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Differential degradation of messenger RNAs in mammalian cells

(tyrosine/alanine/aminotransferases/translation/methylation)

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ABSTRACT **Through the use of an assay that measures cellular capacity for specific enzyme synthesis, mRNA of alanine aminotransferase (EC 2.6.1.2; L-alanine:2-oxoglutarate aminotransferase) was found to be degraded with a half-life of 12–14 hr in cultured Reuber H-35 cells; mRNA of tyrosine aminotransferase (EC 2.6.1.5; L-tyrosine:2-oxoglutarate aminotransferase) has a half-life of 2 hr in the same cells. Rates of degradation of the mRNAs are the same whether new mRNA accumulation is blocked by removal of the steroid inducer or by inhibition of mRNA synthesis (actinomycin). Cycloheximide inhibits the normally rapid turnover of tyrosine aminotransferase mRNA, but agents such as puromycin and sodium fluoride, which disrupt polysome structure, do not alter the turnover rate of the tyrosine and alanine aminotransferase mRNAs. The tyrosine and alanine aminotransferase mRNAs appear to be translated at equivalent rates. The data suggest that the degradation rate of these two mRNAs is determined by the polynucleotide structure of the mRNA molecules at or near the site for ribosome binding and initiation.**

Within rat liver cells, individual species of mRNA are degraded at widely variable rates with functional half-lives ranging from 1 hr for levulinate synthetase mRNA (1) to several days for albumin mRNA (2). The differential stability of individual mRNAs obviously plays an important role in the regulation of protein synthesis, but the mechanisms that control mRNA degradation are undefined and relatively unstudied.

The tyrosine and alanine aminotransferases of rat liver and of minimal deviation hepatoma tissue culture lines are similar enzymes with respect to size, subunit composition, and localization in the soluble fraction of cytoplasmic protein (3, 4). The mRNAs coding for these enzymes should thus be similar with respect to molecular weight and subcellular localization. Steroid hormones induce synthesis of both enzymes, apparently by increasing the cellular content of functional mRNA (5). We describe here studies on the simultaneous degradation of tyrosine and alanine aminotransferase mRNAs in cultured hepatoma cells and on parameters that might account for the differential stability of the two messenger molecules.

EXPERIMENTAL PROCEDURES

Culture of H-35 Cells. Conditions for growth of H-35 cells, a permanent cell line derived from a minimal-deviation hepatoma developed by Reuber (6), have been described (7).

Preparation of Specific Antisera Against Tyrosine Aminotransferase and Alanine Aminotransferase. Tyrosine aminotransferase (EC 2.6.1.5; L-tyrosine:2-oxoglutarate aminotransferase) was purified to homogeneity from rat livers by modifications of a protocol described (8). Alanine aminotransferase (EC 2.6.1.2; L-alanine:2-oxoglutarate aminotransferase) was purified by a modification of the protocol described by Segal and Matsuzawa (9). The purified enzymes were administered to New Zealand rabbits to evoke antibody production as described (8). The gamma globulin fraction was isolated from immunized rabbit sera by precipitation with ammonium sulfate at 40% saturation.

Partial Purification and Immunoprecipitation of Radioactive Tyrosine and Alanine Aminotransferase from H-35 Cells. At appropriate times after cells were labeled with radioactive leucine, they were scraped from the culture-flask surface, collected by centrifugation, and resuspended in 50 mM phosphate buffer (pH 7.6) supplemented with 0.15 M KCl, 5 mM α-ketoglutarate, 1 mM EDTA, 3 μg/ml of pyridoxal phosphate, and 1 mM dithiothreitol. Soluble protein fractions were prepared as described (10). Twenty-five micrograms of each of the aminotransferases were added to each sample as carrier. The samples were then passed through columns packed with 2.0 ml of DEAE-cellulose, type standard 70, which had been preequilibrated with 50 mM phosphate buffer (pH 7.5) supplemented with 5 mM α-ketoglutarate, 1 mM EDTA, 3 μg/ml of pyridoxal phosphate, and 1 mM dithiothreitol. Tyrosine aminotransferase was retained on the columns while 90% of the protein (and the alanine aminotransferase) passed through. The absorbed tyrosine aminotransferase was eluted with buffer adjusted to pH 7.1 and containing 0.4 M KCl. The eluted tyrosine aminotransferase was dialyzed against 50 mM phosphate buffer (pH 7.1) supplemented with the enzyme protectors, heat-treated, and precipitated with antibody as described (10).

The crude alanine aminotransferase samples, which passed through the DEAE-cellulose columns at pH 7.6, were subjected to heat treatment as described by Segal and Matsuzawa (9) and dialyzed against 10 mM phosphate buffer (pH 5.7) supplemented with 1 mM dithiothreitol. The samples were then absorbed onto columns packed with 2 ml of DEAE-cellulose, which had been preequilibrated with the dialysis buffer. The columns were washed and then alanine aminotransferase was eluted with 10 mM phosphate buffer, supplemented with dithiothreitol to 1 mM and disodium sulfate to 25 mM. The eluted alanine aminotransferase was dialyzed against 10 mM phosphate buffer (pH 7.6) plus 1 mM dithiothreitol and finally precipitated with antibody. Tyrosine and alanine aminotransferase activity in each sample was monitored throughout the partial purification procedure; final recovery of both enzymes was consistently between 70 and 75%.

Determination of Peptide Elongation Rate on Specific mRNAs. These measurements were made by the method of Fan and Penman (11). Briefly, radioactive leucine was added simultaneously to a number of flasks of H-35 cells that had been pretreated with 1 μM hydrocortisone. After addition of isotope, cells were harvested periodically and frozen at once in liquid nitrogen. Cells were lysed by alternate freeze-thawing, centrifuged 10 min at 10,000 × g to remove particulate matter, and the supernatant fraction, containing the microsomal and soluble proteins, was taken. Total trichloroacetic acid-insoluble ra-

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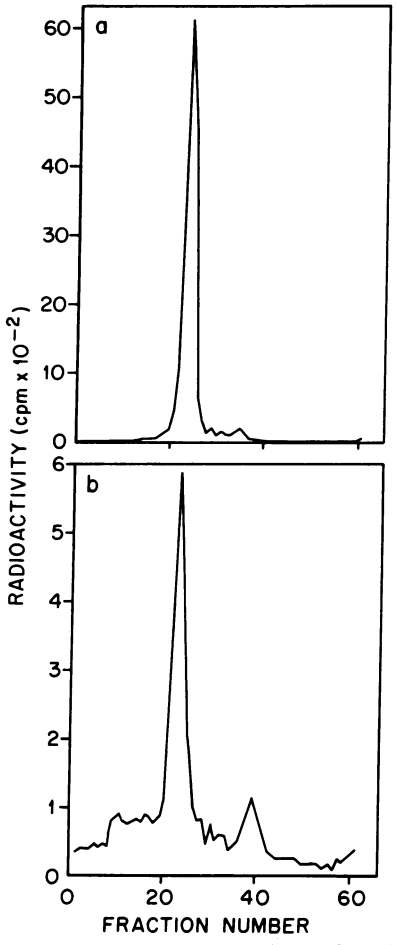

FIG. 1. Sodium dodecyl sulfate gel electrophoresis of the radioactive immunoprecipitates. Migration of the proteins proceeded from left to right. Cells were first treated with hydrocortisone (1 μM) for 20 hr, and for the last 2 hr of this interval [^{3}H]leucine (5 μCi/ml) was also present. The two aminotransferases were partially purified and precipitated with their respective antisera as described above, then dissolved in sodium dodecyl sulfate and subjected to electrophoresis by the method of Weber and Osborne (12). (a) Tyrosine aminotransferase; (b) alanine aminotransferase.

dioactivity (counts per minute in released protein plus radioactive nascent polypeptide chains) was assayed from these fractions. A plot of this radioactivity against time is linear shortly after addition of isotope, and the intercept with the time axis indicates the time at which isotope begins to be incorporated at a linear rate. In separate measurements, cells were treated in the same fashion except that all samples were centrifuged at 40,000 rpm for 30 min to pellet the microsomal fraction and polysomes as well. By immunoprecipitation, appearance of the radioactive tyrosine and alanine aminotransferases in the soluble fractions was monitored as a function of time, and the data were plotted in the same fashion as the total radioactivity data. The horizontal distance on the time axis between the intercept for total (nascent plus released) radioactivity and the intecept for released radioactive enzyme represents the half-time required for the ribosome to transverse the mRNA from the 5′ to 3′ end and to release the completed polypeptide chain.

RESULTS

Specificity of Rabbit Antisera to Tyrosine Aminotransferase and to Alanine Aminotransferase. The degree of specificity of the immune procedures used can be seen from

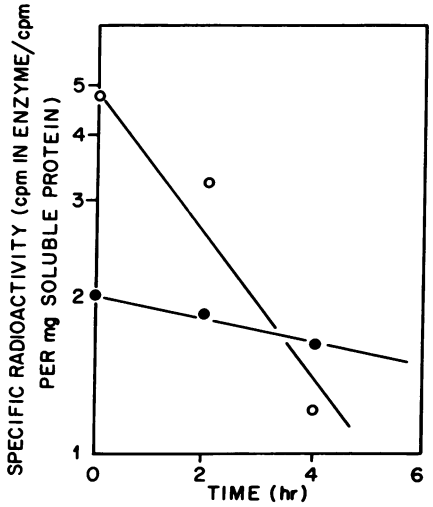

FIG. 2. Degradation of the tyrosine and alanine aminotransferase mRNAs. H-35 cells were grown to near stationary phase and then exposed to 1 μM hydrocortisone in Eagle's basal medium for 18 hr prior to the start of the experiment. At time zero, the steroid-containing medium was decanted; all cells were washed twice with Hanks' balanced salts and then fresh steroid-free medium was added. At zero time, 2 hr, and 4 hr, cells were exposed to 5 μCi/ml of [^{3}H]leucine (specific activity 55 Ci/mmol, from Schwarz/Mann) in leucine-free Eagle's basal medium for 15 min. The tyrosine and alanine aminotransferases were partially purified from the cell lysates and precipitated with antibodies as described in *Experimental Procedures*. Specific radioactivity in tyrosine aminotransferase (×10^{-2}) (○); specific radioactivity in alanine aminotransferase (×10^{-3}) (●).

the data of Fig. 1. Here the enzyme–antibody precipitates, prepared and washed as described (10), were solubilized in sodium dodecyl sulfate and subjected to gel electrophoresis in the presence of that detergent. Radioactivity in the tyrosine aminotransferase–antibody complex is essentially entirely in a single component representing enzyme subunits of 55,000 molecular weight. [A previous report (3) suggested that the tyrosine aminotransferase enzyme possessed four subunits; however, a later report from the same laboratory (13) and our own data indicate that the enzyme has two identical subunits of 55,000 molecular weight.] Precipitation of the alanine aminotransferase is not quite as specific, but most of the radioactivity is found in the 57,000 molecular weight subunits reported for this enzyme (4). Since nonspecific radioactivity as detected by gel electrophoresis is the same as that determined by a second immunoprecipitation procedure described earlier (10), the latter procedure was routinely used in our experiments. The data reported have been corrected for nonspecific radioactivity precipitated.

Turnover of the Tyrosine and Alanine Aminotransferase mRNAs. Intracellular degradation rates were determined by analysis of the rate of synthesis of each enzyme after treatments (inducer withdrawal, treatment with actinomycin) that cause cessation of mRNA synthesis. Fig. 2 shows the loss of mRNA activity for tyrosine and alanine aminotransferase in cells that were first treated with hydrocortisone and then switched to steroid-free medium at time zero. The capacity of cells to synthesize tyrosine aminotransferase decays exponentially with a half-life of about 2 hr; alanine aminotransferase mRNA activity is lost with a half-life of about 14 hr in the same cells. In several experiments of this kind the measured half-lives ranged from 1.5 to 2.2 hr for tyrosine aminotransferase mRNA and from 12 to 14 hr for alanine aminotransferase mRNA.

Turnover of tyrosine aminotransferase mRNA activity in H-35 cells has been analyzed before; these data show that the

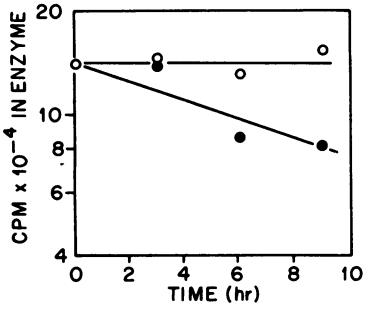

FIG. 3. Degradation of alanine aminotransferase mRNA in actinomycin D-treated cells. H-35 cells were grown to near stationary phase and then placed in fresh medium containing 1 μM hydrocortisone. Twenty-seven hours later (zero time), actinomycin D (0.2 μg/ml) was added to the designated cells. At the indicated times, [^{3}H]leucine (52 Ci/mmol from Schwarz/Mann) was added to a final concentration of 5 μCi/ml and cells were labeled for 1 hr. Alanine aminotransferase was partially purified from cell lysates and precipitated with antibody as described in *Experimental Procedures*. Enzyme from control cells (○); enzyme from actinomycin-treated cells (●).

same half-life is observed after the steady state is perturbed by removal of hydrocortisone from preinduced cells as that previously determined by addition of actinomycin D to induced or to basal state cells (14). As another measure of the turnover rate of alanine aminotransferase mRNA, actinomycin D (0.2 μg/ml) was added to cells that had been pretreated with hydrocortisone. The results of this experiment, depicted in Fig. 3, show that in the presence of actinomycin, alanine aminotransferase synthesis decays with a half-life of about 12 hr, essentially the same as that determined after removal of hydrocortisone. In Fig. 3, the data are presented as "radioactivity in enzyme" rather than "specific radioactivity in enzyme." In the presence of 0.2 μg/ml of actinomycin D, total protein synthesis decays at the same rate as synthesis of alanine aminotransferase, and expression of the data as "specific activity" would yield a horizontal line.

The fact that turnover rates are essentially identical when measured under these two conditions provides evidence that these indirect measurements yield valid estimates of mRNA content, as it is unlikely that parameters determining rates of enzyme synthesis other than mRNA content would be equally affected by such disparate physiological conditions. Also in support of the validity of these measurements is the demonstration that the kinetics of induction and deinduction of tyrosine aminotransferase by glucocorticoids are entirely consistent with a mechanism involving an mRNA undergoing turnover with a half-life of about 2 hr (5).

Since identical half-lives were obtained either by removal of the steroid inducer or by addition of actinomycin D, we generally conducted our turnover measurements using withdrawal of the hormone to perturb the steady-state mRNA pool. This treatment is specific for the enzymes induced by hydrocortisone, including the tyrosine and alanine aminotransferase, and has no detectable effect on total RNA or protein synthesis.

Effect of Cycloheximide and Puromycin on Turnover of Tyrosine Aminotransferase mRNA. Studies on the polarity of nonsense mutations in bacteria have shown that ribosomes exert a protective effect on bacterial mRNA (15). Degradation of bacterial mRNA is enhanced by agents, such as puromycin, which cause release of bound ribosomes from the mRNA, and is inhibited by chloramphenicol, which stabilizes polysome structure by blocking translocation (16–18). We have previously

Table 1. Effect of cycloheximide and puromycin on stability of tyrosine aminotransferase mRNA

	Radioactivity	
	Tyrosine amino-transferase (cpm × 10^{-3})	Total soluble protein (cpm × 10^{-6})
Zero time	26.5	11.8
Control	6.1	8.7
Cycloheximide	14.2	9.4
Puromycin	7.2	8.1

H-35 cells were grown to near stationary phase and then exposed overnight to 1 μM hydrocortisone in Eagle's basal medium. At zero time, the hydrocortisone medium was decanted and the cells were washed twice with Hanks' balanced salts. Fresh steroid-free medium containing 5 μg/ml of cycloheximide, 200 μg/ml of puromycin, or no additive was added to the cells; these concentrations of the inhibitors effectively block (>90%) protein synthesis for at least 3 hr in H-35 cells. Three hours after removal of the steroid, the medium was again decanted from all flasks and cells were washed twice with Hanks' balanced salts. Fresh medium containing no additives was then administered to the cells. At 4 hr, 10 flasks of cells from each of the three experimental groups were labeled for 15 min with 5 μCi/ml of [^{3}H]leucine (specific activity 55 Ci/mmol from Schwarz/Mann) in leucine-free Eagle's basal medium; 10 flasks were labeled in identical fashion at zero time. Tyrosine aminotransferase was partially purified from cell lysates and precipitated with antibody as described in *Experimental Procedures*.

presented kinetic data suggesting that cycloheximide inhibits turnover of tyrosine aminotransferase mRNA in H-35 cells (14). We confirmed this earlier finding directly, using the immunoisotopic technique to follow turnover of tyrosine aminotransferase mRNA in cycloheximide-treated cells. Table 1 shows the results of such an experiment. Overnight exposure to 1 μM hydrocortisone induced synthesis of tyrosine aminotransferase to 0.22% of total protein synthesis, a typical result (5, 7, 14). Four hours after removal of hydrocortisone from preinduced cells, control cells incorporated 25% as much isotope into the enzyme as the zero-time samples, as predicted on the basis of the half-life of 2 hr for tyrosine aminotransferase mRNA. In cells exposed to cycloheximide for a 3-hr period after removal of hydrocortisone, then pulse-labeled after removal of cycloheximide, more than twice as much radioactivity was incorporated into tyrosine aminotransferase as in the controls, indicating a marked inhibition of mRNA turnover during the period of exposure to cycloheximide. However, degradation of tyrosine aminotransferase mRNA is not changed by puromycin treatment; within limits of experimental error, the (ribosome-free) mRNA in puromycin-treated cells was degraded at the same rate as in control cells. The radioactivity incorporated into total soluble protein was approximately the same in all the experimental samples, indicating that the observed radioactivity in tyrosine aminotransferase is a function of mRNA activity, and does not reflect differential incorporation of isotope into the cells in the three experimental groups.

Effect of Fluoride on Turnover of Tyrosine Aminotransferase and Alanine Aminotransferase mRNA. To study further the effect of ribosome binding on mRNA stability, we examined degradation of the tyrosine and alanine aminotransferase mRNAs in fluoride-treated cells. Fluoride inhibits ribosomal initiation in mammalian cells with little or no effect on elongation and termination (19); consequently cellular

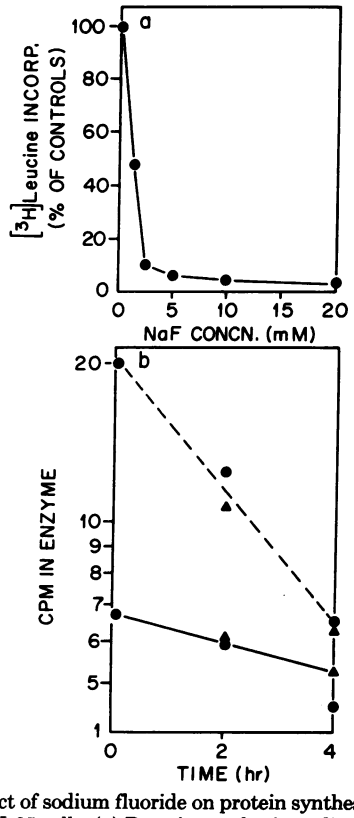

FIG. 4. Effect of sodium fluoride on protein synthesis and mRNA degradation in H-35 cells. (a) Protein synthesis: sodium fluoride was added to individual flasks of H-35 cells to the indicated concentrations. Fifteen minutes after sodium fluoride addition, the cells were labeled with 5 μCi/ml of [^{3}H]leucine for 15 min. Trichloroacetic acid-insoluble radioactivity in the soluble protein fractions was measured and expressed as the percent of radioactivity in protein from untreated cells. (b) Degradation of tyrosine and alanine aminotransferase mRNAs in sodium fluoride-treated cells: H-35 cells were pretreated for 18 hr with 1 μM hydrocortisone in Eagle's basal medium. At zero time, the hydrocortisone medium was decanted and cells were washed twice with Hanks' balanced salts. After washing, fresh steroid-free medium containing either 2.5 mM NaF or no addition was added back to the cells. At 1 hr and at 3 hr the medium in both NaF-treated and control flasks was decanted. After two washes with Hanks' balanced salts, all flasks received fresh medium containing no additives. At zero time, 2 hr, and 4 hr, cells from the control and NaF-treated groups were labeled with 5 μCi/ml of [^{3}H]leucine (specific activity 60 Ci/mmol from Schwarz/Mann) leucine-free Eagle's basal medium for 15 min. The tyrosine and alanine aminotransferases were partially purified and precipitated with their respective antibodies as described in *Experimental Procedures*. Radioactivity ($\times 10^{-3}$) in tyrosine aminotransferase (- - -); radioactivity ($\times 10^{-2}$) in alanine aminotransferase (—). Control cells (●); sodium fluoride-treated cells (▲).

mRNA is in a ribosome-free state several minutes after addition of sodium fluoride to the culture medium. Fig. 4a shows that at a concentration of 2.5 mM, fluoride inhibits 90% of protein synthesis in H-35 cells. Fig. 4b shows that the nonpolysomal tyrosine and alanine aminotransferase mRNAs in fluoride-treated cells are degraded at the same rate as the mRNAs in control cells.

Rate of Peptide Elongation on Tyrosine and Alanine Aminotransferase mRNAs. The data from the puromycin and sodium fluoride experiments provide strong evidence that the turnover rate of an individual mRNA species is not a function of its translation. To eliminate further translation as a factor in determining mRNA stability, we wished to determine if, in cells not treated with metabolic inhibitors, the relatively stable alanine aminotransferase mRNA is translated at a rate different from that of the labile mRNA coding for tyrosine aminotransferase.

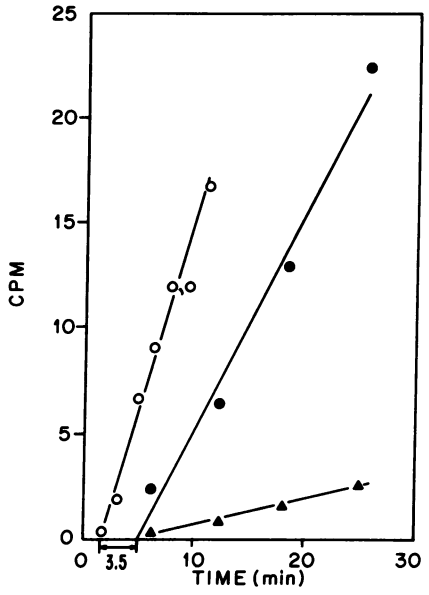

FIG. 5. Rate of peptide elongation on the tyrosine and alanine aminotransferase mRNAs. Cells were pretreated overnight with 1 μM hydrocortisone in Eagle's basal medium. At zero time, the medium in all flasks was replaced with fresh Eagle's basal medium supplemented with hydrocortisone (1 μM) and with 30 μCi/ml of [^{3}H]leucine (specific activity 58 Ci/mmol). At the indicated times, cells were collected. Radioactivity in total protein (nascent plus released polypeptides) and the released tyrosine and alanine aminotransferases were measured as described in *Experimental Procedures*. Radioactivity in total protein ($\times 10^{-4}$) (○); radioactivity in tyrosine aminotransferase ($\times 10^{-2}$) (●); radioactivity in alanine aminotransferase ($\times 10^{-2}$) (▲).

Fig. 5 shows the results of a typical experiment using the method of Fan and Penman (11) to determine translation times of the two mRNAs; within limits of experimental error the transit times are identical. These immunochemical measurements of radioactivity in the specific enzymes demand that the radioactive polypeptides be recognizable by antibody, a complicating element of unknown duration, but which may add significantly to the observed transit times. Despite this complexity, from the identity of the observed transit times it is reasonable to conclude that elongation rates of polypeptide chains are equivalent for the two mRNAs.

The data of Fig. 5, which indicate that the rates of peptide elongation on the tyrosine and alanine aminotransferase mRNAs are essentially identical, also show that synthesis of the tyrosine-specific enzyme is ten times faster than that of the alanine enzyme. This probably reflects a comparable difference in the cellular content of the two mRNAs (see above), but is conceivably related to the number of ribosomes engaged in their translation. However, if this were the case, it is clear that the number of ribosomes must be larger for the tyrosine aminotransferase mRNA than for the mRNA coding for the other enzyme. Thus it cannot be argued that degradation of alanine aminotransferase mRNA is slower because of binding of more ribosomes.

DISCUSSION

Treatment with cycloheximide inhibits degradation of the short-lived tyrosine aminotransferase mRNA in mammalian liver cells (Table 1). We have previously interpreted results of

this sort as suggesting that, as in bacteria, turnover of mammalian mRNAs is linked to their translation (14). However, the present data, demonstrating normal turnover rates of tyrosine aminotransferase mRNA and the longer lived alanine aminotransferase mRNA in the presence of puromycin and sodium fluoride, clearly exclude this interpretation. Each of these inhibitors of translation causes release of bound ribosomes from mRNA; hence ribosome binding cannot be invoked as a determinant of mRNA stability. Further, our translation-time measurements suggest that neither the rate of peptide elongation nor the number of ribosomes bound per unit length of mRNA is related to the rate of mRNA degradation.

The simplest interpretation of these data is that ribosome binding and translation do not play a regulatory role in the stability of tyrosine and alanine aminotransferase mRNA. Rather, the half-lives of these two molecules are determined by the primary structure of the mRNA itself in a region at or near the site for ribosome binding and initiation. In cycloheximide-treated cells, where ribosomal translocation but not binding or initiation is severely restricted, this region of the mRNA near the 5′ terminus may be sterically protected by static ribosomes. Recent evidence indicates that most mRNAs contain methylated, blocked 5′-terminal structures (20–22) that include ribose methylations that could protect against exonucleolytic attack. The possibility that stable mRNAs, such as the alanine aminotransferase messenger, possess blocked 5′ termini whereas labile messengers such as tyrosine aminotransferase mRNA lack such groups, deserves further investigation.

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