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3′ noncoding region of phospho*enol*pyruvate carboxykinase mRNA contains a glucocorticoid-responsive mRNA-stabilizing element

(mRNA turnover/chimeric genes/transfection)

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ABSTRACT **The stabilization of phospho*enol*pyruvate carboxykinase mRNA by glucocorticoids appears to result from the interaction of an induced factor with an RNA element located in the 3′ noncoding sequence of the mRNA. This element can confer glucocorticoid-dependent stabilization upon a heterologous mRNA, and thus strategies developed to investigate the control of mRNA transcription can now be applied to the analysis of hormone-regulated mRNA stabilization.**

The steady-state level of a given mRNA represents the balance of its synthesis, nuclear processing and degradation, transport from nucleus to cytoplasm, and cytoplasmic degradation. Hormones probably regulate all of these steps in mammalian gene expression, but most attention has been given to their role in influencing the transcription of specific genes. A variety of techniques, including DNA transfer using chimeric plasmid constructions, have been employed to show that such effects are mediated through cis-acting DNA elements that, for steroid hormones, bind the hormone–receptor complex. The hormone–receptor complex serves as a trans-acting factor to enhance or repress transcription by mechanisms that are being elucidated.

Several hormones, steroids in particular, are also involved in the regulation of mRNA stability (1) but most of the available information is descriptive. Estrogens affect the stability of several oviduct (2–4) and hepatic (5–8) mRNAs. Glucocorticoids stabilize growth hormone (9, 10) and fibronectin (11) mRNAs and destabilize interleukin 1β (12), type I procollagen (13), and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (14) mRNAs. Apart from the observation that glucocorticoids are thought to stabilize growth hormone mRNA by increasing the length of the poly(A) tail (9), little is known about how hormones regulate mRNA stability.

Analysis of the metabolism of phospho*enol*pyruvate carboxykinase (PEPCK) mRNA provides an approach to the problem of hormonal regulation of mRNA stability. In addition to inducing a rapid 6-fold increase in transcription (15), dexamethasone also induces a 4-fold increase in the stability of PEPCK mRNA. When the 3′ noncoding sequence of PEPCK mRNA was joined to a heterologous mRNA, dexamethasone-induced stability was conferred upon the latter. We conclude that the altered stabilization of PEPCK mRNA is a glucocorticoid-dependent process that requires an RNA element located in the 3′ noncoding sequence of the mRNA.

METHODS

The culture of rat hepatoma line H4IIE (16), the PEPCK competition radioimmunoassay (17), the quantitation of mRNA (18), and the run-on transcription assays (18) were conducted using procedures previously described in detail.

Measurement of PEPCK mRNA Half-Life ($t_{1/2}$). Serum-free medium was added to cell cultures 24 hr before the pulse–chase experiment. Half of the cells were treated with 50 nM dexamethasone for 16 hr prior to and during the chase period; the other half were treated for 4 hr prior to and during the chase. [^{3}H]Uridine (2 mCi, 1 Ci = 37 GBq) was added to 150-cm^2 culture flasks containing 10 ml of medium and incubated for 2 hr. At time zero flasks were washed twice with serum-free medium and then incubated with fresh medium containing 20 mM uridine for various times. The labeled RNA was hybridized to nitrocellulose filter disks bearing an immobilized 5800-base-pair (bp) PEPCK genomic DNA probe (18). Radioactivity specifically bound to the PEPCK filter disk was quantified by liquid scintillation and expressed as a fraction (ppm) of the total [^{3}H]UTP incorporated (18). The same procedure was used to determine the effect of dexamethasone on actin mRNA stability, except that cells were exposed to the hormone for 16 hr, and the labeled RNA was annealed to a 2100-bp rat actin probe (pBAc2995).

Plasmid Constructions. The CATCK fusion gene plasmid pCATCK, consisting of promoter and enhancer elements from simian virus 40 (SV40), the chloramphenicol acetyltransferase (CAT) coding sequences, and 3′ noncoding sequences from PEPCK, was constructed by replacing the SV40 splice and polyadenylylation sequences in plasmid SV2CAT with the analogous sequences from the PEPCK gene. The 1300-bp *Eco*RI–*Bam*HI fragment from pPC112.RI (19) was inserted into the *Bam*HI site of pSV2CAT from which the SV40 splice and polyadenylylation sequences had been deleted by replacing the *Mbo* I–*Bam*HI fragment from pSV2CAT with a *Bam*HI linker. The plasmid pCKTCAT (see Fig. 2*A*) was constructed by inserting the same *Eco*RI–*Bam*HI 3′ noncoding sequence of PEPCK mRNA into the *Bam*HI site of pBLCAT. This vector contains the herpes simplex virus thymidine kinase promoter (positions from −105 to +51 relative to the site of transcription initiation). The composition and construction of the other plasmids used have been described (15). For example, pPG32TKCAT contains a PEPCK gene glucocorticoid response element (from the 5′ flanking sequence between positions −600 and −108) ligated into the *Hin*dIII site of pTKCAT, pPL9 consists of the sequence from position −600 to position +69 of the PEPCK gene ligated to the CAT reporter gene, and TKCAT consists of the herpes simplex virus thymidine kinase promoter sequence (positions −105 to +50) ligated to the CAT reporter gene.

Transfection Protocol and CAT Assays. H4IIE cells, adapted to Dulbecco's modified Eagle's medium containing final concentrations of 5% (vol/vol) newborn calf serum and 5% calf serum, were grown to 60–80% confluence in 150-cm^2 flasks. They were transfected in suspension by incubation

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Abbreviations: PEPCK, phospho*enol*pyruvate carboxykinase; SV40, simian virus 40; CAT, chloramphenicol acetyltransferase; GRS, glucocorticoid-responsive stabilizing element.

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with 2 ml of a calcium phosphate DNA coprecipitate containing 50 μg of reporter gene plasmid DNA, 5 μg of SV2NEO selection marker, and 50 μM chloroquine. The cell suspension was divided equally into two 75-cm^2 culture dishes. After 5 hr, the cells were incubated in medium containing 20% (vol/vol) dimethyl sulfoxide for 5 min and washed once, and then fresh medium was added. After induction for 48 hr, fresh medium containing G418 (500 μg/ml) was added. The medium containing G418 was replaced every 48 hr. After 2–3 weeks, individual foci were cloned. Culture dishes of individual clones were incubated with 0 or 0.5 μM dexamethasone for various times and cell lysates were prepared from control and dexamethasone-treated cells and assayed for CAT activity (15).

RESULTS

Kinetics of PEPCK mRNA Accumulation. A post-transcriptional component in the regulation of PEPCK by dexamethasone was suggested when the kinetics of the induction of transcription of the PEPCK gene, PEPCK mRNA, and PEPCK were measured in H4IIE hepatoma cells (Fig. 1*A*). Transcription of the PEPCK gene increased within 15 min of exposure to dexamethasone and reached its maximal value, 6-fold above the control level, between 30 and 60 min after the addition of the hormone (Fig. 1*A*). This enhanced rate of transcription was not sustained but fell to another steady-state level, about 2-fold above the initial value, and continued there as long as the cells were exposed to the hormone.

In contrast to the monophasic increase in PEPCK gene transcription, a biphasic curve of PEPCK mRNA accumulation was obtained (Fig. 1*A*). The early phase of cytoplasmic PEPCK mRNA accumulation occurred 3–6 hr after the addition of the glucocorticoid, and then PEPCK mRNA declined back toward the basal level. A second increase occurred between 12 and 24 hr and resulted in a marked and prolonged increase of cytoplasmic PEPCK mRNA. This second increase in PEPCK mRNA occurred only in the cytoplasm. Nuclear PEPCK mRNA showed only the first-phase response; the magnitude and duration of the induction of nuclear PEPCK mRNA followed that of PEPCK gene transcription (data not shown).

Finally, a 10-fold increase in PEPCK protein occurred when H4IIE cells were exposed to dexamethasone for 24 hr (from 0.75 to 7.5 nmol of PEPCK per g of total protein; Fig. 1*A*). This increased level was sustained for at least 48 hr in the continued presence of glucocorticoid (data not shown). Thus, although a precursor–product relationship existed between PEPCK transcription and the early-phase PEPCK mRNA response, the latter increase of PEPCK mRNA occurred in the absence of a concomitant increase in transcription. It was apparent that transcription alone could not account for the complex time course of the induction of cytoplasmic PEPCK mRNA by dexamethasone.

Measurement of PEPCK mRNA Stability. The second-phase increase of PEPCK mRNA occurred only after several hours of exposure to dexamethasone when transcription of the gene was at a low level. This suggested that dexamethasone could cause PEPCK mRNA stabilization. The apparent $t_{1/2}$ of PEPCK mRNA was determined by measuring the rate of disappearance of radioactivity from PEPCK mRNA, labeled *in vivo*, in a pulse–chase experiment. The apparent $t_{1/2}$ of β-actin mRNA was measured in the presence and absence of dexamethasone as a control. There was no difference in the rate of degradation of actin mRNA between control cells and those pretreated with dexamethasone, nor was there a difference in the stability of total RNA or polyadenylylated RNA in the presence or absence of dexamethasone (data not shown). H4IIE cells treated with dexamethasone for 4 hr prior to the chase exhibited a biphasic pattern in the $t_{1/2}$ of PEPCK mRNA turnover. The $t_{1/2}$ during the initial phase was 33 min, which is similar to that estimated using a variety of techniques (16, 21, 22). One to 2 hr after the start of the chase period (5–7 hr after the addition of dexamethasone), a marked prolongation of the $t_{1/2}$ was noted (Fig. 1*B*). Beyond this time the $t_{1/2}$ approximated

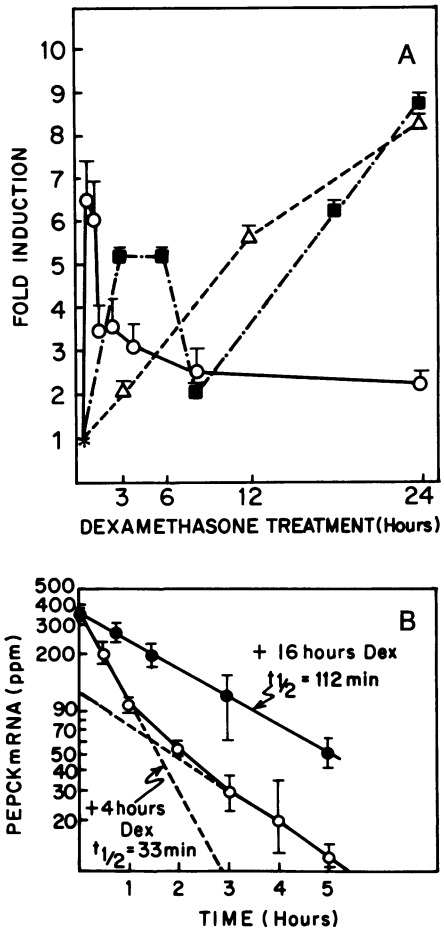

FIG. 1. Comparison of the effects of dexamethasone on PEPCK gene transcription, PEPCK mRNA, and PEPCK induction with its effect on PEPCK mRNA degradation. PEPCK gene transcription, PEPCK mRNA, and PEPCK were measured in H4IIE cells treated with 0.5 μM dexamethasone (Dex) for the time intervals indicated on the abscissa (*A*). The results are expressed as the fold induction over control. Circles, PEPCK gene transcription; Squares, cytoplasmic PEPCK mRNA; triangles, PEPCK. Transcription was measured in ppm of total RNA synthesized as described (18). Actin mRNA, unaffected by dexamethasone, was quantitated in each sample and this was used to correct the PEPCK mRNA value for differences in RNA loading on the gel. PEPCK was corrected for the total protein in each sample. The error bars represent the SEM (n = 2–10), at the various time points. Similar conditions were used to analyze the effect of dexamethasone on PEPCK mRNA degradation. The uridine and UTP pools in control and glucocorticoid-treated H4IIE cells were measured (20) to exclude the possibility that the hormone affected the size of these pools and thus the specific activity of the labeled RNA. The addition of dexamethasone had no effect on the steady-state specific activity of the uridine or UTP pools. The $t_{1/2}$ of PEPCK mRNA was quantified by measuring the disappearance of radioactivity in a pulse–chase assay. The radioactivity (expressed as ppm of total RNA) is shown as a function of the time of the chase period; the error bars represent the SEM (n = 3–7). Radioactivity in PEPCK mRNA was measured in cells treated for 4 hr (○) or 16 hr (●) before initiation of the chase with unlabeled uridine (*B*). The $t_{1/2}$ values were determined from the slope of a single line fitted by linear regression to the data accumulated using cells treated with dexamethasone for 16 hr and from the initial and final slopes of the data obtained using cells treated with dexamethasone for 4 hr prior to the initiation of the chase.

the 112 min measured in cells exposed to the hormone for 16 hr prior to the initiation of the chase. Thus the transition from the basal to the stabilized form of PEPCK mRNA occurred 5–7 hr after the addition of the glucocorticoid. This rate of PEPCK mRNA degradation persisted as long as the H4IIE cells were exposed to dexamethasone.

Identification of a Glucocorticoid-Responsive Stabilizing Element (GRS). To test whether a specific RNA region was involved in the glucocorticoid-mediated stabilization of PEPCK mRNA, we ligated DNA corresponding to the 3′ noncoding region of PEPCK mRNA to the 3′ end of the coding segment of the CAT gene to give the CATCK fusion gene. Stably integrated transformants of H4IIE cells were made using plasmids pSV2CAT and pCATCK (Fig. 2*A*). Transcription of the reporter gene in these vectors is under the control of the SV40 enhancer/promoter, which was not glucocorticoid responsive (Fig. 2 *B* and *C*). The stable transformants and the constitutive promoter provide a constant level of transcription of CAT mRNA throughout the experiments, so changes in mRNA stability should be readily detected as alterations of CAT activity. Four clonal lines of H4IIE cells transfected with the CATCK plasmid exhibited 2.6- to 6.5-fold increases of CAT activity in response to dexamethasone. The induction of CAT activity in two of these clones, CATCK-2 and CATCK-6, is illustrated in Fig. 2*B*. To obviate the possibility that the results obtained from individual clones were artifacts of insertion position, all of the colonies of transformants remaining on the plate after the clones were picked were pooled and analyzed. This pool showed a 3.8-fold induction of CAT activity in response to dexamethasone (Fig. 2*C*), which compares favorably to the response obtained from the individual clones and to the 4-fold increase of endogenous PEPCK mRNA stability caused by the hormone (cf. Figs. 1*B* and 2*C*).

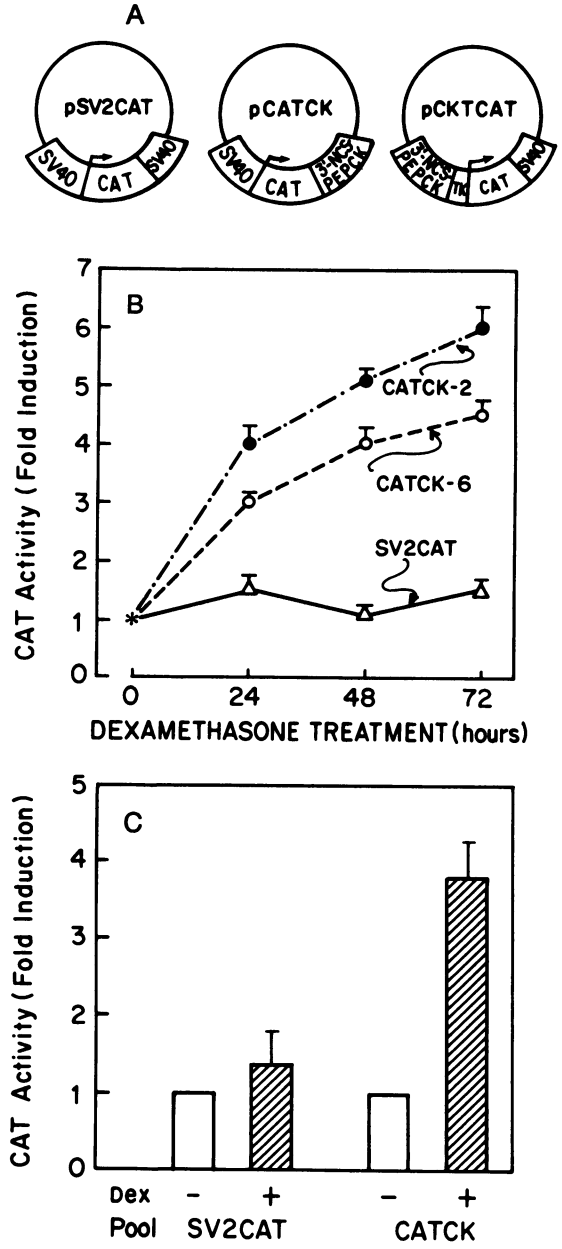

FIG. 2. Stabilization of a CAT–PEPCK fusion mRNA (CATCK) by dexamethasone. Plasmids used in this and subsequent experiments are shown in *A*. The induction of CAT activity by dexamethasone in H4IIE cells stably transfected with pSV2CAT (△) or pCATCK (●, clone 2; ○, clone 6) is shown in *B*. The results are expressed as the fold induction (dexamethasone-treated/untreated cells). After the individual colonies were picked, the colonies remaining on the growth plate were pooled and tested for CAT induction by dexamethasone (*C*). The results are expressed as in *B*.

CATCK mRNA Accumulation. Since the conversion of rapid to slow turnover of PEPCK mRNA required several hours of glucocorticoid treatment, a corresponding lag in the induction of CATCK mRNA should be observed if this mechanism is entirely due to stabilization mediated by the 3′ fragment from PEPCK mRNA. Little change in CATCK mRNA was noted for 8 hr, then an increase began (Fig. 3). In contrast, PEPCK mRNA was markedly increased within 3

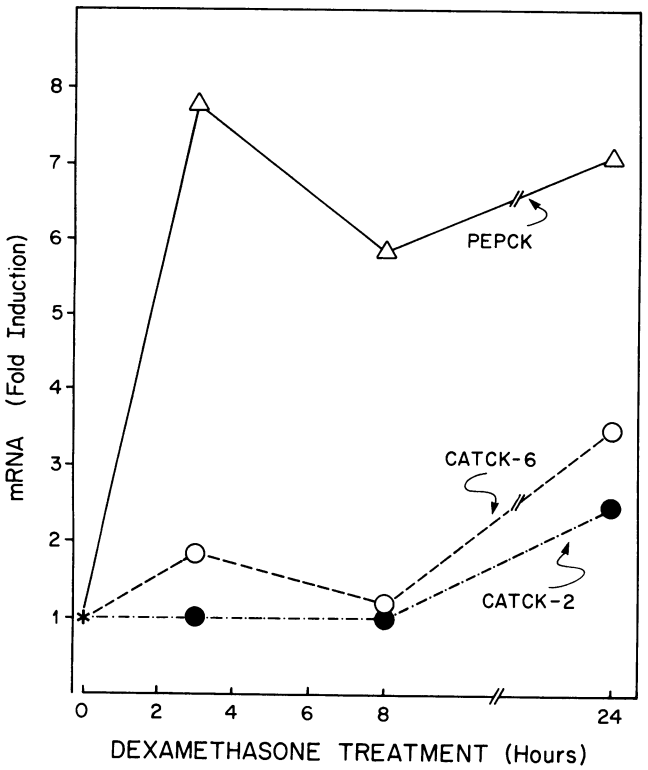

FIG. 3. Kinetics of PEPCK mRNA and CATCK mRNA accumulation. Cultures of two representative H4IIE cell clones stably transfected with the pCATCK plasmid were exposed to 0.5 μM dexamethasone. PEPCK mRNA and CATCK mRNA (measured using the 800-bp *Rsa* I–*Rsa* I fragment of the CAT coding sequence) were quantitated. Actin mRNA was used to correct for differences in RNA loading between samples, as described in Fig. 1. Results represent the mean of duplicate tests and are expressed as the fold-induction compared with control cultures. Triangles, PEPCK mRNA; open circles, CATCK mRNA in H4IIE cell clones CATCK-6; solid circles, CATCK-2.

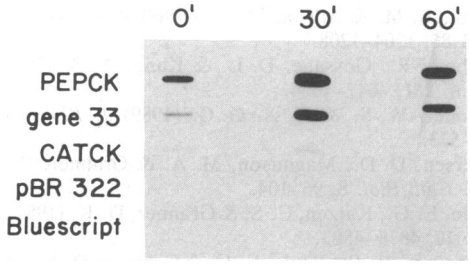

FIG. 4. GRS is not a transcription enhancer. Analysis of the effect of dexamethasone on transcription of the PEPCK and CATCK genes, and on gene *33*, is presented. CATCK-6 cells were incubated in 0.5 μM dexamethasone for 30 or 60 min. Nuclei were then isolated and transcription of these genes (and of the corresponding cloning vectors pBR322 and Bluescript as controls) was measured by the *in vitro* nuclear run-on procedure, except that the hybridization reactions were quantitated by autoradiography.

hr, a response that was qualitatively similar to that seen in the experiment illustrated in Fig. 1*A*. Thus, the induction of CATCK mRNA by glucocorticoids resembled the late-phase induction of PEPCK mRNA. Attempts to demonstrate that the induction of a protein is required for the conversion of the mRNA to the more stable form have, to date, been unsuccessful. Prolonged exposure (6–8 hr) of CATCK-2 and CATCK-6 cells to cycloheximide, at concentrations that block protein synthesis, not only blunted the CATCK mRNA response to dexamethasone but also resulted in a significant decrease of PEPCK mRNA, actin mRNA, and of the incorporation of [^{3}H]uridine into total cell RNA.

Exclusion of Enhancer Function for the GRS. Transcription of the chimeric CATCK gene was compared to that of the endogenous PEPCK gene to exclude the possibility that the 3′ noncoding region was responding to dexamethasone as a transcription enhancer or glucocorticoid-response element. Dexamethasone caused a rapid 4- to 5-fold increase of PEPCK gene transcription in CATCK-6 cells (Fig. 4). Transcription of gene *33*, another gene known to be positively regulated by glucocorticoids in H4IIE cells (23), was also increased. The low level of transcription of the CAT gene in the CATCK-6 cells was unaffected by dexamethasone (Fig. 4).

Further evidence against the 3′ noncoding sequence acting as a glucocorticoid-response element was obtained using a construction (pCKTCAT; Fig. 2*A*) in which this element was ligated on the 5′ side of the transcription initiation site of the CAT reporter. Dexamethasone had no effect on CAT activity in cells transfected with the pCKTCAT construction but did induce CAT activity in cells transfected with plasmids containing a PEPCK gene that was linked by the glucocorticoid-response element to the CAT gene by either the thymidine kinase or PEPCK promoter (Table 1). The GRS worked only when located downstream from the CAT gene, which is consistent with its proposed role.

Table 1. GRS is not a transcription enhancer

Plasmid	Experiments, *n*	CAT activity, fold increase after dexamethasone
pTKCAT	5	1.1 ± 0.1
pCKTCAT	6	1.1 ± 0.1
pPG32TKCAT	6	4.1 ± 1.0
pPL9	6	4.6 ± 0.4

After transient transfection of H4IIE cells with the appropriate plasmid (constructions described in *Methods* and in ref. 15), 0.5 μM dexamethasone was added. Twenty-four hours later the cells were harvested and lysates were assayed for CAT activity. Results are expressed as the fold induction of dexamethasone-treated cultures compared to control cultures.

DISCUSSION

Acute metabolic adaptation is achieved by modification of the activity of key enzymes, whereas chronic adaptation is accomplished through altered rates of enzyme synthesis. The latter, facilitated by changes in the amount of the corresponding mRNA, is the only mechanism involved in the regulation of PEPCK, the rate-controlling enzyme in gluconeogenesis. By regulating independent events in the metabolism of PEPCK mRNA, the stimulatory effect of glucocorticoids produces a gradual increase in the amount of PEPCK until another steady state is achieved. This presumably provides a continuous flux of noncarbohydrate precursors into the gluconeogenic pathway. Transcription, mediated by the two glucocorticoid-response elements located in the DNA sequence that flanks the cap site (15), and degradation, mediated by the GRS located in the 3′ noncoding sequence of the mRNA, are both involved in this important physiologic action of glucocorticoid hormones.

Much has been learned about the turnover of mRNA (1, 24). Most mRNAs in mammalian cells are very stable and have a $t_{1/2}$ measured in hours. Some, however, turn over very rapidly ($t_{1/2}$ = 10–30 min) and in certain instances this process is subject to regulation. It appears that the ends of mRNA molecules govern their stability. The 5′ cap structure in eukaryotic mRNA prevents attack by 5′ exonucleases and the poly(A) tail prohibits the action of 3′ exonucleases. In mRNA molecules with those structures, it is presumed that a single endonucleolytic cut allows exonucleases to attack and digest the entire molecule. Other structures at either end are thought to promote or prevent this initial endonucleolytic action. There is evidence of a role for structures in or associated with the 5′ end of certain eukaryotic mRNAs (25, 32) but most attention has been focused on the 3′ noncoding sequence.

Structures at the 3′ end enhance or diminish the stability of specific mRNAs. The absence of a poly(A) tail is associated with rapid degradation of mRNA (26). Histone mRNAs lack a poly(A) tail but have a sequence near their 3′ terminus that can form a stem–loop structure; this appears to confer resistance to exonucleolytic attack (27). Stem–loop structures in the 3′ noncoding sequence are also critical for the regulation, by iron, of the mRNA encoding the transferrin receptor (28). Other sequences in the 3′ end of certain eukaryotic mRNAs appear to be involved in the destabilization of these molecules. Of particular interest are A+U-rich regions, many of which contain the sequence AUUUA. This sequence appears in mRNAs that have a very short $t_{1/2}$, including mRNAs for granulocyte–macrophage colony-stimulating factor (29), c-*fos* (30), and c-*myc* (31). The importance of this region is underscored by an experiment in which a sequence containing the 3′ noncoding region of the granulocyte-macrophage colony-stimulating mRNA, which includes the AUUUA motif, was added to the β-globin gene. The addition of this region onto the 3′ end of the β-globin mRNA destabilized this transcript (29).

How can what is known about the turnover of eukaryotic mRNA be used to infer the mechanism of action of the GRS? PEPCK mRNA has a poly(A) tail (19), which is a potential site of regulation (9). In addition, the 3′ noncoding sequence contains a 102-nucleotide stretch of alternating purines and pyrimidines, occurring in a predominately UpG pattern, and the numerous palindromes and repeat sequences in this region could form stem–loop structures (19). The sequence motif AUUUA is also present in this region of PEPCK mRNA. Transfection experiments using truncated portions of the 3′ noncoding sequence should enable us to determine whether any of these elements are involved in determining the dexamethasone-regulated stability of PEPCK mRNA or whether some other sequence is involved.

The observation that hormone-regulated control of mRNA stability can be transferred to a heterologous mRNA by a portion of the 3′ noncoding region of PEPCK mRNA provides functional evidence for a GRS. Whether this represents a general mechanism, involved in many of the examples where mRNA stability is regulated by hormones, remains to be established. It is becoming more apparent that cells employ a variety of mechanisms to regulate mRNA stability (1, 24), just as the synthesis of mRNA is controlled in numerous ways. A hormone that affects mRNA transcription and stability, through physically and temporally separate processes, affords the cell flexibility in adapting to a constantly changing environment.

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