CONTROL OF SPECIFIC GENE EXPRESSION EXAMINED IN SYNCHRONIZED MAMMALIAN CELLS

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Abstract.—The control of synthesis of the steroid-inducible enzyme, tyrosine aminotransferase (TAT), has been studied in synchronized cultures of HTC cells, an established line of rat hepatoma cells. During the mitotic and early G1 periods of the generation cycle of cells previously exposed to inducer, TAT synthesis is maximal in the absence of inducer, i.e., synthesis is constitutive. (By contrast, in random cells maximal TAT synthesis is always dependent upon the presence of the inducer.) After the third hour of G1 ("G1[3]"), TAT synthesis is inhibited by a specific posttranscriptional repressor, the formation of which requires RNA synthesis. We speculate that this repressor is antagonized by the steroid inducer.

The degradation of TAT during mitosis and G1 is constant and not affected by cycloheximide or actinomycin D in our experiments.

Adrenal steroids induce a large increase in the rate of synthesis of tyrosine aminotransferase (TAT) in HTC cells, a line of rat hepatoma cells in culture.¹⁻³ TAT can be synthesized during all phases of the cell cycle, but is inducible only during the latter portion of the G1 phase and the entire S phase.⁴

In the present studies we have examined both synthesis and degradation of TAT in HTC cells induced for TAT prior to their synchronization. On the basis of these and earlier observations,¹⁻⁴ we propose that the synthesis of the enzyme is controlled by both a steroid-insensitive transcriptional process and a steroid-sensitive, posttranscriptional mechanism.

Materials and Methods.—Cells were grown as previously described.^{1,2} To obtain induced mitotic cells, we introduced Dexamethasone phosphate (Dex, 10^{-6} M) to random monolayer cultures 24 hr prior to the addition to Colcemid and subsequently selectively harvested the mitotic cells.⁴ Mitotic cells were also obtained in the absence of Colcemid.⁴

TAT was assayed by the method of Diamondstone³⁻⁵ and protein by that of Lowry *et al.*⁶ The rates of synthesis and degradation of TAT were measured by a modified⁷ specific radioimmunoprecipitation technique.³

Cycloheximide was a gift from Upjohn Co. and actinomycin D, Daunomycin, and Mithramycin were obtained from the National Cancer Institute Chemotherapy Center. Neomycin was purchased from Grand Island Biological Co. Mixtures of radioactively labeled amino acids were purchased from New England Nuclear Corp. with specific radioactivities of 116 to 409 mc of ^{14}C /mmole or 1.3 to 55.5 c of ^3H /mmole.

Results.—(1) *TAT synthesis is constitutive in early G1:* The TAT activities in preinduced interphase cells after the removal of inducer, and in preinduced synchronized cells both in the presence and absence of inducer, are illustrated in Figure 1A. As shown, when the inducer and Colcemid were removed from the interphase cells, TAT activity declined promptly with a half time of 4-6 hours.

FIG. 1.—TAT activity during mitosis and G1. Monolayer cultures of HTC cells were induced with Dex, 10^{-6} M, for 24 hr prior to exposure to 2×10^{-7} M Colcemid. After 12 hr of Colcemid treatment, those cells arrested in metaphase were selectively harvested. The adherent interphase cells served as a control population in the experiment in part (A). The populations of synchronized cells (mitotic indices greater than 90%) were washed by centrifugation ($200 \times g$) at 37°C and resuspended in fresh, prewarmed medium containing Neomycin (50 $\mu\text{g}/\text{ml}$) and, when indicated, Dex 10^{-6} M. These suspension cultures (ca. 500,000 cells/ml) were maintained at 37° , and the appropriate drugs were added as indicated. Aliquots were removed at the intervals indicated and chilled. The cells were washed with neutral buffer prior to being quick-frozen and stored for subsequent determinations. TAT specific activities (μmoles of product/min/mg protein) were normalized to an initial value of 100 because, even though the induction in each case was at least 8-fold, the maximally induced levels varied from experiment to experiment.

In the experiment depicted in (A), the population of synchronized cells was washed free of Colcemid at "zero" time. Dex was also removed from one half of the population ($\bigcirc-\bigcirc$), but in the other half Dex (10^{-6} M) was present throughout the experiment ($\bullet-\bullet$). Interphase cells ($\blacksquare-\blacksquare$, population mitotic index 5%) were removed from the same monolayer cultures by a magnetic stirring bar, washed free of Colcemid and Dex, and resuspended in the same medium used for the synchronized population.

In the experiment of (B), the entire synchronized population was washed free of Dex when the Colcemid was removed. The suspension culture was divided into two parts. Two hr after the removal of Colcemid AMD, 0.1 $\mu\text{g}/\text{ml}$, was added to one ($\bullet-\bullet$), and AMD, 5 $\mu\text{g}/\text{ml}$, to the other ($\bigcirc-\bigcirc$). Samples were removed throughout as indicated and assayed for TAT specific activity.

(C) shows an experiment in which cycloheximide, 10^{-4} M, was added to portions of identical populations of preinduced, synchronized cells at 3 times (arrows) after removal of the Colcemid. At 2 hr to a portion of the population AMD (5 $\mu\text{g}/\text{ml}$) was added to prevent the repression of TAT usually seen at G1[3]. To one half of this portion containing AMD, cycloheximide was added 5 hr after the Colcemid removal ($\blacktriangle-\blacktriangle$); the remaining half contained only AMD ($\blacktriangle-\blacktriangle$). One portion of the original synchronized culture received no cycloheximide or AMD ($\triangle-\triangle$) and demonstrated the usual decline of TAT activity after G1[3].

In contrast, in synchronized cells from which inducer and Colcemid were removed, the high level of activity was maintained for the first two hours of G1 before falling. This pattern was also seen in synchronized populations never exposed to Colcemid.

We investigated whether the persistence of the high activity of TAT during early G1 in the absence of inducer reflected constitutive formation of the enzyme. Accordingly, the rates of TAT and total cell protein synthesis were determined in synchronized cells during G1 and in uninduced interphase cells. Lines 1 and 3 of Table 1 give these rates during the first hour of G1 (1 to 2 hours after Colcemid removal), in the presence (experiment I) and absence (experiment II) of inducer. In both, 0.3 per cent of the total radioactivity incorporated was recovered in TAT.⁸ The "absolute rates" of TAT synthesis were also about equal in the presence or absence of inducer. The "differential rate" of TAT synthesis in inter-

TABLE 1. *Rates of TAT synthesis during G1.*

Expt.	Period labeled (hr after Colcemid removal)		"Absolute Rate" (Dpm in TAT/hr/mg protein)		Differential rate of TAT synthesis ^a (×100)
		Dex 10 ⁻⁸ M	³ H × 10 ⁻³	¹⁴ C × 10 ⁻²	
I	1-2	+	13.3	13.6, 14.0	0.34, 0.32, 0.30
	4-5	+	6.8, 6.8		0.14, 0.14
II	1.25-2.0	—	11.7	11.7	0.31, 0.31
	4.25-5.0	—	4.0	3.5	0.07, 0.07
III	Interphase	—	1.3	3.2	0.03, 0.02

Fifteen minutes prior to the labeling, 5-ml aliquots of the indicated synchronized suspension culture were placed in warm 15 × 60-mm Falcon tissue culture dishes and allowed to equilibrate at 37°C. Neutralized radioactive amino acid mixtures were added to each dish (0.5 ml containing 500 μ C of ³H or 50 μ C of ¹⁴C), and the cultures were incubated for the periods indicated. To terminate the labeling, the dishes were chilled on ice. The cells were rapidly collected with the aid of a rubber policeman, washed twice with isotonic buffer in a CW-1 Sorvall cell-washing centrifuge, and quick-frozen. TAT was specifically immunoprecipitated by the modified procedure, and TCA was added to aliquots to precipitate total protein. The radioactivity in each precipitate was determined in a liquid scintillation counter. Double entries on the same line indicate the values for duplicate experiments.

phase cells was 0.03 per cent.⁹ As in asynchronous cells,^{1, 2} the inducer did not affect the rate of total protein synthesis. Thus, prior to the third hour of G1 ("G1[3]") TAT synthesis in cells previously exposed to inducer remains elevated whether inducer is present or not, i.e., synthesis is constitutive.

(2) *Specific inhibition of TAT synthesis begins at G1[3]*: At G1[3], TAT activity suddenly began to decline whether or not Dex was present (Fig. 1A). In the presence of inducer the enzyme activity stabilized at a value about equal to that of fully induced interphase cells. However, in the absence of inducer, the level continued to decline with a half time of 4-6 hours, as in asynchronous cells washed free of inducer (Fig. 1A and ref. 1). Inhibition of TAT synthesis is responsible for the decline of activity which begins at G1[3] (Table 1). The differential rates of TAT synthesis during the fourth hour of G1 were 0.14 per cent in the presence of Dex (line 2) and 0.07 per cent in the absence of inducer (line 4), both considerably lower than the rates prior to G1[3], 0.30-0.34 per cent (lines 1 and 3). The absolute rates of TAT synthesis under these conditions were correspondingly reduced after G1[3]. Since the rates of total protein synthesis before and after G1[3] were approximately the same, the decrease in TAT synthesis which commences at G1[3] (Fig. 1A) is relatively specific. No decrease in the activities of other enzymes occurs at any time during the life cycle.⁴

(3) *RNA synthesis is required for the fall in TAT activity at G1[3]*: We next investigated the role of RNA synthesis in the inhibition of TAT formation that occurs at G1[3]. One hour before G1[3] we added actinomycin D (AMD), 0.1 μ g/ml or 5.0 μ g/ml, to preinduced, synchronized cells. The lower concentration inhibited uridine incorporation into RNA by 75 per cent at 60 minutes but did not prevent the decline of enzyme activity (Fig. 1B). In contrast, AMD at 5 μ g/ml (which at 60 min inhibited uridine incorporation by 90%) completely prevented the decline in TAT activity at G1[3] and maintained the initial high enzyme activity for as long as 12 hours.

Daunomycin¹⁰ (5 μ g/ml) completely inhibited and Mithramycin¹⁰ (5 μ g/ml) partially inhibited the decline of TAT at G1[3]. Thus, the synthesis of RNA is

required for the decline in TAT activity and, as shown below, for the specific inhibition of TAT synthesis which occurs at G1[3].

(4) *A specific repressor of TAT synthesis is formed at G1[3]*: Since at G1[3] TAT synthesis is specifically inhibited and AMD maintains the constitutive level of enzyme activity beyond G1[3], it seemed likely that AMD also maintained the constitutive rate of enzyme synthesis beyond G1[3]. We therefore measured these rates in the presence of AMD (5 $\mu\text{g/ml}$) (Table 2). In experiment I the inducer was present throughout. When AMD was added before G1[3], the rates of TAT synthesis before (line 1) and after G1[3] (line 3) were equal. This contrasts with the data in Table 1 (shown again on line 2 of Table 2), showing that the rate of TAT synthesis declined after G1[3] in the absence of AMD.

TABLE 2. Rates of TAT synthesis during G1 in the presence and absence of AMD.

Expt.	Period labeled (hr after Colcemid removal)	Dex $10^{-8}M$	AMD 5 $\mu\text{g/ml}$	"Absolute Rate" (Dpm in TAT/hr/mg protein)		Differential rate of TAT synthesis ^a ($\times 100$)
				$^3\text{H} \times 10^{-3}$	$^{14}\text{C} \times 10^{-2}$	
I	1-2	+	—	13.3	13.6, 14.0	0.34, 0.30, 0.32
	4-5	+	—	6.8	6.8	0.14
	4-5	+	+		11.8, 10.2	0.30, 0.29
II	1.25-2.0	—	—	11.7	11.7	0.30, 0.31
	4.25-5.0	—	—	4.0	3.5	0.07, 0.07
	4.25-5.0	—	+	8.7		0.27

The footnote to Table 1 describes the methods used. Two hours after Colcemid had been removed from the preinduced synchronized cell cultures, AMD (5 $\mu\text{g/ml}$) was added to the portions indicated, and the incubation was continued in suspension culture until aliquots were dispensed into dishes. The cells were labeled for the periods described in the table. In experiment I Dex was present throughout, but Dex was removed at "zero" time in experiment II.

Experiment II of Table 2 was done in the absence of inducer and again illustrates the reduced rate of TAT synthesis after G1[3] (line 5). However, in the presence of AMD, the elevated rate persists beyond G1[3] (line 6).

In summary, when RNA synthesis is inhibited one hour before G1[3], no repression of TAT synthesis occurs. Since RNA synthesis is required for this repression, we conclude that the repression is due to the formation of a specific repressor rather than the sudden depletion of a factor(s) required for enzyme synthesis.

The relative insensitivity of TAT repressor formation to AMD observed here (5 $\mu\text{g/ml}$ but not 0.1 $\mu\text{g/ml}$ inhibits its formation) recalls the relatively high dose of AMD required to produce "superinduction" of TAT in random HTC cells,² also attributed to the inhibition of the formation of a specific repressor.²

(5) *Degradation of TAT is constant throughout G1 and is not affected by AMD or cycloheximide*: Despite the fact that the rate of TAT synthesis is elevated after G1[3] in the presence of AMD, it might be argued that AMD also maintained the high level of enzyme activity by inhibiting TAT degradation.¹¹ To examine this and to determine whether there is any change in enzyme degradation during G1, we studied TAT degradation in the presence and absence of AMD (5 $\mu\text{g/ml}$) by several methods.

First, we measured the disappearance of enzyme activity before and after G1[3], using cycloheximide to inhibit the formation of new enzyme molecules.

Figure 1C shows the decline of TAT activity when cycloheximide was added to preinduced, synchronized cells one, four, and five hours after removing Colcemid. AMD was added to one of the cultures to prevent the repression of TAT synthesis at G1[3], and cycloheximide was added to this culture at five hours. In each case decay of TAT activity with a half time of four to six hours began immediately after the addition of cycloheximide. As illustrated, the rate of loss of TAT activity in the presence of cycloheximide is quite similar to that which occurs spontaneously at G1[3]. Thus, under these conditions neither AMD nor cycloheximide inhibit TAT degradation.

We also measured the rate of TAT degradation without using inhibitors by following immunologically the loss of radioactivity from previously labeled TAT in HTC cells never exposed to AMD or cycloheximide. As shown in Figure 2A the loss of labeled TAT was exponential with a half time of four to five hours, virtually identical to the values obtained in the presence of cycloheximide (Fig. 1C). When AMD was added two hours after the removal of Colcemid and isotope, the degradation of labeled TAT was not affected, but AMD did prevent the fall of TAT activity which occurs at G1[3] (Fig. 2C). The slower loss of radioactivity from labeled general cell protein (Fig. 2B) also appeared exponential and likewise was not affected by AMD.

Therefore, no change in the rate of degradation of either TAT or total protein occurred during that portion of G1 studied. Furthermore, contrary to previous

FIG. 2.—Loss of labeled TAT during mitosis and G1. As described in the legend of Fig. 1, monolayer cultures of HTC cells were preinduced with Dex and then exposed to Colcemid for 8 hr. A mixture of ^3H -amino acids was added to the medium ($3 \mu\text{C}/\text{ml}$) with Colcemid. Mitotic cells were detached, washed free of Colcemid, Dex, and radioactive amino acids, and were resuspended in fresh, prewarmed medium containing Neomycin ("zero" time). Two hr later the suspension culture was divided into two equal portions. AMD ($5 \mu\text{g}/\text{ml}$) was added to one (arrow). The cultures were maintained at 37°C , and samples were removed from both at the indicated intervals. The cells were immediately washed and quick-frozen. The radioactivity in the TAT of each sample was determined by the immunoprecipitation procedure described in *Materials and Methods*.

(A) is a semilogarithmic plot of the radioactivity in TAT (corrected for the protein concentration in each extract), as a function of the time after removing the Colcemid and radioactive amino acids. The values from the initial culture are represented by (○). The values from the cells exposed to AMD are represented by (●) and those in the absence of AMD by (○).

The radioactivity precipitated from each extract by 10% TCA is displayed in (B), also corrected for protein concentration and plotted semilogarithmically vs. the time after removing radioactive amino acids. The same symbols are used as in part (A).

The relative enzymic specific activities are shown in (C) as a function of time after Colcemid removal. Again the same symbols are used for the initial culture (○) and the cultures with (●) and without AMD (○).

reports,^{11, 12} under these conditions either AMD or cycloheximide affects degradation of TAT in HTC cells.

Discussion.—The TAT repressor inhibits enzyme formation at a site beyond gene transcription: The inhibition of enzyme synthesis by the TAT repressor occurs abruptly at G1[3]. In order for the repressor to act so suddenly by inhibiting TAT gene transcription, the mRNA would necessarily have to be very labile.¹³ This mechanism predicts that the action of TAT repressor should be mimicked by AMD, which also prevents gene transcription.^{14, 15} However, contrary to this prediction (as shown in Fig. 1B and Table 2), AMD (5 μ g/ml) sufficient to inhibit virtually all RNA synthesis does not inhibit the synthesis of the enzyme. In fact, AMD prolongs the period of constitutive synthesis of the enzyme by preventing repressor formation,¹⁶ and under these conditions the TAT messenger appears to be stable. Since the action of TAT repressor is not like that of AMD, the repressor must inhibit enzyme synthesis at a site subsequent to the transcription of the TAT gene.

From the data at hand, we cannot specify the actual posttranscriptional mechanism of action of the TAT repressor. The possibilities include: (a) inhibition of the transport of the specific RNA from nucleus to cytoplasm, (b) direct inhibition of the translation of the mRNA for TAT, (c) promotion of the destruction of either the mRNA for TAT or any other component required specifically for TAT formation.

The role of transcription control in the regulation of TAT synthesis: In addition, certain of our observations indicate that a type of transcriptional repression does exist but that it is not overcome by steroid inducers. For example, in mitotic cells little or no RNA synthesis occurs;^{17, 18} thus, the transcription of the entire genome is repressed. Obviously, this repression is not gene-specific, but one can still examine whether it can be reversed by steroid inducers. However, as expected from the known requirement for RNA synthesis for induction,^{19, 20} Dex does not induce TAT synthesis in mitotic cells. Thus, the repression of the TAT genes during mitosis is not sensitive to inducing steroids. Since TAT synthesis does occur during mitosis, it must be directed by specific template accumulated prior to mitosis.

In addition, TAT synthesis cannot be induced during either G2 or early G1, when RNA synthesis does occur.^{4, 21} We imagine, but have no direct evidence, that the noninducibility of the enzyme during these periods is also a result of the inability to transcribe the TAT gene. This steroid-insensitive gene repression may well be a manifestation of the same mechanism responsible for the general inhibition of transcription during mitosis, but which extends on both sides of the mitotic period to affect the TAT genes somewhat more specifically than the remaining genome.

There might be other types of transcription control specific for the TAT genes and sensitive to inducing steroids. However, despite the fact that this type of regulation is believed to be the most common (e.g., refs. 11, 12, and 22), we have no experimental evidence for its existence.

The role of steroid hormone in TAT induction: The present experiments, as well as earlier work,^{1, 2} show that the biosynthesis of TAT is specifically repressed

by a mechanism(s) operating subsequent to the transcription of DNA into RNA. However, the exact site of steroid action remains obscure.

Concomitant synthesis of RNA is required for enzyme induction,^{1, 2, 19, 20} from which it has been inferred that the inducer promotes an accumulation of enzyme-specific RNA. Since nuclear RNA turns over rapidly (e.g., ref. 23), the steroid could directly stimulate the production of specific RNA or act at a post-transcriptional site to cause its accumulation. For example, inducers might antagonize the posttranscriptional TAT repressor (appearing at G1[3]) which could lead to the accumulation of the TAT-specific RNA by enhancing its transport or preventing its degradation. Although it may be purely coincidental, the appearance at G1[3] of both repressor and inducibility leads us to favor the suggestion that induction results from the antagonism of the posttranscriptional TAT repressor by steroid.

Conclusion.—In uninduced cells, TAT synthesis cannot be induced in early G1, while in preinduced cells, TAT synthesis is constitutive until G1[3]. As proposed above, TAT gene transcription during G2, mitosis, and early G1 is repressed by mechanisms not sensitive to steroid inducers, and the TAT mRNA translated during these periods is presumed to have been formed prior to G2. Since TAT repressor is absent prior to G1[3], we assume that its production is inhibited from G2 until G1[3]. Thus, TAT mRNA would be translated at a derepressed (constitutive) rate from G2 until G1[3]. At G1[3] the repressor appears, after which the steroid is required for the induced rate of TAT synthesis. Because induction requires concomitant synthesis of TAT-specific RNA,^{19, 20} the structural gene for TAT must be active during periods of inducibility and thus must also be transcribed beginning at about G1[3]. Therefore, the structural and regulatory genes for TAT appear to be activated and inactivated coordinately during the cell cycle.

From the evidence accumulated from other inducible metazoan systems,^{24–29} it seems likely that the type of posttranscriptional regulation of gene expression described here is not restricted to hormonal control but occurs quite generally.

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Abbreviations: TAT, tyrosine aminotransferase; HTC cells, hepatoma tissue culture cells; G1[3], third hour of Gap 1; TCA, trichloroacetic acid; AMD, actinomycin D; CH, cycloheximide; Dex, Dexamethasone phosphate.

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TCA/mg protein) obtained by using the modified radioimmunoprecipitation technique are about $1/3$ to $1/4$ of those obtained with the earlier method, but the relative increases on induction are the same (unpublished data).

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