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

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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 G_1 phase of the cell cycle TAT activity cannot be induced by DxP in either cell line; (*c*) 2–3 h into G_1, 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 G_1 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 G_1, 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[1]; 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 immunochemical and isotopic immunoprecipitation techniques.

[1] *Abbreviations used in this paper:* AMD, actinomycin D; DxP, dexamethasone phosphate; E_{sa}, 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 $2-8 \times 10^5$ 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 $2-6 \times 10^5$ 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 $CaCl_2$. 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 G_1 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 $1.5-2.0 \times 10^6$ 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 G_1 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.

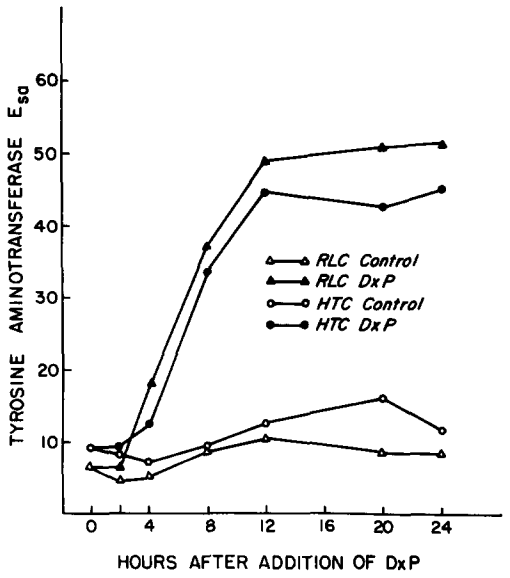

FIGURE 1 TAT induction by DxP in randomly growing HTC and RLC cells. Randomly growing HTC and RLC cells were suspended in S77 medium at concentrations of 400,000 cells/ml. One culture of each cell line served as a control while 10 μM DxP was added to another at time zero. Aliquots of 10^6 cells were taken at the times indicated and frozen. At the completion of the experiment TAT specific activities were determined.

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 G_1. While both random and "early G_1" 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 G_1 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 G_1 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 G_1 than in late G_1 or S, and the decline begins 2–3 h into G_1 ; (*b*)

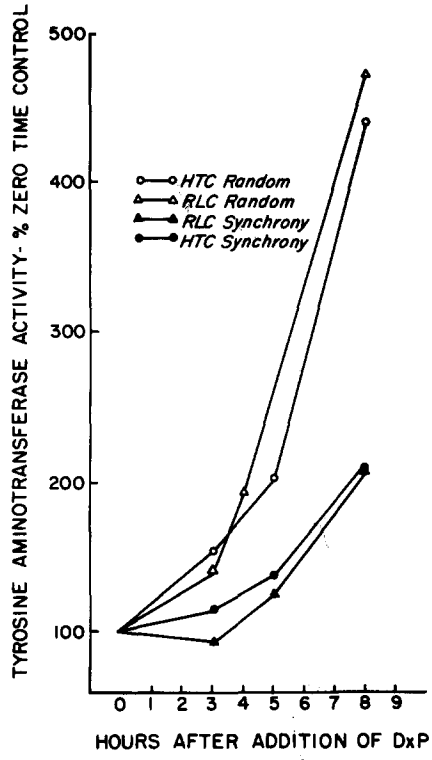

FIGURE 2 TAT induction in randomly growing vs. synchronized cells. Randomly growing HTC and RLC cells were induced as described in Fig. 1. Synchronized HTC and RLC cells were suspended at concentrations of 200,000 cells/ml. 10 μM DxP was added to all cultures at time zero (beginning of G_1 for synchronized cells). Aliquots were taken at times indicated and TAT specific activity calculated as described in Methods.

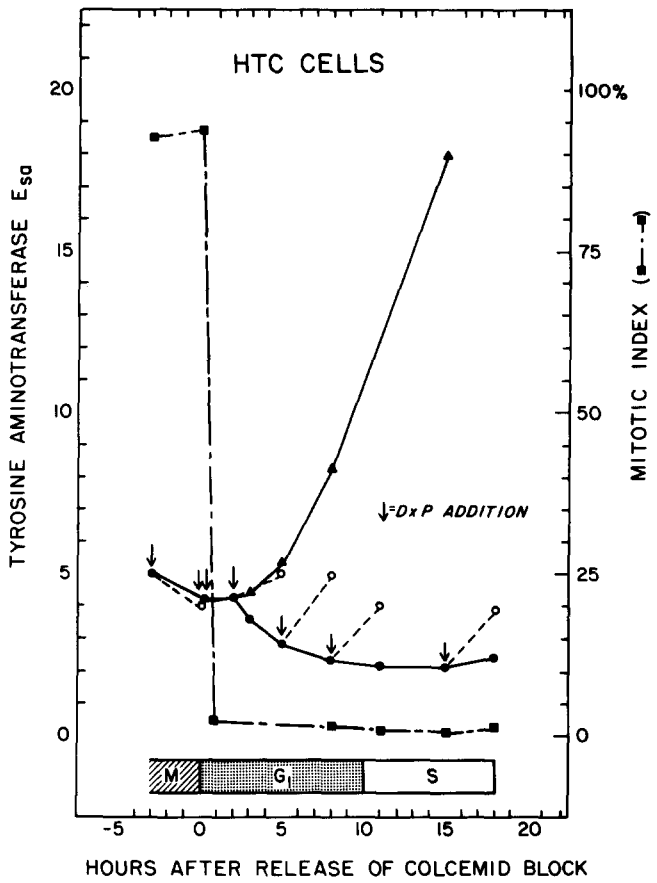

FIGURE 3 TAT induction in synchronized HTC cells. HTC cells synchronized by Colcemid block were examined for inducibility during various phases of the cell cycle. Cells were divided into two flasks each with a concentration of 400,000 cells/ml. One flask served as control and stock for aliquots to be exposed to DxP at 2, 5, 8, and 15 h into the cell cycle as indicated by the single arrow (↓); the other flask had DxP added at the beginning of G_1 as indicated by the double arrow (↓ ↓). Inducibility during mitosis was examined by exposure to DxP in the continued presence of Colcemid. ●——● control; ▲——▲ DxP added at the beginning of G_1 ; o——o DxP added at indicated time in cell cycle.

HTC and RLC cells in M show no response to DxP; (*c*) During early G_1 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 G_1 is the same when the inducer is added at 2 h into G_1 as at time zero (onset of G_1); and (*d*) Addition of DxP later in G_1 or in S results in a rapid response of TAT activity in both cell lines. The rapid increase in TAT activity in late G_1 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 G_1) and sensitive cells (late G_1 and S).

Regulation of TAT Activity in G_1-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 G_1 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 G_1 is due to the absence of some specific regulator, the main-

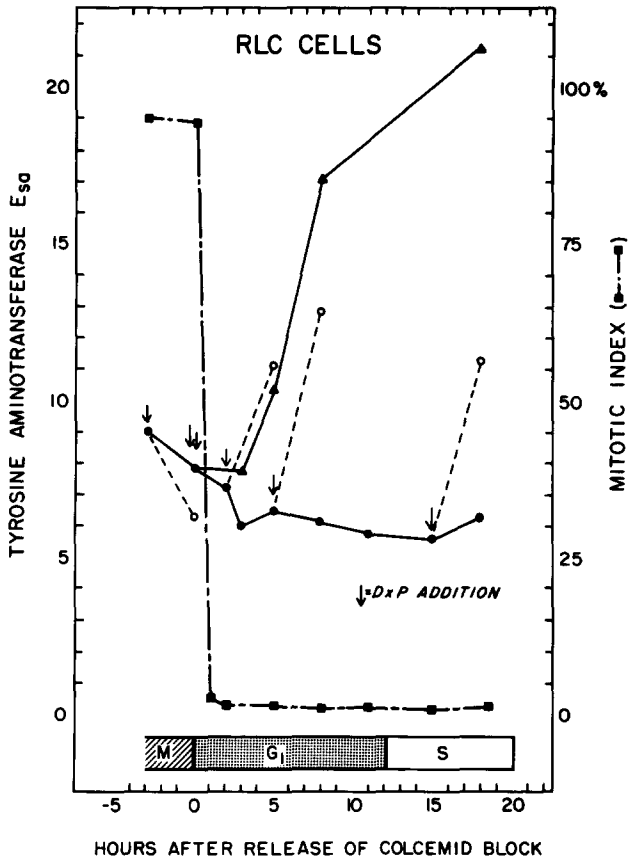

FIGURE 4 TAT induction in synchronized RLC cells. The procedure followed was identical to that described in Fig. 3.

tenance of the induced TAT levels should not depend 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 G_1, TAT activity remains high in both cell lines whether or not DxP is in the medium. About 3 h into G_1 TAT activity declines in parallel in both cell lines until the sixth hour of G_1. At this time enzyme activity levels off in inducer-containing medium (at the level of induced, randomly growing cells) but continues to decline toward the basal level in the control cultures. Studies of randomly growing RLC cells again emphasize the necessity of continued presence of DxP for maintenance of the induced steady-state level of TAT.

Further information about the regulation of TAT activity in G_1 was obtained by using different doses of AMD. This compound inhibits DNA-directed RNA synthesis, and at concentrations of 0.1 μg/ml or greater, completely inhibits TAT induction (24). High concentrations (5 μg/ml) also inhibit 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 "superindiction," has been shown to be due to an enhanced rate of synthesis of TAT (25), and since it occurs when synthesis of TAT messenger 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 G_1, both cultures 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

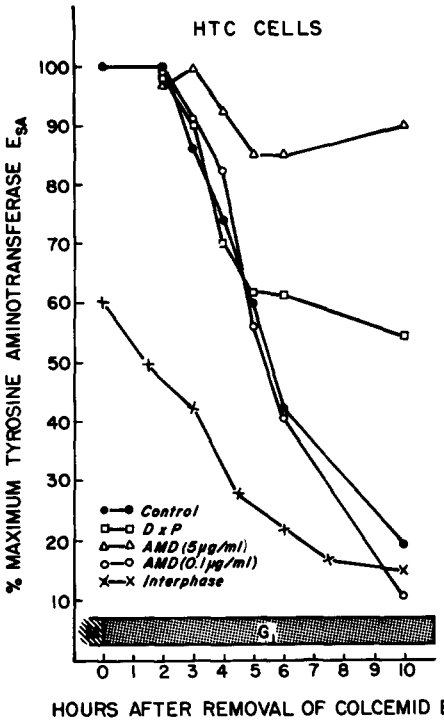

FIGURE 5 TAT activity in G_1-phase HTC cells induced before synchronization. HTC cells were exposed to DxP for 12 h, a period sufficient for maximal induction, before synchronization. After release of the Colcemid block, AMD, either 15 μg/ml or 0.1 μg/mg, was added to appropriate flasks containing 200,000 cells/ml. Nothing was added to the control flask. 1 ml aliquots were taken at the times indicated for determination of TAT specific activity.

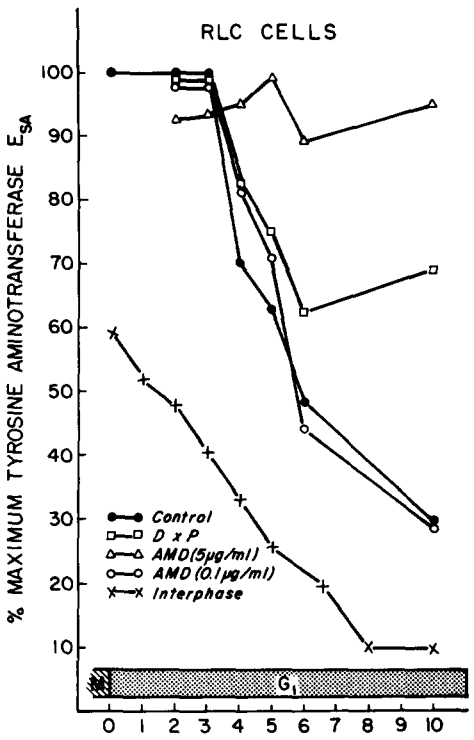

FIGURE 6 TAT activity in G_1-phase RLC cells induced before synchronization. The experimental procedure was the same as described in Fig. 5.

at the maximal or constitutive level throughout the experiment.

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 G_2. 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

F_1 and F_{2a2} histones (protein kinase) increases markedly during S phase (33); also, the peak of TAT activity in early G_1 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 ⅔ of G_1 and S) and steroid-insensitive (M and early G_1) parts.[2] Basal TAT activity is highest in early G_1 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 G_1. 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 cultures. 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.

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[2] Earlier studies with HTC cells indicated that G_2 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 G_2 is substantial, thus precluding precise interpretation of events in G_2-phase RLC cells.

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