Insulin and Glucagon Regulate Cytosolic Phosphoenolpyruvate Carboxykinase (GTP) mRNA in Rat Liver

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SUMMARY

Insulin causes a 7–10-fold decrease of both the mRNA that codes for rat hepatic phosphoenolpyruvate carboxykinase (mRNA^{PEPCK}) and of PEPCK synthesis, provided the animals are made diabetic and fed chow. mRNA^{PEPCK}, measured either by in vitro translation or cDNA hybridization, decreases with a half-time of 30–60 min after insulin treatment. This coordinant decrease, which approximates the half-life of mRNA^{PEPCK} measured in a variety of situations, suggests that insulin acts by decreasing mRNA^{PEPCK} production, and that the hormone does not alter the activity of a fixed amount of this RNA, or enhance its degradation.

Glucagon results in a ninefold induction of mRNA^{PEPCK}. Half-maximal induction occurs with doses between 20–75 μg/100 g body wt and occurs within 30–45 min. Maximal induction requires 150 μg/100 g body wt and occurs about 80 min after a single glucagon injection. N^6,$O^{2'}$-dibutyryl cAMP and a cAMP analogue that is not metabolized, 8-(4-chlorophenylthio)cAMP, induce mRNA^{PEPCK} as effectively as glucagon and with similar kinetics. Since sodium butyrate, adenosine, and dibutyryl cGMP are ineffective inducers, cAMP appears to be the active agent in the hepatocyte. DIABETES 33:328–332, April 1984.

Dietary glucose regulates the release of glucagon and insulin, which in turn provide the major positive and negative hormonal regulation of hepatic gluconeogenesis. The hormonal mechanisms that control gluconeogenesis are temporally separable.[1] A rapid mechanism, due to allosteric regulation and enzyme phosphorylation-dephosphorylation, occurs within seconds or minutes. A slower mechanism, which occurs within hours of a stimulus by insulin or glucagon, involves protein synthesis and probably determines the maximal capacity of the liver to synthesize glucose. This process is due to changes in the concentration of key enzymes, including cytosolic phosphoenolpyruvate carboxykinase (EC 4.1.1.32; PEPCK),[1] a critical enzyme that catalyzes the conversion of oxaloacetate to phosphoenolpyruvate, the committed step in gluconeogenesis.* Since the synthesis of PEPCK is increased by glucagon and, to a lesser extent, by glucocorticoids, and is decreased by insulin,[1] this enzyme is very useful for studying hormonal regulation of gluconeogenesis.

Glucagon was shown to be an inducer in the first study of the hormonal regulation of PEPCK,[2] yet virtually all of the studies since have employed cAMP analogues, particularly Bt_2cAMP, to mimic hepatocellular cAMP and thus act in place of the hormone. A direct comparison of glucagon and Bt_2cAMP action has not been made, hence it is possible that metabolites of the latter, such as butyrate and adenosine, could affect PEPCK induction in a manner unlike that of glucagon. Such a comparison would also address the question of whether Bt_2cAMP acts indirectly, through the release of another inducer, since this would likely result in different kinetics and extent of induction.

Considerable progress has been made in elucidating the molecular mechanisms involved in the regulation of PEPCK. Hanson and co-workers, and our group, have shown that cAMP analogues increase transcription of the PEPCK gene, which in turn results in an increase of nuclear and cytoplasmic mRNA^{PEPCK}, then of PEPCK synthesis.[3,4] There has not been a thorough study of hepatic mRNA^{PEPCK} levels after glucagon treatment, and no direct comparison of such with rates of PEPCK synthesis.

Insulin also affects PEPCK gene transcription. A decrease in mRNA^{PEPCK} synthesis in cultured H4IIE hepatoma cells is followed by decreases of nuclear and cytoplasmic RNA, then by decreased PEPCK synthesis.[4] An analysis of the effects

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*Abbreviations used: PEPCK, phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32); mRNA^{PEPCK}, messenger RNA coding for PEPCK; poly(A)$^+$RNA, RNA containing a polyadenylated tail; and Bt_2cAMP, N^6,$O^{2'}$-dibutyryl cylic AMP.

of insulin on rat liver mRNA^{PEPCK} amount and activity, and PEPCK synthesis measured in the same tissue samples, has not been presented. Such studies should establish whether or not the results obtained in hepatoma cells reflect the mechanism involved in normal liver.

MATERIALS AND METHODS

Materials. Streptozotocin was a gift from the Upjohn Company, Kalamazoo, Michigan. Crystalline porcine insulin was a gift from Dr. Ronald Chance, Lilly Research Laboratories, Indianapolis, Indiana. Reticulocyte lysate translation kits with [^{35}S]methionine (>500 Ci/mmol) were purchased from New England Nuclear, Boston, Massachusetts. Other radioisotopes were purchased from New England Nuclear or Amersham Corp., Arlington Heights, Illinois. All chemicals were of analytic grade. Nitrocellulose filters (BA85) were from Schleicher and Schuell (Keene, New Hampshire).

Animals. CD strain male rats (100–125 g) were purchased from Charles River Breeding Laboratories, Wilmington, Massachusetts. Diabetes mellitus was induced according to the following protocol. Streptozotocin, dissolved in 100 mM sodium citrate, pH 4.5, was injected i.p. at a dose of 75 mg/kg body wt into animals that had been fasted for 24 h. They were fasted for an additional 5 h, allowed to eat ad libitum for 5 h, fasted for 12 h, then given a second streptozotocin injection of 19 mg/kg body wt. Five days later, a blood glucose determination was performed. Animals were considered to be severely diabetic if the blood glucose was greater than 400 mg/dl. Approximately 80% of the treated animals met this criterion, and were used for experimentation. The remaining 20% developed diabetes that was of an intermediate level (blood glucose 200–400 mg/dl) and had variable responses to insulin. They were not used in the experiments reported here. Insulin was injected i.p. at the doses indicated in each experiment. One unit of insulin is approximately 42 μg or about 7 nm.

Assay of in vivo PEPCK synthesis and mRNA^{PEPCK} translational activity. PEPCK synthesis in rat liver was measured by injecting rats with [^{35}S]methionine and immunoprecipitating PEPCK as described by Beale et al.[5] Total cell poly(A)$^+$RNA was isolated from 300-mg portions of each of the rat livers as outlined previously.[5] mRNA^{PEPCK} activity was determined using reticulocyte lysate translation kits purchased from New England Nuclear. The details of this assay have also been described previously.[5] The activity of mRNA^{PEPCK} is expressed as a percentage of total mRNA translational activity (i.e., [radioactivity in PEPCK/radioactivity in TCA-precipitable material] × 100).

Analysis of mRNA^{PEPCK} by hybridization assay. mRNA^{PEPCK} was quantitated using the dot blot hybridization assay[6] under conditions we described previously,[7] except that a filtration manifold (from Bethesda Research Labs) was used for applying RNA samples to the nitrocellulose filter. The hybridization results are expressed in integrator units (absorbance at 550 nm × cm^2) obtained by a Beckman DU-8 densitometer scan of an autoradiogram. These units depend on several factors, including the sensitivity setting of the densitometer, the amount of poly(A)$^+$RNA per spot, the concentration and specific activity of the probe, and the time of exposure of the autoradiogram. We optimized each of these factors to insure that the integrated image density is directly proportional to the amount of mRNA^{PEPCK} present.[7] The dot blot thus provides a measure of the relative amounts of mRNA^{PEPCK} in samples assayed together. The hybridization probe was a 400 base-pair Rsa I/Hae III or a 200 base-pair Alu I/Alu I restriction fragment isolated from pPC2.[7] It was labeled by nick translation[8] to a specific activity of 1–2 × 10^8 cpm/μg.

RESULTS

Glucagon induces hepatic mRNA^{PEPCK}. Since glucagon is a physiologic inducer of PEPCK, it should cause an increase of mRNA^{PEPCK} that is equivalent to the increase of PEPCK. Cimbala et al.[9] demonstrated that glucagon increases mRNA^{PEPCK}, as measured by in vitro translational activity, but the amount of this mRNA was not quantitated and a dose-response or time-course was not shown. In the experiment shown in Figure 1A, rats were injected with various amounts of glucagon and mRNA^{PEPCK} was quantitated by dot blot hy-

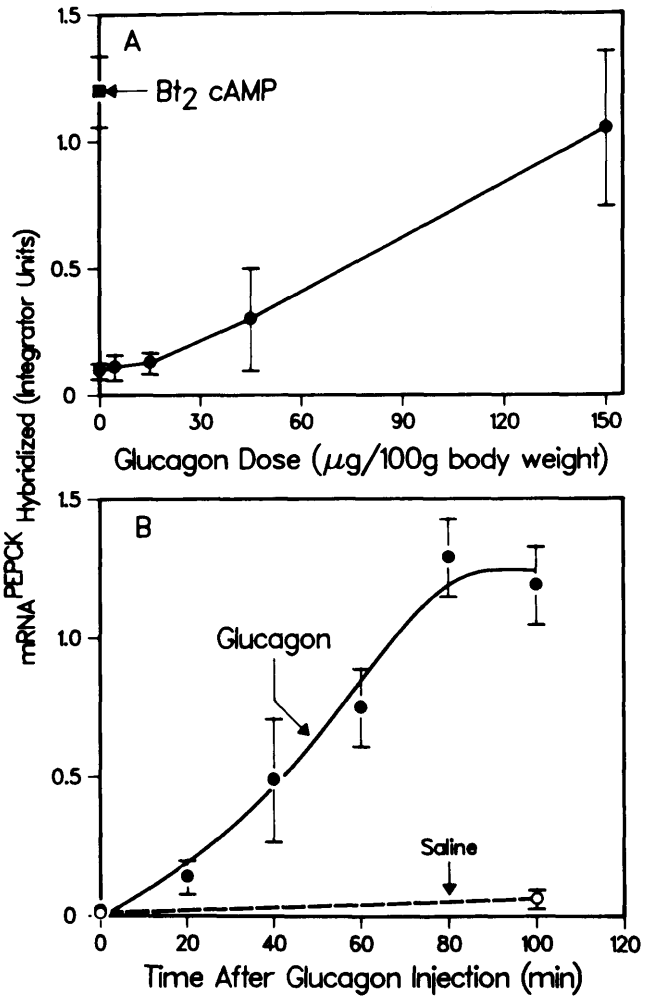

FIGURE 1. Effect of glucagon on mRNA^{PEPCK} concentration. Normal rats were fasted, then fed glucose by gavage to decrease PEPCK synthesis, then glucagon was injected. Panel A represents an experiment in which various amounts of glucagon were injected i.p. and the rats killed 90 min later. The level of RNA^{PEPCK} achieved by the injection of a maximally effective dose of Bt$_2$cAMP plus theophylline (5 mg each/100 g body wt) is shown as a filled square. In the experiment illustrated in panel B, glucagon, 150 μg/100 g body wt, was injected and the rats killed at the indicated times. The amount of mRNA^{PEPCK} was quantitated by dot blot hybridization as described in MATERIALS AND METHODS. Each point represents the mean ± SE of three rats.

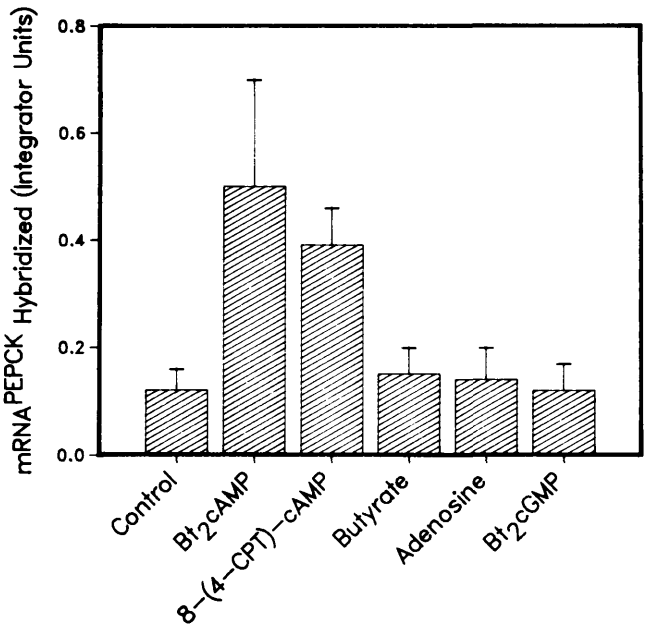

FIGURE 2. Specificity of induction by cAMP analogues. Normal rats were fasted, then fed glucose as described in Figure 1. Two hours later, various compounds were injected i.p. in 2 ml of saline. Doses per 100 g body wt were: Bt_2cAMP, 5 mg; 8-(4-chlorophenylthio)cAMP (8-CPT-cAMP), 0.2 mg; sodium butyrate, 5 mg; adenosine, 5 mg; and Bt_2cGMP, 5 mg. Two hours after these injections the animals were killed, poly(A)$^+$RNA was extracted, and $mRNA^{PEPCK}$ amount was determined as described in MATERIALS AND METHODS. The data are expressed in densitometer integrator units. Each value is the mean ± SE of three rats per group.

bridization. A maximally effective dose of 150 μg/100 g body wt resulted in a ninefold stimulation, while the concentration of glucagon required to elicit a half-maximal response varied between 20 and 75 μg/100 g body wt in separate experiments. A dose of 500 μg/100 g body wt did not cause a greater increase than that seen with 150 μg/100 g body wt. The time course of induction following a single injection of glucagon is shown in Figure 1B. A significant increase occurred at 20 min and the maximum level was reached at 80 min.

If Bt_2cAMP mimics the effects of glucagon on the hepatocyte, the magnitude and kinetics of the induction of $mRNA^{PEPCK}$ by both agents should be very similar, if not identical. Indeed, the kinetics and the maximal induction of $mRNA^{PEPCK}$ by glucagon was matched by that of Bt_2cAMP (Figure 1A, and cf. Figure 1B with refs. 3 and 7).

The cAMP moiety of Bt_2cAMP induces $mRNA^{PEPCK}$. Although the injection of Bt_2cAMP results in increased $mRNA^{PEPCK}$ levels in rats, the possibility that this effect is due to one or more of the many metabolites of this compound[10] has not been tested. We injected rats with a variety of compounds to test this possibility, and the results are shown in Figure 2. Bt_2cAMP and 8-(4-chlorophenylthio)cAMP were effective inducers, as they increased hybridizable $mRNA^{PEPCK}$ from the basal value of 0.11 ± 0.06 to 0.50 ± 0.20 and 0.40 ± 0.04 integrator units, respectively. Dibutyryl cGMP, adenosine, and butyrate had little or no effect; $mRNA^{PEPCK}$ values ranged from 0.10 ± 0.05 to 0.15 ± 0.05 integrator units after the injection of these agents. Control levels of $mRNA^{PEPCK}$ were higher than usual in this experiment, probably due to metabolism of the glucose that was given to deinduce $mRNA^{PEPCK}$ 4 h before killing, thus the extent of the increase was diminished. Nonetheless, these results demonstrate that the cAMP moiety is responsible for the induction of $mRNA^{PEPCK}$ and that adenosine and butyrate, the principal end metabolites of Bt_2cAMP, are of little consequence.

Insulin represses PEPCK synthesis by decreasing the amount of $mRNA^{PEPCK}$. Insulin has been shown, in separate studies, to decrease both the activity and amount of hepatic $mRNA^{PEPCK}$ in diabetic rats.[9,11] This indicates that the decrease of PEPCK synthesis is caused, at least in part, by a corresponding decrease of $mRNA^{PEPCK}$. However, the decrease in $mRNA^{PEPCK}$ was not compared with the decrease in PEPCK synthesis in the same animals. As shown in Table 1, a single injection of insulin decreased both $mRNA^{PEPCK}$ activity and PEPCK synthesis approximately threefold 90 min after the injection; $mRNA^{PEPCK}$ decreased from 0.32% to 0.11% of total mRNA, and PEPCK synthesis decreased from 2.22% to 0.86% of total protein synthesis. $mRNA^{PEPCK}$ activity and PEPCK synthesis remained constant in fasted rats whether or not the animals were treated with insulin (see Table 1). The rats had to be fed in order to observe this insulin effect, probably because feeding prevents an insulin-induced hypoglycemia that would cause glucagon release and thus obscure the effect of insulin.[9] We next injected diabetic rats with insulin and measured PEPCK synthesis in vivo, $mRNA^{PEPCK}$ activity, and the amount of $mRNA^{PEPCK}$ in separate samples of each liver (Figure 3.). There were proportionate six- to sevenfold decreases of both the in vivo synthesis of PEPCK and of the activity of $mRNA^{PEPCK}$ throughout a 2-h time course (panel A). In this experiment, $mRNA^{PEPCK}$ activity was 0.16% of the total mRNA activity at time zero and decreased to 0.11% within 1 h and to 0.02% 2 h after the initial insulin injection. The corresponding values for the in vivo synthesis of PEPCK were 1.9%, 1.5%, and 0.29% of the total cytosolic protein synthesized. We then determined whether the decrease of $mRNA^{PEPCK}$ activity was due entirely to a decrease of the amount of $mRNA^{PEPCK}$. As shown in Figure 3B, the i.p. injection of insulin at hourly intervals resulted in parallel, 10-fold decreases of both the amount (from an average of 1.9 integrator units to 0.16 integrator units) and the activity of $mRNA^{PEPCK}$ [from 0.25% of total poly(A)$^+$RNA activity to 0.02%]. There is, thus, an excellent correlation be-

TABLE 1
Effect of insulin deficiency on the response of $mRNA^{PEPCK}$ and PEPCK synthesis to glucose

Condition	$mRNA^{PEPCK}$ activity (% of total mRNA activity)	PEPCK synthesis (% of total)
Fed chow	0.32 ± 0.03	2.22 ± 0.42
Fed chow + insulin	0.11 ± 0.03	0.86 ± 0.06
Fasted	0.32 ± 0.06	1.70 ± 0.34
Fasted + insulin	0.30 ± 0.05	1.16 ± 0.09

Diabetes was induced by streptozotocin as described in the text. Animals were either fasted for 24 h or fed rat chow ad libitum before being killed. Where indicated, regular insulin (4.5 U/100 g body wt) was injected i.p. 90 min before killing. Measurements of $mRNA^{PEPCK}$ activity and PEPCK synthesis are described in the text. Results are presented as the mean ± SE of five rats for each condition.

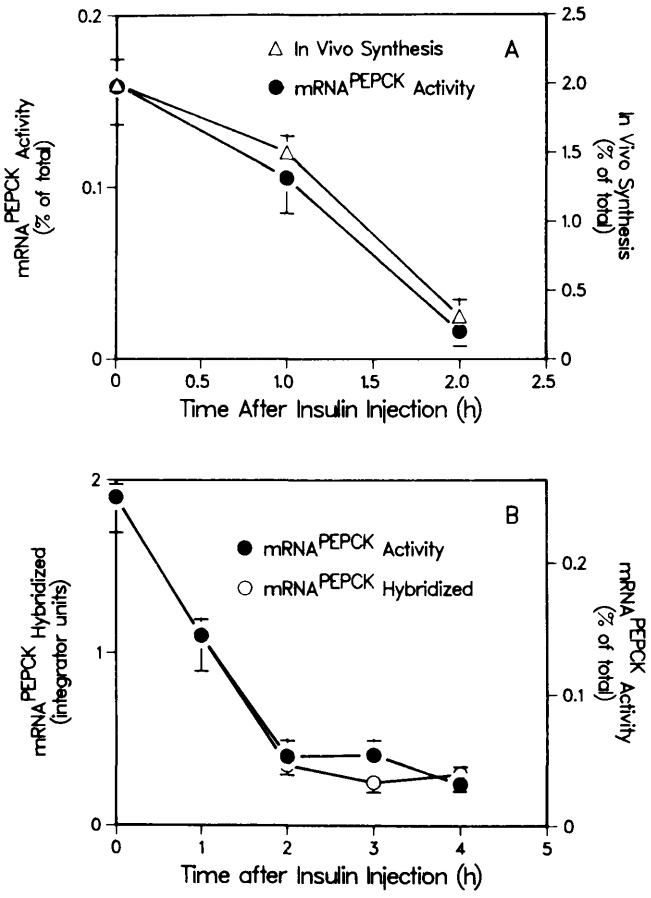

FIGURE 3. Correlation of mRNA^{PEPCK} activity and amount with PEPCK synthesis in rat liver after insulin treatment. Rats, made diabetic by streptozotocin as described in MATERIALS AND METHODS, were fed chow ad libitum. Relative rates of PEPCK synthesis, mRNA^{PEPCK} activity, and mRNA^{PEPCK} amount were determined as described in the text. The rate of synthesis of PEPCK is expressed as a percentage of the total cytosolic proteins synthesized, mRNA^{PEPCK} amount as densitometer integration units, and mRNA^{PEPCK} activity as a percentage of the total in vitro translational products synthesized from poly(A)$^+$RNA. In the experiment illustrated in panel A, crystalline insulin, at a dose of 2.5 U/100 g body wt, was given at time zero and a second dose of 0.8 U/100 g body wt was given 1 h later. Values are the mean ± SE of four animals. In the experiment shown in panel B, crystalline insulin was injected at doses of 0.8 U/100 g body wt at time zero and 0.3 U/100 g body wt each hour thereafter. Rats were killed at the indicated times, poly(A)$^+$RNA was extracted, and mRNA^{PEPCK} activity and amount were quantitated. Each value is the mean ± SE of six animals.

tween the effects of insulin on mRNA^{PEPCK} amount, mRNA^{PEPCK} activity, and PEPCK synthesis. Multiple injections were necessary to achieve the sustained repression shown in Figure 1B, as the effect of a single injection of insulin was transient (data not shown).

DISCUSSION

Glucagon is the predominant inducer of PEPCK,[1] an action presumably achieved through an increase of intracellular cAMP. Various cAMP derivatives have been employed to mimic glucagon action on PEPCK but, while the validity of this approach is generally accepted, a direct, detailed comparison of the effects of glucagon and cAMP analogues on mRNA^{PEPCK} and PEPCK synthesis has not been conducted. The observation that glucagon and Bt_2cAMP or 8-(4-chlorophenylthio)cAMP cause equivalent increases of mRNA^{PEPCK} (Figures 1 and 2) with similar kinetics (cf. Figure 1B with refs. 3 and 7) supports the belief that these analogues are suitable substitutes for glucagon at the cellular level. This is important, since permanent hepatoma cell lines that both bind glucagon and express PEPCK have not been found. In the available cell lines, one has to either elevate endogenous cAMP using a nonspecific adenylate cyclase stimulator such as cholera toxin or forskolin, or use cAMP analogues that can penetrate the plasma membrane. The data in Figures 1 and 2, coupled with the known effectiveness of cAMP analogues in cultured cell systems,[13,14] suggest that cAMP analogues do not stimulate PEPCK synthesis by promoting the release of one or more other hormones, which, in themselves, are the proximate inducers. A different hormone would likely increase PEPCK to a different extent and with different induction kinetics.

Dibutyryl cAMP, the most frequently used analogue, is rapidly metabolized into a number of compounds that could themselves augment or inhibit PEPCK synthesis.[10] Two moles of butyrate are liberated from each mole of Bt_2cAMP, and this compound has been shown to affect the induction of several proteins,[15,16] including PEPCK (unpublished observation). Adenosine, another major metabolite, affects many cellular processes, including DNA synthesis, cell proliferation, and enzyme activity.[7] We, therefore, compared the action of glucagon with that of an analogue that is not metabolized, 8-(4-chlorophenylthio)cAMP. The fact that this compound induces mRNA^{PEPCK}, and that butyrate, adenosine, and Bt_2cGMP are ineffective, suggests that the cAMP moiety itself is active. Taken collectively, these data indicate that glucagon acts via cAMP to increase PEPCK synthesis, presumably because transcription of the gene is enhanced.[3,4]

The separation of insulin effects from those of counterregulatory hormones or substrates, which themselves affect a given response, poses a major challenge in studying insulin action in animals. For this and other reasons enumerated below, systems simpler than intact animals have been employed. For example, in recent studies we have shown that: (1) insulin, in picomolar concentrations, decreases the synthesis of PEPCK in H4IIE hepatoma cells; (2) the effect is direct and does not involve any other hormone intermediates; (3) the effect is exerted in a few minutes; (4) the effect is mediated by the insulin receptor; and (5) the effect is probably due to a selective decrease in the amount of cytoplasmic mRNA^{PEPCK}.[18] In a more recent study, we confirmed this last point and demonstrated that cytoplasmic mRNA^{PEPCK} decreases because of the specific inhibition, by insulin, of PEPCK gene transcription.[4] These studies, many of which could not have been done in animals, offer quite a detailed explanation of how insulin works in hepatoma cells. Still, the relevance of such studies to the physiologic situation in intact animals must be established.

In some tissues, insulin activates phosphodiesterase and decreases the concentration of cAMP. Thus it is possible that the level of mRNA^{PEPCK} is determined by the balance between glucagon and insulin, as reflected by the intracellular cAMP level. We have excluded this possibility in two ways in studies to be published elsewhere. First, insulin repressed mRNA^{PEPCK} in H4IIE hepatoma cells treated with a variety of

nonmetabolizable cAMP analogues. In another experiment, H4IIE cells were treated with various concentrations of forskolin to increase endogenous cAMP. In these cells, insulin did not affect the intracellular concentration of the nucleotide, or the activity ratio of cAMP-dependent protein kinase, yet it was fully able to repress $mRNA^{PEPCK}$. This action of insulin is, thus, not due to changes of cAMP concentration.

The studies reported in this paper illustrate that the general features of $mRNA^{PEPCK}$ regulation in liver and H4IIE cells are the same. Insulin decreases hepatic $mRNA^{PEPCK}$ when quantitated either by an in vitro activity assay or by specific cDNA hybridization, which measures the amount of the RNA. This observation speaks against an action in which insulin alters the activity of a specific mRNA without changing its amount. The decrease occurs with a $t_{\frac{1}{2}}$ of 30–60 min (Figure 3B) and is similar to the $t_{\frac{1}{2}}$ of $mRNA^{PEPCK}$ determined under a variety of conditions in rat liver[5,7,19] and in cultured hepatoma cells.[20] Thus, regulation of the rate of cytoplasmic $mRNA^{PEPCK}$ turnover can probably be eliminated as a major site of regulation by insulin. It seems probable that the major effect of insulin is to alter the production of hepatic $mRNA^{PEPCK}$, presumably through an effect on gene transcription, as noted in H4IIE cells.[4]

In addition to PEPCK, there is now evidence that insulin affects the synthesis of albumin,[21] amylase,[22] and pyruvate kinase[23] by changing mRNA levels. If, as has been suggested, insulin regulates the synthesis of specific proteins by affecting the activity of ribosomes and related translation factors,[12] or by modifying the structure of mRNAs and thereby altering their translational efficiency,[24] these must be minor effects for PEPCK, but they could represent major regulatory mechanisms for other insulin-regulated proteins.

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