Rapid Changes in the Concentration of Phosphoenolpyruvate Carboxykinase mRNA in Rat Liver and Kidney

EFFECTS OF INSULIN AND CYCLIC AMP*

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Starvation and diabetes both caused a marked increase in the concentration of hepatic phosphoenolpyruvate carboxykinase mRNA while the administration of insulin to diabetic rats or refeeding glucose to starved animals caused a marked reduction in the levels of enzyme mRNA as measured by hybridization using a cDNA probe. The administration of dibutyryl cAMP to a starved-refed rat caused an 8-fold induction of phosphoenolpyruvate carboxykinase mRNA in 1 h. Triaminolone plus acidosis induced the level of enzyme mRNA in kidney 3-fold within 6 h, however, starvation for 24 h had only marginal effects. In all of the above conditions, the levels of phosphoenolpyruvate carboxykinase mRNA measured by hybridization assay agreed well with the relative levels of translatable mRNA for the enzyme.

The half-time of phosphoenolpyruvate carboxykinase mRNA, determined after the administration of either a-amanitin or cordycepin to starved animals, was approximately 40 min. However, cycloheximide either alone or together with cordycepin, not only prevented the decrease in phosphoenolpyruvate carboxykinase mRNA sequence abundance, but induced it 2-fold. Cycloheximide itself, when injected into 21-day fetal rats in utero caused an induction of enzyme mRNA equal to that noted when dibutyryl cAMP was administered.

The mRNA for phosphoenolpyruvate carboxykinase is approximately 2.8 kb in length, but nuclei from the liver of diabetic rats contain a number of putative precursor RNA species for the enzyme, up to 6.5 kb in size, all containing a poly(A) tail. Two hours after refeeding glucose to a starved rat, these nuclear RNA species could no longer be detected by hybridization to our cDNA probe.

The mechanism underlying the hormonal regulation of the cytosolic form of P-enolpyruvate carboxykinase (GTP) (EC 4.1.1.32) in both rat liver and kidney have been extensively studied (1). This enzyme is generally considered one of the regulatory steps in the gluconeogenic pathway (2) and its synthesis rate is acutely regulated by a number of hormones (1). P-enolpyruvate carboxykinase in liver and kidney are immunologically similar (3, 4) but each enzyme has a unique pattern of hormonal regulation. For example, translatable mRNA levels for hepatic but not renal P-enolpyruvate carboxykinase are markedly increased by administration of $N^\circ,D^\circ$-dibutyryladenosine 3':5'-monophosphoric acid (4), whereas acidosis induces the concentration of enzyme mRNA in kidney but not liver (4). Translatable P-enolpyruvate carboxykinase mRNA in both tissues can be induced by glucocorticoids (5, 6), but the extent of this induction in liver is lower than noted after Bt2cAMP administration (6). Conversely, insulin, which lowers the hepatic cAMP concentration, will rapidly induce hepatic P-enolpyruvate carboxykinase mRNA (7), but has no effect in the kidney.

To elucidate the mechanisms responsible for the hormonally regulated alterations in hepatic and renal P-enolpyruvate carboxykinase synthesis, we have constructed a cDNA clone using partially purified enzyme mRNA (8). In the present study we have used this clone to examine the hormonal regulation of P-enolpyruvate carboxykinase mRNA levels. Our results indicate that the concentration of P-enolpyruvate carboxykinase mRNA can be rapidly altered by various hormonal stimuli. We also noted a number of putative precursors of mature P-enolpyruvate carboxykinase mRNA present in the nuclei (but not cytosol) from rat liver. The concentration of these precursors could be rapidly altered by dietary and hormonal stimuli in the same manner as mature, cytosolic P-enolpyruvate carboxykinase mRNA, suggesting that induction and deinduction of enzyme mRNA is primarily a nuclear event.

MATERIALS AND METHODS

Chemicals—[$\text{32P}$]dCTP (400-600 Ci/mmol) and L-[1$\text{35S}$]methionine (1000 Ci/mmol) were obtained from New England Nuclear or Amersham Searle. DNA polymerase I and reticulocyte lysate translation systems were products of New England Nuclear. DNase I was purchased from Worthington Biochemicals. Nitrocellulose paper (BAS$\text{i}$) was obtained from Schleicher and Schuell. Dextran sulfate was purchased from Pharmacia, deoxynucleotides from Boehringer Mannheim, cesium chloride (optical grade) from Varilac Chemical Co., and guanidinium thiocyanate from Fluka. Formamide (MCB Manufacturing Chemists, Inc.) was deionized before use with Bio-Rad AG 50X-8 resin (8). Salmon testis DNA, lysozyme, streptozotocin, a-amanitin, cordycepin, insulin, and $N^\circ,D^\circ$-dibutyryl cyclic AMP were purchased from Sigma.

*The abbreviations used are: Bt$_2$cAMP, dibutyryl cAMP; $N^\circ,D^\circ$-dibutyryladenosine 3':5'-monophosphoric acid; poly(A$\text{+}$)RNA, RNA containing a polyadenylic acid sequence; IX SSC, 0.15 $\text{M NaCl}, 0.015 \text{ M Na citrate}$.  

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_P-enolpyruvate Carboxykinase mRNA_

**Animals**—Male, Charles River CD rats weighing 200–350 g were fed ad libitum with Wayne “Lab Block.” Diabetes was induced by intravenous injection of streptozotocin (60 mg/kg of body weight) as previously described (7). The blood glucose concentration of the diabetic animals averaged 381 ± 12 mg/ml (n = 59). Insulin (5 units/kg of body weight) was injected intraperitoneally, with a glucose (5 g/kg of body weight) at the same time to prevent hypoglycemia (Table I). Animals which were “refed” were first starved for 24 h and then given glucose (5 g/kg of body weight) by gastric intubation and then killed at the times specified. Drugs were dissolved in 154 mM NaCl and injected intraperitoneally as previously described (1) at the following doses (per kg of body weight): a-aminitin, 1 mg; BSA-MP and theophylline, 25 mg each; cordycepin, 30 mg, and cycloheximide, 10 mg. To induce renal _P-enolpyruvate carboxykinase_, rats were injected with triamcinolone (32 mg/kg of body weight) and then made acidic by NHCl gavage (10 ml/kg of body weight) (4).

**RNA Extraction**—Total RNA was extracted from rat liver or kidney with phenol/m-cresol/water (76:11:13) containing 0.07% (w/v) hydroxyquinoline as described by Nienhuis et al. (9) and Nelson et al. (10). Poly(A)RNA was obtained by oligo(dT)-cellulose chromatography as previously described (1).

**Translational Analysis**—Poly(A)RNA for the determination of translational activity was assayed using the wheat germ extract system (7). Briefly, 5 μg of poly(A)RNA were translated for 90 min in a total volume of 300 μl at 25 °C. Total protein synthesis was determined after deacylation with 0.2 N KOH and precipitation in cold, 10% trichloroacetic acid. Incorporation of[^35S]methionine into total protein was linear with respect to RNA concentration and incubation time. _P-enolpyruvate carboxykinase_ synthesis was quantitated by immunoprecipitation using techniques described previously (10).

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**—Total protein synthesis and the immunoprecipitated enzyme synthesized in the wheat germ translation system were separated by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis in a 9% slab gel as described by Maizel (11). Gels were dried, autoradiographed using Kodak SB5 X-ray film, and the relative band intensities quantitated by densitometry (12).

**Nick Translation of Plasmid DNA**—Two recombinant plasmids containing a DNA segment complimentary to _P-enolpyruvate carboxykinase_ RNA were used in these studies (8). One of these plasmids (pPCK1) is 228 bases in length, and the other (pPCK2) is 606 bases in length and contains the complete pPCR1 sequence. Both of these plasmids were radiolabeled in _vitro_ by nick translation essentially as described by Maniatis et al. (13). Briefly, 0.5 milliunit of activated [d'PJdCTP each of the four nucleotides, 11 mM MgCl₂, 19 mM D-mercaptoethanol and 22 mM Tris-Cl, pH 8. After 2 min at room temperature, 5.4 units of DNA polymerase I were added and the reaction continued for 60 min at 14 °C. The reaction was stopped by the addition of 10 mM EDTA, 0.5 M NaCl, and the radiolabeled plasmid recovered by chromatography through Sephadex G-100 in 0.5 M NaCl, 0.05 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5. The plasmid collected with the void volume was labeled to the extent of 0.5–1.0 × 10⁶ cpm/μg and was used that day for Northern blotting. For dot blot analysis the nick translation assay was scaled up to ensure at least 0.4 μg of plasmid/filter.

**Northern Analysis of RNA**—Poly(A)*RNA was denatured in 2.2 M formaldehyde, 50% formamide for 5 min at 65 °C (14), applied to a 0.6% agarose gel containing 2.2 M formaldehyde, 20 mM 4-morpholinolineethanesulfonic acid, pH 7.0, 5 mM NaOAc, and 1 mM EDTA, and separated by electrophoresis at no greater than 1 V/cm of gel for at least 5 h. The gel was stained with 2.5 μg/ml of ethidium bromide in 0.5 M NH₄ acetate, and photographed with UV light to establish migration of ribosomal RNA. The gel was then treated with 50 mM NaOH for 30 min, neutralized with 2 washes of 20X SSC for 20 min each, and transferred to nitrocellulose (15) in 20X SSC for at least 12 h. After transfer, RNA was fixed by baking the paper for 2 h at 80 °C. The nitrocellulose filters were prehybridized or at least 1 h at 50 °C in a medium containing 90% formamide, 10% dextran sulfate, 5X SSC, 1.5 mg/ml of sheared, fragmented salmon testis DNA, 0.5% sodium dodecyl sulfate, 0.02% ficol, 0.02% polyvinylpyrrolidone, and 1.5 mg/ml of glycine (16). Hybridization was performed in an identical mixture except that 0.25–0.5 μg of plasmid DNA (0.25–0.5 × 10⁶ cpm) was added after being heat denatured (by boiling with the salmon testis DNA) for 5 min. Hybridization was allowed to proceed for 36 h. The nitrocellulose paper was washed with 50% formamide, 5X SSC, 0.2% ficol, 0.2% polyvinylpyrrolidone at 37 °C and autoradiograms prepared by the method of Swanson and Shank (12).

**Quantitation of the Northern Blots**—Relative exposures were obtained from each Northern gel. The density of the 2.8 kb band was previously identified as _P-enolpyruvate carboxykinase_ mRNA (8) was evaluated by densitometry using conditions where the density of the film image was proportional to the exposure time. Integration units have been normalized in each experiment, setting the control for each condition at a value of 1.0.

**Quantitation of Relative RNA Content by Dot Blot Analysis**—The dot-blot procedure used in this study is essentially that described by Kafatos et al. (17). Nick translation products were pre-wet with 20X SSC and allowed to air dry. Incremental amounts of each poly(A)*RNA to be analyzed (between 0.1 and 0.7 μg as measured spectrophotometrically) were spotted in 5X SSC within a defined square on the filter and the filters were baked at 80 °C for 2 h. Prehybridization and hybridization conditions were identical with those described for Northern analysis. Equal amounts of radiolabeled plasmid were added to each filter. After hybridization and washing, the locations of the RNA spots were determined by exposure of the filters to x-ray film and squares of identical sizes were cut out around the RNA. Radioactivity was determined by liquid scintillation and corrected for background by subtraction of radioactivity found on blank squares cut from the filter. To compare RNA preparations, the slope of the linear signal response between micrograms of RNA bound and counts per min hybridized was normalized to counts per min μg of RNA.

**Isolation of Nuclear RNA**—Livers were isolated from rats treated as indicated in Fig. 1 and homogenized in 5 volumes of 0.25 M sucrose and 1.5% citric acid, pH 2.3, containing 0.5% Triton X-100 (18). The homogenate was layered onto a 10-mI cushion of 1.8 M sucrose with 1.5% citric acid and 5 ml of 1.0 M sucrose with 1.5% citric acid and 0.5% Triton X-100 and centrifuged for 30 min at 2000 × g. The nuclear pellet was suspended in 15 ml of the original homogenization buffer, layered onto a 10-mI cushion of 1.0 M sucrose with 1.5% citric acid and 0.5% Triton X-100, and centrifuged for 10 min at 600 × g. The nuclear pellet from this step was resuspended in 8 ml of 4 M guanidinium thiocyanate containing 0.5% sodium N-lauroyl sarcosine, 20 mM sodium EDTA, and 0.2 M β-mercaptoethanol, pH 7.0, to extract RNA.

Cytosol RNA was obtained from the 0.25 and 1.0 M sucrose layers that remained after the first centrifugation of the homogenate. After centrifugation for 30 min at 15,000 × g, the RNA was extracted from the resulting pellet by dissolving it in 10 ml of 5 M guanidinium thiocyanate, containing 15% sodium N-lauroyl sarcosine, 30 mM sodium EDTA, 0.2 M β-mercaptoethanol, and 150 mM NaHCO₃.

**Size of P-enolpyruvate Carboxykinase mRNA**—The size of the mature _P-enolpyruvate carboxykinase_ mRNA is 2.8 kb (Fig. 1). This mRNA, prolactin mRNA, albumin mRNA, and 18S and 28S rRNAs as outlined under “Materials and Methods” PEPC, phosphoenolpyruvate carboxykinase; 18S and 28S, ribosomal RNA; kb, kilobases.
was determined using RNA extracted from bovine pituitary, rabbit reticulocytes, and liver from 17-day fetal and adult rats as outlined previously (10). The RNA was separated by size by electrophoresis in 0.8% agarose, 2.2 M formaldehyde as described above. Specific cDNA probes for bovine prolactin (19), rabbit globin (20), rat albumin (21), and rat cytosolic P-enolpyruvate carboxykinase (pCK2) were nick translated and hybridized to the RNA by procedures outlined in detail above.

RESULTS

Effect of Insulin on P-enolpyruvate Carboxykinase mRNA Sequence Abundance—The rapid loss of P-enolpyruvate carboxykinase mRNA was examined in more detail in the insulin-treated diabetic rat using both Northern analysis and the dot-blot assay. The dot-blot procedure (17) detects all hybridizable RNA species in a given sample. Within a defined range of RNA, there is a direct proportion between the amount of P-enolpyruvate carboxykinase mRNA in the bound sample and the radioactivity in pPCK1 which hybridizes to that RNA (Fig. 2). The slope of this line changes as the levels of P-enolpyruvate carboxykinase mRNA are altered (Fig. 2), and changes in the relative levels of P-enolpyruvate carboxykinase mRNAs are reflected as the fold difference in the slopes of the lines, reported as counts per min in pPCK1 bound/µg of RNA spotted. This assay easily and quickly detects changes as small as 2-fold. For the studies reported here, examination of 0.1 to 0.7 µg of RNA resulted in a nonsaturating, linear response during both induction (diabetic) or deinduction (diabetic plus insulin) of P-enolpyruvate carboxykinase mRNA (Fig. 2).

The second assay used to assess relative changes in RNA content was Northern analysis (16). Poly(A) + RNA (usually 5 µg) from each of the conditions under examination was separated by electrophoresis on a denaturing formaldehyde-agarose gel and then transferred to nitrocellulose. After hybridization with [32P]pCK1, the radioactive profile was determined by autoradiography (Fig. 3) and the peak density of the 2.8 kb band previously identified as P-enolpyruvate carboxykinase mRNA was evaluated by densitometry (Fig. 3). Comparison of results using dot-blot or Northern analysis are presented in Table 1. Injection of insulin into a diabetic rat caused a loss of about 70% of translatable P-enolpyruvate carboxykinase mRNA from the liver in 2 h. This loss is accompanied by an identical decrease in hybridizable enzyme RNA when assayed either by dot-blot or by Northern blotting.

Induction of P-enolpyruvate Carboxykinase mRNA in Rat Liver and Kidney—Induction of hepatic P-enolpyruvate carboxykinase is due to an elevated rate of enzyme synthesis (22, 23), which is accompanied by a marked increase in translatable mRNA for the enzyme (23). Previous studies using inhibitors of DNA transcription (24) or of mRNA processing (25) suggest that this increase in translatable mRNA for hepatic P-enolpyruvate carboxykinase was due to a stimulation of transcription of the gene for the enzyme. In Fig. 4, we demonstrate that starvation for 24 h caused a 9-fold elevation of enzyme mRNA in rat liver as detected by hybridization to our cDNA probe. Similarly, in the diabetic rat the relative sequence abundance of hepatic P-enolpyruvate carboxykinase mRNA increased 8-fold (Fig. 4) as compared to normal animals.

![Fig. 3. Northern analysis of P-enolpyruvate carboxykinase mRNA under conditions of induction and deinduction. Poly(A) + RNA was prepared from animals as described in Fig. 2. Five µg of poly(A) + RNA were denatured with 2.2 M formaldehyde, 50% formamide at 60 °C for 5 min and applied to a 0.8% agarose gel containing 2.2 M formaldehyde. Following electrophoresis for 3 h, the RNA was transferred to nitrocellulose with 20X SSC overnight at 4 °C. Hybridization conditions are described under "Materials and Methods." Lanes 1-4, four preparations of RNA from control, non-diabetic rat livers. Lanes 5-8, five preparations of RNA from diabetic livers. Lanes 10-13, five preparations of RNA from diabetic rats injected with insulin for 2 h. The origin is marked by an arrow and the major bands are 2.8 kb in length.](http://www.jbc.org/)

![Table 1](http://www.jbc.org/)

| Condition | Translatable | Hybridization assay |
|-----------|--------------|---------------------|
|           | cpm/µg RNA   | Dot-blot | Northern (integration units) |
| Diabetes  | 0.39 ± 0.06 (9) | 1055 ± 79 (7) | 17.44 ± 3.5 (5) |
| Diabetes + insulin | 0.11 ± 0.02 (5) | 313 ± 34 (7) | 5.49 ± 1.4 (5) |

![Fig. 2. Linear response of the dot-blot assay under conditions of P-enolpyruvate carboxykinase mRNA induction and deinduction. RNA was extracted from the livers of diabetic rats and from diabetic rats injected with insulin (5 units/kg of body weight) for 2 h. Increasing amounts of the poly(A) + RNA (from 0.1 to 0.7 µg) was spotted on nitrocellulose. Seven points were used for each RNA preparation. The nitrocellulose paper was baked and hybridized against [32P]pCK1 as described under "Materials and Methods." Individual spots were cut from the paper and the radioactivity determined. Each point represents the mean ± S.E. for seven RNA preparations.](http://www.jbc.org/)
Liver preparations are presented as the average of at least four extractions and the results are presented as the average fold induction assayed from the pooled poly(A)'RNA.

The concentration of P-enolpyruvate carboxykinase mRNA was evaluated by densitometric scanning of Northern blot exposure. Liver preparations are presented as the average of at least four extractions ± S.E. Kidneys were pooled (4–32 kidneys/RNA extraction) and the results are presented as the average fold induction assayed from the pooled poly(A)'RNA.

Rat kidney contains relatively higher levels of P-enolpyruvate carboxykinase mRNA than is found in the liver (25). The synthesis of the enzyme in rat kidney is not responsive to Bt2cAMP (4) and, as found in this study, its relative mRNA sequence abundance was not altered significantly after 24 h of starvation (Fig. 4) or by diabetes (not shown). This is consistent with our earlier finding that renal P-enolpyruvate carboxykinase is not stimulated by diabetes in the absence of acidosis (4). The injection of triamcinolone as well as the induction of metabolic acidosis by the administration of NH4Cl by gavage caused a 4-fold enhancement of renal P-enolpyruvate carboxykinase mRNA sequence abundance over control values (Fig. 4) within 6 h. This is similar to the 4.5-fold induction in translatable mRNA for the renal enzyme reported by Lyndenjian and Hanson (5) after the same treatment.

Rapid Alterations in Hepatic P-enolpyruvate Carboxykinase mRNA Sequence Abundance—The levels of enzyme mRNA can be rapidly modulated during both induction and deinduction. Feeding a rat starved for 24 h caused a decrease in hepatic P-enolpyruvate carboxykinase mRNA sequence abundance to approximately 15% of the starved value after only 2 h (Fig. 5). On the other hand, the injection of Bt2cAMP into a rat that was starved for 24 h and refed carbohydrate for 2 h, resulted in a 5-fold stimulation in the level of hybridizable mRNA for the enzyme after 1 h (Fig. 5). These data demonstrate that Bt2cAMP can stimulate the accumulation of hepatic P-enolpyruvate carboxykinase mRNA which in previous studies (23) was shown to be in a translatable form. Also, these results indicate that refeeding causes a rapid loss of enzyme mRNA and not just conversion into an inactive, untranslated form (26).

P-enolpyruvate Carboxykinase mRNA Turnover—Both α-amanin and cordycepin, at concentrations which inhibit RNA synthesis by 95%, caused a rapid decrease in the levels of hybridizable mRNA for P-enolpyruvate carboxykinase present in the livers of rats starved for 24 h (Fig. 6). The levels of enzyme mRNA decreased to only 30% of that present in the livers of starved rats 90 min after the injection of cordycepin. Similar results were obtained after injection of α-amanitin. Also, the half-time of enzyme mRNA after administration of either inhibitor was approximately 50 min (Fig. 6). Alternatively, the injection of cycloheximide or cycloheximide plus cordycepin caused a doubling in sequence abundance of P-enolpyruvate carboxykinase mRNA within the first 30 min of the experiment and it remained at this elevated level during the final hour of the time course (Fig. 6). Because this increase in enzyme mRNA occurs so rapidly, it could be due to cycloheximide stimulation of RNA polymerase II (27). It is known that the synthesis of P-enolpyruvate carboxykinase mRNA can be very rapidly increased by cAMP (Fig. 5) so that the cycloheximide-induced doubling of enzyme mRNA such as noted in Fig. 6 could occur within 10 min. This induction of P-enolpyruvate carboxykinase mRNA may not be immediately blocked by cordycepin due to a relatively slower rate of cordycepin uptake by the liver. The ability of cycloheximide to stimulate the accumulation

![Fig. 4. Long term induction of P-enolpyruvate carboxykinase mRNA. Poly(A)⁺RNA was extracted from the livers and kidneys of rats which had been either starved for 24 h, made diabetic with streptozotocin injection, or been injected with (22 mg/kg of body weight) triamcinolone, force-fed was evaluated by densitometric scanning of Northern blot exposure. Each point represents the mean ± S.E. of at least four RNA preparations.](http://www.jbc.org/)

![Fig. 5. Rapid alteration in P-enolpyruvate carboxykinase mRNA concentrations. Poly(A)⁺RNA was extracted from control rats, which had been fasted 24 h, rats which had been force-fed glucose for 2 h, and rats which received an injection of Bt2cAMP plus theophylline 2 h after the glucose feeding. Levels of enzyme mRNA were estimated by densitometric scanning of a Northern blot exposure. Each point represents the mean ± S.E. of at least four RNA preparations.](http://www.jbc.org/)

![Fig. 6. The effect of inhibitors of RNA and protein synthesis on the levels of P-enolpyruvate carboxykinase mRNA. Rats were fasted for 24 h and then injected with α-amanitin (1 mg/kg), cordycepin (30 mg/kg), cycloheximide (10 mg/kg), or cycloheximide plus cordycepin. At the indicated time they were killed and poly(A)⁺RNA was extracted from the liver. Relative levels of P-enolpyruvate carboxykinase mRNA were determined by densitometric tracing of a Northern blot exposure. Each point is the mean ± S.E. of three RNA preparations.](http://www.jbc.org/)
of P-enolpyruvate carboxykinase mRNA can be noted more clearly in studies using fetal rats (Fig. 7). We detected very low levels of hybridizable mRNA for the enzyme in poly(A)-containing RNA isolated from the livers of 21-day fetal rats injected with saline in utero. However, the injection of Bt,cAMP caused a rapid accumulation of hybridizable mRNA for P-enolpyruvate carboxykinase in the livers of fetal rats after only 2 h (Fig. 7). Furthermore, when cycloheximide and Bt,cAMP were injected together there was a large increase in enzyme mRNA sequence abundance, which was 3.6-fold greater than the increase noted with Bt,cAMP alone. Injection of cycloheximide alone also raised the levels of hybridizable mRNA for P-enolpyruvate carboxykinase to those noted after Bt,cAMP administration alone.

Alterations in Nuclear mRNA for P-enolpyruvate Carboxykinase from Rat Liver: Evidence for Putative Precursor RNA—Livers from adult, diabetic rats, from fasted rats refed carbohydrate for 2 h, or from rats fed a protein-free diet were fractionated into cytosol and nuclei as outlined under “Materials and Methods.” Total RNA was isolated from both cytosol and nuclei and the sequence abundance of P-enolpyruvate carboxykinase mRNA determined by Northern blotting (Fig. 8). Lanes 1 and 2 show the enzyme mRNA present in the cytosol and nuclei, respectively, with mature P-enolpyruvate carboxykinase mRNA at 2.8 kb as indicated by the arrow. The cytosol contained a single detectable band whereas there were a number of mRNA species detectable in the nuclei which hybridized with our probe. One of these species was about the size of the mature enzyme mRNA, but there were as many as 4 larger bands, the largest of which was approximately 6.5 kb. There were also a number of bands smaller than the mature mRNA. These RNA species, possibly breakdown products, have been observed in numerous other nuclear preparations and appear to have a regular and highly specific size distribution. Both the putative P-enolpyruvate carboxykinase mRNA precursors and degradation products were polyadenylated but were not observed in cytosolic RNA (data not shown).

Glucose refeeding to an animal starved for 24 h caused a rapid loss in enzyme mRNA (within 2 h) from both the nucleus and cytosol (lanes 3 and 4, Fig. 8), with an equal loss in abundance of all of the nuclear RNA species, including the mature enzyme mRNA. A diet free of protein (lanes 5 and 6, Fig. 8) also markedly reduced the abundance of P-enolpyruvate carboxykinase mRNA in both nuclei and cytosol.

Livers taken from 21-day fetal rats were fractionated into nuclei and cytosol and total RNA isolated. Northern blotting indicated the presence of a low level of hybridizable P-enolpyruvate carboxykinase mRNA in the cytosol and no detectable message in the nuclei (lanes 7 and 8, Fig. 8). Similarly there were no RNA bands either larger or smaller than the mature 2.8 kb mRNA which hybridized with our cDNA probe. However, 2 h after delivery of the animals, appreciable levels of P-enolpyruvate carboxykinase mRNA had accumulated in the cytosol (lane 9, Fig. 8) and a predominant RNA species at 3.0 kb was apparent in the nuclei (lane 10, Fig. 8). Also, the appearance of both larger and smaller bands of RNA were noted in the liver nuclei from newborn rats, but their intensity was far less than the mature 2.8 kb species. A comparison of the relative sequence abundance of the mature enzyme mRNA to putative precursors (or degradation products) in nuclei from the adult and fetus suggests that during the initial expression of the P-enolpyruvate carboxykinase gene the rate of accumulation of mature enzyme mRNA is more rapid than the accumulation of larger, putative precursor RNA species.
DISCUSSION

There is extensive evidence demonstrating hormonally induced alterations in P-enolpyruvate carboxykinase mRNA in rat liver (1) and kidney (5) but most of these studies have measured the levels of translatable mRNA in vitro using either the reticulocyte or wheat germ translation system. The results of the present study in which the levels of P-enolpyruvate carboxykinase mRNA are measured by direct hybridization techniques, clearly show a parallel between the sequence abundance of enzyme mRNA and the translatability in vitro. For example, the injection of insulin into a diabetic rat causes a 70% decline in translatable mRNA for the hepatic enzyme in 2 h and an identical 70% decrease in sequence abundance for the message as determined by either Northern blotting or dot-blot techniques. From these results, and numerous other experiments carried out in our laboratory and not reported here, we conclude that our earlier measurements of translatable P-enolpyruvate carboxykinase mRNA accurately measures the relative levels of mRNA for the enzyme.

Effect of Insulin and Bt2cAMP on P-enolpyruvate Carboxykinase mRNA—The administration of insulin to diabetic rats also causes a rapid decay in the levels of P-enolpyruvate carboxykinase mRNA. Within 2 h after insulin injection the sequence abundance of the mRNA detectable by Northern analysis declined to barely detectable levels (Fig. 3). This effect correlates directly with a rapid decline in the rate of enzyme synthesis in the livers of diabetic rats after the administration of insulin (35). The rapidity of the effect on insulin on the decay of P-enolpyruvate carboxykinase mRNA noted in this study suggests that the hormone blocks the synthesis of new enzyme mRNA by altering transcription or mRNA maturation. Since the half-life of enzyme mRNA after insulin injection is similar to that noted after the administration of either a-amanitin or cordycepin, it may be appropriate to suggest analogies between the effect of the inhibitors and the mechanism of action of the hormone. However, it is not established whether insulin acts directly or via some effect on hepatic cAMP levels. Recently, Andreone et al. (28) presented evidence for a direct effect of insulin on the turnover of P-enolpyruvate carboxykinase mRNA using hepatoma cells in culture. This effect was achieved at relatively low insulin concentrations and appeared to be mediated via an insulin receptor. They also suggest that insulin alters the rate of P-enolpyruvate carboxykinase synthesis at a point prior to mRNA translation. Whether insulin lowers the levels of P-enolpyruvate carboxykinase mRNA directly or acts by altering the intracellular concentration of cAMP, the half-time of enzyme synthesis in Reuber H35 cells after the removal of Bt2cAMP (\(t_\text{1/2} = 4\) min) (29) is the same as that caused by insulin injection into a diabetic rat (30). The effects of Bt2cAMP on the induction of P-enolpyruvate carboxykinase mRNA are remarkably rapid. The injection of the cyclic nucleotide into starved-re fed rats will induce the sequence abundance of enzyme mRNA almost 5-fold in 1 h (Fig. 4). We have recently investigated the effect further and have shown that Bt2cAMP causes an 8-fold stimulation of transcription of the gene for P-enolpyruvate carboxykinase by isolated rat liver nuclei within 20 min. The transcription rate then decreases by 60 min after the administration of Bt2cAMP. Furthermore, there is a parallel increase in nuclear precursor RNA detectable by hybridization with our cDNA probe. These precursors, ranging in length from 6 to 2.8 kb, have a maximum accumulation in the nucleus within 20 min after Bt2cAMP administration and subsequently decrease in sequence abundance by 60 min. However, the levels of cytosolic P-enolpyruvate carboxykinase mRNA increases during the entire 60-min period. 2 It is clear from these studies that cAMP can rapidly alter the transcription of the P-enolpyruvate carboxykinase gene by a mechanism as yet poorly understood.

P-enolpyruvate Carboxykinase mRNA Turnover—The half-life of total P-enolpyruvate carboxykinase mRNA in rat liver measured after blocking polymerase II with a-amanitin, or after inhibiting mRNA maturation with cordycepin, is approximately 40 min. Enzyme mRNA is apparently degraded since, within 2 h after administration of these inhibitors we can detect only low levels of P-enolpyruvate carboxykinase mRNA sequences using our specific cDNA probes. The sequence abundance of mRNA for albumin or for B-actin, as well as the levels of total translatable mRNA do not change significantly during the 2-h period following the administration of the inhibitors (data not shown). This suggests that the relatively short half-life of P-enolpyruvate carboxykinase mRNA as measured in this study is not due to some generalized effect of either a-amanitin or cordycepin on total mRNA degradation.

The effect of cycloheximide on enzyme induction was first noted by Ernst et al. (31) in their studies of tyrosine aminotransferase mRNA. They reported a 5.8-fold increase in the levels of tyrosine aminotransferase mRNA 4 h after the administration of cycloheximide. In contrast to our work with P-enolpyruvate carboxykinase mRNA (10), they also noted a 7-fold induction in tyrosine aminotransferase mRNA levels with puromycin. They concluded that the best analogy for this type of finding was the “superinduction” of fibroblast interferon mRNA which appears to be regulated by a short lived repressor protein. Alternatively, they also suggested the cycloheximide could act by freezing the polysome-mRNA complex, therefore rendering it less susceptible to cytoplasmic degradation. Nelson et al. (10) presented evidence in favor of the latter effect of cycloheximide based primarily on the finding that cycloheximide, but not puromycin, will prevent the normal decline in the levels of P-enolpyruvate carboxykinase mRNA caused by the administration of cordycepin. It is thus possible that the association of enzyme mRNA with ribosomes protects the message from degradation and that any factor which enhances the initiation of mRNA translation will increase the half-life of a specific mRNA.

Effects of Bt2cAMP and Cycloheximide on Enzyme mRNA in Fetal Rat Liver—There is no P-enolpyruvate carboxykinase activity detectable in the cytosol of fetal rat liver. The enzyme appears initially at birth but can be induced in utero by the administration of Bt2cAMP or glucagon (32). We could measure only marginal rates of enzyme synthesis in the livers of fetal rats and low levels of translatable P-enolpyruvate carboxykinase mRNA (33). It is apparent from the present study that there is a low but detectable concentration of enzyme mRNA in fetal rat livers, which can be markedly increased within 2 h after the injection of Bt2cAMP directly into the fetal rats in utero. What is surprising is that cycloheximide is as effective as Bt2cAMP administration in inducing the levels of P-enolpyruvate carboxykinase mRNA and that there is a synergistic effect when both compounds are administered together (Fig. 7). It is possible that cycloheximide stabilizes the low level of enzyme mRNA present in fetal rat liver, against degradation as discussed above. Alternatively, cycloheximide may increase the level of camp in fetal liver (or in adult liver) or directly stimulate gene transcription due to an induction in the activity of RNA polymerase II as suggested by Lindell and Duffy (27).
kinase mRNA—The results of the present study demonstrate the existence of multiple species of hepatic P-enolpyruvate carboxykinase mRNA which are larger than the mature form of the enzyme mRNA. These larger forms of mRNA are most likely precursors which are processed to the mature, functional mRNA. We detect up to five distinct bands ranging from approximately 6.5 kb to the mature message at 2.8 kb. Since P-enolpyruvate carboxykinase from rat liver contains 635 amino acids (34) it only requires an mRNA coding sequence of 1.9 kb, rather than the 2.8 kb of mature enzyme mRNA detected by hybridization to our cDNA probe. The larger putative precursor RNA species appear to be largely concentrated in the nucleus, since total cytoplasmic RNA contained no detectable bands which hybridize with P-enolpyruvate carboxykinase cDNA.

There is evidence for the existence of precursors for a number of mRNAs including growth hormone mRNA (35), prolactin mRNA (36), globin mRNA (37), immunoglobulin mRNA (38), and ovalbumin mRNA (39). Recently, Snead et al. (40) have reported that the entire 3.0 kb gene for uro-tergolin is expressed into primary RNA transcripts which are subsequently processed into a mature, 465-nucleotide long mRNA by splicing. In these studies the largest precursor detected by hybridization was the size of the entire uro-tergolin gene. Hoffman et al. (36) have also reported a large, 14 kb precursor mRNA which is the size of the complete prolactin mRNA gene. Perhaps the best studied is the precursor mRNA for globin which is a continuous transcript of the globin gene and contains both introns and exons (37). The size of the gene for rat cytosol P-enolpyruvate carboxykinase mRNA is not yet known so that the relationship between our largest putative precursor and the rate of RNA processing in the livers of newborn rats (Fig. 8). This requires measurements of the rate of synthesis or the breakdown of one form to another.

The presence of putative precursors for P-enolpyruvate carboxykinase mRNA in the nucleus suggests that alterations in the processing of these precursors may regulate the levels of the mature enzyme mRNA. This suggestion could be of particular interest for P-enolpyruvate carboxykinase mRNA, since it undergoes acute alterations in its concentration in response to hormones. We can show that all of the precursor mRNA bands decrease in parallel after insulin administration to diabetic rats (Fig. 8), suggesting some type of coordinate control of the levels of all of the precursors. Also, during the initial expression of the gene for P-enolpyruvate carboxykinase in the first 2 h after birth, the mature 2.8 kb length mRNA is present in the nucleus with very low levels of precursor bands detectable. It is possible that processing of these nuclear precursors is hormonally controlled and that the rate of RNA processing in the livers of newborn rats exceeds the rate of gene transcription so that nuclear precursors do not accumulate in the nucleus relative to the mature mRNA. However, Northern analysis only provides information on the relative concentration of the various P-enolpyruvate carboxykinase mRNA species but does not indicate their rate of synthesis or the breakdown of one form to another. This requires measurements of de novo synthesis of enzyme mRNA; studies which are currently in progress in our laboratory.

We have also noted bands of lower molecular weight than mature P-enolpyruvate carboxykinase mRNA which hybridize with our probe. These bands are found only in the nucleus, contain a poly(A) segment, and are not detected in cytoplasmic fractions of rat livers. These smaller molecular weight species disappear during P-enolpyruvate carboxykinase deinduction after refeeding carbohydrates to starved rats (Fig. 8). We are presently analyzing the kinetics of labeling of both the large and small nuclear RNA bands in order to more definitively establish their relationship to mature P-enolpyruvate carboxykinase mRNA.
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