REGULATION OF TYROSINE AMINOTRANSFERASE ACTIVITY IN TWO LIVER-DERIVED PERMANENT CELL LINES

LARRY SELLERS and DARYL GRANNER

From the Veterans Administration Hospital and the Division of Endocrinology, Department of Internal Medicine, University of Iowa, Iowa City, Iowa 52242

ABSTRACT
The regulation of tyrosine aminotransferase (TAT) activity has been examined in two liver-derived heteroploid cell lines. One (hepatoma tissue culture cells [HTC]) was derived from a hepatoma, the other (rat liver culture cells [RLC]) was derived from normal liver. The two cell lines show the following striking similarities in the control of this specific protein: (a) The kinetics of TAT induction by dexamethasone phosphate (DxP) are similar in randomly growing cells of both lines; (b) During mitosis and early G1 phase of the cell cycle TAT activity cannot be induced by DxP in either cell line; (c) 2–3 h into G1, when both lines become sensitive to inducer, basal enzyme activity declines to a new steady-state level; (d) Preinduced cells collected in mitosis show approximately twice the level of TAT activity as fully induced, randomly growing cultures and this activity is maintained in early G1 with or without the inducer; and (e) Inhibition of RNA synthesis by 5 µg/ml of actinomycin D in preinduced, synchronized cells allows TAT activity to remain at constitutive levels throughout G1, even in the absence of inducer. These results are presented in support of a previously described model which states that glucocorticoid hormones exert posttranscriptional control of the synthesis of specific proteins in mammalian cells.

INTRODUCTION
The induction of the hepatic enzyme tyrosine aminotransferase (TAT; EC 2.6.1.5) by glucocorticoid hormones has been one of the most intensively studied model systems of mammalian protein regulation. Lin and Knox first demonstrated a several-fold induction of TAT by glucocorticoids in rat liver (1). Subsequently, this induction has been documented in perfused rat liver (2–4), fetal liver organ culture (5), and at least four different tissue culture cell lines, namely: hepatoma tissue culture cells (HTC), Thompson et al. (6); H4-II-E, Pitot et al. (7); rat liver culture cells (RLC), Gerschenson et al. (8); and mouse hepatoma culture cells (MHC), Richardson et al. (9). The increase in TAT activity is the result of an increased rate of synthesis of the enzyme in liver (10) and HTC cells (11) as shown by immunohistochemical and isotopic immunoprecipitation techniques.

1 Abbreviations used in this paper: AMD, actinomycin D; DxP, dexamethasone phosphate; Eiso, enzyme-specific activity; HTC, hepatoma tissue culture cells; MHC, mouse hepatoma culture cells; mRNA, messenger ribonucleic acid; RLC, rat liver culture cells; TAT, tyrosine aminotransferase.
Regulatory mutants, which were instrumental in elucidating control mechanisms in bacteria, are generally not available for such studies in mammalian cells. Valuable information has, however, been gained from investigations of TAT synthesis and induction in the various phases of the generation cycle of HTC cells (12, 13). Data from these studies of synchronized HTC cells played a major role in the formulation of a model which postulates that glucocorticoid hormones act at a posttranscriptional site (14). Recently it has been suggested that this model may apply to the regulation of a variety of inducible mammalian enzymes (15).

This model of control of specific gene expression is based on data obtained from one cell line, HTC cells, which was derived from a "minimal deviation" hepatoma. It has been reported that enzyme regulation in hepatomas may not be the same as in normal liver (16). Furthermore, regulation of the same enzyme may vary from one cell line to another (17) or within clones of the same cell line (18, 19). Thus, if the posttranscriptional hypothesis is to be accepted as a general model even for steroid regulation of TAT activity, data from another cell line, preferably derived from normal liver, should be studied in comparison to HTC cells.

The results of this study indicate that regulation of TAT activity in the RLC cell line, which was derived from normal rat liver, is virtually the same as in the HTC cells, thereby adding credence to the posttranscriptional concept of steroid hormone action.

MATERIALS AND METHODS

Materials

L-tyrosine, pyridoxal phosphate, α-ketoglutarate, Tricine, and actinomycin D (AMD) were purchased from Calbiochem, San Diego, Calif.; Swim's S77 powder, aceto-orcein, and Colcemid were obtained from Grand Island Biological Co., Grand Island, N. Y.; bovine serum and fetal calf serum from the St. Louis Serum Company, St. Louis, Mo. Bovine serum albumin was purchased from Armour Pharmaceutical Co., Chicago, Ill. Dexamethasone phosphate (DxP) was a gift from Merck Chemical Div., Merck & Co., Inc., Rahway, N. J.

Cell Culture

HTC cells, derived from the ascites form of Morris hepatoma 7288c (6), were grown in suspension culture in Swim's medium 77 containing bovine and fetal calf sera at final concentrations of 5% each, and buffered with 50 mM Tricine (N-tris[hydroxymethyl]methylglycine, Calbiochem). Cultures were incubated at 37°C in room air. HTC cells have a doubling time of approximately 22 h and grow exponentially between cell densities of 2–8 × 10^5 cells/ml. RLC cells, established in culture after dissociating normal rat liver with sodium tetraphenylborate (8), were maintained in suspension culture in the same medium. Under these conditions, these cells have a doubling time of about 30 h and are in exponential growth between cell densities of 2–6 × 10^5 cells/ml. Cells of both lines from stationary cultures gave the same results.

Synchronization of Cells

Exponentially growing HTC or RLC cells were placed in fresh S77 medium containing 2.41 mM CaCl₂. 5 × 10^6 cells were then inoculated into each of several Blake bottles. 12–24 h later the medium was decanted and fresh medium containing 0.1 μM Colcemid was added. Mitotic cells were collected after 5–10 h exposure to Colcemid by placing the bottles in the upright position and decanting the medium off the surface opposite that to which the cells were attached. The cells were then either maintained in mitosis by the continued presence of Colcemid or were allowed to enter G₁ by removing the Colcemid by centrifuging the cells at 75 g for 5 min and then washing them twice with fresh S77 medium. Cells remaining attached to the glass were considered to be interphase or nonmitotic cells. Colcemid had no effect on TAT induction in such cells. HTC cells usually yielded 1.5–2.0 × 10^6 mitotic cells per bottle, and RLC cells about 1 × 10^6 cells per bottle.

The mitotic index (number of mitotic cells/total number of cells counted) in all synchrony experiments was determined by counting at least 200 cells stained with 2% aceto-orcein. The degree of synchronization was considered adequate to proceed with the experiment when the mitotic index was equal to or greater than 90%. Within an hour after removing the Colcemid the mitotic index ordinarily fell to less than 5%, indicating a rapid, synchronous entry into the G₁ phase of the cell cycle.

Assays

Protein concentration was determined by the method of Lowry et al. (20), using bovine serum albumin as the standard. TAT was assayed according to the method of Diamondstone (21) as described by Hayashi et al. (22). One unit of activity represents the formation of 1 μmol of product (p-hydroxyphenylpyruvic acid) per minute.
RESULTS

Kinetics of TAT Induction in Randomly Growing and Synchronized HTC and RLC Cells

Fig. 1 illustrates the response of randomly growing cultures of both RLC and HTC cells to the addition of 10 µM DxP, a concentration which gives maximal induction. After a short lag period, TAT activity begins to increase, and by 3 h the values are always above the basal level. TAT activity continues to increase for 12 h, at which time the peak of induction has occurred, with enzyme levels 4- to 6-fold higher than basal activity. This new steady-state level is maintained for 24-36 h if inducer is kept in the medium. Thus, no difference was seen in the kinetics of TAT induction in randomly growing cultures of HTC and RLC cells.

TAT induction in randomly growing cells was then compared to induction in synchronized cells, in which the DxP was added at the beginning of G₁. While both random and “early G₁” cells ultimately show a 4- to 6-fold increase in TAT activity (see below, Figs. 3 and 4), Fig. 2 shows that the lag period before TAT activity begins to increase is about twice as long in early G₁ HTC and RLC cells as in randomly growing cultures.

TAT Induction in Synchronized HTC and RLC Cells

The delayed responsiveness to DxP in cells synchronized in G₁ as compared to randomly growing cells led to a systematic study of TAT inducibility during the various phases of the HTC and RLC cell cycle. The results shown in Figs. 3 and 4 can be summarized as follows: (a) In both cell lines basal TAT activity is higher in mitosis (M) (time 0 in Figs. 3 and 4) and early G₁ than in late G₁ or S, and the decline begins 2-3 h into G₁; (b)
HTC and RLC cells in M show no response to Dxp; (c) During early G1, there is a period of 2-3 h when both HTC and RLC cells are refractory to Dxp, as regards TAT induction. This statement is based on the fact that TAT activity 5 h into G1 is the same when the inducer is added at 2 h into G1 as at time zero (onset of G1); and (d) Addition of Dxp later in G1 or in S results in a rapid response of TAT activity in both cell lines. The rapid increase in TAT activity in late G1 or S suggests that the lag period of 2-3 h seen in randomly growing cells may be due at least in part to a mixed population of steroid resistant cells (M and early G1) and sensitive cells (late G1 and S).

**Regulation of TAT Activity in G1-Phase HTC and RLC Cells**

The results discussed above show that Dxp has no effect on TAT activity during M and the early stages of G1 in HTC and RLC cells. Since the constant presence of the Dxp is required for maintenance of the induced rate of TAT synthesis in randomly growing HTC cells (23), it became of interest to study the requirement for steroid in previously induced HTC and RLC cells, during the refractory periods of the cell cycle. If the lack of response to Dxp during M and early G1 is due to the absence of some specific regulator, the main-
tenance of the induced TAT levels should not de-
"pend on inducer at these times. This has been
found in earlier experiments with HTC cells (12,
13). Fig. 5 confirms these results in HTC cells
and Fig. 6 shows similar findings in RLC cells.

During the first 2–3 h of G1, TAT activity re-
mains high in both cell lines whether or not DxP is
in the medium. About 3 h into G1 TAT activity
declines in parallel in both cell lines until the sixth
hour of G1. At this time enzyme activity levels off
in inducer-containing medium (at the level of in-
duced, randomly growing cells) but continues to
decline toward the basal level in the control cul-
tures. Studies of randomly growing RLC cells
again emphasize the necessity of continued pres-
ence of DxP for maintenance of the induced steady-
state level of TAT.

Further information about the regulation of
TAT activity in G1 was obtained by using different
doses of AMD. This compound inhibits DNA-di-
rected RNA synthesis, and at concentrations of 0.1
µg/ml or greater, completely inhibits TAT induc-
tion (24). High concentrations (5 µg/ml) also in-
hibit induction if added before the inducer, but
when added to previously induced cells causes a
further rise in TAT activity (6). This phenomenon,
known as "superinduction," has been shown to be
due to an enhanced rate of synthesis of TAT (25),
and since it occurs when synthesis of TAT messen-
ger ribonucleic acid (mRNA) cannot occur, this
has been interpreted as a posttranscriptional effect
(see below).

Figs. 5 and 6 show a differential effect of low and
high concentrations of AMD in both HTC and
RLC cells. Again, for the first 3 h of G1, both cul-
tures maintain constitutive TAT activity. After
this time the cultures containing 0.1 µg/ml AMD
fall toward basal levels, and at 10 h are essentially
the same as control cultures. In cultures containing
5 µg/ml AMD, and no DxP, TAT activity remains

---

**Figure 4** TAT induction in synchronized RLC cells. The procedure followed was identical to that de-
scribed in Fig. 3.
**DISCUSSION**

The mammalian cell generation cycle consists of an ordered series of events demarcated by the periods of DNA synthesis (S phase) and mitosis (M). In addition to the off-on regulation of these events, rates of general protein and RNA synthesis vary markedly within the cell cycle. This suggests the presence of distinct, albeit-less absolute, regulatory mechanisms which might be exploited to study the control of specific enzymes.

Several patterns of enzyme activity in relation to the cell cycle have been reported. Most, such as lactate dehydrogenase and fumarase in KB cells (26), deoxycytidine monophosphate deaminase in HeLa cells (27), thymidylate kinase in HeLa cells (28), thymidine kinase in HeLa and mouse L cells (28, 29), ornithine transaminase in Chang’s liver cells (30), and lactate dehydrogenase, glucose-6-phosphate dehydrogenase, and alcohol dehydrogenase in HTC cells (12) increase in activity steadily through the cell cycle with a peak in late S or early G2. Klevecz has postulated that this pattern may reflect an escape from stringent regulation of enzyme activity due to the heteroploid nature of all these cell lines (17). He reported periodic peaks in lactate dehydrogenase and glucose-6-phosphate dehydrogenase activity in Don C cells (17, 31), a diploid line, and Friedman et al. have shown that ornithine carboxylase activity in the same cell line has peaks in early S, late S, and M phase (32). It is also possible that periods of enzyme synthesis might exist in heteroploid cells, but be obscured because of gene dosage effects for example. Two examples of specific control of enzyme activity in the heteroploid HTC cell line can be cited to argue that the activity of certain enzymes is regulated and can be studied in heteroploid cells.

We have previously shown that phosphorylation of
F1 and F2αd histones (protein kinase) increases markedly during S phase (33); also, the peak of TAT activity in early G1 with subsequent fall noted in both HTC and RLC cells reported in this paper and the variable sensitivity to glucocorticoid hormones (12) is yet another contrast to the pattern noted above.

Studies of enzyme induction have afforded considerable insight into the mechanism of action of steroid hormones. In general, these studies have been interpreted in terms of the Jacob-Monod model of bacterial gene regulation (34). By analogy the steroid (inducer) is assumed to interact with and antagonize a specific gene repressor. The result is an increased rate of synthesis of specific mRNAs which then are translated into the specific proteins. Control would thus be exerted at the level of transcription.

A different suggestion for the mechanism of action of glucocorticoid hormones has resulted from the intensive study of TAT induction in HTC cells. It is suggested that such steroids interfere with the action of a labile inhibitor (product of a regulatory gene) which itself represses the translation, and presumably also enhances the degradation of the TAT mRNA. This model has been described in detail (14) and was elaborated upon more recently (15).

Several of the essential features of this model were derived from studies of synchronized HTC cells (12, 13, 35). This work confirms these studies and illustrates the same phenomena in RLC cells, another TAT-inducible cell line of different origin.

In RLC cells, as in the HTC line, the cell cycle can be divided into steroid-sensitive (last 2/3 of G1 and S) and steroid-insensitive (M and early G1) parts. Basal TAT activity is highest in early G1 in both lines and declines at about the time steroid sensitivity appears. Preinduced cells collected in M phase have constitutive levels of TAT activity, and this is not affected by removal of inducer or by addition of AMD (concentrations which completely inhibit TAT induction if added before or with the inducer) until 2-3 h into G1. After this time TAT levels in inducer-free cells fall to the basal value whereas those in inducer-containing media decline to the level of induced random

*Earlier studies with HTC cells indicated that G2 is part of the steroid-insensitive portion of the cell cycle (35). RLC cells have a somewhat longer cycle than HTC cells (~30 h compared to ~22) and asynchrony in late S and G2 is substantial, thus precluding precise interpretation of events in G2-phase RLC cells.*

A concentration of AMD which causes super-induction maintains TAT activity at the constitutive level in both HTC and RLC lines.

One interpretation of these findings, as before (14), is that during the inducible phases of the cell cycle, both the regulatory and structural genes are active and the inducer is effective because of the presence of the regulatory gene product. The inducer is presumed to stabilize TAT mRNA, thus allowing for its increased accumulation and an increased rate of synthesis of TAT itself. During the noninducible phases neither gene is transcribed.

The product of the regulatory gene is assumed to be labile, and with a shorter half-life than the TAT mRNA. Hence, within a short time after entering the noninducible phase, the inducer is ineffective, and in preinduced cells TAT is synthesized at the constitutive rate even in the absence of steroid.

Recently Palmiter and Schimke showed that the superinduction of the oviduct secretory proteins ovalbumin, conalbumin, ovomucoid, and lysozyme is due to increased rates of synthesis of these proteins (36). Since the total amount of mRNA (ovalbumin) did not increase, they postulated the following model of posttranscriptional regulation based on differential stability of mRNAs. Secretory protein mRNAs are long-lived in relation to general mRNA and hence constitute a greater proportion of total mRNA after inhibition of RNA synthesis by AMD. The secretory protein mRNAs can then be translated at an increased rate because they can more favorably compete for factors which are rate limiting for protein synthesis, i.e., initiation factors (36).

The finding that the regulation of a specific enzyme, TAT, is identical in a cell line derived from normal liver and one from a hepatoma should allow for more precise testing of these models of posttranscriptional regulation of mammalian enzyme synthesis.

Larry W. Sellers is a predoctoral trainee supported by U. S. Public Health Service training grant no. 5-T01-AM05080-17. This work was supported by Veterans Administration research funds and by U. S. Public Health Service grant CA12191.

Received for publication 2 April 1973, and in revised form 1 August 1973.

REFERENCES

1. Lin, E. C. C., and W. E. Knox. 1958. Adaption of rat liver tyrosine-α-ketoglutarate transaminase. J. Biol. Chem. 233:1186.

L. SELLERS AND D. GRANNER Regulation of Tyrosine Aminotransferase Activity 343
10. Kenney, C. B., and F. T. Kenney. 1968. Regulation of tyrosine-α-ketoglutarate transaminase in rat liver. VII. Hormonal effects on synthesis in the isolated, perfused liver. J. Biol. Chem. 243:2926.

5. Wicks, W. D. 1969. Induction of hepatic enzymes by adenosine 3′,5′-monophosphate in organ culture. J. Biol. Chem. 244:3941.

6. Thompson, E. B., G. M. Tomkins, and J. E. Curran. 1966. Induction of tyrosine-α-ketoglutarate transaminase by steroid hormones in a newly established tissue culture cell line. Proc. Natl. Acad. Sci. U. S. A. 56:2296.

7. Pitot, H. C., C. Peraino, P. A. Morse, Jr., and V. R. Potter. 1964. Hepatoma in tissue culture compared with adapting liver in vitro. Natl. Cancer Inst. Monogr. 13:229.

8. Gerschenson, L. E., M. Anderson, J. Molson, and T. Okigaki. 1970. Tyrosine transaminase induction by dexamethasone in a new rat liver cell line. Science (Wash. D. C.). 170:859.

9. Richardson, U. I., A. H. Tashjian, Jr., and L. Levine. 1969. Establishment of a clonal strain of hepatoma cells which secrete albumin. J. Cell Biol. 40:236.

10. Kenney, F. T. 1962. Induction of tyrosine-α-ketoglutarate transaminase in rat liver. IV. Evidence for an increase in the rate of enzyme synthesis. J. Biol. Chem. 237:3495.

11. Granner, D. K., S. Hayashi, E. B. Thompson, and G. M. Tomkins. 1968. Stimulation of tyrosine aminotransferase synthesis by dexamethasone phosphate in cell culture. J. Mol. Biol. 35:291.

12. Martin, D., G. M. Tomkins, and D. K. Granner. 1969. Synthesis and induction of tyrosine aminotransferase in synchronized hepatoma cells in culture. Proc. Natl. Acad. Sci. U. S. A. 62:248.

13. Martin, D., G. M. Tomkins, and M. Bressler. 1969. Control of specific gene expression examined in synchronized mammalian cells. Proc. Natl. Acad. Sci. U. S. A. 63:842.

14. Tomkins, G. M., T. D. Gelehrter, D. K. Granner, D. Martin, H. H. Samuelis, and E. B. Thompson. 1969. Control of specific gene expression in higher organisms. Science (Wash. D. C.). 166:1474.

15. Tomkins, G. M., B. B. Levinson, J. D. Baxter, and L. DeHelesien. 1972. Further evidence for posttranscriptional control of inducible tyrosine aminotransferase synthesis in cultured hepatoma cells. Nat. New Biol. 239:9.
populations of HeLa cells. Nature (Lond.) 207: 176.

29. LITTLEFIELD, J. W. 1966. The periodic synthesis of thymidine kinase in mouse fibroblasts. Biochim. Biophys. Acta. 114:398.

30. VOLPE, P. 1969. Derepression of ornithine-δ-transaminase synchronized with the life cycle of HeLa cells cultivated in suspension. Biochim. Biophys. Res. Commun. 34:190.

31. KLEVECZ, R. R., and F. H. Ruddle. 1968. Cyclic changes in enzyme activity in synchronized mammalian cell cultures. Science (Wash. D.C.). 159:634.

32. FRIEDMAN, S. J., R. A. BELLANTONE, and E. S. CANELLAKIS. 1972. Ornithine decarboxylase activity in synchronously growing Don C cells. Biochim. Biophys. Acta. 261:188.

33. BALHORN, R., J. BORDWELL, L. SELLERS, D. K. GRANNER, and R. CHALKLEY. 1972. Histone phosphorylation and DNA synthesis are linked in synchronous cultures of HTC cells. Biochem. Biophys. Res. Commun. 46:1326.

34. JACOB, F., and J. MONOD. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 3:318.

35. MARTIN, D. W., JR., and G. M. TOMKINS. 1970. The appearance and disappearance of the post-transcriptional repressor of tyrosine aminotransferase synthesis during the HTC cell cycle. Proc. Natl. Acad. Sci. U. S. A. 65:1064.

36. PALMITER, R. D., and R. T. SCHIMKE. 1973. Regulation of protein synthesis in chick oviduct. III. Mechanism of ovalbumin “superinduction” by actinomycin D. J. Biol. Chem. 248:1502.