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# Regulation of Tyrosine Aminotransferase Messenger Ribonucleic Acid in Rat Liver. Effect of Cycloheximide on Messenger Ribonucleic Acid Turnover†

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ABSTRACT: Tyrosine aminotransferase messenger ribonucleic acid (mRNA) activity in rat liver was rapidly increased 3–6-fold following in vivo administration of hydrocortisone acetate, dibutyryladenosine cyclic 3′,5′-phosphate, or the protein synthesis inhibitor cycloheximide. Treatment with the steroid hormone or cyclic nucleotide in combination with cycloheximide resulted in levels of tyrosine aminotransferase mRNA 10–20-fold greater than control values. These changes in mRNA activity were not accompanied by changes in albumin mRNA or total liver template activity. The rapid decline in tyrosine aminotransferase mRNA activity following cordycepin inhibition of de novo RNA synthesis was prevented by cycloheximide treatment. This protection was not observed when pactamycin was substituted for cycloheximide, demonstrating that the inhibition of protein synthesis per se was not responsible for the stabilization of tyrosine aminotransferase mRNA. Based upon the effects of cycloheximide and pactamycin on rat liver polysome structure, it is concluded that the cycloheximide-mediated increase in tyrosine aminotransferase mRNA activity is the result of stabilization of the mRNA molecule which renders the message less susceptible to inactivation and degradation in the cytoplasm. The action of cycloheximide is very specific for tyrosine aminotransferase, phosphoenolpyruvate carboxykinase, and probably several other mRNAs that code for minor liver proteins that turn over rapidly in response to hormonal or metabolic stimuli.

A general feature of steroid hormone and adenosine cyclic 3′,5′-phosphate (cyclic AMP)[1] action in target tissues is an increase in the activity of specific enzymes following hormone treatment. The induction of enzyme activity is due principally to an increase in the rate of enzyme synthesis as a result of a rise in the level of functional mRNA coding for the enzyme (Higgins & Gehring, 1980; Rosenfeld & Barrieux, 1979). One of the best characterized examples of this phenomenon is the regulation of tyrosine aminotransferase mRNA by glucocorticoids and cyclic AMP in rat liver (Ernest & Feigelson,

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[1] Abbreviations: $Bt_2cAMP$, $N^6$,$O^{2'}$-dibutyryladenosine cyclic 3′,5′-phosphate; $NaDodSO_4$, sodium dodecyl sulfate; cyclic AMP, adenosine cyclic 3′,5′-phosphate; Tris, tris(hydroxymethyl)aminomethane.

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1979). While both compounds increase the level of translatable mRNA coding for tyrosine aminotransferase, the mechanism(s) by which the steroid hormone and cyclic nucleotide stimulate this mRNA activity is (are) not known.

The most direct site of action would be at the level of the structural gene where glucocorticoids or cyclic AMP might enhance tyrosine aminotransferase gene transcription. Evidence for this type of action has been presented for the glucocorticoid-dependent expression of mouse mammary tumor virus RNA (Ringold, 1979) and cyclic AMP regulation of phosphoenolpyruvate carboxykinase in rat liver (Yoo-Warren et al., 1981; Beale et al., 1982). However, in other instances, the accumulation of hormonally induced mRNAs appears to involve posttranscriptional and possibly translational actions (Palmiter & Carey, 1974; Wicks, 1974).

We reported recently that cycloheximide and other protein synthesis inhibitors were capable of stimulating tyrosine aminotransferase mRNA activity in rat liver (Ernest & Feigelson, 1978b). It was suggested that cycloheximide might block the synthesis of a short-lived protein repressor of tyrosine aminotransferase gene transcription. Alternatively, the drug might inhibit turnover of the mRNA template either directly by stabilizing the tyrosine aminotransferase mRNA–ribosome complex or indirectly by inhibiting the synthesis of a labile protein necessary for tyrosine aminotransferase mRNA degradation. The net effect of any of these actions would be an increase in the level of functional tyrosine aminotransferase mRNA in the liver following treatment with protein synthesis inhibitors.

In the present report, cycloheximide was used in combination with glucocorticoids and cyclic AMP to assess the processes which control the concentration of tyrosine aminotransferase mRNA in rat liver. When administered simultaneously with the natural inducers, cycloheximide increased tyrosine aminotransferase mRNA activity above the level achieved by treatment with the steroid hormone or cyclic nucleotide alone. This increase was the result of a stabilization of the mRNA template by cycloheximide.

## Experimental Procedures

*Chemicals.* $N^6$,$O^{2'}$-Dibutyryladenosine cyclic 3′,5′-phosphate ($Bt_2cAMP$), theophylline, cordycepin, cycloheximide, and oligo(dT)–cellulose were purchased from Sigma Chemical Co. Hydrocortisone acetate (Cortef acetate) and pactamycin were supplied by the Upjohn Co. L-[$^{35}$S]Methionine (>1000 Ci/mmol) was a product of New England Nuclear Co.

*Animals.* Sprague-Dawley (CD) rats (100–150 g) were supplied by Charles River Breeding Laboratories. They were maintained under a 12-h light–dark cycle and fed commercial chow. Fasted animals (24 h) were used in all the experiments. Each group consisted of at least three animals, and all experiments were started between 9 and 10 a.m. $Bt_2cAMP$ (30 mg/kg body weight) and theophylline (30 mg/kg) were dissolved together in saline and injected intraperitoneally. Cordycepin (15 mg/kg), cycloheximide (3 mg/kg), and pactamycin (3 mg/kg) were also prepared in saline and injected by the same route. Hydrocortisone acetate (50 mg/kg) was injected directly from suspension as supplied.

*Preparation and Assay of mRNA.* Poly(A+) RNA was isolated from frozen liver samples by guanidine hydrochloride extraction (Chirgwin et al., 1979) and oligo(dT)–cellulose column chromatography (Krystosek et al., 1975). Tyrosine aminotransferase and albumin mRNA were quantitated by translation in the rabbit reticulocyte lysate system (Pelham & Jackson, 1976) followed by immunoprecipitation of the in vitro synthesized, radioactive proteins by monospecific antibodies against tyrosine aminotransferase and rat serum albumin. The antigen–antibody complexes were isolated by adsorption to inactivated *Staphylococcus aureus* cells (Ivarie & Jones, 1979). Immunoprecipitates were analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis, and the gels were sliced and counted as described previously (Ernest & Feigelson, 1978a,b). Poly(A+) RNA concentrations were chosen so that the counts per minute incorporated into each protein was proportional to the amount of tyrosine aminotransferase or albumin mRNA added to the translation system.

Table I: Effect of Hormones and Cycloheximide on Tyrosine Aminotransferase and Albumin mRNA Activity in Rat Liver[a]

| treatment | catalytic activity[b] | mRNA activity: total[c] | mRNA activity: TAT[d] | mRNA activity: albumin[e] |
|---|---|---|---|---|
| saline | 10.6 | 1.36 | 410 | 11.4 |
| hydrocortisone | 55.8 | 1.42 | 2740 | 11.3 |
| $Bt_2cAMP$ | 42.3 | 1.28 | 1850 | 10.6 |
| cycloheximide | 7.2 | 1.35 | 1240 | 11.2 |
| hydrocortisone + cycloheximide | 7.8 | 1.27 | 8720 | 10.8 |
| $Bt_2cAMP$ + cycloheximide | 8.0 | 1.23 | 4540 | 10.9 |

[a] The concentrations of hormones and inhibitors administered intraperitoneally were the following: saline, 0.14 M NaCl; hydrocortisone acetate, 50 mg/kg body weight; $Bt_2cAMP$/theophylline, 30 mg of each/kg; cycloheximide, 3 mg/kg. At 2 h after $Bt_2cAMP$ treatment or 4 h after glucocorticoid treatment, the animals were killed and their livers frozen in liquid $N_2$. Total template activity and tyrosine aminotransferase (TAT) and albumin mRNA activities were determined as described under Experimental Procedures. [b] Units of tyrosine aminotransferase per milligram of protein. [c] cpm of [$^{35}$S]methionine incorporated into total protein × $10^{-6}$ per microgram of poly(A+) RNA. [d] cpm of [$^{35}$S]methionine incorporated into TAT per $10^6$ cpm in total protein. [e] cpm of [$^{35}$S]methionine incorporated into albumin per $10^3$ cpm in total protein.

Total mRNA template activity was determined by acid precipitation of the $^{35}$S-labeled proteins produced in the translation reaction. Total translation products were analyzed on an 8–15% NaDodSO$_4$–polyacrylamide gradient gel and visualized by fluorography (Laskey & Mills, 1975).

*Polysome Preparation and Analysis.* Polysomes were prepared from frozen liver samples by the magnesium precipitation procedure (Palmiter, 1974). Samples containing 5–10 $A_{260}$ units of polysomes were layered over 0.5–1.5 M linear sucrose gradients containing 25 mM Tris-HCl, pH 7.5, 25 mM NaCl, and 5 mM $MgCl_2$. The samples were centrifuged at 283000$g$ for 90 min at 2 °C. After centrifugation, the gradients were fractionated and monitored continuously at 254 nm by using a flow cell and a Gilford recording spectrophotometer.

## Results

Glucocorticoids and cyclic AMP are well-known inducers of several enzymes and their mRNAs in rat liver. The effect of these compounds on tyrosine aminotransferase catalytic and mRNA activity is shown in Table I. A 6.7-fold increase in mRNA activity was accompanied by a 5.3-fold increase in enzyme activity 6 h after treatment with hydrocortisone acetate. A comparable increase in catalytic and mRNA activity was observed 2 h after the administration of $Bt_2cAMP$. As reported previously, the protein synthesis inhibitor cycloheximide can also induce rat liver tyrosine aminotransferase mRNA activity in vivo (Ernest & Feigelson, 1978b). Under conditions in which protein synthesis was inhibited by 95%, cycloheximide tripled tyrosine aminotransferase mRNA ac-

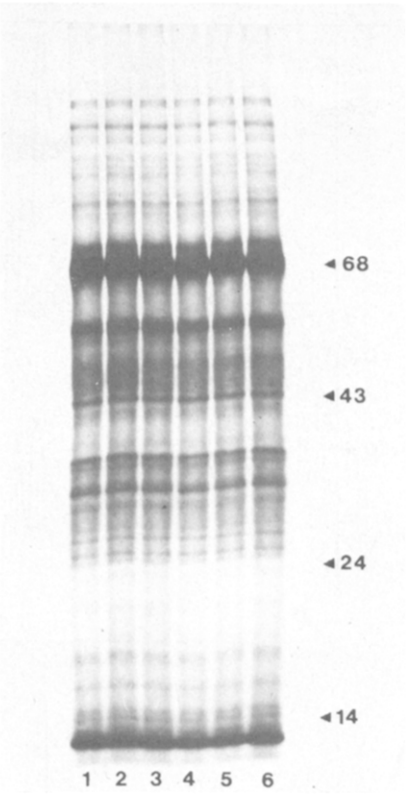


FIGURE 1: Total translation products coded for by rat liver poly(A+) RNA from hormone- and cycloheximide-treated animals. The experimental conditions were the same as those described in Table I. Samples of the translation reactions were analyzed on an 8–15% $NaDodSO_4$–polyacrylamide gradient gel and visualized by fluorography. Lane 1, saline; lane 2, cycloheximide; lane 3, hydrocortisone; lane 4, hydrocortisone + cycloheximide; lane 5, $Bt_2cAMP$; lane 6, $Bt_2cAMP$ + cycloheximide. The numbers to the right of the fluorograph are the molecular weights ($\times 10^{-3}$) of a set of standard proteins run on the same gel.

tivity within 2 h (Table I). Since de novo protein synthesis was completely arrested by the drug, no increase in enzyme activity was observed. A greater than 20-fold increase in tyrosine aminotransferase mRNA activity was observed within several hours after treatment with hydrocortisone and cycloheximide as shown in Table I. A smaller increase was seen when $Bt_2cAMP$ and cycloheximide were tested together.

So that it could be determined if the increase in tyrosine aminotransferase mRNA activity is a specific response to cycloheximide, the effect of the drug on total mRNA template activity and the level of albumin mRNA was assessed. Albumin was chosen because it is a major, long-lived liver protein, in contrast to tyrosine aminotransferase. It is clear from the data in Table I that cycloheximide alone or in combination with the steroid hormone or cyclic AMP did not significantly alter the total liver mRNA template activity during the time period in which tyrosine aminotransferase mRNA activity was increased. Likewise, the activity of albumin mRNA was not affected by any of the treatments listed in Table I. In addition, a $NaDodSO_4$–polycrylamide gel profile of the total translation products coded for by the various mRNA preparations did not reveal any major changes in the activity of other liver mRNAs (Figure 1). These findings suggest that the cycloheximide-mediated increase in tyrosine aminotransferase mRNA activity in the presence of glucocorticoids or $Bt_2cAMP$ is not a general phenomenon. Rather, it appears to be highly specific for tyrosine aminotransferase and probably several other mRNAs coding for proteins which represent only a small fraction of the total liver protein population (Nelson et al., 1980).

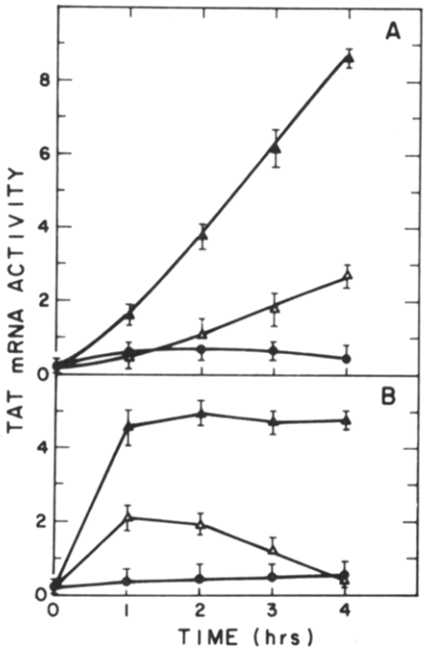


FIGURE 2: Time course of tyrosine aminotransferase (TAT) mRNA induction by hormones and cycloheximide in rat liver. The experimental conditions were the same as those described in Table I except animals were killed at the indicated times after treatment. The data are expressed as the mean ± standard error for three separate experiments using three animals per time point. (A) Saline (●), hydrocortisone (△), and hydrocortisone + cycloheximide (▲). (B) Saline (●), $Bt_2cAMP$ (△), and $Bt_2cAMP$ + cycloheximide (▲).

For further investigation of the mechanism of cycloheximide action, the time course of the increase in tyrosine aminotransferase mRNA activity was examined. The effect of cycloheximide on the kinetics of mRNA induction by hydrocortisone is shown in Figure 2A. Following a 1-h delay, the steroid hormone alone promoted a linear increase in tyrosine aminotransferase mRNA activity. When cycloheximide was administered simultaneously, the lag period disappeared and tyrosine aminotransferase mRNA began to accumulate immediately. Over the next several hours, the increased rate of induction in the presence of cycloheximide resulted in a level of tyrosine aminotransferase mRNA severalfold greater than that elicited by hydrocortisone alone. A similar response was observed for $Bt_2cAMP$ shown in Figure 2B. However, the transient nature of the induction by the cyclic nucleotide provided a clue to the mechanism of action of cycloheximide. Treatment with $Bt_2cAMP$ alone resulted in a rapid induction of liver tyrosine aminotransferase mRNA activity which peaked within 1 h and declined to basal levels after 3–4 h. Cycloheximide, when administered simultaneously with $Bt_2cAMP$, maintained the induced level of mRNA activity over the course of the experiment. This suggested that cycloheximide was stabilizing tyrosine aminotransferase mRNA activity in rat liver.

So that this hypothesis could be tested, the half-life of tyrosine aminotransferase mRNA was measured in the presence of cordycepin to block de novo mRNA synthesis (Noguchi et al., 1978; Olson et al., 1980). If cycloheximide stabilizes tyrosine aminotransferase mRNA activity, it should be reflected in an increase in the half-life of the mRNA. Since tyrosine aminotransferase mRNA levels that are substantially lower than adult basal levels fall below the limit of detectability of the translational assay, the half-life experiments were performed by using animals that were preinduced with $Bt_2cAMP$ or hydrocortisone. Following the induction period, cordycepin or cordycepin and cycloheximide were adminis-

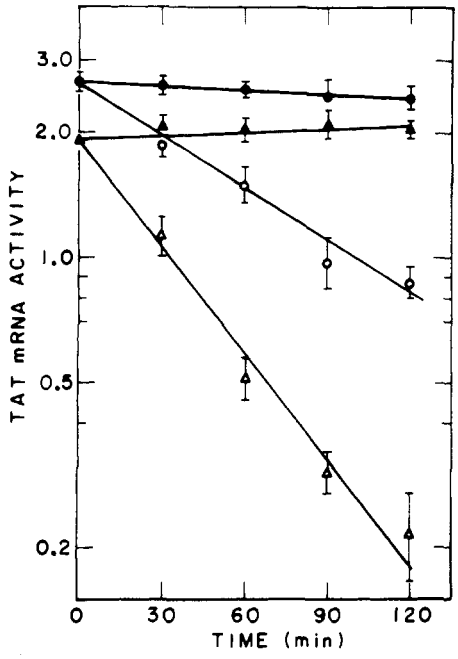


FIGURE 3: Turnover of rat liver tyrosine aminotransferase (TAT) mRNA in the presence and absence of cycloheximide. Animals were preinduced for 2 h with $Bt_2cAMP$ or for 4 h with hydrocortisone. Following preinduction (time zero), cordycepin (15 mg/kg body weight) or cordycepin + cycloheximide was administered. Animals were killed at the indicated times, and tyrosine aminotransferase mRNA activity was determined. The data are expressed as the mean ± standard error for three separate experiments using three animals per time point. Circles, hydrocortisone induced: +cordycepin (○) or +cordycepin and cycloheximide (●). Triangles, $Bt_2cAMP$ induced: +cordycepin (△) or +cordycepin and cycloheximide (▲).

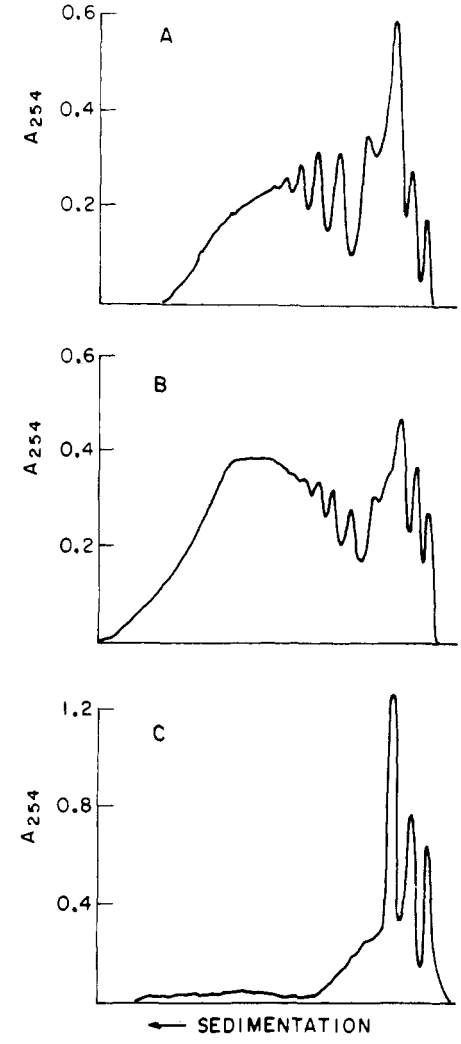


FIGURE 4: Profile of liver polysomes prepared from rats treated with cycloheximide or pactamycin. Animals were treated with saline (A), cycloheximide (B), or pactamycin (C) for 2 h. Polysomes were prepared from frozen liver samples by magnesium precipitation and analyzed on sucrose gradients as described under Experimental Procedures.

tered. The concentration of cordycepin used in these experiments inhibited RNA synthesis by 92% on the basis of labeled percurosr incorporation. Tyrosine aminotransferase mRNA activity was determined at 30-min intervals after treatment with the inhibitors. The results are shown in Figure 3. In the absence of cycloheximide and following the arrest of de novo RNA synthesis by cordycepin, tyrosine aminotransferase mRNA activity declined rapidly with a half-life of 1.3 h in hydrocortisone-treated animals and 45 min in animals induced with $Bt_2cAMP$. When cycloheximide was administered at the same time RNA synthesis was inhibited, tyrosine aminotransferase mRNA was completely stabilized at the induced level of activity. These results demonstrate that cycloheximide prevents the inactivation or degradation of tyrosine aminotransferase mRNA activity in the liver cell cytoplasm.

As an inhibitor of polypeptide elongation, cycloheximide treatment results in polysome aggregation increasing the density of ribosomes on mRNA molecules (Pestka, 1971). As noted in the introduction, aggregation of the ribosome–mRNA complex by cycloheximide might serve to physically protect tyrosine aminotransferase mRNA from cytoplasmic turnover. This would result in the maintenance of induced levels of tyrosine aminotransferase mRNA even in the absence of de novo mRNA synthesis.

So that this hypothesis could be tested, the protein synthesis inhibitor pactamycin was used in place of cycloheximide. In contrast to cycloheximide action, pactamycin, which inhibits initiation of protein synthesis, leads to dissociation of polysome complexes (Pestka, 1971). Figure 4 shows the sucrose density gradient profiles of rat liver polysomes prepared from animals treated with cycloheximide or pactamycin. On the basis of the areas under the peaks, 32% of the ribosomal material from the control polysome preparation was present as 80S monosomes and 40S and 60S subunits (Figure 4A). Treatment with cycloheximide reduced the monomer and subunit fraction to 14% and promoted a general shift in the profile toward heavier polysomes (Figure 4B). Most of the tyrosine aminotransferase mRNA activity was associated with large polyribosomes (larger than hexasomes) in control liver. No major change in the size distribution was observed after cycloheximide treatment, although, as expected, cycloheximide polysomes contained 2.5–3 times more tyrosine aminotransferase mRNA activity than the control. Pactamycin treatment caused an almost complete dissociation of liver polysomes into monosomes and subunits (Figure 4C).

When pactamycin was substituted for cycloheximide in the half-life experiments, no stabilization of glucocorticoid- or cyclic AMP induced tyrosine aminotransferase mRNA activity was observed as shown in Table II. Despite an 84% inhibition of protein synthesis by pactamycin, the drug did not prevent the decline in tyrosine aminotransferase mRNA activity 2 h after RNA synthesis was blocked by cordycepin. In fact, pactamycin appeared to further enhance the decline in tyrosine aminotransferase mRNA activity compared to cordycepin treatment alone. Apparently, the cycloheximide-mediated stabilization of tyrosine aminotransferase mRNA is not a consequence of the inhibition of protein synthesis per se.

Table II: Effect of Protein Synthesis Inhibitors and Cordycepin on Tyrosine Aminotransferase mRNA Activity in Rat Liver[a]

| treatment | mRNA activity | |
|---|---|---|
| | total[b] | TAT[c] |
| hydrocortisone preinduced | | |
| +cordycepin | 1.39 | 970 |
| +cordycepin and cycloheximide | 1.35 | 2680 |
| +cordycepin and pactamycin | 1.32 | 440 |
| $Bt_2cAMP$ preinduced | | |
| +cordycepin | 1.31 | 360 |
| +cordycepin and cycloheximide | 1.22 | 1760 |
| +cordycepin and pactamycin | 1.37 | 230 |

[a] Animals were induced for 2 h with $Bt_2cAMP$ or for 4 h with hydrocortisone acetate. Following induction, cordycepin (15 mg/kg body weight) and cycloheximide (3 mg/kg) or pactamycin (3 mg/kg) were administered. Animals were killed 2 h after cordycepin treatment and their livers frozen in liquid $N_2$. Total template, activity and TAT mRNA activity were determined as described under Experimental Procedures. [b] cpm of [$^{35}$S]methionine incorporated into total protein $\times 10^{-6}$ per microgram of poly(A+) RNA. [c] cpm of [$^{35}$S]methionine incorporated into TAT per $10^6$ cpm in total protein.

Rather, it is concluded that cycloheximide action is the result of protection of the mRNA molecule, possibly in the form of aggregated polysomes, which renders the message less susceptible to inactivation and degradation in the cytoplasm.

## Discussion

As noted previously, the possible mechanisms by which cycloheximide increases tyrosine aminotransferase mRNA activity in rat liver can be classified into two categories. The first is inhibition of the synthesis of a short-lived protein that regulates the activity of tyrosine aminotransferase mRNA. This regulatory protein might inhibit tyrosine aminotransferase gene transcription or posttranscriptional processing, or it might stimulate cytoplasmic turnover of the mRNA molecule. Inhibition of the synthesis of the regulatory protein by cycloheximide would result in an increase in the level of functional tyrosine aminotransferase in the liver cell. This type of control has been implicated in the deinduction and superinduction of fibroblast interferon, where a labile protein appears to repress or inactivate interferon mRNA (Sehgal et al., 1977; Cavalieri et al., 1977). A similar model has been proposed for the turnover of phosphoenolpyruvate carboxykinase mRNA in rat liver (Faliks et al., 1980).

The second category involves a more direct effect of the protein synthesis inhibitor on tyrosine aminotransferase mRNA. By interruption of the normal ribosome cycle, cycloheximide could stabilize the ribosome–tyrosine aminotransferase mRNA complex, thereby protecting the mRNA from cytoplasmic inactivation or degradation. Evidence for this type of action has been presented also for phosphoenolpyruvate carboxykinase mRNA (Nelson et al., 1980).

The results presented in this report demonstrate, first, that cycloheximide is capable of stabilizing tyrosine aminotransferase mRNA in the cytoplasm. Stabilization was observed during the natural decline in tyrosine aminotransferase mRNA activity following $Bt_2cAMP$ induction (Figure 2B) or when de novo RNA synthesis was arrested by cordycepin (Figure 3). The fact that cycloheximide further increases mRNA activity when administered in combination with glucocorticoids or $Bt_2cAMP$ indicates that the mechanism of cycloheximide action (mRNA stabilization) is probably different from the means by which the steroid hormone and cyclic AMP induce tyrosine aminotransferase mRNA activity.

So that the mechanisms cited above could be distinguished, four different protein synthesis inhibitors have thus far been tested for their effect on tyrosine aminotransferase mRNA. Two of the inhibitors, cycloheximide and emetine, promote polysome aggregation as a consequence of their mechanisms of action (Grollman, 1968). Both were good inducers of tyrosine aminotransferase mRNA activity (Ernest & Feigelson, 1978b). The other two inhibitors, pactamycin and puromycin, cause a breakdown of polysome structure (Pestka, 1971). Pactamycin did not stabilize tyrosine aminotransferase mRNA activity (Table II), while puromycin was as effective as cycloheximide or emetine (Ernest & Feigelson, 1978b). Since puromycin and pactamycin inhibited labeled precursor incorporation into protein to the same extent (78% and 84%, respectively), it seems unlikely that a labile protein regulator of tyrosine aminotransferase mRNA is involved. However, since both drugs also promote polysome dissociation, the results are difficult to reconcile with a model based on polysome stabilization. One possibility has emerged from a consideration of the mechanism of action of pactamycin and puromycin.

Pactamycin inhibits initiation by interfering with the binding of initiator tRNA to the initiation complex. It binds to the 40S ribosome subunit and alters the structure of the initiation complex, causing destabilization and dissociation. At higher concentrations, the drug inhibits translocation (MacDonald & Goldberg, 1970).

In contrast, puromycin, through its resemblance to the aminoacyladenyl end of aminoacyl-tRNA, inhibits protein synthesis by competing with aminoacyl-tRNA for its binding site on the 60S ribosome subunit. In place of aminoacyl-tRNA, puromycin accepts nascent peptides, causing premature release of incomplete peptide chains (Pestka, 1971). It has been suggested that since aminoacyl-tRNA is intimately involved in the ribosome cycle, replacement of aminoacyl-tRNA and release of peptidyl-tRNA by puromycin might loosen the association between the subunits so that ribosomes fall off mRNA, leading to dissociation of the polysome complex. However, ribosome translocation continues in the presence of puromycin; a new chain is initiated presumably with the amino acid specified by the codon next to the point of interruption (Williamson & Schweet, 1965). Since puromycin will act again in subsequent steps, the initiation of new peptide chains ends abortively in the production of short, puromycin-linked, oligopeptide fragments (Morris et al., 1963). The random distribution of the N-terminal amino acids of these oligopeptides (Williamson & Schweet, 1965) indicates that initiation can begin anywhere along the mRNA molecule. Either ribosomes re-form from subunits at random points along the message or more likely, following puromycin release, the 60S subunit dissociates and new 60S subunits reassociate with 40S subunits that remain attached to the mRNA (Kaempfer & Meselson, 1969). It has been shown by Kaempfer and Meselson that puromycin almost instantaneously stimulates this type of ribosomal subunit exchange along mRNA.

Thus, the mechanism by which pactamycin and puromycin dissociate polysomes may account for their opposite effects on tyrosine aminotransferase mRNA activity. In the presence of puromycin, enough 40S subunits or 80S ribosomes may remain attached to tyrosine aminotransferase mRNA to protect it against inactivation even though the polysome complex has been disrupted. As an inhibitor of initiation but not translocation, pactamycin treatment would result in the accumulation of free ribosomal subunits and unprotected tyrosine aminotransferase mRNA.

The cycloheximide-mediated increase in tyrosine aminotransferase mRNA activity has been observed only in intact rats (Ernest & Feigelson, 1978b; Hofer & Sekeris, 1978). No

change in tyrosine aminotransferase mRNA activity was detected in hepatoma tissue culture (HTC) cells treated with cycloheximide alone or in combination with dexamethasone (Olson et al., 1980). It was suggested that the cycloheximide effect may be a consequence of the release of endogenous inducers (e.g., glucocorticoids, catecholamines, or glucagon) as part of a general stress response to the drug. This seems unlikely for two reasons. First, cycloheximide increases tyrosine aminotransferase mRNA activity above the levels induced by exogenous glucocorticoids or cyclic AMP, suggesting a mechanism of action that is different from that of the natural inducers. And second, similar studies using adrenalectomized rats yielded the same results as those reported here for intact animals (data not shown). At present, the reason for the absence of a cycloheximide response in HTC cells is unknown.

The question of the specificity of the effect of cycloheximide on rat liver mRNA deserves further comment. The data in Table I and Figure 1 demonstrate that there is no significant change in total template activity or albumin mRNA activity or the spectrum of translation products coded for by mRNA prepared from control and cycloheximide-treated animals. However, there is at least one other rat liver mRNA whose activity is stabilized by cycloheximide (Nelson et al., 1980; Faliks et al., 1980). Phosphoenolpyruvate carboxykinase mRNA is a short-lived message ($t_{1/2}$ = 40 min) that represents only a small fraction of the total liver mRNA activity and is inducible by glucocorticoids and $Bt_2$cAMP. Nelson et al., studying phosphoenol pyruvate carboxykinase mRNA turnover, reported that cycloheximide treatment produced a 5-fold increase in mRNA activity in rat liver over a period of 4 h. A further increase was observed when $Bt_2$cAMP and the protein synthesis inhibitor were administered simultaneously. Puromycin was ineffective when used in place of cycloheximide. It also did not prevent the rapid decay in phosphoenolpyruvate carboxykinase mRNA activity when RNA synthesis was blocked with cordycepin. This same group recently reported that these effects on phosphoenolpyruvate carboxykinase mRNA activity were accompanied by corresponding changes in the number of phosphoenolpyruvate carboxykinase mRNA sequences measured by cDNA hydribization (Cimbala et al., 1982). It was concluded that cycloheximide treatment served to stabilize polysome structure and thereby protect phosphoenolpyruvate carboxykinase mRNA from inactivation and degradation.

Employing virtually the same experimental procedures, Faliks et al. (1980) reported that cycloheximide and pactamycin were equally effective in preventing the decline in phosphoenolpyruvate carboxykinase mRNA activity following insulin or cordycepin treatment of diabetic rats. Neither cycloheximide nor pactamycin alone had any effect on mRNA activity. It was concluded by these investigators that the turnover of phosphoenolpyruvate carboxykinase mRNA is dependent upon ongoing protein synthesis but independent of polysome structure. They suggested the possible existence of a system for mRNA inactivation in which one or more labile proteins participate.

While both groups agree that cycloheximide is capable of stabilizing phosphoenolpyruvate carboxykinase mRNA, each suggests a different mechanism based upon the effects of pactamycin and puromycin. In this instance, pactamycin but not puromycin can substitute for cycloheximide in stabilizing phosphoenolpyruvate carboxykinase mRNA activity.

The results of the studies on tyrosine aminotransferase mRNA and phosphoenolpyruvate carboxykinase mRNA suggest that the stabilization of mRNA activity by protein synthesis inhibitors is probably restricted to a limited number of mRNAs coding for minor liver proteins which turnover rapidly ($t_{1/2}$ < 2 h) in response to hormonal or metabolic stimuli. The short half-life of these mRNAs makes them particularly sensitive to the stabilizing action of cycloheximide (Berlin & Schimke, 1965). The majority of liver protein mRNAs, which turn over much more slowly, would be much less sensitive to cycloheximide.

Alternatively, the specificity of cycloheximide action might be related to the efficiency of translation of the message. For example, cycloheximide could stabilize those mRNAs that are less efficiently translated (having fewer ribosomes per mRNA molecule) compared to messages that are more efficiently translated by virtue of having more ribosomes per molecule of mRNA. Cycloheximide is known to enhance the utilization of inefficient mRNAs by slowing down polypeptide chain elongation without interfering with initiation (Lodish, 1971). This phenomenon has been observed in mouse sarcoma 180 ascites cells where a substantial proportion of the ribosomes and cellular mRNA occurs as small, inactive aggregates [20–70S particles, monosomes, and small polysomes (≤ tetramers) (Geoghegan et al., 1979)]. Individual mRNAs were distributed in different proportions between active polysomes and small aggregates. Some, but not all, of the mRNAs could be driven almost completely into polysomes upon incubation of cells with cycloheximide. However, it seems unlikely that this type of action could account for tyrosine aminotransferase mRNA stabilization since the message activity in control liver was already associated with large polyribosomes and no major size shift was observed after cycloheximide treatment.

While the precise mechanism of stabilization remains to be established, the results clearly demonstrate the complexity of the effects that can occur when protein synthesis inhibitors are used to study the regulation of specific mRNAs. This is due, in part, to the fact that the mechanism and precise mode of action of most inhibitors have not been definitively established (Pestka, 1971). On the other hand, changes in the stability of these mRNAs, in response to protein synthesis inhibitors, emphasize the role that cytoplasmic turnover can play in regulating the level of translatable mRNA in the cell.

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# Enzyme Digestion of Intermediates of Excision Repair in Human Cells Irradiated with Ultraviolet Light†

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ABSTRACT: During excision repair of DNA in ultraviolet-irradiated human fibroblasts there was an alteration of protein–nucleic acid interactions that resulted in a nearly 2-fold increase in sensitivity of the repair patch to digestion by micrococcal nuclease. This was followed by a decrease in nuclease sensitivity of the repair patch during the reassembly of chromatin structure, which had a half-time of about 30 min. This decrease in nuclease sensitivity was not influenced by the presence of cycloheximide during the labeling period. The mechanism of reassembly of the DNA repair patch into a nucleosomal structure is therefore different from reassembly of newly replicated DNA strands and does not require de novo protein synthesis. Treatment of UV-irradiated human fibroblasts with 1-β-D-arabinofuranosylcytosine (ara-C) resulted in a dose-dependent inhibition of DNA repair synthesis and an accumulation of incomplete patches with 3′-OH termini. Maximum (65%) inhibition of DNA repair synthesis occurred at ≥25 μM ara-C. Continuous incubation of cells for up to 4 h in the presence of ara-C also resulted in a dose-dependent increase in micrococcal nuclease sensitivity of the DNA repair patch. Reassembly of the nucleosomal structure therefore occurs after synthesis of the DNA repair patch and its ligation into the strand. Treatment with ara-C also resulted in a 2.5–10-fold increase in the susceptibility of the repair patch in DNA and chromatin to enzymatic digestion by exonuclease III. However, in ara-C-treated cells the amount of the repair patch susceptible to exonuclease III digestion was 3-fold greater in purified DNA than in isolated nuclei. These results, in conjunction with the results from digestion by micrococcal nuclease, suggest that although there is an alteration in the protein–nucleic acid interactions in the newly synthesized repair patch, they are not completely eliminated. Incubation of ultraviolet-irradiated cells with [$^3$H]ara-C resulted in the incorporation of [$^3$H]ara-C into purified DNA. Exonuclease III removed only 48% of the [$^3$H]ara-C, indicating that ara-C did not block completion of ligation at every repair site. Estimates of the number of repair events from the number of single-strand breaks that accumulate in the presence of ara-C will therefore be in error by as much as a factor of 2, and use of this inhibitor for quantitative comparisons of repair between different cell types will be unreliable.

Excision repair of ultraviolet (UV)[1]-induced damage to DNA involves the synthesis of short patches throughout the genome. When irradiated cells have been incubated with [$^3$H]thymidine, which labels these repair patches, it has been shown that repaired DNA undergoes a transition from being initially highly susceptible to enzymatic digestion to eventually having the same susceptibility to digestion as bulk chromatin (Bodell & Cleaver, 1981; Smerdon & Lieberman, 1978, 1980). This structural transition represents one of the final steps of

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[1] Abbreviations: UV, ultraviolet; ara-C, 1-β-D-arabinofuranosylcytosine; HU, hydroxyurea; dThd, thymidine; dCyd, 2′-deoxycytidine; Tris, tris(hydroxymethyl)aminomethane.

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