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Kinetics of Steroid Induction and Deinduction of Tyrosine Aminotransferase Synthesis in Cultured Hepatoma Cells

(specific mRNA turnover/steroid action/computer simulations)

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ABSTRACT **The specific rate of synthesis of tyrosine aminotransferase (EC 2.6.1.5; L-tyrosine:2-oxoglutarate aminotransferase) is used as a measure of the level of functional, cytoplasmic, tyrosine aminotransferase-specific mRNA in cultured rat hepatoma cells. An analysis of the kinetics of change in this rate after the addition or withdrawal of glucocorticosteroids sets an upper limit on the half-life of the enzyme-specific mRNA of 1–1.5 hr, whether or not steroid is present. The inactivation rate of the enzyme mRNA is independent of the growth condition of the cells, occurring equally rapidly in the presence or absence of serum or insulin, both of which induce tyrosine aminotransferase in these cells. The implications of these results for the mechanism of steroid induction are discussed.**

The glucocorticoid induction of tyrosine aminotransferase (EC 2.6.1.5; L-tyrosine:2-oxoglutarate aminotransferase) in cultured rat hepatoma (HTC) cells has been advanced as a model system for effector-mediated control of gene expression in mammalian cells (1). A variety of indirect experiments suggest (6, 7) that the steroid increases the level of mRNA specific for the enzyme (2–10). This conclusion is consistent with studies in other steroid responsive systems where mRNA increases have been measured directly (11–14).

Whether steroids promote the formation or slow the degradation of cytoplasmic mRNA remains unresolved. Previous attempts to assess the effects of steroid hormones on the stability of inducible mRNAs have relied upon the RNA synthesis inhibitor, actinomycin D (15), which is now known to affect both protein synthesis and mRNA stability (16, 17), or have used systems in which mRNA metabolism is complicated by cell proliferation or degeneration (18). Steroids have no measurable effects on gross macromolecular metabolism or on the growth of HTC cells (2), and we report here studies of stability of mRNA of tyrosine aminotransferase using kinetic methods that avoid the use of inhibitors.

The apparent absence of translational effects of steroids on synthesis of the enzyme (6, 7) suggests that the level of active cytoplasmic aminotransferase mRNA can be estimated by the rate of enzyme synthesis *in vivo*. Assuming that mRNA synthesis is zero-order, that mRNA inactivation is first-order with respect to mRNA concentration, and that the steroid acts very rapidly to modify the rate constant for either synthesis or inactivation, the half-time for the ensuing change in mRNA level will be equal to the half-life of the mRNA during the change (19). Therefore, the kinetics of induction and deinduction of rates of synthesis of the aminotransferase can give estimates of the stability of functional aminotransferase mRNA in the presence and absence of glucocorticoids. Since a mRNA stabilization mechanism for steroid action would entail roughly a 10-fold difference between the half-times for induction and deinduction, while a mechanism regulating mRNA production would produce no such difference, the kinetic approach should be able to distinguish between these two possible modes of steroid action.

Abbreviations: Aminotransferase, tyrosine aminotransferase; HTC cells, cultured rat hepatoma cells; BSA medium, serum-free Swim's S-77 medium containing 5 mg/ml of bovine serum albumin.

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METHODS

HTC cell stocks were grown in modified Swim's S-77 medium with 10% calf serum (5, 20). Experiments were performed on cells conditioned overnight in serum-free S-77 containing 5 mg/ml of bovine serum albumin (Armour Fraction V) (BSA medium). Recent flow microfluorometric studies suggest that this conditioning procedure blocks cells in the G1 phase of the cell cycle (Steinberg *et al.*, *Cell*, in press). Hormonal treatments are described in figure legends.

For determinations of aminotransferase synthetic rates, culture samples were concentrated by centrifugation to about 10^7 cells per ml and incubated for 15 min at 37° in medium containing 100 μCi/ml of L-[4,5-^{3}H]leucine (New England Nuclear). In some experiments we were able to reduce the sample size from 200 to 50 ml by lowering the concentration of nonradioactive leucine in the labeling medium from the normal 200 to 10 μM. Incorporations were stopped by adding ice-cold phosphate-buffered saline, and the cells were centrifuged, washed once, and frozen as drained pellets. Extracts were prepared, particulate material was removed by high-speed centrifugation, and radioactivity in immunoprecipitable aminotransferase was determined after purification through heating and DEAE-cellulose chromatography steps, as described (5, 21). Assays for total protein and for aminotransferase enzymic activity have also been described (5, 20). In some experiments cells were lysed with 0.5% (v/v) Nonidet P-40 (Shell Chemicals) instead of by sonication, and the lysate volumes were reduced from 2.5 to 0.7 ml. Use of freshly degassed DEAE-cellulose resulted in reproducible enzyme recoveries greater than 80%.

Rates of enzyme synthesis are corrected for enzyme recoveries and are expressed as the percentage of radioactivity incorporated into total soluble proteins to correct for variations in incorporation. Immunoprecipitations were performed in duplicate and counts in "background" second immunoprecipitates were subtracted from the first immunoprecipitates (21). Total protein radioactivity was greater than 10^7 cpm per sample; corrected background immunoprecipitates were

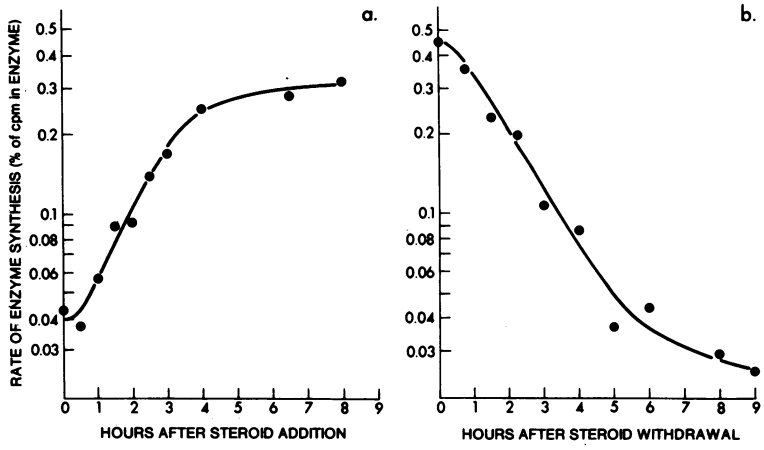

FIG. 1. Kinetics of steroid-mediated changes in tyrosine aminotransferase synthesizing activity. (a) *Induction.* 1 μM dexamethasone (Sigma) was added to a culture preconditioned overnight in BSA medium. Samples were removed and labeled for 15 min with [^{3}H]leucine; the radioactivity in immunoprecipitable aminotransferase was determined. Rates of enzyme synthesis were expressed as the percent of radioactivity in total soluble protein. (b) *Deinduction.* HTC cells preinduced about 16 hr in BSA medium containing 0.1 μM dexamethasone were centrifuged, washed once, and resuspended in steroid-free BSA medium. The differences in fully induced and basal rates of aminotransferase synthesis apparent in Figs. 1a and b and 2b reflect variability inherent in different batches of HTC cells.

generally less than 0.01% of this total. In one experiment, immunoprecipitations were performed with the normal carrier aminotransferase but the antibody against the enzyme was replaced with highly purified sheep IgG and rabbit antibody against sheep IgG in concentrations to give as much immunoprecipitated protein as the enzyme/anti-enzyme reactions. These controls gave backgrounds similar to the enzyme/anti-enzyme second precipitates.

Kinetic simulations were performed on a PDP 12 computer (Digital Equipment Corp.) and plotted with a Zeta flat bed plotter (Zeta Research Inc.).

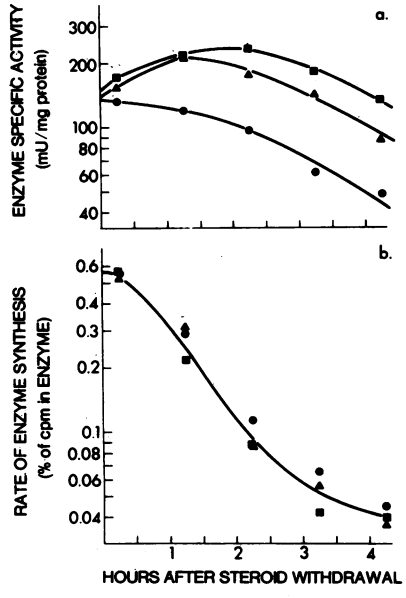

FIG. 2. Effects of serum and insulin on the kinetics of deinduction of tyrosine aminotransferase. Cells were preinduced as in Fig. 1b, then washed and resuspended in either BSA medium (●), BSA medium containing 5 μg/ml of insulin (Calbiochem, bovine B grade, 25.9 USP units/mg) (▲), or S-77 medium with 10% calf serum (■). Samples were assayed for enzyme synthesizing activity as in legend of Fig. 1.

RESULTS AND DISCUSSION

Fig. 1a shows the results of a typical induction experiment in which the change in rate of specific aminotransferase synthesis was followed after the addition of the synthetic glucocorticoid, dexamethasone. Consistent with earlier indirect studies (4), there is a lag of about 30 min before a change in enzyme synthesizing activity is observed; the rate of enzyme synthesis then increases with a half-time of about 3 hr to a new plateau level. In repetitions of this experiment, the magnitude of the induction has varied from about 8- to 20-fold, but the essential features of the kinetics have remained the same. Washing the cells and resuspending them in fresh medium prior to the induction (to mimic the conditions used in deinduction; see legend to Fig. 1b) did not markedly affect the course of the reaction. Fig. 1b shows that after removal of steroid from fully induced cells, aminotransferase synthesis falls exponentially to a new basal steady-state level with little if any delay.

Although serum and insulin enhance the specific enzymic activity of the aminotransferase (refs. 22 and 23; Fig. 2a), neither the relative rate of enzyme synthesis nor the rate of decline of enzyme synthesizing ability is sensitive to these agents (Fig. 2b). Therefore, in contrast to the stability of the enzyme protein itself (20), the functional stability of aminotransferase mRNA is apparently independent of cell growth (for which serum is obligatory). This is consistent with findings for bacterial mRNAs showing that functional stability is independent of growth rate at a fixed temperature (24, 25).

To analyze the kinetics of aminotransferase mRNA induction and deinduction more fully, we used a model for mRNA metabolism discussed in detail by Kafatos (26) which has satisfactorily described changes in specific radioactivity of total cellular mRNAs after addition of labeled precursors (27). As illustrated in Fig. 3, functioning cytoplasmic aminotransferase mRNA is maintained at steady-state levels by balancing production with degradation. Addition or removal of steroid is imagined to alter rapidly either the synthetic (K_s) or degradative (K_d) rate constant for the mRNA, leading eventually to a new steady state (at steady state, $[M] = K_s/K_d$). The model does not specify which events in

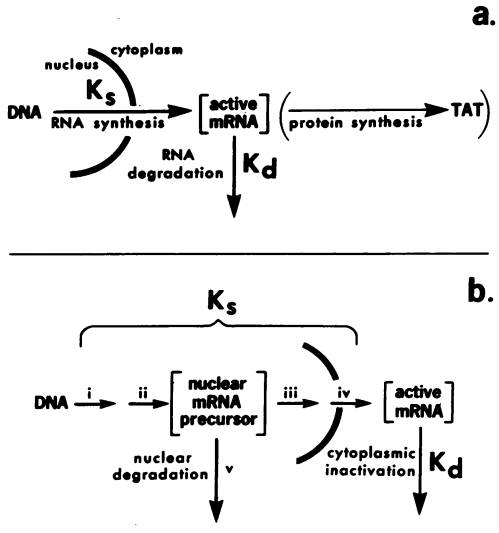

FIG. 3. Schematic pathway for tyrosine aminotransferase (TAT) mRNA metabolism. (a) The level of active cytoplasmic enzyme mRNA is determined by synthesis, represented by the zero-order rate constant K_s, and by degradation, characterized by the first-order rate constant K_d. (b) The production of active cytoplasmic enzyme mRNA, summarized by the rate constant K_s in (a), is shown to consist of several potentially regulable steps indicated by roman numerals.

synthesis and inactivation of cytoplasmic mRNA are regulable, and, as indicated in Fig. 3b, K_s may encompass a number of processes, including maturation and transport of nuclear mRNA as well as transcription.

The model is expressed mathematically by Eq. [1], where [M] is the concentration of enzyme mRNA; K_s, the zero-order synthesis constant; K_d, the first-order degradation constant; and t, time:

$$d[\mathrm{M}]/dt = K_s - K_d[\mathrm{M}] \qquad [1]$$

For transition from one steady-state mRNA level (M_0) to another (M_∞), Eq. [1] can be solved to give [M] as a function of time ([M](t)):

$$[\mathrm{M}](t) = M_\infty + (M_0 - M_\infty)\exp(-K_d t) \qquad [2]$$

Eq. [2] can be rearranged to give the linear form:

$$\log\{([\mathrm{M}](t) - M_\infty)/(M_0 - M_\infty)\} = -K_d t/2.303 \qquad [3]$$

Thus, plots of induction or deinduction data in the form log $\{([\mathrm{M}](t) - M_\infty)/(M_0 - M_\infty)\}$ against time should yield straight lines with negative slopes equal to $K_d/2.303$ for the two processes. If steroid changes the rate of production of enzyme mRNA, the data for induction and deinduction should lie on the same line when plotted in this way; if steroid stabilizes the mRNA, however, the negative slope for the deinduction line should be 8 to 20 times greater than that for the line representing the induction.

Fig. 4 shows data from three induction and five deinduction experiments plotted using the formulation suggested above; the values for [M](t) are taken as the relative rates of aminotransferase synthesis at given times after induction or deinduction. There is clearly some variability from experiment to experiment, and statistical difficulties (arising from the increased significance given to estimates of initial and final rates of enzyme synthesis) preclude a heavy reliance on this

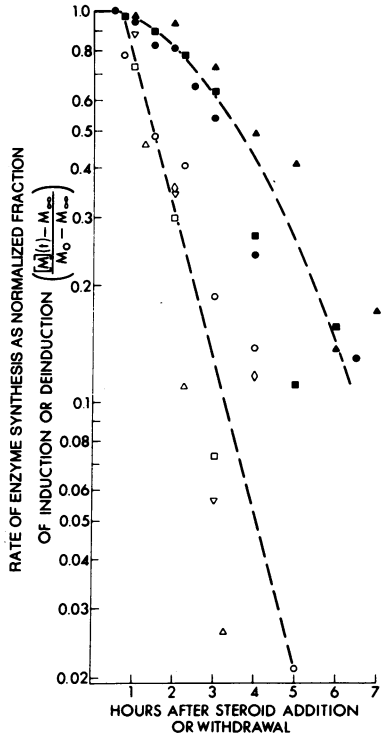

FIG. 4. Replotted kinetic data for aminotransferase synthesizing activity after steroid administration or withdrawal. For each set of experimental data, the best available estimates of basal and fully induced rates of aminotransferase synthesis were used for initial and final levels of enzyme mRNA (M_0 and M_∞, respectively). Intermediate levels, ([M](t)), taken as rates of enzyme synthesis during induction or deinduction, were used to generate values of the expression $([\mathrm{M}](t) - M_\infty)/(M_0 - M_\infty)$, which were plotted on a logarithmic scale against time. *Closed* and *open symbols* represent, respectively, induction and deinduction. Different symbols represent different experiments. The dashed lines are included merely as visual aids.

plot. Nevertheless, it can be seen that although the deinduction data show the expected exponential decline after an initial lag of 20–40 min, the induction data follow more complex kinetics, showing a slow approach to a limiting slope comparable in magnitude to that seen for the deinductions. These induction kinetics conform to neither of the simple models considered above, since both predict a simple linear change in log $\{([\mathrm{M}](t) - M_\infty)/(M_0 - M_\infty)\}$ as a function of time. The initial 20- to 40-min lag observed for both induction and deinduction is considerably longer than the time required for nuclear uptake or release of steroid (28), and we will discuss its possible implications below.

To reconcile the observed kinetics with our simple models for steroid regulation, we used computer simulations to explore the possibility that the steroid effect on K_s or on K_d is itself time-dependent. In Fig. 5a we assume that K_s remains constant and the steroid mediates a time-dependent change in K_d; in Fig. 5b we assume that steroid regulates K_s (data replotted from Fig. 1). With either model we ascertained that the upper limit for aminotransferase mRNA stability is determined by the kinetics of induction (in fact, a lower limit for K_d is given by the magnitude of the limiting slope of the induction curve plotted as in Fig. 4). From our experiments, this maximal enzyme mRNA half-life is in the range of 1–1.5 hr. To obtain a reasonable fit to the experimental data using

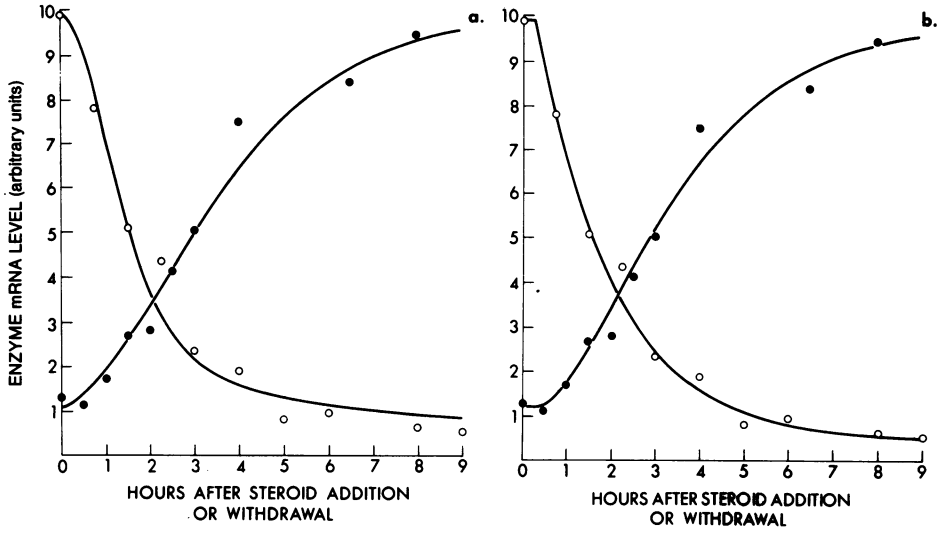

FIG. 5. Computer simulations of induction and deinduction of aminotransferase mRNA levels assuming a steroid-mediated, time-dependent change in the rate constant for either its degradation (a) or production (b). Data are replotted in linear form from the induction (●) and deinduction (○) experiments shown in Fig. 1 (normalized to give the same fully induced levels of aminotransferase synthesis). Standard numerical integration techniques were used to simulate inductions and deinductions using the general equation: $d[M]/dt = K_s - K_d[M]$, where either K_d or K_s was altered in a time-dependent manner following the equation:

$$K(t) = K_\infty + (K_0 - K_\infty) \exp [-L(t - t_0)].$$

The initial and final values of K (K_0 and K_∞, respectively) were calculated from the steady-state levels of [M], using the relationship $K_s = K_d[M]$; t_0 represents an initial lag during which $K(t) = K_0$; and L is a rate constant for the change in K [which can be expressed in terms of a half-time, $t_{1/2} = \ln (2)/L$]. The values used for M_0 and M_∞ were within experimental error of the measured basal and induced rates of aminotransferase synthesis from these experiments; values for the invariable constant (K_s or K_d), the half-time for change in the variable constant (K_d or K_s), and the initial lag (t_0) were changed systematically to give the most reasonable fits to both the induction and deinduction data. (a) For induction, the half-life of aminotransferase mRNA increases from about 9.6 min to 1.5 hr with a $t_{1/2}$ for the change of 0.65 hr; for deinduction the half-life decreases from 1.5 hr to about 4 min with a $t_{1/2}$ of 8.5 hr. The initial lag for both processes is assumed to be less than 1 min. (b) For induction and deinduction, aminotransferase mRNA half-life is 1.2 hr, and a 20-min initial lag precedes both processes. For deinduction, the $t_{1/2}$ for the change in K_s is negligible, while for induction it is 1.2 hr.

a mRNA stabilization model (Fig. 5a) we had to assume that deinduction is considerably slower than would be predicted by the steady-state mRNA half-life and that the kinetics are determined primarily by the slow rate of change of K_d ($t_{1/2}$ for the change in K_d about 8.5 hr); induction proceeds after a shorter, but still considerable, transition period for K_d. We could not find parameters that improved the fit to the late deinduction data without sacrificing the close fit to the intermediate time points. The mRNA production model for steroid regulation gave theoretical curves redrawn in Fig. 5b. In these simulations we assumed an initial 20-min lag before either induction or deinduction has begun and a constant mRNA half-life of 1.2 hr. To fit the induction data, we had to assume, in addition, that steroid increased K_s in a time-dependent manner with a half-life for the transition of about 1.2 hr (using an exponential change analogous to that for M in Eq. 2).

While the data are neither sufficiently precise nor extensive enough to allow valid statistical discrimination between the two sets of simulations, we favor the production model. It gives a somewhat better fit to the data (although changes in the form of the time-dependent function for K_d change can also be used to improve the fit), it requires only one significant modification of the simple production model (namely, the time-dependence of K_s change during induction), and it is consistent with the widespread finding of nuclear localization of steroid as a prerequisite of steroid action. Furthermore, since an upper limit of aminotransferase mRNA half-life is set at about 1.5 hr by the rapid induction kinetics, the mRNA stabilization mechanism would require that the functional half-life of aminotransferase mRNA, in the absence of steroid, be approximately 5–12 min, almost two orders of magnitude shorter than estimated half-lives of average poly(A)-containing cellular mRNAs (17, 27) and considerably shorter than those for even the most rapidly metabolized animal cell messengers yet studied.

The conclusion that steroids act by stimulating the production of specific cytoplasmic mRNAs, while localizing the site of steroid action to the nucleus, does not unambiguously define the mechanism of steroid action since, as indicated in Fig. 3b, several potentially regulable processes intervene between transcription and cytoplasmic mRNA appearance. The binding of receptor-steroid complexes to DNA (29) *in vitro* suggests a role of steroids in regulating transcription. Since 10–30 min are required for processing poly(A)-containing eukaryotic mRNAs (27, 30), a transcriptional effect of glucocorticoids would account for the lags preceding both induction and deinduction. However, considering the rapid nuclear uptake of steroid, a simple transcriptional model does not account for the very slow onset of induction relative to deinduction (Fig. 4). If this asymmetry is not artifactual (e.g., a result of cell handling), it may imply additional rate-limiting steps between nuclear localization of steroid and enhancement of transcription.

An alternative production model, compatible with the observed kinetic asymmetry, is that steroid regulates the

degradation of nuclear precursors of aminotransferase mRNA. Such a steroid-mediated stabilization would lead to increased nuclear pools of these RNAs which, by stochastic sampling, would result in an increase in cytoplasmic aminotransferase mRNA. Using Eq. [2] (above) to describe changes in concentration of nuclear mRNA sequences (where "K_d" includes terms for both degradation and transport to the cytoplasm), this model generates a slow accumulation of nuclear aminotransferase mRNA precursors on induction and a more rapid diminution on deinduction. Unlike the transcriptional model, this formulation predicts a degree of asymmetry between induction and deinduction kinetics equal to the extent of induction. In this model a further zero-order mRNA processing is still required to explain the absolute 20- to 40-min lag seen in both induction and deinduction.

Conclusions

These studies show that induction and deinduction of tyrosine aminotransferase synthesizing activity are rapid, suggesting a short aminotransferase mRNA half-life in the presence or absence of steroid inducers. This relative instability of aminotransferase mRNA compared with the marked stability of bulk cellular mRNA (17, 27) indicates that eukaryotic cells can effect rapid changes in some metabolic functions by regulating mRNA production; indeed, while kinetics cannot unambiguously determine the site of steroid action, our results suggest that steroids can accelerate mRNA production.

It should be noted that the ambiguities of the kinetic approach used here also apply to direct measurements of mRNA accumulation. Moreover, methodological and other difficulties pose potentially greater problems than the assumption used here that aminotransferase mRNA levels are directly proportional to aminotransferase synthetic rates. Unambiguous definition of the steroid-regulated step in mRNA production awaits a technique for assaying label in aminotransferase mRNA.

From measurements of the mRNA content of HTC cells, the relative rate of aminotransferase synthesis, and the estimated molecular weight of mRNA required to encode the 50,000-dalton polypeptide chain of tyrosine aminotransferase (Steinberg, unpublished results), we conclude that a steroid-induced HTC cell contains about 1000 molecules of aminotransferase mRNA. Coupled with the observed half-life (about 72 min), this implies an induced aminotransferase mRNA production rate of about 10 molecules per cell per minute, a value in close agreement with those determined for a number of mRNAs encoding proteins comprising major proportions of their cells' composition, including ovalbumin, hemoglobin, chymotrypsinogen, and coccoonase zymogen (18, 26). This striking similarity in production rates for mRNAs for major cell constituents and for aminotransferase mRNA, which comprises less than a half percent of HTC cell mRNA activity, lends added support to Kafatos' contention (26) that differential mRNA stability is an important factor in determining the relative levels of various cell messengers.

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