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Regulation of Hepatoma Tissue Culture Cell Tyrosine Aminotransferase Messenger Ribonucleic Acid by Dexamethasone†

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ABSTRACT: A maximally effective concentration of dexamethasone causes an 8–10-fold increase in the steady-state values of $mRNA^{TAT}$ [from 0.04 to 0.40% of total poly(A+) RNA activity], the in vivo rate of synthesis of tyrosine aminotransferase (0.02–0.19%), and tyrosine aminotransferase catalytic activity (9–90 milliunits/mg) in rat hepatoma tissue culture (HTC) cells. Concentrations of dexamethasone which result in different steady-state levels of induction of tyrosine aminotransferase result in varying, but always proportional, changes in these three functions. Finally, variant HTC cells, which have a lower basal level of tyrosine aminotransferase than wild type cells and in which tyrosine aminotransferase is not induced in response to glucocorticoids, have proportionately lower basal $mRNA^{TAT}$ levels and show no change in the latter following treatment with dexamethasone. Inasmuch as there is a linear relationship between tyrosine aminotransferase catalytic and mRNA activities at steady state under a variety of different experimental conditions, we conclude that the concentration of $mRNA^{TAT}$ is the primary determinant of the intracellular concentration of this protein. This induction is an extremely rapid process since $mRNA^{TAT}$ increases within 30 min after the addition of the inducer. Ongoing RNA synthesis is required since the induction by dexamethasone can be prevented by the simultaneous addition of actinomycin D or cordycepin. The increase in $mRNA^{TAT}$ activity occurs in the presence of inhibitors of protein synthesis such as cycloheximide and emetine, indicating that the mRNA increase is not tightly coupled to its translation or to the synthesis of another protein.

A number of systems, most of which involve enzyme induction, have been used to study glucocorticoid hormone action. Of these, one of the most intensively studied is the induction of tyrosine aminotransferase (EC 2.6.1.5; L-tyrosine:2-oxoglutarate aminotransferase). The addition of glucocorticoid hormones results in an increase in the specific rate of synthesis of the proteins in both liver and HTC cells[1] (Kenney, 1962; Granner et al., 1968). In 1968 it was predicted that tyrosine aminotransferase induction involved a "precursor"

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[1] Abbreviations used: $mRNA^{TAT}$, the messenger ribonucleic acid which codes for tyrosine aminotransferase; poly(A+) RNA, RNA containing polyadenylate residues at the 3′ terminus; HTC cells, hepatoma tissue culture cells; SAC, *Staphylococcus aureus*, Cowan strain I; $NaDodSO_4$, sodium dodecyl sulfate.

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substance, presumably $mRNA^{TAT}$, which accumulated even in the presence of inhibitors of protein synthesis (Peterkofsky & Tomkins, 1968). Direct confirmation of this hypothesis awaited the advent of cell-free protein translation systems which can be used to quantitate the levels of functional, specific mRNAs. By use of such cell-free systems, an increase in functional hepatic $mRNA^{TAT}$ activity following injection of rats with hydrocortisone (Roewekamp et al., 1976; Diesterhaft et al., 1977; Nickol et al., 1976, 1978) has been demonstrated.

Within the past year several groups, including our own, have been able to quantitate $mRNA^{TAT}$ activity isolated from HTC cells (Beck et al., 1978; Rether et al., 1978; Hofer & Sekeris, 1978). The studies presented in this paper describe the induction of tyrosine aminotransferase in more detail, test some of the predictions developed earlier using less direct techniques, and begin to explore why certain variant HTC cells are resistant to the action of glucocorticoid hormones.

Experimental Procedures

Cell Culture. Clone 4-1 of HTC cells was grown in suspension culture in Swim's medium 78 supplemented with Tricine (0.06 M), cystine (6×10^{-5} M), and 0.5% (v/v) each of bovine and fetal calf sera. The variant HTC cell lines 263E and 268E were cultured in the same medium supplemented with 2.5% (v/v) each of the sera.

Tyrosine Aminotransferase Assay. Tyrosine aminotransferase catalytic activity was determined by using a modification of the Diamondstone assay as previously described (Granner & Tomkins, 1970). One unit of tyrosine aminotransferase catalyzes the formation of 1 μmol of product per min at 37 °C. Protein content was determined by using bovine serum albumin as a standard (Lowry et al., 1951).

Purification of Tyrosine Aminotransferase and Tyrosine Aminotransferase Antibody Production. Tyrosine aminotransferase was purified by the procedure of Hargrove et al. (1980) to a specific activity of 500 units/mg of protein or greater and injected into New Zealand white rabbits wherein a highly specific antibody was formed. The antiserum was partially purified by precipitation with ammonium sulfate at 40% saturation and was quantitated by using an immunotitration assay (Granner et al., 1968). Fluorescent tyrosine aminotransferase was prepared by using highly purified rat liver tyrosine aminotransferase labeled with 2-methoxy-2,4-diphenyl-3(2*H*)-furanone by the method of Weigele et al. (1973).

Immunoprecipitation of Tyrosine Aminotransferase. Staphylococcus aureus, Cowan strain I (SAC), was used as an immunoadsorbant as described by Kessler (1975). For the in vivo rate of synthesis experiments, aliquots of labeled supernatant in NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, and 0.02% NaN_3) containing 0.05% Nonidet P-40 (NP-40) and 2 mM leucine and adjusted to pH 7.4 were incubated for 15 min at 23 °C with 15–30 μL of antibody which had been diluted 1:25 or 1:50 with incubation buffer (NET buffer containing 0.05% NP-40, 2 mM leucine, and 1 mg/mL bovine serum albumin and adjusted to pH 7.4). Fifty microliters of the SAC reagent was added, and the mixture was incubated at 23 °C for 10 min. The samples were centrifuged at 2000*g* for 5 min, and the pellet was washed 3 times with incubation buffer. The SAC pellet was then heated for 5 min at 90 °C in dissociating buffer (10% glycerol, 5% 2-mercaptoethanol, 2% $NaDodSO_4$, and 62 mM Tris-HCl, pH 6.8; Laemmli, 1970). The sample was again centrifuged, and the supernatant was subjected to electrophoresis on 10% $NaDodSO_4$–polyacrylamide gels (Laemmli, 1970). The gels were frozen, cut into 2-mm slices, dissolved in 0.5 mL of 30% H_2O_2, and counted in a Beckman scintillation counter. The net radioactivity in the tyrosine aminotransferase peaks was quantitated by subtracting the counts in the seven slices adjacent to each side of the peak as background. The in vivo rate of tyrosine aminotransferase synthesis is expressed as the radioactivity in tyrosine aminotransferase divided by that in the trichloroacetic acid precipitable cell extract.

Immunoprecipitation of the cell-free translation products was performed by a modified procedure in which the extracts (containing 0.05% NP-40) and 100 milliunits of carrier tyrosine aminotransferase were first treated with SAC to remove nonspecific absorbing molecules. This results in significantly decreased background levels of radioactivity but does not decrease the amount of tyrosine aminotransferase immunoprecipitated (Noguchi, unpublished experiments). Following removal of this SAC reagent, the subsequent immunoprecipitation reaction was performed as described above.

Poly(A+) RNA Isolation. Poly(A+) RNA was isolated by a method essentially as described by Iynedjian & Hanson (1977) and modified by Diesterhaft et al. (1977). Approximately 1.5×10^8 cells were homogenized in a Kontes glass homogenizer in 10 mL of extraction buffer containing 50 mM Tris, pH 9.0, 1 mM EDTA, pH 7.5, 100 mM NaCl, and 0.5 mg/mL heparin. One milliter of 10% $NaDodSO_4$ was then added, and the solution was again homogenized. Ten milliliters of phenol (saturated with extraction buffer without heparin) and chloroform (1:1) was added, and the solution was shaken at room temperature for 10 min and then placed on ice for 5 min. The samples were centrifuged at 10000*g* for 10 min at 4 °C, and the aqueous phases were pooled and precipitated overnight in 0.5 M NaCl and 2 volumes of cold ethanol. The resulting pellet was incubated with 5 mL of proteinase K buffer (10 mM Tris, pH 7.5, 0.5% $NaDodSO_4$, and 100 μg/mL proteinase K) at 25 °C for 30 min (Hilz et al., 1975). The samples were then extracted with 2 mL of chloroform twice and precipitated with 0.5 M NaCl and 2 volumes of cold ethanol. These pellets were extracted 2 times with cold 3 M sodium acetate, pH 6.0, on ice (Palmiter, 1974) and chromatographed twice on oligo(dT)–cellulose columns to isolate poly(A+) RNA (Aviv & Leder, 1972). The samples were again precipitated in 0.5 M NaCl and 2 volumes of cold ethanol. The poly(A+) RNA was diluted in 10 mM NaCl and stored in liquid nitrogen at a concentration of 1 mg/mL.

In Vivo Rate of Tyrosine Aminotransferase Synthesis. This assay is a modification of that described by Granner et al. (1968). Thirty minutes prior to the termination of an experiment, 30-mL aliquots of cells at $\sim 10^6$ cells/mL were centrifuged at 200*g* for 4 min and the cell pellets were resuspended in 2 mL of the original medium. Eighty microcuries of [^{3}H]leucine was added, and the cells were incubated at 37 °C for 30 min. After the cell pellets were washed, the cells were resuspended in 1.5 mL of NET buffer containing 0.05% NP-40 and 2 mM leucine and adjusted to pH 7.4. The cells were sonicated and then centrifuged at 105000*g* for 60 min to obtain the supernatants which were used in the immunoprecipitation reaction. Portions of the uncentrifuged cell extract were taken for determinations of tyrosine aminotransferase enzyme activity, protein, and the radioactivity incorporated into total protein (determined by precipitating 25–50-μL portions of the uncentrifuged extract with 10% trichloroacetic acid containing 2–10 mM leucine).

Cell-Free Translation. Rabbit reticulocyte lysates were prepared as previously described (Palmiter, 1973) and stored in aliquots in liquid nitrogen. Thawed lysates were supplemented with 1 mM $CaCl_2$, 0.177 mM amino acids except

leucine, 0.01 mM hemin, 0.58 mM $MgCl_2$, 117 mM KCl, and 11.7 mM creatine phosphate as final concentrations. Fifty microgram per milliliter micrococcal nuclease was added for 10 min at 20 °C to degrade endogenous mRNA (Pelham & Jackson, 1976) and then was inactivated by the addition of EGTA to a final concentration of 2 mM. Two hundred fifteen microliters of the lysate was then added to a mixture containing 5 μg of poly(A+) RNA and 25 μCi of [^{3}H]leucine, and the sample was incubated at 26 °C for 90 min. The reaction volume was increased to 580 μL by the addition of 10 mM Tris, pH 7.5, and 5-μL aliquots were precipitated with 25% trichloroacetic acid containing 2 mM leucine for the determination of the [^{3}H]leucine in total protein (Pelham & Jackson, 1976). The remaining material was centrifuged at 45000 rpm for 45 min in a TY65 rotor (Beckman), and 5-μL aliquots were taken for the determination of the [^{3}H]leucine incorporated into trichloroacetic acid precipitable released protein chains. The remaining material was used for immunoprecipitation of tyrosine aminotransferase as described above.

Materials. Dexamethasone phosphate was a gift from Merck Sharp & Dohme Research Laboratory, L-[4,5-^{3}H]-leucine (60 Ci/mmol) was obtained from New England Nuclear Co., proteinase K was from Beckman Instruments, Inc., oligo(dT)–cellulose, Type III, was from Collaborative Research, Inc., and micrococcal nuclease was from P-L Biochemicals. Polyacrylamide gel electrophoresis reagents were obtained from Bio-Rad Laboratories, and Nonidet P-40 was from Particle Data Laboratories, Inc. The triamcinolone for enzyme preparation was a gift from Lederle Laboratories. Tissue culture medium was purchased from Gibco, and sera were from St. Louis Serum Co. The *S. aureas*, Cowan strain I, was obtained from the American Type Collection, and all other reagants were obtained from Sigma Chemical Co.

Results

Translation of HTC Cell mRNA in a Rabbit Reticulocyte Lysate Cell-Free System. By use of the micrococcal nuclease treated reticulocyte lysate system (Pelham & Jackson, 1976), [^{3}H]leucine incorporation into total trichloroacetic acid insoluble material increases for at least 90 min and is linear with HTC cell poly(A+) RNA concentrations between 5 and 40 μg/mL (data not shown). These results are similar to those found by using livers from uninduced and hydrocortisone-treated rats as the sources of exogenous poly(A+) RNA (Diesterhaft et al., 1977). There is no detectable difference in the ability of equal amounts of poly(A+) mRNA isolated from uninduced and dexamethasone-treated cultures to be translated. Therefore, we conclude that the addition of dexamethasone to HTC cells does not cause a general increase in total mRNA activity.

Characterization of the Translation Product. One major peak of radioactivity is detected when the released protein chains from the cell-free translation assay are immunoprecipitated with antibody directed against tyrosine aminotransferase and then electrophoresed on $NaDodSO_4$–acrylamide gels (Figure 1). The [^{3}H]leucine-labeled tyrosine aminotransferase synthesized in cell-free systems supplemented with poly(A+) RNA isolated from uninduced or dexamethasone-treated HTC cells migrates identically on $NaDodSO_4$ gels, comigrates with tyrosine aminotransferase which has been partially purified from HTC cells, and also migrates with tyrosine aminotransferase which has been purified from rat liver and labeled with the fluorescent compound 2-methoxy-2,4-diphenyl-3(2*H*)-furanone. These data indicate that HTC cell mRNA added to the rabbit reticulocyte lysate system directs the synthesis of tyrosine aminotransferase which is indistinguishable from that made in intact HTC cells or in rat liver.

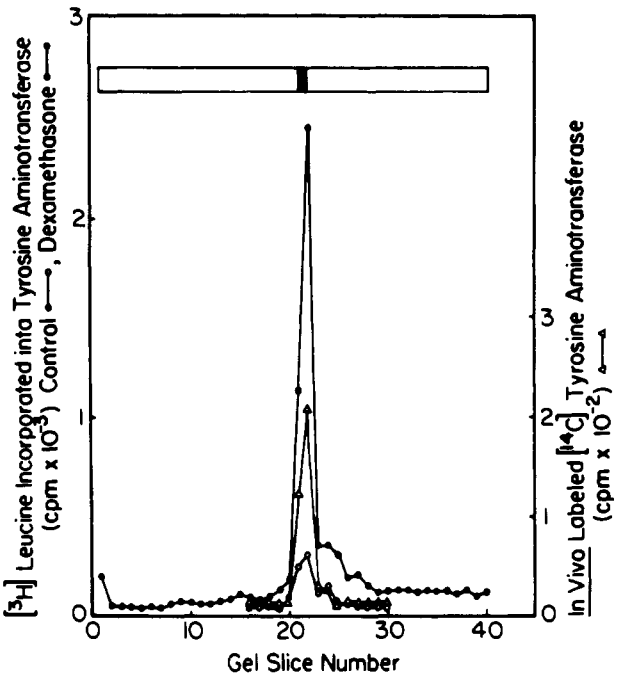

FIGURE 1: Characterization of tyrosine aminotransferase synthesized in vitro. Poly(A+) RNA isolated from uninduced (○) or dexamethasone-treated (●) HTC cells was translated in the cell-free system, and the released chains of tyrosine aminotransferase in each were immunoprecipitated along with purified, in vivo ^{14}C-labeled tyrosine aminotransferase (△) by using antibody directed against tyrosine aminotransferase. After immunoprecipitation, fluorescent-labeled tyrosine aminotransferase was added to the immunoprecipitation product and the samples were electrophoresed on companion gels. Migration of the fluorescent tyrosine aminotransferase was located by UV light and is represented by the band in the gel in the inset. The gels were sliced and dissolved in 30% H_2O_2, and the radioactivity in the slices was determined.

Kinetics of Induction. Several years ago we showed that there was a period of ~1 h before the rate of synthesis of tyrosine aminotransferase increased after addition of dexamethasone, whereas detectable increases in enzymic activity in the same cells could only be seen after ~2 h (Granner et al., 1970). We have confirmed these earlier findings (see parts A and B of Figure 2) and have in addition investigated the kinetics of mRNA^{TAT} induction in the same cell extracts.

Functional mRNA^{TAT} activity comprises ~0.03% of the total mRNA activity in HTC cells, and this activity increases within ~30 min after the addition of dexamethasone (Figure 2C). As with catalytic activity and the in vivo rate of synthesis, this increase continues until a new steady-state level of functional mRNA^{TAT} activity is reached, at which point it constitutes ~0.25% of the total mRNA activity. This increase, like that for enzyme synthesis and catalytic activity, is also maintained for at least 24 h if the glucocorticoid hormone is present (data not shown).

Figure 2D is a graphic display of parts A–C of Figure 2 and shows that the increase in functional mRNA^{TAT} precedes the increase in the in vivo rate of synthesis of tyrosine aminotransferase and this, in turn, precedes the increase in tyrosine aminotransferase catalytic activity. This is the sequence expected if there is a "precursor–product" relationship between mRNA^{TAT}, the rate of tyrosine aminotransferase synthesis, and the appearance of tyrosine aminotransferase catalytic activity.

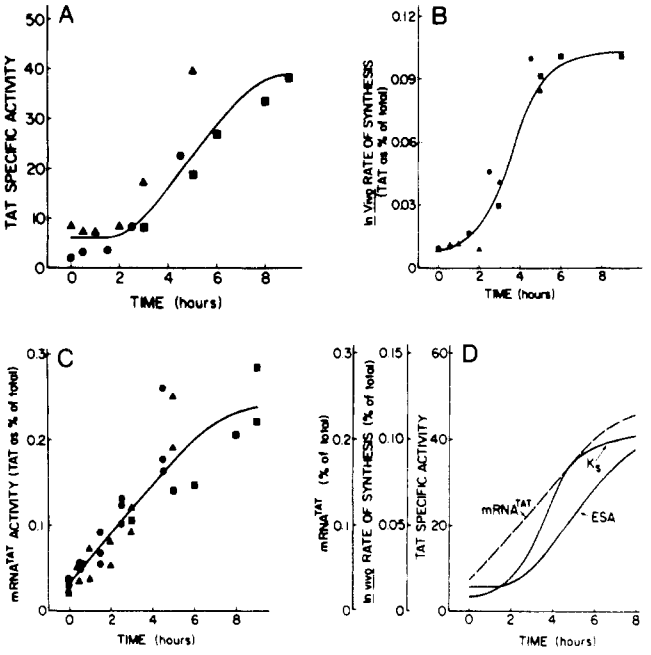

FIGURE 2: Induction of tyrosine aminotransferase by dexamethasone. Dexamethasone was added to HTC cells and, at the times indicated, assays for tyrosine aminotransferase catalytic activity (A), the in vivo rate of tyrosine aminotransferase synthesis (B), and functional $mRNA^{TAT}$ activity (C) were performed. The different symbols represent data from three separate experiments. In panel A the data represent the mean value of duplicate assays. In panels B and C the symbols represent each assay performed. Panel D is a composite of the data in panels A, B, and C.

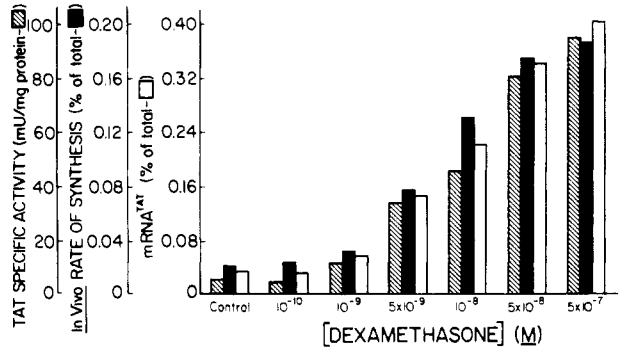

FIGURE 3: Induction of tyrosine aminotransferase by various concentrations of dexamethasone. The various concentrations of dexamethasone indicated were added to HTC cells for 12 h. At that time tyrosine aminotransferase catalytic activity (▨), the in vivo rate of synthesis (■), and functional $mRNA^{TAT}$ activity (□) were determined on the same aliquots of HTC cells.

Dexamethasone Concentration and $mRNA^{TAT}$ Activity. The induction of tyrosine aminotransferase catalytic activity increases in proportion to the amount of hormone in the culture medium until the maximal level for a particular steroid is reached (Samuels & Tomkins, 1970). As is seen in Figure 3, 10^{-10} M dexamethasone has no effect on tyrosine aminotransferase enzyme activity whereas larger concentrations result in progressively greater levels of induction. Optimal induction is reached between 10^{-7} and 5×10^{-7} M concentrations of dexamethasone, and the maximally induced value of 90 milliunits/mg of protein is 10 times greater than the basal level measured in this experiment. As tyrosine aminotransferase catalytic activity increases with higher concentrations of dexamethasone, there is a proportionate increase in the in vivo rate of synthesis of tyrosine aminotransferase from 0.02 to 0.19% of the total protein synthesized and also

Table I: Effect of Inhibitors of Protein and RNA Synthesis on $mRNA^{TAT}$[a]

treatment	TAT catalytic act. (milliunits/mg of protein)	$mRNA^{TAT}$ as % of total mRNA
control	8.8 ± 0.7	0.03 ± 0.003
+cycloheximide, 0.1 mM	3.0	0.03
+actinomycin D, 1 μg/mL	6.8	0.04
dexamethasone	25.9 ± 1.6	0.16 ± 0.01
+cycloheximide, 0.1 mM	2.3 ± 0.5	0.10 ± 0.01
+cycloheximide, 0.1 mM, last 2 h only	9.8	0.14
+actinomycin D, 1 μg/mL	9.9 ± 1.1	0.04 ± 0.02

[a] The inhibitors, at concentrations which result in at least a 95% inhibition of protein and RNA synthesis, were added to HTC cells either alone (control) or with the simultaneous addition of 5×10^{-7} M dexamethasone. After a 4-h incubation at 37 °C, tyrosine aminotransferase enzyme and $mRNA^{TAT}$ activities were determined as described under Experimental Procedures. The one exception to this was the sample in which cycloheximide was added to the cultures for the last 2 h of the 4-h incubation with dexamethasone. Values represent means ± standard errors of the mean when at least three different samples were assayed. Other numbers represent means of duplicate assays.

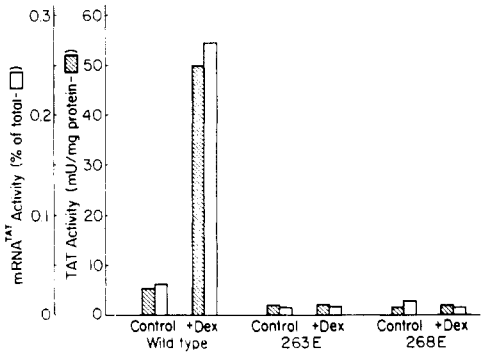

FIGURE 4: Tyrosine aminotransferase induction by dexamethasone in wild type and variant HTC cells. Cultures of wild type HTC cells and of the two variant cell lines were divided into two portions. Dexamethasone at a 5×10^{-7} M concentration was added to one culture, and the other served as the control. Twenty-four hours later, samples were taken for determination of tyrosine aminotransferase catalytic and $mRNA^{TAT}$ activities, as described under Experimental Procedures.

a proportionate increase in the functional mRNA activity for tyrosine aminotransferase (0.04–0.40% of the total mRNA activity). The concentration of dexamethasone that gives half-maximal induction of these three functions is $(6–9) \times 10^{-9}$ M, a value very similar to the estimated K_d of the glucocorticoid receptor for dexamethasone (Rousseau & Schmit, 1977).

Effect of Inhibitors of Protein and RNA Synthesis on $mRNA^{TAT}$. Cycloheximide decreases tyrosine aminotransferase enzyme activity from 9 to 3 milliunits/mg of protein when incubated with uninduced cells for 4 h (Table I). This is expected if tyrosine aminotransferase degradation continues to occur in the absence of ongoing protein synthesis. Tyrosine aminotransferase mRNA activity levels, however, were not affected by cycloheximide during this time period, even though the methods used are capable of detecting a decrease from the basal level (see Figure 4). Thus, maintenance of the basal level of $mRNA^{TAT}$ does not require ongoing translation, at least for this period of time.

When dexamethasone is added to HTC cell cultures for 4 h, there is a threefold induction of tyrosine aminotransferase enzyme activity and a fivefold increase in $mRNA^{TAT}$ activity.

The disparity in the extent of induction of enzyme and mRNA^{TAT} activities occurs because the new steady state has not been achieved at 4 h (see parts A and C of Figure 2). The addition of cycloheximide at the same time as the dexamethasone prevents the increase in catalytic activity; however, mRNA^{TAT} activity levels increased as much as threefold. In one experiment cycloheximide was added to cells only during the last 2 h of the 4-h induction with dexamethasone (Table I). Enzyme activity remained at uninduced levels, but mRNA^{TAT} activity achieved nearly maximal levels (0.14%) and was higher than that in the cultures treated for the entire 4 h with cycloheximide (0.10%). In these experiments functional tyrosine aminotransferase mRNA did not appear to increase to the same extent as it did in cultures treated with dexamethasone alone, suggesting some dependence of RNA synthesis, processing, transport, or turnover on protein synthesis (Palmiter et al., 1976; McKnight, 1978). Emetine, another protein synthesis inhibitor, at a concentration of 0.06 mM gave similar results (data not shown).

Two inhibitors of RNA synthesis were also tested. Actinomycin D had little effect on tyrosine aminotransferase activity and mRNA^{TAT} activity in uninduced cultures. Simultaneous addition of dexamethasone and the inhibitor to the culture for a 4-h incubation prevented any increase in mRNA^{TAT} and enzyme activity, indicating that in HTC cells, as in liver, ongoing RNA synthesis is required for the increase in mRNA^{TAT} seen after dexamethasone addition (Table I). Qualitatively similar results were obtained by using cordycepin in a parallel set of experiments. On the assumption that the translational assay is sensitive enough to detect small changes and since the data are expressed as a ratio, the lack of a decrease in mRNA^{TAT} activity following actinomycin D treatment could result if mRNA^{TAT} and total mRNA turn over at the same rate. Since this is unlikely, the data suggest that RNA and/or protein synthesis may be required for turnover of this RNA.

Noninducible HTC Cell Variants. Several lines of variant HTC cells which show little or no induction of tyrosine aminotransferase activity by gluococorticoids have been isolated. These lines were selected by serial cloning of the lowest inducing subclones of wild type HTC cells, and no selective pressure was used to produce this phenotype (Thompson et al., 1977). Various tests, including antibody precipitation, indicate that the enzymic activity present in these cells is tyrosine aminotransferase. Also, the glucocorticoid receptor mechanism seems to be intact in these cells (Thompson et al., 1979). Basal tyrosine aminotransferase catalytic activity in lines 263E and 268E, which we have adapted to grow in suspension culture, is ~2 milliunits/mg of protein as compared to 8–10 milliunits/mg in wild type HTC cells (Figure 4). As noted previously, 5×10^{-7} M dexamethasone does not induce tyrosine aminotransferase enzyme activity in these variant cells, whereas a ninefold increase is seen in wild type cells. Comparable differences were seen when functional mRNA^{TAT} activity was quantitated. The basal mRNA^{TAT} activity of 0.01–0.02% in the variants was lower than the 0.03–0.04% level noted in wild type cells. The addition of dexamethasone for 24 h did not alter functional mRNA^{TAT} levels in variant HTC cells and produced the usual increase in the wild type cells (Figure 4). Total mRNA activity was the same in the variant HTC cells as in the wild type (data not shown).

Test for an Inhibitor of Translation in Variant HTC Cells. The lack of a response in the variant HTC cells could be due to the fact that they contain an inhibitor of functional tyrosine aminotransferase mRNA activity which copurifies with the poly(A+) RNA. Mixing experiments were done to test this possibility. Poly(A+) RNA isolated from uninduced and induced wild type HTC cells and from the two variant cell lines was translated to determine the tyrosine aminotransferase mRNA activity of each, as were various combinations of these. In all cases the mRNA^{TAT} activity is that which is expected if the average of the two mRNA^{TAT} activities which were mixed is calculated. This is consistent with the idea that the lack of steroid induction in these cells is not due to an inhibitor of translation.

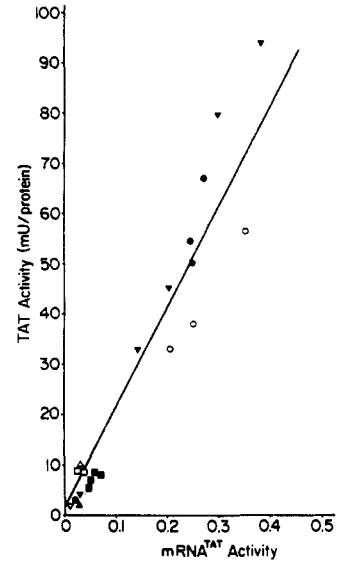

FIGURE 5: Comparison of steady-state values of tyrosine aminotransferase catalytic and mRNA^{TAT} activities. Steady-state levels of mRNA^{TAT} activity are plotted on the abscissa, and the corresponding catalytic activities are plotted on the ordinate. The slope of the line was determined by linear regression, and a correlation coefficient of 0.96 was calculated.

Relationship of Tyrosine Aminotransferase Catalytic Activity to mRNA^{TAT} Activity. Although there is a difference in the rate of appearance of increases in tyrosine aminotransferase catalytic activity and mRNA^{TAT} activity (Figure 2), the data obtained at steady state (Figures 2–4) suggests a remarkably direct relationship between these two processes. This can be better illustrated by comparing tyrosine aminotransferase enzyme specific activity with mRNA^{TAT} activity. Figure 5 shows that there is a direct proportionality between these two activities.

Discussion

Three different experiments indicate that, after the addition of dexamethasone to HTC cells, the rate of tyrosine aminotransferase synthesis measured in vivo and the enzyme catalytic activity increase in direct proportion to the increase in functional mRNA^{TAT}. First, at the new steady-state level of tyrosine aminotransferase which follows the addition of a maximally inducing concentration of dexamethasone, there are concomitant increases in all three of these activities (Figure 2). In this particular experiment a 9- to 10-fold increase was seen in each. Next, the observation that different concentrations of dexamethasone cause, at steady state, proportionate changes in mRNA^{TAT} activity and in the rate of synthesis and catalytic activity of the protein (see Figure 3) provides another example in support of the primary role of mRNA^{TAT} in the induction of this protein. Finally, the observation that dexamethasone-resistant variants have a lower tyrosine aminotransferase catalytic activity and a proportionally lower mRNA^{TAT} level in the basal state again lends support to this

position (see Figure 4). These observations, presented in summary form in Figure 5, which shows the linear relationship between tyrosine aminotransferase catalytic and mRNA activities, suggest that an increase in functional $mRNA^{TAT}$ activity, by whatever mechanism, is sufficient to account for the increased rate of tyrosine aminotransferase synthesis seen during glucocorticoid induction. Translation and/or post-translational control do not seem to be quantitatively important.

The effect of the steroid inducer is extremely rapid, since an increase in functional $mRNA^{TAT}$ is seen at 30 min, the earliest time at which samples were examined. It may be even shorter than this in view of the relative lack of sensitivity of cell-free translation assays. This response is at least as rapid as that caused by hydrocortisone induction of $mRNA^{TAT}$ in rat liver (Nickol et al., 1978; Diesterhaft et al., unpublished experiments) and of tryptophan oxygenase mRNA (DeLap & Feigelson, 1978; Tuohimaa et al., 1976) and is much more rapid than the induction of α_2u-globulin (Feigelson et al., 1978). The only response that appears to be more rapid than this is the induction of mouse mammary tumor virus in GR cells by dexamethasone (Ringold et al., 1975) which is maximal within 15 min of steroid addition as detected by a very sensitive hybridization technique.

The sequence of an increase in functional $mRNA^{TAT}$ activity followed by an increase in the in vivo rate of synthesis of the protein and finally by an increase in the specific activity of tyrosine aminotransferase suggests a precursor–product relationship (Figure 2D). This experiment also suggests that a considerable period of time elapses between the increase in mRNA activity and the increase in the in vivo rate of tyrosine aminotransferase synthesis. While there is a distinct increase in $mRNA^{TAT}$ activity by 30 min, at least another 30 min elapses before the in vivo rate of synthesis increases, clearly in excess of the 8–10-min ribosomal transit time estimated for tyrosine aminotransferase (Roper & Wicks, 1978). This difference could be real or could be due to a number of experimental difficulties. It is possible that the assay used to calculate the in vivo rate of synthesis is insensitive and thus that the time differential is overestimated. It is also not clear how the translation assays which are used to estimate functional mRNA activity relate to in situ conditions. For example, some $mRNA^{TAT}$ might be sequestered in an inactive form and, during the isolation procedure used for obtaining the poly(A+) RNA, inactive or less active product might be converted to a more active form. Finally, part of this difference could be attributed to the fact that the $mRNA^{TAT}$ must compete for ribosomal initiation factors with other mRNAs already being translated in vivo, but $mRNA^{TAT}$ does not have this problem in the in vitro assay since endogenous mRNA activity has been depleted by micrococcal nuclease. Any of these possibilities or some combination would account for the different rate of increase of $mRNA^{TAT}$ activity and in vivo rate of tyrosine aminotransferase synthesis.

It is not possible to establish what the mechanism of the increase in $mRNA^{TAT}$ acticity is by using the in vitro translation assay. Such an increase could result from an increase in $mRNA^{TAT}$ synthesis as a direct transcriptional effect or could result from a selective increase in the transport, processing, or activation of the mRNA. In theory, an increase could result from selective mRNA stabilization. On the assumption of a $t_{1/2}$ for this mRNA of ~60 min (Nickol et al., 1978; Land et al., 1978), turnover would virtually have to cease to account for the increase in activity that was observed. Although unlikely, our data do not absolutely exclude this possibility. Indirect support of a transcriptional site of action is provided by the fact that continuing RNA synthesis is required for the induction of $mRNA^{TAT}$ by dexamethasone (Table I).

Inhibitors of protein synthesis result in quite different effects. In 1968 Peterkofsky & Tomkins (1968) showed that dexamethasone led to the accumulation of a product that was sensitive to inhibition by RNA synthesis inhibitors but not to inhibitors of protein synthesis; they postulated that this substance was $mRNA^{TAT}$ (Peterkofsky & Tomkin, 1968). Studies reported in this paper offer direct confirmation of this hypothesis in that $mRNA^{TAT}$ accumulates in the presence of either cycloheximide or emetine (Table I). The inhibitors caused no increase in $mRNA^{TAT}$ by themselves, an observation that is of interest because of several recent reports that protein synthesis inhibitors increase functional tyrosine aminotransferase and tryptophan oxygenase mRNAs in untreated and steroid-treated rats (Hofer & Sekeris, 1978; Ernest et al., 1978; DeLap & Feigelson, 1978). It was suggested that short-lived regulatory proteins may regulate the activity of these specific mRNAs or that there is an increase in mRNA half-life in the presence of cycloheximide, i.e., that ongoing translation is needed for degradation. These experiments were performed by using intact animals; thus, the inhibitor may, perhaps through a general stress response, be causing the release of one or more other inducers of tyrosine aminotransferase such as glucocorticoids, catecholamines, or glucagon.

Inhibition of protein synthesis prevents the glucocorticoid-mediated increase of α_2u-globulin mRNA (Chen & Feigelson, 1979) and of tryptophan oxygenase mRNA (DeLap & Feigelson, 1978) and the estrogen-mediated induction of ovalbumin and conalbumin (McKnight, 1978). This suggests that one or more mediator proteins are involved in each of these induction processes and that the steroid effect is indirect to that extent. The induction of $mRNA^{TAT}$ by dexamethasone is likely to represent a direct effect of the steroid since it is very rapid and is not dependent on protein synthesis.

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Reaction of 5,5′-Dithiobis(2-nitrobenzoic acid) with Myosin Subfragment One: Evidence for Formation of a Single Protein Disulfide with Trapping of Metal Nucleotide at the Active Site†

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ABSTRACT: Treatment of rabbit skeletal myosin chymotryptic subfragment one (SF_1) in the presence of MgADP with a twofold molar excess of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) at 0 °C, pH 8.0, results in complete ATPase inactivation. Inactivation occurs in two phases that ultimately result in the modification of three SH groups. In the first phase there is a rapid reaction ($t_{1/2} \sim 10$ min) of DTNB with two SH groups which leads to activation of the Ca^{2+}-ATPase and inactivation of the K^+-EDTA-ATPase. Only one of these fast-reacting SH groups is believed essential for activity. In the second phase ($t_{1/2} \sim 2$ h), a thionitrobenzoic acid (TNB) group blocking one of the fast-reacting SH groups is displaced by a neighboring thiol believed to be the critical thiol called SH-2 to form a cystine disulfide bond. This latter reaction resulted in the loss of all Ca^{2+}-ATPase activity with concomitant trapping of MgADP at the active site ($t_{1/2,\text{off rate}} \sim 6$ days). Treatment of fully inactivated SF_1 with dithioerythritol reduced the disulfide, reduced the remaining TNB-SF_1 mixed disulfide, and released MgADP with the full recovery of all ATPase activity. In this reaction the remaining TNB was released more rapidly than ATPase activity was recovered, suggesting it plays a noncritical role in the original inactivation. Prior treatment of SF_1 with DTNB in the presence of MgADP was found to protect against subsequent modification by *N,N′*-(*p*-phenylene)dimaleimide or cobalt phenanthroline complexes. These reagents which have cross-linking spans of 12–13 and 3–5 Å, respectively, cross-link two critical thiol groups per active site in myosin [Reisler, E., Burke, M., Himmelfarb, S., & Harrington, W. F. (1974) *Biochemistry 13*, 3837; Wells, J. A., Werber, M. M., & Yount, R. G. (1979) *Biochemistry 18*, 4800]. The apparent formation of a disulfide bond between the cross-linked SH groups means they can move as close as 2.0 Å from each other. This demonstrates the large range of movement in the myosin molecule in response to binding of magnesium nucleotide. Furthermore, these studies extend prior observations [Wells, J. A., & Yount, R. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4966] that metal nucleotide is trapped at the active site during magnesium nucleotide stimulated inactivation by thiol cross-linking reagents.

Myosin and its active proteolytic subfragments double-headed heavy meromyosin and single-headed SF_1[1] are well-known to undergo spectroscopically sensitive conformational changes during the MgATP hydrolytic cycle [Werber et al., 1972; Morita, 1967; Murphy, 1974; Bagshaw & Trentham,

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[1] Abbreviations used: SF_1, chymotryptic subfragment one; pPDM, *N,N′*-(*p*-phenylene)dimaleimide; phen, 1,10-phenanthroline; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoic acid; DTE, dithioerythritol; AMP-PNP, adenyl-5′-yl imidodiphosphate; TNB-SF_1, SF_1 inactivated in the presence of MgADP and a twofold excess of DTNB as described under Materials and Methods; MalNEt, *N*-ethylmaleimide.

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