RNA (15 dishes) from three separate experiments using dexamethasone and 24 ± 6 pg/μg of RNA (31 dishes) from eight separate experiments using 8-Br-cAMP. The data represent the means ± standard errors obtained from eight to 13 dishes for the experiments using dexamethasone and 13 to 33 dishes for the experiments using 8-Br-cAMP. *Inset*, time course data after approach to steady state analysis. The TH mRNA levels presented on the *y*-axis are as follows: *X*, TH mRNA levels at any time point; *Xo*, steady state TH mRNA levels in control cells; *Xi*, steady state TH mRNA levels in cells treated for 24 hr with either dexamethasone or 8-Br-cAMP. For the experiments using dexamethasone, linear regression analysis ($r^2$ = 0.99) estimates $t_{½}$ of 7.7 hr. For the experiments using 8-Br-cAMP, linear regression analysis ($r^2$ = 0.96) estimates $t_{½}$ of 2.5 hr.

TABLE 4

**Summary of the half-life values for TH mRNA measured by either approach to steady state kinetic analysis or pulse-chase analysis**

Apparent half-life values were determined as described in the text, in untreated PC18 cells or cells treated with 0.1 μM dexamethasone, 1 mM 8-Br-cAMP, or 0.1 mM 8-CPT-cAMP. The results represent means ± standard errors (or mean ± range for *n* = 2) obtained from the number of experiments designated in parentheses. Data were analyed by one-way analysis of variance, followed by two-tailed *F* test of planned comparisons.

| | Apparent half-life values | |
|---|---|---|
| | Approach to steady state analysis | Pulse-chase analysis |
| | *hr* | |
| Control | 8.6 ± 0.4 (*n* = 4) | 8.6 ± 4.4 (*n* = 2) |
| Dexamethasone | 8.0 ± 0.6 (*n* = 3) | 8.0 ± 2.2 (*n* = 2) |
| 8-Br-cAMP | 3.1 ± 1.1[a] (*n* = 3) | ND[b] |
| 8-CPT-cAMP | 3.3 ± 1.2[a] (*n* = 2) | 8.5 ± 1.5 (*n* = 2) |

[a] $p < 0.005$, compared with control values.
[b] ND, not determined.

by 8-Br-cAMP (data not shown). If the assumptions of the approach to steady state kinetic model are met (see Discussion), then this difference in the time courses for TH mRNA induction by these two different drugs suggests that the half-life of TH mRNA is different in PC18 cells treated with cAMP analogs than in those treated with dexamethasone. Approach to steady state analysis estimates an apparent $t_{½}$ of approximately 3 hr in PC18 cells treated with either 8-Br-cAMP or 8-CPT-cAMP (Fig. 3; Table 4).

In order to estimate an apparent $t_{½}$ for TH mRNA in control PC18 cells using approach to steady state kinetic analysis, cells were treated with either 0.1 μM dexamethasone for 24 hr or 1 mM 8-Br-cAMP for 12 hr, and then the medium containing the inducing agent was removed and replaced with fresh medium lacking the inducing agent. Cells were harvested for measurement of TH mRNA at different times after the washout of the inducing agents. In order to demonstrate that the washout procedure was effective for the 8-Br-cAMP-treated cells, cAMP levels were measured in PC18 cells before and 15 min after the washout. cAMP was measured by the radioimmunoassay procedure of Harper and Brooker (30). Elevated cAMP levels decreased back to levels approaching those observed in control cells 15 min after the removal of the drug (cAMP levels, in nmol/mg of protein: control cells, 0.10 ± 0.01; before washout, 3.5 ± 0.3; 15 min after washout, 0.20 ± 0.03; means ± standard errors from three to five dishes). We did not measure directly the effectiveness of the washout in the dexamethasone-treated cells; however, because TH mRNA levels decreased dramatically after removal of the medium containing dexamethasone (Fig. 4), it was reasonable to assume that the washout of the steroid hormone was essentially effective. These deinduction time courses demonstrated that TH mRNA levels remained relatively unchanged for 3–6 hr and then decreased slowly to levels approaching those observed in control cells by 20–30 hr after removal of the inducing agents (Fig. 4). Approach to steady state analysis estimates an apparent $t_{½}$ of 6–9 hr for TH mRNA in control PC18 cells independently of whether 8-Br-cAMP or dexamethasone was used as an inducing agent (Fig. 4).

**Determination of TH mRNA stability using pulse-chase analysis of thiolabeled TH mRNA.** In order to estimate TH mRNA stability in control and drug-treated PC18 cells by a different method than approach to steady state analysis, pulse-chase studies were performed by thiolabeling the RNA in the PC18 cells using 4-thiouridine and then chasing the thiolabel using high concentrations of uridine and cytidine. The loss in the amount of thiolabeled TH mRNA over time after the chase yields an estimate of the apparent $t_{½}$ of TH mRNA in the cells. After the addition of unlabeled uridine and cytidine (time 0 in Fig. 5), thiolabeled TH mRNA levels increased slightly. This paradoxical increase was observed to varying degrees in all the pulse-chase experiments, reaching a maximum between 3 and 9 hr. Presumably, this 3–9 hr period represented the time required for the chase conditions (10 mM uridine and 10 mM cytidine) to dilute out the thiolabeled UTP from the cellular UTP pools. Consequently, apparent $t_{½}$ values were calculated from the data representing the time points after this initial 3–9-hr effective chase period. In dexamethasone-treated cells thiolabeled TH mRNA levels decreased at a rate that predicted an apparent $t_{½}$ of 6–10 hr (Fig. 5; Table 4). Similarly, thiolabeled TH mRNA levels decreased with an

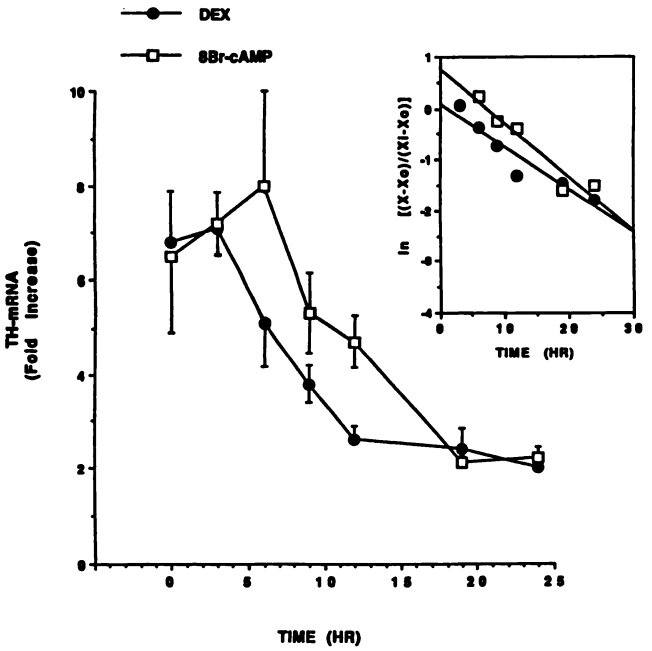


Fig. 4. Time courses for the deinduction of TH mRNA levels after the removal of dexamethasone or 8-Br-cAMP from the culture medium. Cells were treated for 12 hr with 1 mM 8-Br-cAMP or for 24 hr with 0.1 μM dexamethasone (*DEX*). The medium was then removed and replaced with fresh medium lacking the inducing agent. Cells were then harvested at the appropriate times after this change in medium. TH mRNA levels were expressed as the fold increases over control levels measured in untreated cells; control levels were 22 ± 5 pg/μg RNA (24 dishes) averaged over four different experiments. The TH mRNA levels presented at different time points in the figure represent the means ± standard errors obtained from eight to 18 dishes averaged over at least two separate experiments with each inducing agent. *Inset*, time course data after approach to steady state analysis. The TH mRNA levels presented on the *y*-axis are as follows: *X*, TH mRNA levels at any time point; *Xo*, steady state TH mRNA levels in control cells; *Xi*, steady state TH mRNA levels in cells treated for 12 hr with 8-Br-cAMP or for 24 hr with dexamethasone. Linear regression analysis estimates $t_{½}$ of 8.3 hr ($r^2 = 0.91$) for deinduction after removal of dexamethasone and $t_{½}$ of 6.6 hr ($r^2 = 0.91$) for deinduction after removal of 8-Br-cAMP.

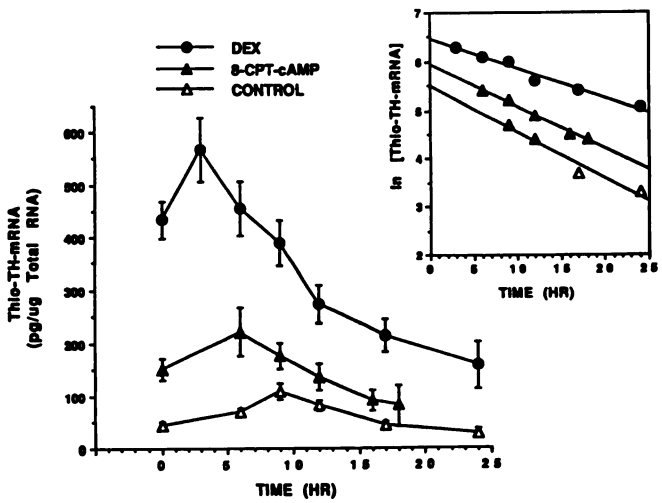


Fig. 5. Pulse-chase analysis of TH mRNA stability in untreated PC18 cells and cells treated with dexamethasone or 8-CPT-cAMP. Cells were cultured in the presence of 1 μM dexamethasone (*DEX*) or 0.1 mM 8-CPT-cAMP for 24 or 12 hr, respectively. Control cells remained untreated during this time period. 4-Thiouridine (0.1 mM) was then added to the medium. One hour later the medium was changed and the chase was initiated by the addition of 10 mM uridine and 10 mM cytidine. When appropriate, inducing agents remained in the medium during both the pulse-labeling and the chase. Cells were harvested at the initiation (0 time point in the figure) and at different times after the initiation of the chase. Thiolabeled TH mRNA is expressed as pg/100 μg of total cellular RNA applied to the BioGel 501 column. Each value represents the mean ± standard error from five to eight dishes averaged over at least two separate experiments. *Inset*, logarithmic transformation of these data according to first-order kinetic analysis of the degradation rates. Linear regression analysis estimates the $t_{½}$ values for thiolabeled TH mRNA as follows: control cells, 5.7 hr ($r^2 = 0.99$); dexamethasone-treated cells, 10 hr ($r^2 = 0.98$); 8-CPT-cAMP-treated cells, 7.7 hr ($r^2 = 0.99$).

apparent $t_{½}$ of approximately 7–10 hr in PC18 cells that were treated with 0.1 mM 8-CPT-cAMP (Fig. 5; Table 4). When pulse-chase studies were performed on untreated PC18 cells, an apparent $t_{½}$ of approximately 8 hr for thiolabeled TH mRNA was estimated.

## Discussion

TH mRNA levels are induced in a concentration-dependent manner by the synthetic glucocorticoid dexamethasone ($EC_{50} \approx 3$ nM) and by the cAMP analogs 8-CPT-cAMP ($EC_{50} \approx 5$ μM) and 8-Br-cAMP ($EC_{50}$ was not determined). These concentration-response curves for dexamethasone and 8-Br-cAMP are in excellent agreement with those reported previously for the effects of these inducing agents on TH protein levels in PC18 cells (13). In a previous study (15) cAMP-elevating agents and glucocorticoids were shown to induce TH mRNA in rat pheochromocytoma PC8b cells at least partially by stimulating the TH gene; however, it was not clear in that earlier study whether the increases in TH gene transcription rate totally accounted for the increases in TH mRNA levels. In the present study the effects of these inducing agents on TH mRNA levels and TH gene transcription rate have been carefully compared to determine whether these effects are transcriptional or post-transcriptional.

The transcription rate of the TH gene was estimated by two different procedures; 1) nuclear run-on assays were used to measure directly the relative TH gene transcription rates in nuclei derived from PC18 cells and 2) pulse-labeling experiments using 4-thiouridine were used to measure the relative rates of synthesis of thiolabeled TH mRNA in intact PC18 cells. Using the nuclear run-on assay, we have shown that the relative transcription rate of the TH gene increases rapidly (within 20 min) after the addition of either dexamethasone or cAMP analogs to the culture medium. The transcription rate remains elevated for at least 24 hr during continual exposure to these inducing agents. These time courses are similar to those reported for the effects of dexamethasone or forskolin on TH gene transcription rate in PC8b cells (15). *In vivo* studies have shown that the TH gene in rat adrenal medulla is stimulated 2–3-fold within 10 min after the injection of nicotine and remains elevated when nicotine is administered repeatedly (5). Because nicotine elevates blood glucocorticoid levels and raises cAMP in rat adrenal medulla, it is possible that these inducing agents at least partially mediate this response of the gene *in vivo*.

In cells treated with either 1 mM 8-Br-cAMP or 0.1 mM 8-CPT-cAMP, TH mRNA levels increase 3–4-fold after at least 6 hr of treatment (Figs. 1 and 3). These fold increases agree reasonably well with the fold increases in relative TH gene transcription rate observed in cells treated with the cAMP

analogs (Fig. 2; Table 1). In contrast, in cells treated with dexamethasone TH mRNA levels increase approximately 10-fold after 24 hr of treatment (Figs. 1 and 3), whereas relative TH gene transcription rate as measured by the nuclear run-on assay increases only 3–4-fold in dexamethasone-treated cells (Fig. 2; Table 1). In order to investigate further this discrepancy, the effects of these inducing agents on the rate of synthesis of TH mRNA in intact PC18 cells have been evaluated. The relative rate of synthesis of TH mRNA is increased 3–4-fold after 12 hr of treatment with 8-CPT-cAMP and approximately 12-fold after 24 hr of treatment with dexamethasone. These fold increases in TH mRNA rates of synthesis are in excellent agreement with the fold increases in TH mRNA levels observed at these time points.

The results discussed above support the hypothesis that the major effect of cAMP and glucocorticoid on TH mRNA levels is on the transcription rate of the TH gene. The reason that dexamethasone elevates the apparent transcription rate of the TH gene by only 3–4-fold, as measured by the nuclear run-on assay, but elevates TH mRNA synthesis in the intact PC18 cells by 12-fold, as measured by pulse-labeling, is not clear. Inherent limitations of the nuclear run-on assay suggest a number of possible explanations for this discrepancy. This assay measures the relative number of RNA polymerases bound to a specific gene in the intact cell, presumably reflecting the initiation rate for the gene. Thus, in addition to increasing the initiation rate for the TH gene 3–4-fold, dexamethasone may also regulate the elongation rate of the primary TH gene transcript (as has been demonstrated for the phosphoenolpyruvate carboxykinase gene) (31), the rate of processing of the primary transcript, or the stability of nuclear TH RNA transcripts. These effects would not be measured by the nuclear run-on assay but would be measured by the pulse-labeling technique using the intact cells.

In contrast to the relatively large number of studies that have investigated the transcriptional regulation of TH gene expression, very few studies have tested the possibility that TH mRNA levels may be regulated by post-transcriptional processes and, to our knowledge, no accurate estimation of the half-life of TH mRNA is available in the literature. Vyas *et al.* (19) have attempted to measure TH mRNA half-life in PC12 cells after blockade of transcription with actinomycin D. Their preliminary analysis does not permit an accurate estimate of the $t_{1/2}$ value but suggests that TH mRNA is very stable in the PC12 cells ($t_{1/2} \approx$ 10–16 hr). Their work also suggests that phorbol esters may regulate the stability of TH mRNA. In a more recent report Craviso *et al.* (18) have shown that the nicotinic agonist dimethylphenylpiperazinium elevates TH mRNA in bovine adrenal chromaffin cells for prolonged periods of time (1–4 days), even though TH gene transcription rate increases for only 1–2 hr. These results are consistent with the hypothesis that nicotinic receptor stimulation elevates TH mRNA by both transcriptional and post-transcriptional processes.

In the present study we have measured the apparent $t_{1/2}$ of TH mRNA in PC18 cells and evaluated the effects of dexamethasone and cAMP analogs on this apparent $t_{1/2}$. A number of approaches to measure TH mRNA half-life have been attempted in our laboratory. Actinomycin D (at a concentration necessary to inhibit total RNA synthesis by 90%) is toxic to the PC18 cells. Hence, measuring the decline of TH mRNA levels after the addition of actinomycin D yields variable results that are difficult to interpret.

Approach to steady state kinetic analysis is a technically simple method to estimate mRNA half-life values; however, it assumes a specific kinetic model (28). The rate constants are assumed to be zero-order for synthesis, $k_s$, and first-order for degradation, $k_d$, and these constants are assumed to change rapidly from the values that determine the initial steady state level of the mRNA in the control cells to the values that determine the steady state level that exists in cells treated with inducing agents. Based upon these assumptions, this analysis predicts that the rate of change from initial to induced steady state levels depends only on the value of the $k_d$ for the specific mRNA at the new (induced) steady state. Thus, the steady state $k_d$ value for TH mRNA in PC18 cells treated with inducing agents is calculated from the time course of change in TH mRNA levels from control to induced steady states. Similarly, the basal $k_d$ is calculated from the time course of change in TH mRNA levels from induced to basal steady states during deinduction.

For the induction time courses most of the assumptions discussed above are reasonably well satisfied. The nuclear run-on assays demonstrate that the $k_s$ increases relatively rapidly (within 20 min) after the addition of cAMP analogs or dexamethasone to the culture medium. Also, the level of TH mRNA in the untreated cells remains constant over the 24-hr time period used in these experiments, and the level of TH mRNA increases to a new constant steady state after 6 or 24 hr of treatment with either 8-Br-cAMP or dexamethasone, respectively (14) (Fig. 3). Thus, the level of TH mRNA at time 0 relative to the addition of inducing agent represents an initial steady state value and the level of TH mRNA at 12–24 hr represents a new induced, steady state value. This analysis predicts apparent $t_{1/2}$ values for TH mRNA of approximately 8 hr for cells treated with dexamethasone and approximately 3 hr for cells treated with either 8-Br-cAMP or 8-CPT-cAMP.

In contrast, in the deinduction studies relative TH gene transcription rate, as measured by the nuclear run-on assay, decreases back to control values relatively slowly after the removal of either dexamethasone or 8-Br-cAMP (within 1–3 hr) (data not shown). This slow change in TH gene transcription rate is presumably a reflection of the time required to dephosphorylate or disassemble the transcriptional factors that have been recruited to stimulate the TH gene. Alternatively, it may be due to slow diffusion of the inducing agents out of the cell; however, in the case of 8-Br-cAMP, this latter possibility is unlikely, because cAMP levels decrease back to approximately control levels within 15 min after the washout of the cAMP analog. Nevertheless, it is likely that this slow change in $k_s$ accounts for the 3–6-hr lag that occurs before the decline in TH mRNA levels in the deinduction time courses (see Fig. 4). With these caveats in mind, the approach to steady state analysis of these deinduction kinetics estimates an apparent $t_{1/2}$ value of approximately 6–9 hr for TH mRNA in control PC18 cells.

We have also used classical pulse-chase procedures using 4-thiouridine as a labeling agent to measure TH mRNA stability. First-order kinetic analysis of the decline in thiolabeled TH mRNA levels estimates an apparent $t_{1/2}$ value of approximately 8 hr for TH mRNA in cells treated with either dexamethasone or 8-CPT-cAMP. The apparent $t_{1/2}$ value for TH mRNA meas-

ured by pulse-chase analysis in untreated PC18 cells is also approximately 8 hr. These apparent $t_{1/2}$ values measured by pulse-chase analysis may be slight underestimates of the true $t_{1/2}$ values, due to the dilution of the chased thiolabeled TH mRNA during cell division. PC18 cells divide approximately once every 30 hr at the cell density used in these experiments. If we correct the apparent $t_{1/2}$ values measured by pulse-chase analysis (Table 4) for cell division, as described by Rodgers *et al.* (28) [$t_{1/2}$ = apparent $t_{1/2}$ ($T_d$/($T_d$ − apparent $t_{1/2}$)), where $T_d$ is the division rate of the cells (30 hr)], the corrected $t_{1/2}$ values are as follows: control cells, 14 ± 9 hr; dexamethasone-treated cells, 11 ± 4 hr; 8-Br-cAMP-treated cells, 11 ± 2 hr. However, even these corrected values represent only estimates, because reutilization of thiolabeled UTP during the chase period may result in an overestimation of the true $t_{1/2}$ values. Pertinent to these sources of error, it is important to note that 1) neither dexamethasone nor 8-Br-cAMP affects PC18 cell division rate and 2) the incorporation of thiolabeled UTP into total cellular RNA is similar in control cells and in treated cells (Table 2), suggesting that the thio-UTP pools in the PC18 cells are not affected by these inducing agents. Hence, even though the apparent $t_{1/2}$ value for TH mRNA measured by the pulse chase analysis may be only a rough estimate of the true $t_{1/2}$ value in the PC18 cells, the pulse-chase results support the hypothesis that prolonged treatment with either dexamethasone or 8-Br-cAMP does not affect the rate of degradation of TH mRNA.

Considering the limitations of the two different protocols used to measure TH mRNA stability, the apparent $t_{1/2}$ values for TH mRNA in untreated PC18 cells and in cells treated with dexamethasone are in excellent agreement, regardless of which protocol is used (Table 4). Taken together, the data suggest that the apparent $t_{1/2}$ for TH mRNA under basal culture conditions is approximately 6–9 hr and that dexamethasone does not exert an apparent effect on the stability of TH mRNA in PC18 cells. In contrast, the effect of cAMP analogs on TH mRNA stability is more complex. The pulse-chase analysis predicts that 8-CPT-cAMP does not alter TH mRNA stability in cells treated for 12 hr with this drug. However, the approach to steady state analysis suggests that the apparent $t_{1/2}$ of TH mRNA is significantly decreased in PC18 cells treated with cAMP analogs. This discrepancy in the apparent $t_{1/2}$ values predicted by pulse-chase analysis or approach to steady state analysis for TH mRNA in cAMP-treated cells may be due to the inherent limitations of these protocols. This possibility seems unlikely, because the two protocols estimate similar apparent $t_{1/2}$ values for untreated and dexamethasone-treated cells. Alternatively, the approach to steady state analysis measures changes in $k_d$ that occur very rapidly after treatment of the cells with the inducing agent, whereas the pulse-chase analysis measures $k_d$ values after the cells have been exposed to the inducing agent for a prolonged period of time. Consequently, it is possible that the elevation of cAMP rapidly decreases the stability of TH mRNA but that this effect is transient, such that, after prolonged exposure to elevated cAMP, TH mRNA stability returns to that observed under basal conditions. This paradoxical decrease in TH mRNA stability may work to elevate TH mRNA to a new steady state level more rapidly after the elevation of cAMP than would occur if the $t_{1/2}$ for TH mRNA maintained its normal value of 6–9 hr. Pertinent to this hypothesis, it is interesting to note that, even though TH gene transcription rate is elevated only 2–3-fold after 6–24 hr of exposure to these cAMP analogs, the transcription rate increases transiently 4–5-fold after exposure to these drugs (Fig. 2). This relatively large transient stimulation of the TH gene is also observed in PC7e cells treated with forskolin (15). It is possible that this relatively large transient stimulation of the TH gene compensates for the transient decrease in TH mRNA stability elicited by cAMP-elevating agents. Further work is required to test this hypothesis. However, it is interesting to note that adrenal medullary TH mRNA levels *in vivo* are elevated rapidly (within 3 hr) by nicotine or stress (5, 17). These results suggest that the half-life of TH mRNA in the adrenal medulla of rats treated with these stimuli may be similar to that observed in the PC18 cells treated with cAMP analogs.

Finally, the results of the experiments using cycloheximide suggest that protein factors that are rapidly degraded play a role in maintaining the basal rate of transcription of the TH gene in the PC18 cells. In agreement with these results, Lewis and Chikaraishi (32) stated that cycloheximide lowers the basal transcription rate of the TH gene in PC7e cells, even though their results were not presented in detail. Interestingly, cycloheximide does not lower TH mRNA levels in PC18 cells treated for 4 hr with the drug. This lack of effect is presumably due to the relatively long half-life of TH mRNA. In contrast, Stachowiak *et al.* (33) have shown that cycloheximide slightly elevates TH mRNA in bovine adrenal chromaffin cells after 12 hr of treatment. It is not clear from their results whether shorter incubations with cycloheximide induce TH mRNA, nor have they measured the effect of the protein synthesis inhibitor on TH gene transcription rate. Our results suggest that measuring the effect of a protein synthesis inhibitor on TH mRNA levels may not be representative of its effect on TH gene transcription rate.

A number of studies have recently shown that inducible transcription factors that bind to the AP1 site of the TH gene promoter may mediate the response of the gene to different inducing agents (33, 34). Our studies using cycloheximide in PC18 cells do not rule out the hypothesis that inducible transcription factors may be involved in the regulation of the TH gene by cAMP or glucocorticoid. However, our studies suggest that the immediate effects of these inducing agents are directly on the TH gene and do not require ongoing protein synthesis. The major apparent effect of cycloheximide in the PC18 cells is on the basal transcription rate of the TH gene.

In summary, our results suggest that the primary mechanism by which glucocorticoid and cAMP elevate TH mRNA is via stimulation of the TH gene and that this stimulation does not require ongoing protein synthesis. Glucocorticoid may also regulate other processes involved in the rate of synthesis of TH mRNA; however, this conclusion requires further investigation. The apparent $t_{1/2}$ of TH mRNA in PC18 cells is approximately 6–9 hr. Glucocorticoid and prolonged elevation of cAMP do not significantly alter the stability of TH mRNA. However, our results suggest that a rapid but transient effect of cAMP may be to decrease the stability of TH mRNA.

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