The induction, desensitization and de-induction of tyrosine aminotransferase by 8-bromo-cyclic AMP in rat hepatoma cells

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Addition of 1–3 mM-8-bromo-cyclic AMP to monolayer cultures of H-4 rat hepatoma cells resulted in a rapid but short-lived increase in tyrosine aminotransferase (EC 2.6.1.5) activity. The transient nature of this induction is due to desensitization to 8-bromo-cyclic AMP. Throughout this time course of induction and desensitization, removal of 8-bromo-cyclic AMP resulted in a rapid and significant decrease in tyrosine aminotransferase activity, a process referred to as ‘de-induction’ in this study. We showed that the changes in tyrosine aminotransferase activity in its induction, desensitization and de-induction by 8-bromo-cyclic AMP were directly attributable to changes in the synthesis rate of the protein, and the amount of translatable and hybridizable mRNA encoding for tyrosine aminotransferase (mRNA^{TAT}). We further showed that this desensitization was specific to cyclic AMP. First, only active analogues of cyclic AMP and agents which increased cellular concentrations of cyclic AMP elicited this desensitization. Second, the desensitized cells were refractory only to the effects of 8-bromo-cyclic AMP; dexamethasone and insulin induced the tyrosine aminotransferase activity in the 8-bromo-cyclic AMP-desensitized cells in a manner similar to that of the controls. Studies on the metabolism of 8-bromo-cyclic AMP suggest that neither its degradation nor the accumulation of its primary metabolite, 8-bromoadenosine, played a significant role in modulating the expression of tyrosine aminotransferase during the time course of action of 8-bromo-cyclic AMP. These results provide evidence for a specific pretranslational mode of action of cyclic AMP in the control of tyrosine aminotransferase expression in its desensitization and de-induction, in addition to the early phase of induction.

INTRODUCTION

The addition of an active analogue of cyclic AMP (e.g. dibutyryl cyclic AMP or 8-bromo-cyclic AMP) to hepatoma-cell culture or its injection into rats causes a rapid and transient increase in the expression of tyrosine aminotransferase. We previously reported that 8-bromo-cyclic AMP increases the amount of translatable mRNA^{TAT} in H-4 rat hepatoma cells; this effect can account for the increase in the synthesis rate and activity of tyrosine aminotransferase during the early phase of induction of the enzyme [1]. Further direct evidence for a pretranslational mode of action of cyclic AMP in the regulation of tyrosine aminotransferase induction was obtained by using the cloned cDNA^{TAT} probe to quantify the amount of mRNA^{TAT} in rat liver after injections of dibutyryl cyclic AMP [2]. Finally, there is also evidence that dibutyryl cyclic AMP injected into rats causes a rapid increase in the transcription rate of mRNA^{TAT} as assayed in isolated rat liver nuclei run-off experiments [3].

These results demonstrate that cyclic AMP increases the transcription rate of the tyrosine aminotransferase gene, and that this effect of cyclic AMP accounts for the early phase of induction of tyrosine aminotransferase activity in both rat liver and rat hepatoma cells. However,

the biochemical event(s) subserving the transiency of the induction (whether exhaustion of the inducer, desensitization of the cells, or a secondary post-transcriptional effect of cyclic AMP on tyrosine aminotransferase expression) remains to be determined. For example, it has been reported that repeated injection of dibutyryl cyclic AMP into adrenalectomized rats modifies the translational activity of a basal amount of mRNA^{TAT} [4,5].

To gain a better understanding of the biochemical processes underlying the transitory nature of the cyclic AMP-evoked induction of tyrosine aminotransferase and to determine whether cyclic AMP has multiple, perhaps temporally separated, effects in the control of tyrosine aminotransferase expression, we examined the regulation of tyrosine aminotransferase expression by the addition and re-addition of 8-bromo-cyclic AMP to and its removal from H-4 hepatoma cells. We determined the rates of synthesis and degradation of tyrosine aminotransferase, and quantified the amount of translatable and hybridizable mRNA^{TAT} to evaluate if they, either singly or in combination, could account for the observed changes in tyrosine aminotransferase activity during its desensitization and de-induction, in addition to the early phase of induction. We further analysed the metabolism of 8-bromo-cyclic AMP, and identified and

Abbreviations used: mRNA^{TAT}, mRNA for tyrosine aminotransferase; mRNA^{PEPCK}, mRNA for phosphoenolpyruvate carboxykinase.

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quantified its metabolites to determine if they may contribute to the regulation of tyrosine aminotransferase during the course of action of 8-bromo-cyclic AMP.

MATERIALS AND METHODS

Materials

8-Bromo-cyclic AMP and 8-bromo-cyclic [^3H]AMP were prepared respectively from cyclic AMP and cyclic [^3H]AMP by methods described by Muneyama *et al.* [6]. T.l.c. plates used for the analysis of cyclic nucleotides were obtained from Eastman Kodak Co., Buffalo, NY, U.S.A., and were developed in a solvent system of butanol-ol/acetic acid/water (5:2:3, by vol.). Rabbit antiserum against purified rat liver tyrosine aminotransferase was prepared as previously described [1]. Rabbit antiserum against rat liver phosphoenolpyruvate carboxykinase was generously supplied by Dr. P. Iynedjian [7]. A slant of *Escherichia coli* 5K harbouring the plasmid pcTAT-3, a 600-base-pair cDNA clone of tyrosine aminotransferase spliced into the *Pst*I site of pBR322, was kindly provided by Dr. Gunther Schutz of the German Cancer Research Centre, Heidelberg, Germany [2]. The phosphodiesterase inhibitor RO-1724/1 was a gift from Dr. W. E. Scott of Hoffmann-La Roche, Nutley, NJ, U.S.A. Forskolin was obtained from Calbiochem, San Diego, CA, U.S.A. Other chemicals and biochemicals, unless specified, were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Tissue-culture supplies were obtained from Gibco, Grand Island, NY, U.S.A.

Cell culture

The H-4 rat hepatoma cell line is a derivative of line H4-II-E of Pitot *et al.* [8], adapted to grow *in vitro* from the Reuber H-35 hepatoma. The cell line used in this study was obtained from Dr. A. Pardee of the Sidney Farber Cancer Institute, Harvard Medical School. The cells were grown as monolayer cultures in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) foetal-calf serum, 50 units of penicillin/ml and 50 μg of streptomycin/ml. Cells were subcultured at confluency by trypsin treatment and were seeded at a density of 0.1×10^4 – 1×10^4 cells/cm 2 .

For the experiments described in this study, H-4 hepatoma cells at an early stationary phase of growth were serum-deprived by incubating them in serum-free DMEM for 12–16 h before the start of an experiment. All experiments were carried out under serum-free conditions.

Induction and assay of tyrosine aminotransferase activity

8-Bromo-cyclic AMP was added to designated plates of H-4 hepatoma cells to a final concentration as indicated (generally in the range 1–3 mM, which in dose-response studies were shown to give a near-maximal to maximal increase in tyrosine aminotransferase activity). Cells were harvested at predetermined time intervals; samples of the cell homogenates were used to assay for tyrosine aminotransferase activity by procedures previously described [9,10]. Protein concentration was determined by the method of Lowry *et al.* [11], with bovine serum albumin as the standard.

Determination of the rates of synthesis and degradation of tyrosine aminotransferase

To determine the relative rate of synthesis of tyrosine aminotransferase during the course of its transient induction, desensitization and de-induction by 8-bromo-cyclic AMP, cells were pulse-labelled with 80–100 μCi of [^{35}S]methionine/ml (sp. radioactivity 1000–2000 Ci/mmol) in methionine-free DMEM during the last 20 min of incubation before harvest. The relative rate of synthesis of tyrosine aminotransferase, determined by the incorporation of [^{35}S]methionine into tyrosine aminotransferase (measured by the immunoprecipitation technique described previously [1]), was expressed as a percentage of the radioactivity incorporated into total soluble protein.

A pulse-chase technique was used to measure the turnover rate of tyrosine aminotransferase by methods described previously [1]. Briefly, cells were labelled with 20–30 μCi of [^{35}S]methionine/ml for 4 h; after a 30 min wash-out period, the rate of decay of ^{35}S -labelled tyrosine aminotransferase was measured as a function of the time of chase with excess methionine.

Cytoplasmic RNA extraction and cell-free translation

Cytoplasmic RNA was extracted from H-4 hepatoma cells by the procedures of Ricciardi *et al.* [12] as previously described [1]. Concentration of RNA solution was determined by taking readings of A_{260} and A_{280} and assuming that 1 mg of RNA/ml gave 25 A_{260} units [13]. Cell-free translation of cytoplasmic RNA was carried out as described by Pelham & Jackson [14] and Hunt *et al.* [15], in a message-dependent rabbit reticulocyte-lysate protein-synthesis system. The reaction mixture (total volume 20 μl) contained 400 μg of cytoplasmic RNA/ml, 625 μCi of [^{35}S]methionine/ml (sp. radioactivity 1000–2000 Ci/mmol) and reticulocyte lysate supplemented with 10 mM-phosphocreatine, 100 mM-potassium acetate, 0.5 mM-magnesium acetate, 0.05 mM of all of the amino acids except methionine, and 0.5 mM-dithiothreitol. The translation *in vitro* was carried out at 30 °C for 90 min. The amount of radioactivity incorporated into the 50000-Da tyrosine aminotransferase relative to that incorporated into total translational product was determined by procedures described previously [1].

Determination of the amount of mRNA^{TAT} by hybridization to [^{32}P]cDNA probe

A cDNA probe of tyrosine aminotransferase (pcTAT-3) was used to quantify the amount of hybridizable mRNA^{TAT} in a dot-blot analysis. The utility of the pcTAT-3 in determining the amount and size of mRNA^{TAT} has previously been published [2].

Various amounts of RNA (up to 20 μg) in a constant volume (5 μl) were spotted on to a $20 \times \text{SSC}$ ($1 \times \text{SSC} = 0.15 \text{ M-NaCl}/0.015 \text{ M-sodium citrate}$, pH 7.0)-pretreated and dried nitrocellulose membrane [16,17]. The membrane was baked for 2 h at 80 °C *in vacuo* and then incubated for 60 min at 45 °C in a prehybridization mixture containing 50% (v/v) formamide, $5 \times \text{TEN}$ ($1 \times \text{TEN} = 10 \text{ mM-Tris}$, pH 7.4, 1 mM-EDTA and 100 mM-NaCl), 0.1 M-sodium phosphate (pH 7.0), 0.1% sodium pyrophosphate, 0.1% SDS, 50 μg of denatured salmon sperm DNA/ml, and $2.5 \times \text{Denhardt's solution}$ ($1 \times \text{Denhardt's solution} = 0.2\%$

each of bovine serum albumin, polyvinylpyrrolidone 360 and Ficoll 400) [18]. The membrane was then transferred to a hybridization solution composed of $> 10^6$ c.p.m. of denatured nick-translated pcTAT-3 ($> 10^8$ c.p.m./ μ g of DNA)/ml and 10% dextran in the prehybridization mixture supplemented with $1 \times$ Denhardt's solution. Hybridization was carried out at 45°C overnight. The membrane was then washed three times at 50°C in $0.1 \times \text{TEN}/0.1\%$ sodium pyrophosphate/ 10 mM -sodium phosphate (pH 7.0)/ 0.1% SDS, followed by a wash at room temperature with $0.1 \times \text{TEN}$. The membrane was air-dried and exposed to X-ray film with an intensifying screen at -70°C . For quantification of the relative amount of radioactivity of the dots, the fluorogram was scanned with a densitometer, and the A_{600} readings were used to determine the relative abundance of mRNA^{TAT} . A control experiment comparing this densitometric method of measurement with the method of liquid-scintillation spectrometry revealed a correlation coefficient of 0.998 for dots with $< 0.8 A_{600}$ unit above the film's background.

Phosphodiesterase assay

Phosphodiesterase activity present in homogenates of the H-4 hepatoma cells was assayed by measuring the production of $[\text{H}]$ adenosine from cyclic $[\text{H}]$ AMP by methods previously described [19] (exogenous nucleotidase was used to cleave the phosphate from $5'$ - $[\text{H}]$ AMP, the initial product of the phosphodiesterase reaction when cyclic $[\text{H}]$ AMP was used as the substrate).

RESULTS

Fig. 1 depicts the time course of change in the activity and synthesis rate of tyrosine aminotransferase activity on the addition and re-addition of 8-bromo-cyclic AMP to, and its removal from, monolayer cultures of H-4 hepatoma cells. The result is representative of a large number of experiments carried out over a 1-year period. The addition of 1 mM -8-bromo-cyclic AMP to H-4 hepatoma cells resulted in a rapid and transient increase in the activity and synthesis of tyrosine aminotransferase (Fig. 1). Re-addition of fresh 8-bromo-cyclic AMP to the cell culture after the induction had reached a peak failed to evoke increases in the activity and synthesis of tyrosine aminotransferase in a manner comparable with that of the untreated cells (Fig. 1). Furthermore, conditioned media recovered from cells at 16 h after addition of 1 mM -8-bromo-cyclic AMP, a time when tyrosine aminotransferase activity had fallen back to a basal value, induced tyrosine aminotransferase activity in control cell cultures (results not shown). Together, these results suggest that the transitory, rather than sustained, induction of tyrosine aminotransferase by 8-bromo-cyclic AMP is due to desensitization of the cells towards stimulation by 8-bromo-cyclic AMP. At all time points after the addition of 8-bromo-cyclic AMP, removal of the inducer gave a marked and rapid decrease in the activity and synthesis rate of tyrosine aminotransferase (Fig. 1), a process referred to as 'de-induction' in this study. Analysis of the turnover of tyrosine aminotransferase in control ($t_1 = 1.8 \pm 0.2 \text{ h}$), 8-bromo-cyclic AMP-induced ($2.2 \pm 0.4 \text{ h}$), desensitized ($1.8 \pm 0.3 \text{ h}$) and de-induced ($2.0 \pm 0.3 \text{ h}$) cell cultures revealed no significant difference. These results suggest that the primary action of 8-bromo-cyclic AMP in the

Fig. 1. Time course of effect of 8-bromo-cyclic AMP on the synthesis rate and activity of tyrosine aminotransferase in its induction, desensitization and de-induction

(a) Fluorogram illustrating the incorporation of $[\text{H}]$ -methionine into the 50000-Da tyrosine aminotransferase at various times of treatment with 1 mM -8-bromo-cyclic AMP. (b) Determination of the time course of change in the rate of synthesis (▲, △) and activity (●, ○) of tyrosine aminotransferase. The relative rate of synthesis of tyrosine aminotransferase was determined by pulse-labelling cells, at various time points of treatment with 8-bromo-cyclic AMP, with $100 \mu\text{Ci}$ of $[\text{H}]$ -methionine/ml for 20 min immediately before harvest. Samples were processed for immunoprecipitation of tyrosine aminotransferase; the methods of immunoprecipitation and the specificity of the antiserum against tyrosine aminotransferase were previously described [1]. The bands other than the 50000-Da tyrosine aminotransferase were also observed when the immunoprecipitation reaction was done with the pre-immune serum [1]. Samples 1, 2, 3, 4 and 5 in (a) and (b) were obtained from cells incubated with 1 mM -8-bromo-cyclic AMP for 0, 2, 4, 8 and 16 h respectively (▲ in Fig. 1b). Sample 6 was from cells treated for 16 h with 1 mM -8-bromo-cyclic AMP, followed by the re-addition and incubation with 1 mM -8-bromo-cyclic AMP for 2 h more (—△ in Fig. 1b; desensitized). Samples 7 and 8 represent cells from which 8-bromo-cyclic AMP was removed 16 h after its addition and followed by incubation in inducer-free medium for an additional 2 and 6 h respectively (—△ in Fig. 1b; de-induced). Corresponding changes in the activity of tyrosine aminotransferase at various time points after the addition (●) and re-addition (—○) of 8-bromo-cyclic AMP to, and its removal from (—○), cultures of the H-4 hepatoma cells are illustrated in Fig. 1(b). The positions of the M_r markers and their M_r values and of tyrosine aminotransferase (TAT) are indicated.

Fig. 2. Autoradiogram illustrating the concerted induction, desensitization and de-induction of translatable messages encoding for tyrosine aminotransferase and phosphoenolpyruvate carboxykinase by 8-bromo-cyclic AMP in H-4 hepatoma cells

Groups of five 100 mm-diam. plates of cells were used for each of the conditions. Samples 1–8 represent RNA from cells treated under conditions as described in the legend of Fig. 1. Total cytoplasmic RNA was isolated and translated in a message-dependent rabbit reticulocyte-lysate protein-synthesis system as previously described [1]. The translation of mRNA^{TAT} and mRNA^{PEPCK} to their respective protein products was monitored by the techniques of immunoprecipitation, SDS/polyacrylamide-gel electrophoresis and fluorography. The specificity of the phosphoenolpyruvate carboxykinase antiserum, provided by Dr. P. Iynedjian, was previously described [7]. The arrows identify the positions on the gel of the 50000-Da tyrosine aminotransferase (TAT) and the 65000-Da phosphoenolpyruvate carboxykinase (PEPCK). The amounts of translatable mRNA^{TAT} in samples 1–8 were 0.0354, 0.0811, 0.0523, 0.0530, 0.014, 0.016, 0.010 and 0.013% of that of total translatable message activity. The corresponding values for mRNA^{PEPCK} were 0.0510, 0.211, 0.172, 0.136, 0.068, 0.082, 0.02 and 0.01% respectively.

control of tyrosine aminotransferase expression in its induction, desensitization and de-induction is on the synthesis rather than the degradation of the protein.

In separate experiments, the amount of mRNA encoding tyrosine aminotransferase was determined by cell-free translation in a rabbit reticulocyte lysate system (Fig. 2) and by hybridization to a cDNA probe of tyrosine aminotransferase (Fig. 3). Results showed that 8-bromo-cyclic AMP gave a rapid and transient increase in the amount of translatable (Fig. 2, samples 1–5) and hybridizable (Fig. 3, samples 1–5) mRNA^{TAT}; re-addition of 8-bromo-cyclic AMP 16 h after its initial addition failed to elicit another increase in the amount of the message (Figs. 2 and 3, sample 6); lastly, removal of 8-bromo-cyclic AMP 16 h after its initial addition caused a further decrease in the amount of translatable (Fig. 2, samples 7 and 8) and hybridizable mRNA^{TAT} (Fig. 3, samples 7 and 8). It is noteworthy that changes in the amount of hybridizable mRNA^{TAT} are directly correlated with the amount of translatable mRNA^{TAT} and the synthesis rate of tyrosine aminotransferase (Fig. 3).

Using a double-immunoprecipitation protocol with antibodies against tyrosine aminotransferase and phosphoenolpyruvate carboxykinase, we showed that 8-

Fig. 3. Quantification of the amount of mRNA^{TAT} by dot-blot hybridization, and its correlation with the synthesis rate of tyrosine aminotransferase and the activity of mRNA^{TAT}

(a) Autoradiogram of a dot-blot of mRNA^{TAT}. Increasing amounts of RNA (1–5 μ l) in a constant volume (5 μ l) were spotted on to a nitrocellulose membrane. Samples 1–8 represent RNA from cells treated under conditions identical with those described in the legends of Figs. 1 and 2. For quantification of the amount of mRNA^{TAT}, the amount of RNA present in the serially diluted samples applied to the nitrocellulose membrane was plotted against the intensity of the dot on the autoradiogram. The slope of the line was calculated by linear regression and normalized against the slope of the control. Using this method, we obtained values of 1, 3.75, 3.0, 1.9, 1.29, 1.4, 0.55 and 0.25 relative units of hybridizable mRNA^{TAT} for samples 1–8 of the autoradiogram illustrated in Fig. 3(a). (b) Correlation of the relative amount of hybridizable mRNA^{TAT} and the relative rate of synthesis of tyrosine aminotransferase. Results for the synthesis rate were obtained from Fig. 1 ($r = 0.977$). (c) Correlation of the amounts of hybridizable and translatable mRNA^{TAT}. Results for the amount of translatable mRNA^{TAT} were from Fig. 2 ($r = 0.887$).

bromo-cyclic AMP regulated the amount of translatable message encoding for phosphoenolpyruvate carboxykinase and tyrosine aminotransferase in a similar manner, although the absolute amount of mRNA^{PEPCK} was significantly greater than that of mRNA^{TAT} (Fig. 2). Previous studies have provided clear evidence of a transcriptional effect of cyclic AMP in the control of phosphoenolpyruvate carboxykinase gene expression.

Experiments were done to determine the specificity of the desensitization process and to evaluate if the metabolism of 8-bromo-cyclic AMP may contribute to the observed changes in tyrosine aminotransferase

Table 1. Specificity of agents that caused the desensitization of tyrosine aminotransferase induction

H-4 cells were incubated with the various compounds for 20 and 22 h in Expts. 1 and 2 respectively. Cells were then rinsed twice with phosphate-buffered saline (150 mM-NaCl/10 mM-sodium phosphate, pH 7.4), and fresh medium with and without 8-bromo-cyclic AMP was added to the cells to test for sensitivity towards stimulation by 8-bromo-cyclic AMP. Cells were incubated for 4 h before harvest. Results are expressed as the fold increase in tyrosine aminotransferase activity in the 8-bromo-cyclic AMP-induced cell culture over that of the control (i.e. with and without 8-bromo-cyclic AMP, respectively, during the last 4 h of incubation).

Expt. no.	Treatment	Increase in TAT activity (fold)
1. 20 h pre-treatment	Control	3.3
	3 mM-8-bromo-cyclic AMP	0.8
	5 mM-dibutylryl cyclic AMP	1.1
	5 mM-AMP	3.4
	10 mM-Butyrate	3.8
2. 22 h pre-treatment	Control	4.6
	1 mM-8-bromo-cyclic AMP	1.3
	1 mM-8-Bromo-cyclic AMP+0.7 mM-RO 20-1724/1	1.0
	20 μ M-Forskolin+0.7 mM-Ro 20-1724/1	1.1
	1 mM-Cyclic AMP	3.1
	100 μ M-8-bromoadenosine	4.8

expression in its induction and desensitization. Results in Table 1 show that only active analogues of cyclic AMP, including 8-bromo-cyclic AMP and dibutylryl cyclic AMP, and agents that increase cellular cyclic AMP concentration by activating adenylate cyclase and inhibiting phosphodiesterase, were able to evoke this desensitization phenomenon. Structurally related compounds, such as 5'-AMP (5 mM), butyrate (10 mM), cyclic AMP (1 mM) or 8-bromoadenosine (100 μ M), had no effect either in inducing tyrosine aminotransferase or in evoking the desensitization of tyrosine aminotransferase induction towards 8-bromo-cyclic AMP. In addition, the results in Table 2 showed that the 8-bromo-cyclic AMP-desensitized cells were refractory only to homologous inducers (e.g. 8-bromo-cyclic AMP or dibutylryl cyclic AMP); dexamethasone and insulin gave 2–4-fold increase in tyrosine aminotransferase activity in the desensitized cells, a magnitude comparable with that of the untreated controls.

The hydrolysis of cyclic AMP to 5'-AMP is catalysed by phosphodiesterase. In several previous studies, it was demonstrated that the activity of phosphodiesterase can undergo adaptive regulation. Cyclic AMP-mediated induction of phosphodiesterase activity has been reported in L-cell fibroblasts [20], 3T3 cells [21], S-49 lymphoma cells [22], C6 glioma cells [23], astrocytoma cells [24] and

Table 2. Induction of tyrosine aminotransferase by 8-bromo-cyclic AMP, dexamethasone and insulin in control and 8-bromo-cyclic AMP-pretreated (desensitized) H-4 rat hepatoma cells

Control and 8-bromo-cyclic AMP-pretreated (1.5 mM, 16 h) cultures were used. Cells were rinsed once and replenished with fresh medium. 8-Bromo-cyclic AMP, dexamethasone and insulin were added to designated plates of cells, to final concentrations of 1 mM, 1 μ M and 3 munits/ml respectively. Cells were incubated for 6 h at 37 °C and then harvested. Results are expressed as the fold increase in tyrosine aminotransferase in the inducer-treated cells over that of the controls during the last 6 h of incubation.

Treatment . . . Cells	Increase in TAT activity (fold)		
	8-Bromo-cyclic AMP (1 mM)	Dexa-methasone (1 μ M)	Insulin (3 munits/ml)
Control	3.2	3.5	2.4
8-Bromo-cyclic AMP-pretreated	0.9	3.3	2.6

neuroblastoma cells [25]. These results raise the possibility that enhanced metabolism of 8-bromo-cyclic AMP may account for, at least in part, the desensitization of tyrosine aminotransferase induction to 8-bromo-cyclic AMP. In this connection, we assayed for phosphodiesterase activity in extracts of control and 8-bromo-cyclic AMP-desensitized (1 mM for 16 h) H-4 hepatoma cells under conditions that allowed for assessment of the high- K_m and low- K_m enzyme activities. The phosphodiesterase activities present in extracts of the control and of the 8-bromo-cyclic AMP-desensitized cells were 17.2 ± 2 and 16.2 ± 1.2 pmol of cyclic AMP hydrolysed/min per mg of protein when assayed with 0.5 μ M-cyclic AMP (low- K_m enzyme activity), and were 234 ± 25 and 178 ± 32 pmol/min per mg of protein when assayed with 100 μ M-cyclic AMP (high- K_m enzyme activity).

The possibility that metabolites of 8-bromo-cyclic AMP may modulate tyrosine aminotransferase activity during the course of action of 8-bromo-cyclic AMP was evaluated. T.l.c. analysis of medium and extract of cells incubated with 1 mM-8-bromo-cyclic [3 H]AMP (sp. radioactivity 2.5 Ci/mol) revealed that the primary metabolite of 8-bromo-cyclic [3 H]AMP under these experimental conditions was 8-bromo[3 H]adenosine. A 16 h incubation with 1 mM-8-bromo-cyclic [3 H]AMP resulted in the accumulation of 6–17 μ M-8-bromo-[3 H]adenosine in the incubation medium, depending on the state of confluency of the cell cultures used. This amounted to less than 2% of the 8-bromo-cyclic [3 H]AMP added. This accumulation of 8-bromo-adenosine in the medium was inhibited by 89% by the addition of 0.7 mM of the phosphodiesterase inhibitor RO 20-1724/1. The amount of radioactivity present in cell extracts represented only approx. 0.1–0.2% of the total radioactivity added, of which only a trace amount was identified as 8-bromoadenosine.

Analysis of the effects of 8-bromoadenosine on tyrosine aminotransferase activity showed that it gave a dose-

Fig. 4. Effect of 8-bromoadenosine on the control and 8-bromo-cyclic AMP-induced tyrosine aminotransferase activity in H-4 hepatoma cell

Increasing concentrations of 8-bromoadenosine were added to H-4 hepatoma cells with and without 1 mM-8-bromo-cyclic AMP. Cells were incubated for 4 h at 37 °C before harvest for tyrosine aminotransferase assay. The concentration of 8-bromoadenosine needed to give 50 % inhibition (I_{50}) of the 8-bromo-cyclic AMP-dependent increase in tyrosine aminotransferase activity was calculated to be 135 μ M. The results represent means of five separate experiments; the bars represent S.D. Inset: result obtained from a representative experiment demonstrating the 8-bromoadenosine-dose-dependent inhibition of basal (—) and 8-bromo-cyclic AMP-induced (+) tyrosine aminotransferase activities in H-4 hepatoma cells.

dependent decrease in both the basal and 8-bromo-cyclic AMP-induced tyrosine aminotransferase activity in H-4 hepatoma cells (Fig. 4). The average concentration of 8-bromoadenosine, determined from five separate experiments, needed to give 50 % inhibition (I_{50}) of the 8-bromo-cyclic AMP-dependent increase in tyrosine aminotransferase activity was 135 μ M. This concentration of 8-bromoadenosine was significantly higher than that found in the culture medium of cells incubated with 1 mM-8-bromo-cyclic AMP for 16 h (< 20 μ M). In separate experiments, we determined that 8-bromoadenosine selectively increased the degradation rate of tyrosine aminotransferase without decreasing its rate of synthesis; the half-life of tyrosine aminotransferase in the control and in 100 μ M-8-bromoadenosine-treated H-4 cell cultures was 1.8 ± 0.3 h and 0.9 ± 0.2 h respectively. This effect of 8-bromoadenosine on the turnover of tyrosine aminotransferase may underlie its inhibition of tyrosine aminotransferase activity (Fig. 4). 8-Bromoadenosine had little or no effect on the general pattern and rate of total protein synthesis, nor did it significantly affect the degradation rate of total soluble proteins (results not shown).

Together, these results suggest that 8-bromo-cyclic AMP has a specific and intrinsic effect on the regulation of tyrosine aminotransferase expression during its induction, desensitization and de-induction, and that neither its degradation by phosphodiesterase nor the accumulation of its metabolite contributed significantly to the regulation of tyrosine aminotransferase expression under these conditions.

DISCUSSION

In this study, we present our observation of three temporally distinct effects of 8-bromo-cyclic AMP on the regulation of tyrosine aminotransferase expression in H-4 rat hepatoma cells; these effects are induction, desensitization and de-induction.

The possibility that the metabolism of 8-bromo-cyclic AMP and the accumulation of its metabolites account for or contribute to changes in tyrosine aminotransferase activity was investigated. There was no difference in the phosphodiesterase activity of control cells and of cells treated with 1 mM-8-bromo-cyclic AMP for 16 h (desensitized), suggesting that the transient induction of tyrosine aminotransferase activity and its desensitization cannot be attributed to enhanced degradation of the inducer. 8-Bromoadenosine was identified as the major, if not the only, catabolite of 8-bromo-cyclic AMP in both the medium and the extract of cells treated with 8-bromo-cyclic AMP, and it seemed possible that it could contribute to the observed transient induction and desensitization of tyrosine aminotransferase activity by 8-bromo-cyclic AMP. This hypothesis seemed particularly relevant, since at least some of the biological effects of cyclic nucleotides have been shown to be attributed to their metabolites. For example, the growth-inhibitory effects of dibutyryl cyclic AMP in hepatoma cells is primarily attributable to its adenosine catabolites [26]. In another study, it was reported that the 5'-AMP derivatives of both 8-bromo-cyclic AMP and N^6 -monobutyryl cyclic AMP are as potent as the cyclic nucleotides in inhibiting growth of six different cell lines [27]. In our study, we found that pharmacological doses of 8-bromoadenosine increased the turnover rate of tyrosine aminotransferase. In addition, we found that the following compounds, listed in an order of decreasing potency, inhibited tyrosine aminotransferase activity and increased the degradation of the enzyme in a manner similar to that of 8-bromoadenosine: papaverine $\gg$ 8-bromoadenosine $>$ N^6 -(L-2-phenylisopropyl)adenosine = 8-bromo-AMP $>$ 3-isobutyl-1-methylxanthine = adenosine. The inclusion of 5 μ M-erythro-9-(2-hydroxy-3-nonyl)adenine, a potent inhibitor of adenosine deaminase, had no effect by itself, but potentiated the effects of adenosine in inhibiting tyrosine aminotransferase activity, lowering the I_{50} from 1 mM to 200 μ M. Similar results have been obtained by other investigators. For example, in HTC hepatoma cells, theophylline, 3-isobutyl-1-methylxanthine, RO 20-1724/1 and papaverine increase tyrosine aminotransferase turnover [28]. Methylthioinosine, a cytotoxic purine nucleoside, was also found to decrease tyrosine aminotransferase activity in H-35 hepatoma cells by specifically increasing the degradation of tyrosine aminotransferase [29]. The mechanism of action of these agents remains to be determined.

It is clear, however, that under our experimental conditions the accumulation of 8-bromoadenosine cannot account for the desensitization of tyrosine aminotransferase induction by 8-bromo-cyclic AMP. (i) At the normal concentration found in medium of cells incubated with 1 mM-8-bromo-cyclic AMP (< 20 μ M), 8-bromoadenosine had only a marginal effect on tyrosine aminotransferase activity. (ii) The 8-bromo-cyclic AMP-evoked changes in tyrosine aminotransferase activity during its induction, desensitization and de-induction

were directly attributable to corresponding changes in the relative synthesis rate of the protein, whereas 8-bromoadenosine specifically increased the degradation rate of tyrosine aminotransferase without decreasing its rate of synthesis. (iii) The phosphodiesterase inhibitor RO 20-1724/1 (0.7 mM) inhibited the accumulation of 8-bromoadenosine by > 89 %, yet it had no significant effect on the pattern or time course of the 8-bromo-cyclic AMP-evoked transient induction and desensitization of tyrosine aminotransferase in H-4 hepatoma cells.

In our analysis, we found that the 8-bromo-cyclic AMP-evoked changes in tyrosine aminotransferase activity in its induction, desensitization and de-induction can be directly attributed to corresponding changes in its rate of synthesis and in the amount of mRNA^{TAT}. Our results do not provide evidence for a translational effect of cyclic AMP in the control of tyrosine aminotransferase expression. Furthermore, the possibility that cyclic AMP may have temporally separated effects on the pre-translational and translational events of tyrosine aminotransferase expression [4,5] cannot be supported by the results of this study. The thesis of a pretranslational regulation of tyrosine aminotransferase expression by 8-bromo-cyclic AMP is also supported by corresponding changes in the amount of translatable message encoding phosphoenolpyruvate carboxykinase under these experimental conditions. Previous studies from several laboratories have provided clear evidence that cyclic AMP exerts its primary effect on phosphoenolpyruvate carboxykinase induction at the nuclear, gene-transcription, level [30–32].

It has not escaped our attention that the desensitization of tyrosine aminotransferase induction may be attributable to changes in the amount and activity of cyclic AMP-dependent protein kinase. Quantification of the regulatory and catalytic subunits of cyclic AMP-dependent kinase showed significant decreases in extracts obtained from 8-bromo-cyclic AMP-treated H-4 hepatoma cells as compared with that of the untreated control. Thus the amounts of the regulatory-subunit protein, as determined by the photoactivated incorporation of 8-azido-cyclic [³²P]AMP, in cytosol of H-4 cells pretreated with 1 mM-8-bromo-cyclic AMP for 4 and 16 h, were 65 % and 32 % of that of the untreated control respectively. The corresponding values for the catalytic-subunit protein, assayed by the total amount of cyclic AMP-dependent phosphotransferase activity, were 40 % and 20 % of that of the untreated control. It is conceivable that this decrease in cytosolic cyclic AMP-dependent protein kinase may underlie the attenuated induction of tyrosine aminotransferase by 8-bromo-cyclic AMP in cells that were pretreated with the analogue. However, our analysis was limited to cytosolic cyclic AMP-dependent protein kinase; the possibilities that the kinase may be translocated from the cytosol to nuclear fraction, or that the decrease in cytosolic cyclic AMP-dependent protein kinase may represent an artifact *in vitro*, need to be carefully evaluated.

Portions of this work were submitted as part of the Ph.D. thesis of J.D.S. This work was supported by research grants from the National Science Foundation (PCM 84-41349 and DCB 84-17775), and Busch Memorial Funds.

REFERENCES

1. Culpepper, J. A. & Liu, A. Y.-C. (1983) *J. Biol. Chem.* **258**, 13812–13819
2. Scherer, G., Schmid, W., Strange, C. M., Rowekamp, W. & Schutz, G. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7205–7208
3. Hashimoto, S., Schmid, W. & Schutz, G. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6637–6641
4. Granner, D., Noguchi, T. & Diesterhaft, M. (1981) *Adv. Cyclic Nucleotide Res.* **14**, 259–537
5. Noguchi, T., Diesterhaft, M. & Granner, D. (1982) *J. Biol. Chem.* **257**, 2386–2390
6. Muneyama, K., Bauer, R. J., Schuman, D. A., Robins, R. K. & Simon, L. N. (1971) *Biochemistry* **10**, 2390–2395
7. Iynedjian, P. & Hanson, R. W. (1977) *J. Biol. Chem.* **252**, 655–662
8. Pitot, H. C., Peraino, C., Morse, P. A. & Potter, V. R. (1964) *Natl. Cancer Inst. Monogr.* **12**, 229–246
9. Diamondstone, T. I. (1966) *Anal. Biochem.* **16**, 395–401
10. Liu, A. Y.-C. (1980) *J. Biol. Chem.* **255**, 4421–4429
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
12. Ricciardi, R. P., Miller, J. S. & Roberts, B. E. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4927–4931
13. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning*, p. 468, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
14. Pelham, H. R. B. & Jackson, R. J. (1976) *Eur. J. Biochem.* **67**, 247–256
15. Hunt, T., Vanderhoff, G. & London, I. M. (1972) *J. Mol. Biol.* **66**, 471–481
16. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5201–5205
17. Thomas, P. S. (1982) *Methods Enzymol.* **100**, 255–266
18. Denhardt, D. T. (1966) *Biochem. Biophys. Res. Commun.* **23**, 641–646
19. Thompson, W. J., Terasaki, W. G., Epstein, P. M. & Strada, S. J. (1979) *Adv. Cyclic Nucleotide Res.* **10**, 69–92
20. Maganiello, V. & Vaughan, M. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 269–273
21. D'Armiento, M., Johnson, G. S. & Pastan, I. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 459–462
22. Bourne, H. R., Tomkins, G. M. & Dion, S. (1973) *Science* **183**, 952–954
23. Schwartz, J. P. & Passonneau, J. V. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3844–3848
24. Uzunov, P., Shein, H. M. & Weiss, B. (1972) *Science* **180**, 304–306
25. Liu, A. Y.-C., Chan, T. & Chen, K. Y. (1981) *Cancer Res.* **41**, 4579–4587
26. Hargrove, J. L. & Granner, D. K. (1982) *J. Cell. Physiol.* **111**, 232–238
27. Martin, T. F. J. & Kowalchuk, J. A. (1981) *Science* **213**, 1120–1122
28. Stellwagen, R. H., Sailor, R. D. & Kohli, K. K. (1977) *Biochem. Biophys. Res. Commun.* **78**, 1162–1167
29. Koontz, J. W. & Wicks, W. D. (1984) *J. Biol. Chem.* **259**, 929–934
30. Cimbala, M. A., Lamers, W. H., Nelson, K., Monahan, J. E., Yoo-Warren, H. & Hanson, R. W. (1982) *J. Biol. Chem.* **257**, 7629–7636
31. Lamers, W. H., Hanson, R. W. & Meisner, H. M. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5137–5141
32. Chrapkiewicz, N. B., Beale, E. C. & Granner, D. K. (1982) *J. Biol. Chem.* **257**, 14428–14432