Regulation of Rat Liver Phosphoenolpyruvate Carboxykinase (GTP) Messenger Ribonucleic Acid Activity by $N^6,O^{2'}$-Dibutyryladenosine 3',5'-Phosphate†

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ABSTRACT: $N^6,O^{2'}$-Dibutyryladenosine 3',5'-phosphate (Bt_2cAMP) induces the synthesis of the gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32), in rat liver by increasing the activity of messenger ribonucleic acid (mRNA) coding for this enzyme ($mRNA^{PEPCK}$) more than 20-fold (from <0.01% to >0.20% of total mRNA activity) as determined by using in vitro translation systems which measure only active $mRNA^{PEPCK}$. The increase in $mRNA^{PEPCK}$ activity could result from increased synthesis, increased processing, or decreased inactivation rates. Actinomycin D and cordycepin inhibit $mRNA^{PEPCK}$ induction by 89% and 70%, respectively, a result that indicates a requirement for ongoing RNA synthesis but that does not distinguish which of these steps is regulated by cAMP. We have employed a kinetic approach, not involving RNA synthesis inhibitors, to determine the half-life of $mRNA^{PEPCK}$ both during a period of deinduction following glucose feeding and during a subsequent induction by Bt_2cAMP. An estimated half-life of 20 ± 5 min during both of these periods indicates that Bt_2cAMP has no effect on the rate of inactivation of $mRNA^{PEPCK}$. We conclude that Bt_2cAMP effects the increase in activity of $mRNA^{PEPCK}$ by promoting its synthesis or processing.

Synthesis of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) is regulated by a wide variety of agents in numerous tissues (Tilghman et al., 1976). For example, synthesis of the hepatic enzyme is increased by insulin deficiency, fasting, glucagon, Bt_2cAMP,[1] and glucocorticoids and is inhibited by insulin treatment or carbohydrate feeding. In contrast, the renal enzyme is induced by acidosis, fasting, or glucocorticoids but is not affected by insulin, glucagon, or Bt_2cAMP. Thus, phosphoenolpyruvate carboxykinase provides an excellent model for studies of the molecular action of many metabolic and hormonal stimuli. In addition, the striking differences among various tissues with respect to the regulation of this enzyme should afford systems amenable to the study of differentiation.

The present study deals with the regulation of phosphoenolpyruvate carboxykinase by cAMP in the rat liver. Iynedjian & Hanson (1977) showed that $mRNA^{PEPCK}$ activity increases following a single injection of Bt_2cAMP. Recently, a more detailed analysis from the same laboratory has demonstrated that the rate of synthesis of phosphoenolpyruvate carboxykinase remains proportional to the activity of $mRNA^{PEPCK}$ under a variety of experimental manipulations, establishing conclusively that Bt_2cAMP induces the synthesis of this protein by increasing the activity of $mRNA^{PEPCK}$ (Nelson et al., 1980). This specific increase in $mRNA^{PEPCK}$ activity could arise through a number of mechanisms, including increased synthesis or processing,[2] or via a decreased rate of inactivation. We have estimated the turnover rate of $mRNA^{PEPCK}$, without the use of inhibitors of RNA synthesis, during deinduction by glucose and during subsequent induction by Bt_2cAMP. We suggest that Bt_2cAMP increases the rate of $mRNA^{PEPCK}$ synthesis or processing, thereby leading to its accumulation and the consequent increased synthesis of phosphoenolpyruvate carboxykinase. Some results have been published in abstract form (Beale et al., 1980).

Materials and Methods

Animals. Male CD strain rats, 100-125-g body weight, were purchased from Charles River Breeding Laboratories. Adrenalectomized rats were given normal saline in place of drinking water. All animals received Wayne Lab-Blox ad lib. and were fasted 18-22 h before the experiments were started. Experiments with adrenalectomized rats were conducted 6 days after surgery. Glucose was given by gavage (500 mg in 1.0 mL of water) between 7 and 10 a.m. on the day of the experiment, and Bt_2cAMP treatments, when given, were administered 2 h after the glucose feeding as described by Iynedjian & Hanson (1977). The rats received Bt_2cAMP plus theophylline (30 mg each/kg of body weight) in 1.0 mL of phosphate-buffered saline (150 mM sodium chloride and 10 mM sodium phosphate, pH 7.4) or phosphate-buffered saline alone by intraperitoneal injection at times appropriate to each experiment. Actinomycin D (1.0 mg/kg of body weight) and cordycepin (16 mg/kg of body weight) were injected intraperitoneally in 0.5 mL of phosphate-buffered saline 30 min before the injection of Bt_2cAMP plus theophylline. Radioactive amino acids were given, when indicated, by intraperitoneal injection 30 min before the animals were killed by decapitation, and the livers were quickly removed, frozen in liquid nitrogen, and stored at -60 °C until use.

Isolation of Poly(A)+ RNA. All solutions were treated with 0.05% diethyl pyrocarbonate and autoclaved or filter-sterilized before use. Glassware was washed with dichromate/sulfuric acid, rinsed with deionized water, and baked at 200 °C for

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[1] Abbreviations used: Bt_2cAMP, $N^6,O^{2'}$-dibutyryladenosine 3',5'-phosphate; $mRNA^{PEPCK}$, messenger ribonucleic acid coding for phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32); $mRNA^{total}$, mRNA coding for total hepatic protein; poly(A)+ RNA, polyadenylated RNA; $NaDodSO_4$, sodium dodecyl sulfate; Tes, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; GSH, glutathione; IDP, inosine 5'-diphosphate; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

[2] The term processing is meant to include any postsynthetic modifications such as splicing, capping, addition of a poly(A) tail, or methylation at internal sites [see Revel & Groner (1978)].

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2 h before use. RNA was extracted from a 300-mg portion of each frozen liver as described by Diesterhaft et al. (1980). Poly(A)+ RNA was dissolved in 10 mM sodium chloride at a final concentration of 250–300 μg/mL and stored at −60 °C until use.

Assay of mRNA^{PEPCK}. mRNA^{PEPCK} activity was determined by using reticulocyte lysate translation kits purchased from New England Nuclear. Assays were carried out in a total volume of 17 μL in autoclaved 1.5-mL conical polypropylene tubes. Each reaction mixture contained 0.4 mM magnesium acetate, 100 mM potassium acetate (in addition to endogenous magnesium and potassium in the lysate), and 30–35 μCi of [^{35}S]methionine. Poly(A)+ RNA was added in a volume of 1.0 μL to give a final concentration of 10–20 μg/mL. The mixture was incubated for 90 min at 26 °C and terminated by diluting to 100 μL with ice-cold 10 mM Tris-HCl, pH 7.5, containing 0.2 μg of carrier phosphoenolpyruvate carboxykinase (partially purified, by DEAE-cellulose chromatography, to 30 μg of enzyme/mg of total protein). Total incorporation of [^{35}S]methionine into protein (mRNA^{total}) was determined in duplicate 5-μL aliquots by trichlorocetic acid precipitation as described below. The incorporation of radioactive material in the absence of exogenous mRNA (<10% of the total) was determined in parallel assays and subtracted from mRNA^{total} activities. Incorporation of label into phosphoenolpyruvate carboxykinase was measured in the remaining 90 μL by immunoprecipitation as described below. mRNA^{PEPCK} activity is expressed as a percent of mRNA^{total} activity [i.e., (radioactivity in phosphoenolpyruvate carboxykinase/radioactivity in trichloracetic acid precipitable material) × 100]. There were no consistent differences in mRNA^{total} activities following the treatments described in this paper.

In Vivo Synthesis of Phosphoenolpyruvate Carboxykinase. Portions (1.5 g) of frozen liver from rats injected with [^{3}H]-leucine or [^{35}S]methionine (1.0 mCi/kg of body weight) 30 min before killing were homogenized in a 7-mL Dounce homogenizer with 3 mL of a buffer containing 10 mM sodium phosphate, pH 7.4, 0.15 M sodium chloride, 5 mM sodium EDTA, and 1 mM dithiothreitol. The homogenate was centrifuged 10 min at 3000*g*, and the supernatant was then centrifuged 45 min at 45000 rpm in a Beckman 50.3 Ti rotor. The lipid layer was discarded, and the cytosol was stored at −60 °C until use. Incorporation of radioactive amino acid into total protein and into phosphoenolpyruvate carboxykinase, respectively, was determined by trichloroacetic acid precipitation of 5-μL aliquots and immunoprecipitation of 500-μL aliquots as described below. Results are expressed as (radioactivity in phosphoenolpyruvate carboxykinase/radioactivity in trichloroacetic acid precipitate) × 100.

Determination of Radioactivity in Phosphoenolpyruvate Carboxykinase and Total Protein. Cowan strain I of *Staphylococcus aureus* was obtained from American Type Culture Collection and was grown and processed as described by Kessler (1975). Immunoprecipitation was accomplished essentially as described by Hargrove et al. (1980) except that 0.7% Triton X-405 was included in all solutions. For minimization of nonspecific binding of radioactivity, each sample to be immunoprecipitated was presorbed with *Staphylococcus* cells. Sheep antibody, specific for phospholenolpyruvate carboxykinase, was added to each translation sample or cytosol in amounts sufficient to bind 2 or 100 μg of enzyme, respectively. The immunoprecipitated protein was extracted from the *Staphylococcus* cells with 75 μL of $NaDodSO_4$ sample buffer containing 5 μg of fluorescent bovine serum albumin [prepared as described by Weigele et al. (1973), as a marker to localize phosphoenolpyruvate carboxykinase] and subjected to polyacrylamide gel electrophoresis (Laemmli, 1970). For quantitative purposes, the gels were sliced, dissolved in 30% hydrogen peroxide, and radioactivity was measured using Ready Solv HP (Beckman) scintillation cocktail. Slab gel electrophoresis followed by fluorography with EN^{3}HANCE (New England Nuclear) was used for qualitative purposes. Two-dimensional gels were done as described by O'Farrell (1975). Aliquots (5 μL) of the translation mixtures or of ^{3}H-labeled cytosols were placed into 0.5 mL of 0.5 M sodium hydroxide plus 0.25 M hydrogen peroxide and incubated 15 min at 37 °C. Protein was precipitated by the addition of 0.5 mL of 25% trichloroacetic acid containing 2 mM leucine or methionine (where appropriate), and samples were incubated 30 min on ice. The precipitates were collected by filtration on 24-mm glass fiber filters (Whatman GF/A). The filters were placed in scintillation vials, incubated 30 min at 60 °C with 0.5 mL of Protosol, and assayed for radioactivity in 10 mL of Econofluor (New England Nuclear).

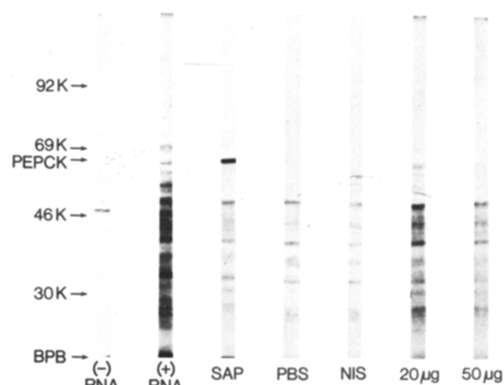

FIGURE 1: Fluorogram of [^{35}S]methionine-labeled translation products synthesized in a mRNA-dependent reticulocyte lysate system and separated by $NaDodSO_4$–polyacrylamide gel electrophoresis. Hepatic poly(A)+ RNA was isolated from a fasted–glucose-fed rat treated 90 min with Bt_2cAMP plus theophylline (3 mg each/100 g of body weight) as described under Materials and Methods. Following translation, 90-μL aliquots were immunoprecipitated and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis on slab gels and then fluorography as described in the text. Other aliquots (5 μL) were electrophoresed directly, without prior immunoprecipitation. The positions of five standards including pure [^{3}H]phosphoenolpyruvate carboxykinase (PEPCK) are marked by arrows at the left. The standards include phosphorylase B (92000), bovine serum albumin (69000), ovalbumin (46000), and carbonic anhydrase (30000). Bromophenol blue (BPB) was used as the dye marker. The electrophoretic tracks are as follows: (−) RNA, total products from translation with no added poly(A)+ RNA; (+) RNA, total products synthesized from hepatic poly(A)+ RNA; SAP, products immunoprecipitated with sheep anti-phosphoenolpyruvate carboxykinase. Tracks designated as PBS and NIS are sham immunoprecipitations where phosphate-buffered saline or nonimmune sheep serum, respectively, were substituted for anti-phosphoenolpyruvate carboxykinase. 20 μg and 50 μg indicate tracks where 20 or 50 μg of pure phosphoenolpyruvate carboxykinase, respectively, was added as competitor prior to immunoprecipitation.

Results

Characterization of Translation Products Synthesized in Vitro in the Reticulocyte Lysate System. The mRNA dependency of the translation system and the specificity of the immunoprecipitation reaction were assessed by $NaDodSO_4$ slab gel electrophoresis and fluorography as shown in Figure 1. When mRNA was omitted from the translational assay two major products (47000 and <10000 M_r) were seen. Several minor products, not visible in this reproduction, were also observed. The addition of rat liver poly(A)+ RNA resulted in the appearance of a large number of translational

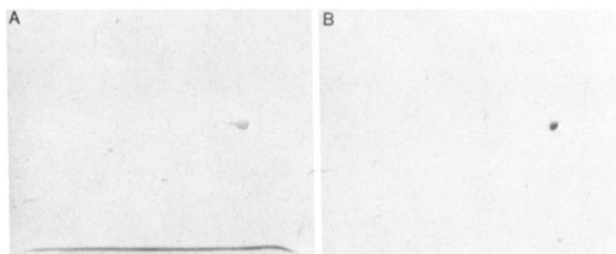

FIGURE 2: Two-dimensional polyacrylamide gel analysis of authentic phosphoenolpyruvate carboxykinase and immunoprecipitated translation product. Poly(A)+ RNA was prepared and translated, and the products were immunoprecipitated as described under Materials and Methods. 5 μg of phosphoenolpyruvate carboxykinase, purified as described in the supplementary material (see paragraph at end of paper regarding supplementary material), was added, and the sample was subjected to two-dimensional analysis as described by O'Farrell (1975). Isoelectric focusing in pH gradient of 4–7 was the first dimension, and $NaDodSO_4$–polyacrylamide gel electrophoresis in a 10% gel was the second dimension. The gel was fixed, stained in Coomassie blue, then dried, and subjected to fluorography as described in the text. (Panel A) Stained gel; (panel B) fluorogram. The figure is oriented so that the pH gradient is shown with the basic pH to the right in the horizontal dimension.

products, of which only a small fraction (0.3% of the total radioactivity) is phosphoenolpyruvate carboxykinase. Immunoprecipitation of this sample using sheep anti-phosphoenolpyruvate carboxykinase resulted in a substantial enrichment in a band which comigrates with authentic phosphoenolpyruvate carboxykinase (SAP lane). A large number of other bands were consistently seen in the immunoprecipitated samples. This could be due to nonspecific binding of other translation products or to a lack of specificity of the antibody. When antibody was omitted or nonimmune sheep serum was used (PBS or NIS lanes, respectively), only the presumptive phosphoenolpyruvate carboxykinase band was lost. Furthermore, the addition of excess pure phosphoenolpyruvate carboxykinase prior to the addition of antibody resulted in the competitive loss of only the band assumed to be phosphoenolpyruvate carboxykinase. Some of the other bands may be phosphoenolpyruvate carboxykinase fragments which do not bind to antibody directed against native phosphoenolpyruvate carboxykinase. This possibility is unlikely since none of the other bands appear to be repressed by glucose or induced by Bt_2cAMP (data not shown). The contaminating bands seem to be present in approximately the same proportion as they are represented in the total translational products. Immunoprecipitation a second time resulted in identical patterns except that the phosphoenolpyruvate carboxykinase band was not present (data not shown). We conclude from these results that the antibody is specific and quantitatively binds the enzyme, although $NaDodSO_4$–polyacrylamide gel electrophoresis is a necessary step in our assay of radioactivity incorporated into phosphoenolpyruvate carboxykinase.

The identification of the immunoprecipitated translation product as phosphoenolpyruvate carboxykinase was also assessed by two-dimensional polyacrylamide gel analysis by the method of O'Farrell (1975). As shown in Figure 2, the radioactive translation product (panel B) comigrated with authentic phosphoenolpyruvate carboxykinase (panel A). Apparent heterogeneity of isoelectric points was observed with both the authentic enzyme and the immunoprecipitated translation product. The most basic form ($pI \simeq 6.8$) was in greatest abundance and five progressively less abundant forms were seen at lower isoelectric points. The proportion of the more acidic forms was lower in the translation product than in the purified enzyme. The multiple peptides could be due

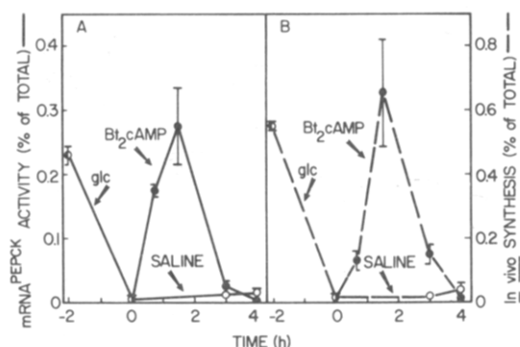

FIGURE 3: Time course following glucose intubation and a single Bt_2cAMP injection in adrenalectomized rats. Rats were fasted 18–22 h and then fed 500 mg of glucose by gavage 2 h prior to the start of the experiment. At time zero rats were injected with 3 mg of Bt_2cAMP plus 3 mg of theophylline per 100 g of body weight in 1.0 mL of saline. Rats were injected with 125 μCi of [^{3}H]leucine/100 g of body weight 30 min prior to being killed. The in vivo synthesis of phosphoenolpyruvate carboxykinase was measured by immunoprecipitation from a liver cytosol. Poly(A)+ RNA was extracted from a portion of each liver and was assayed for $mRNA^{PEPCK}$ activity in a reticulocyte lysate system as described under Materials and Methods. Results for in vivo synthesis and $mRNA^{PEPCK}$ activity are each expressed as the radioactivity incorporated into phosphoenolpyruvate carboxykinase as a percentage of the radioactivity incorporated into trichloroacetic acid precipitable material. (A) (—) $mRNA^{PEPCK}$ activity; (B) (- - -) in vivo synthesis of phosphoenolpyruvate carboxykinase: (◐) fasted animals; (◑) fasted animals fed glucose (glc) for 2 h; (○) fasted–glucose-fed animals treated with saline; (●) fasted–glucose-fed animals treated with Bt_2cAMP plus theophylline. The error bars are standard error of the means with 3 rats per point.

to several species of enzyme differing in charge due to modifications such as phosphorylation or acetylation or could be due to the presence of contaminants. We are as yet unable to assess which of these is correct. Taken together, these data suggest that phosphoenolpyruvate carboxykinase is synthesized in the translational assay and provide further evidence that sheep anti-phosphoenolpyruvate carboxykinase specifically binds this enzyme.

Phosphoenolpyruvate Carboxykinase Induction in Intact and Adrenalectomized Rats. We have compared $mRNA^{PEPCK}$ activity and enzyme synthesis in vivo by using fasted–refed intact rats treated with single injections of Bt_2cAMP. $mRNA^{PEPCK}$ activity increased from undetectable levels to a peak of 0.11% of total mRNA activity 45 min after treatment and declined rapidly thereafter. In comparison, the in vivo synthesis of phosphoenolpyruvate carboxykinase increased from 0.04% to 1.1% of total protein synthesis 90 min after Bt_2cAMP injection (data not shown graphically). These data are quantitatively very similar to those from another laboratory (Iynedjian & Hanson, 1977; Nelson et al., 1980). Glucocorticoid hormones are known to increase phosphoenolpyruvate carboxykinase synthesis (Shrago et al., 1963); thus, it is possible that the observed Bt_2cAMP-stimulated increase in $mRNA^{PEPCK}$ activity could be due to release of adrenal steroids. Adrenalectomized rats were fasted overnight and then given glucose by gavage prior to treatment with Bt_2cAMP (Figure 3), to test this possibility. Both mRNA activity (Figure 3A) and in vivo synthesis (Figure 3B) decreased from fasting levels (0.23% and 0.53% of total, respectively) to <5% of these values within 2 h following glucose feeding. Injection of these fasted–refed animals with Bt_2cAMP resulted in rapid increases in mRNA activity and in vivo synthesis, with maximal values of 0.28% and 0.66% of total, respectively, occurring 90 min after treatment. Four hours after Bt_2cAMP administration, $mRNA^{PEPCK}$ activity and in vivo synthesis had both decreased to values below the saline-treated controls. We

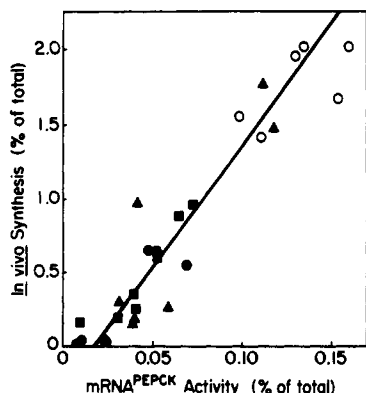

FIGURE 4: Relationship of mRNA^{PEPCK} activity to the in vivo synthesis of phosphoenolpyruvate carboxykinase under steady-state conditions. Rats were fasted for 18 h and then given glucose (5 g/kg of body weight) by gavage 4 h prior to killing. Bt_2cAMP plus theophylline, when given, was injected intraperitoneally (30 mg each/kg of body weight) 2 h after gavage and again 1 h later. Actinomycin D (1.0 mg/kg of body weight) and cordycepin (16 mg/kg of body weight) were injected 30 min prior to Bt_2cAMP injection. mRNA^{PEPCK} activity and phosphoenolpyruvate carboxykinase synthesis in vivo were measured as described under Materials and Methods. The best-fit line was determined by the method of least squares, and it had a correlation coefficient (r^2) of 0.90. Each point represents data from an individual rat: (●) glucose-fed controls; (○) Bt_2cAMP; (▲) Bt_2cAMP plus cordycepin; (■) Bt_2cAMP plus actinomycin D.

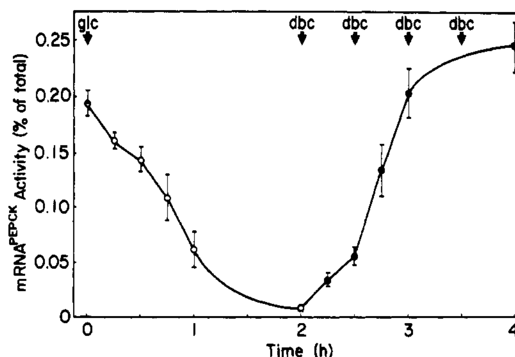

FIGURE 5: Time course of mRNA^{PEPCK} deinduction following glucose feeding and induction following Bt_2cAMP treatment. Intact rats were fasted 18 h and then given 500 mg of glucose in 1 mL of water by gavage at time zero (shown by the arrow labeled glc). In a separate experiment intact rats were fasted, fed glucose, and treated with Bt_2cAMP plus theophyline at 2 h and every 30 min thereafter as shown by the arrows labeled dbc. Poly(A)+ RNA was extracted from livers of rats killed at the indicated times and assayed for mRNA^{PEPCK} activity as described under Materials and Methods. Symbols: fasted animals (◉); fasted–glucose-fed rats (○); fasted glucose-fed Bt_2cAMP-treated rats (●).

consistently noted that the activity of mRNA^{PEPCK} increased slightly prior to but commensurate with in vivo synthesis, and proportionate decreases were observed with each parameter following glucose feeding. Thus, the observed effects are not mediated by adrenal hormones since virtually identical results are obtained with normal and adrenalectomized rats.

Previous studies relating mRNA^{PEPCK} activity to the synthesis rate of the enzyme have been done following single Bt_2cAMP injections and thus have not been steady-state measurements. Nelson et al. (1980) reported that multiple injections of Bt_2cAMP could maintain mRNA^{PEPCK} activity, but they did not measure in vivo synthesis of the enzyme under these conditions. The results in Figure 4 are the composite of several different treatment conditions, all at steady state, showing mRNA^{PEPCK} activity vs. the synthesis of the enzyme in vivo. The fact that steady states occur under these treatment conditions (glucose feeding and Bt_2cAMP treatment in the presence and absence of actinomycin D or cordycepin) was verified in separate time-course experiments where mRNA^{PEPCK} activities remained constant for at least 4 h (data not shown). Each point represents measurements from a single rat, and the best-fit line was determined by the method of least squares. The correlation coefficient (r^2) of 0.90 indicates a linear relationship between mRNA^{PEPCK} levels and enzyme synthesis. It should be noted that the line does not intersect at the origin. It may be that our assay overestimates mRNA^{PEPCK} activity by a constant amount or alternatively, that there is a small pool of competent but untranslated mRNA^{PEPCK} in vivo. The data presented above (Figures 3 and 4) and by others (Iynedjian & Hanson, 1977; Nelson et al., 1980) support the hypothesis that change in mRNA^{PEPCK} activity is the primary determinant of the cAMP-induced change in phosphoenolpyruvate carboxykinase synthesis in rat liver and that ongoing RNA synthesis is necessary for this response.

Measurement of mRNA^{PEPCK} Turnover during Deinduction by Glucose and Induction by Bt_2cAMP. Alterations in mRNA^{PEPCK} activity following Bt_2cAMP treatment could be due to changes in its rate of synthesis, processing, or inactivation. In order to understand the mechanism of cAMP action in this system, it is necessary to assess which step is regulated. At the present time direct measurements are not possible without complementary DNA probes. However, we have indirectly estimated the turnover rate of mRNA^{PEPCK} during both deinduction by glucose and, subsequently, induction by Bt_2cAMP. Berlin & Schimke (1965) pointed out that, following an inducing or deinducing stimulus, the rate at which a protein goes from an initial to a final steady-state level is equivalent to its degradation rate since synthesis is zero order but degradation is first order. This principle has been applied to the measurement of the half-life ($t_{1/2}$) of proteins (Berlin & Schimke, 1965; Segal & Kim, 1963) and mRNA (Nickol et al., 1978). It is important to note that this approach does not require the use of inhibitors of mRNA synthesis; thus, it avoids potential nonspecific effects of such drugs. The time course of the deinduction of mRNA^{PEPCK} following the feeding of glucose to fasted rats and the subsequent induction by Bt_2cAMP is shown in Figure 5. mRNA^{PEPCK} activity decreased as early as 15 min after gavage; it continued to decrease to 30% of the initial activity after 60 min and was almost undetectable after 2 h. The initial lag (~30 min) may be due to the time required for glucose to reach its site of action and stimulate a maximal response. When Bt_2cAMP was given 2 h after the glucose feeding, the induction curve followed a reciprocal pattern. There was an initial lag (~30 min), followed by an increase to 0.2% of total mRNA activity 1 h after, and then to 0.24% 2 h after, the first Bt_2cAMP injection. Little, if any, further increase occurred beyond 2 h after the initial Bt_2cAMP treatment, suggesting that a new steady state had been attained (data not shown). These results indicate that major changes in the rate of inactivation of mRNA^{PEPCK} do not occur during its induction by cAMP. The curves shown in Figure 5 were replotted in their integrated forms (Segal & Kim, 1963; Nickol et al., 1978), and the results are seen in Figure 6. In this type of plot, exponential changes are linear and the negative slope of this line is equivalent to the degradation rate constant, k, of mRNA^{PEPCK}. Half-life, $t_{1/2}$, can be calculated from the relationship $t_{1/2} = \ln 2/k$. Both the induction and deinduction plots are nonlinear during the first 30 min, probably as a result of the time required for glucose and Bt_2cAMP to exert their maximal effects, and then both assume indistinguishable slopes. The estimated mRNA^{PEPCK} half-life is 20 ± 5 min and is unchanged by Bt_2cAMP

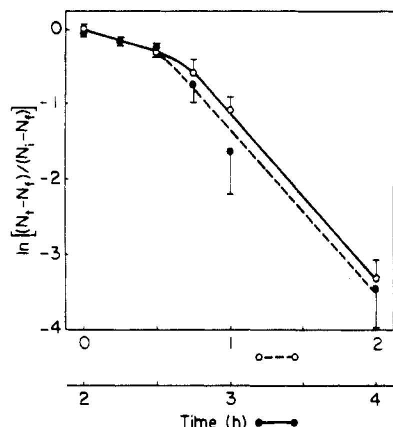

FIGURE 6: Kinetic determination of the half-life of mRNA^{PEPCK}. The data shown in Figure 5 were plotted in integrated form as described by Nickol et al. (1978). N_t is mRNA^{PEPCK} at the indicated time; N_i and N_f are initial and final steady-state levels of mRNA^{PEPCK}, respectively. N_i and N_t during deinduction (O) were assumed to be 0.19% and 0.0%, respectively, whereas the corresponding values during induction (●) were 0.0% and 0.25%. The negative slope (equivalent to the degradation rate constant, k) of the linear portions of these curves is 0.035 min^{-1} for both curves.

treatment following glucose feeding.

Discussion

The primary efect of Bt_2cAMP in the induction of phosphoenolpyruvate carboxykinase in rat liver is to increase mRNA^{PEPCK} activity since there is a direct correlation between changes in the activity of this mRNA and the rate of synthesis of the protein (Iynedjian & Hanson, 1977; Nelson et al., 1980; this study). Although studies have been presented to suggest that the degradation rate of mRNA^{PEPCK} is not altered during Bt_2cAMP-stimulated induction, such studies involved the use of inhibitors of RNA synthesis (Nelson et al. 1980). Numerous conflicting results regarding the mechanism of induction suggest caution in the interpretation of results obtained with inhibitors (Tilghman et al., 1976). We have estimated the turnover rate of mRNA^{PEPCK} without the use of inhibitors. Our observations suggest that the rate of inactivation of mRNA^{PEPCK} is not detectably altered by cAMP (Figure 6). Indeed, if cAMP-stimulated induction were solely mediated by a change of $t_{1/2}$, the 30-fold increase seen in Figure 5 would require a corresponding change in half-life from 20 to 600 min (Berlin & Schimke, 1965). Our data constitute the first measurements of mRNA^{PEPCK} turnover made during cAMP-stimulated induction of phosphoenolpyruvate carboxykinase synthesis without the use of transcriptional inhibitors. Turnover rate measurements were done without the use of inhibitors of transcription in this study for two important reasons. First, we wanted to measure mRNA^{PEPCK} turnover during induction by Bt_2cAMP, and inhibitors block this induction (Iynedjian & Hanson, 1977). Second, we wanted to avoid the potential problems inherent in the use of such drugs. For example, actinomycin D apparently stabilizes mRNA^{PEPCK} in rat liver since phosphoenolpyruvate carboxykinase synthesis decreases with a half-life of 4 h following actinomycin D treatment (Tilghman et al., 1974). In contrast, enzyme synthesis decreases with a half-life of 1 h following cordycepin treatment, and a $t_{1/2}$ of 30 min was observed when fasted rats were fed chow (Tilghman et al., 1974), presumably due to a decrease in mRNA^{PEPCK}. Recently, Neslon et al. (1980) estimated a half-life of 40 min for mRNA^{PEPCK} in rat liver by using cordycepin and α-amanitin but urged caution in the interpretation of such data due to potential nonspecific effects of such drugs. The time course of deinduction following glucose feeding reported here (Figure 5) is in excellent agreement with that reported by Kioussis et al. (1978). These investigators estimated a $t_{1/2}$ for mRNA^{PEPCK} of 45 min but included the initial lag in their estimation. A half-life of ~20 min can be calculated from their data if this lag time is omitted.

The factors which determine the rate of turnover of specific mRNAs in eukaryotic cells are not presently known. The fact that different mRNAs exhibit strikingly varied half-lives even in the same tissue provides an interesting molecular problem. For example, short-lived messengers (half-lives of minutes to hours) such as those coding for phosphoenolpyruvate carboxykinase (this study; Nelson et al., 1980) and tyrosine aminotransferase (Nickol et al., 1978) are present with long-lived messengers (half-lives of several days) such as that coding for serum albumin (Wilson & Hoagland, 1967) in liver cells. Such differences could be determined by structural aspects of the mRNAs which confer variable sensitivities to degradative enzymes (Revel & Groner, 1978). It is also interesting to compare the 20-min $t_{1/2}$ of mRNA^{PEPCK} in liver with an apparent $t_{1/2}$ of 4 h in rat kidney. This latter value was inferred from the time course of phosphoenolpyruvate carboxykinase synthesis induction during ammonium chloride induced acidosis (Iynedjian et al., 1975). A greater stability would result in a substantial reduction in the synthetic demand in the kidney in order to maintain a given level of mRNA^{PEPCK}. This difference could be related to the regulatory differences between the liver and kidney enzymes.

The increase in mRNA^{PEPCK} activity might conceivably be the indirect result of a purely translational action of Bt_2cAMP if an increase in the rate of initiation of translation protects mRNA^{PEPCK} from degradation (Iynedjian & Hanson, 1977; Nelson et al., 1980; Kioussis et al., 1978). Such an effect would be manifested as an increase in the $t_{1/2}$ of mRNA^{PEPCK} following Bt_2cAMP induction. Nelson et al. (1980) argued against this possibility since mRNA^{PEPCK} decreased with identical half-lives in fasted rats treated with inhibitors of RNA synthesis and in rats fed glucose. This conclusion assumes that the mechanism of deinduction due to feeding involves a decrease in cAMP levels and that the inhibitors do not affect cAMP or its actions, but the validity of these assumptions has not been verified. Since the $t_{1/2}$ measurements in this report do not involve the use of inhibitors, it is not necessary to invoke such assumptions. Thus, cAMP probably induces phosphoenolpyruvate carboxykinase synthesis by increasing the rate of mRNA^{PEPCK} synthesis or processing, thereby leading to the accumulation of mRNA^{PEPCK} which is rate limiting for protein synthesis.

We have presented data supporting the hypothesis that Bt_2cAMP induces phosphoenolpyruvate carboxykinase synthesis by increasing the activity of mRNA^{PEPCK}. Since cAMP has no discernible effect on mRNA^{PEPCK} turnover ($t_{1/2}$ = 20 ± 5 min), mRNA^{PEPCK} synthesis and/or processing are probably increased by Bt_2cAMP. Confirmation of this hypothesis awaits the availability of complementary DNA probes which will allow direct measurement of mRNA^{PEPCK} synthesis and degradation. The exact mechanism of Bt_2cAMP-stimulated mRNA^{PEPCK} induction remains unknown. It is possible that the induction could be indirect, involving a substance liberated from another tissue, but the most likely possibility, adrenal steroids, has been eliminated. Moreover, induction occurs in Reuber H-35 hepatoma cells in culture (our unpublished observation). RNA synthesis is required but protein synthesis is not (Nelson et al., 1980), suggesting that Bt_2cAMP increases the expression of the gene without inducing regu-

latory proteins. Presumably, cAMP-dependent protein kinase is involved in a phosphorylation reaction with some protein involved in $mRNA^{PEPCK}$ synthesis, or, alternatively, Bt_2cAMP might act via a binding protein as in bacteria (Pastan & Adhya, 1976).

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Supplementary Material Available

An appendix describing phosphoenolpyruvate carboxykinase purification, amino acid analysis, and antibody preparation and assay (11 pages). Ordering information is given on any current masthead page.

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