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# Isolation and characterization of the gene coding for cytosolic phospho*enol*pyruvate carboxykinase (GTP) from the rat

(R-loop mapping/Southern blots/cDNA clones/DNA sequence/*in vitro* transcription)

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**ABSTRACT** **The gene for cytosolic phospho*enol*pyruvate carboxykinase (GTP) [GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32] from the rat was isolated from a recombinant library containing the rat genome in phage λ Charon 4A. The isolated clone, λPCK1, contains the complete gene for phospho*enol*pyruvate carboxykinase and ≈7 kilobases (kb) of flanking sequence at the 5′ end and 1 kb at the 3′ terminus. Restriction endonuclease mapping, R-loop mapping, and partial DNA sequence assay indicate that the gene is ≈6.0 kb in length (coding for a mRNA of 2.8 kb) and contains eight introns. Southern blotting of rat DNA digested with various restriction enzymes shows a pattern predicted from the restriction map of λPCK1. A control region at the 5′ end of the gene contained in a 1.2-kb restriction fragment was isolated and subcloned into pBR322. This segment of the gene contains the usual transcription start sequences and a 24-base sequence virtually identical to the sequence found in the 5′-flanking region of the human proopiomelonocortin gene, which is known to be regulated by glucocorticoids. The 1.2-kb fragment of the phospho*enol*pyruvate carboxykinase gene can be transcribed into a unique RNA fragment of predicted size by an *in vitro* transcription assay.**

Phospho*enol*pyruvate carboxykinase (GTP) [GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32; *P-enol*pyruvate carboxykinase] is a key regulatory enzyme in hepatic gluconeogenesis, and its synthesis rate is controlled by a number of hormones including glucagon (acting through cAMP) (1, 2), cAMP (1), insulin (2, 3), epinephrine (4), glucocorticoids (5), and thyroid hormone (6). Of particular importance are: cAMP, which can induce the synthesis rate of *P-enol*pyruvate carboxykinase from rat liver cytosol 8-fold in 90 min (7); insulin, which causes an equally rapid deinduction of enzyme synthesis when administered to diabetic animals (2); and glucocorticoids, which also stimulate the synthesis of the enzyme (5). These rapid changes in the synthesis of hepatic *P-enol*pyruvate carboxykinase are accompanied by equally rapid changes in the sequence abundance of the mRNA coding for the enzyme (8, 9). Some of us have shown (9), using isolated nuclei from rat liver, that cAMP can cause a 7-fold stimulation of the transcription rate of the gene for *P-enol*pyruvate carboxykinase within 20 min. This increase in the transcription rate of the gene was accompanied by the appearance of eight separate RNA precursors for the enzyme in the nucleus, the sequence abundance of which paralleled the changes in the transcription of the *P-enol*pyruvate carboxykinase gene.

In order to analyze the mechanisms responsible for these alterations in gene expression, we isolated the gene for rat cytosolic *P-enol*pyruvate carboxykinase and determined key features in its structural organization. Examination of sequence homologies with other hormonally regulated genes uncovered a putative control region contained in flanking sequences at the 5′ end of the gene.

## MATERIALS AND METHODS

**Materials.** The following nucleotides and reagents were purchased from New England Nuclear or Amersham: [$\alpha$-$^{32}$P]dCTP (400–600 Ci/mmol; 1 Ci = $3.7 \times 10^{10}$ Bq), [$\alpha$-$^{32}$P]UTP (410 Ci/mmol), Gene Screen, and DNA polymerase I. Nitrocellulose (BA-85) was from Schleicher & Schuell; nucleotides and dextran sulfate were from Boehringer Mannheim and Pharmacia, respectively. The restriction endonucleases used in this study were from New England Bio-Labs, New England Nuclear, Bethesda Research Laboratories, and Amersham and were used according to their instructions. HeLa cells were kindly supplied by D. Goldthwait (Case Western Reserve University), and the pSmaF fragment of adenovirus 2 (Ad2) virus DNA was a gift of A. Weil (University of Iowa). M. Weiss (Centre Nationale de la Recherche Scientifique, Gif-sur-Yvette, France) generously provided us with the rat hepatoma cell lines Fao and H5. Buffalo rat liver cells were obtained from the American Type Culture Collection.

**Rat Genomic Library.** The rat genomic library was a gift from J. T. Bonner (California Institute of Technology). The library was constructed by Sargent *et al.* (10) from DNA extracted from the livers of adult male Sprague–Dawley rats. This DNA was partially digested with *Hae* III and *Alu* I, *Eco*RI linkers were added, and then the fragments were ligated into the phage λ vector, Charon 4A (11).

**cDNA Clones.** A cDNA clone (pPCK10) of rat cytosolic *P-enol*pyruvate carboxykinase of ≈2.6 kilobases (kb) (mature mRNA is 2.8 kb) was isolated from a rat kidney cDNA library by using as a probe a cDNA clone (pPCK2) of ≈600 base pairs (bp) isolated previously in this laboratory (8). Comparisons of several restriction sites and partial sequence data (not shown) established that pPCK2 includes sequences contained within pPCK10 and is located at the 3′ end of the *P-enol*pyruvate carboxykinase gene (see Fig. 2).

**Screening of Phage λ Genomic Library.** Approximately 200,000 phage (10,000 per plate) were tested for *P-enol*pyruvate carboxykinase genomic sequences with pPCK2 by the plaque hybridization procedure of Benton and Davis (12). The most intensely hybridizing plaques were selected for further puri-

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Abbreviations: *P-enol*pyruvate, phospho*enol*pyruvate; kb, kilobase; bp, base pair; Ad2, adenovirus 2; MMTV, mouse mammary tumor virus.

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fication and analysis. DNA was extracted from these phage by the plate lysate method (13) and was used for restriction mapping.

**Subcloning of λPCK1 Fragments into pBR322.** *Eco*RI and *Bam*HI fragments of the *P-enol*pyruvate carboxykinase genomic clone, λPCK1, were subcloned into the corresponding restriction sites of pBR322 by ligating the fragments to the complementary ends of phosphatase-treated pBR322 under standard conditions (14). Clones containing fragments of λPCK1 were selected by Southern blotting. These included a 7-kb *Bam*HI fragment, two *Eco*RI fragments of 5.4- and 2.0-kb length, and a *Hin*dIII/*Bam*HI fragment of 1.2 kb located at the 5′ end of the 7-kb *Bam*HI fragment. The 7-kb *Bam*HI fragment (pPCK-B7.0) was subsequently shown, by R-loop mapping, to contain the entire gene for *P-enol*pyruvate carboxykinase, whereas the 1.2-kb *Bam*HI/*Hin*dIII fragment (pPCK-BH1.2) includes the transcription control regions of the 5′ flanking sequence of the gene.

**Restriction Endonuclease Analysis.** Several of the genomic subclones indicated above and λPCK1 were extensively studied by restriction endonuclease mapping and Southern blotting. DNA from these clones (≈2 μg) was digested with various enzymes and analyzed by electrophoresis on a 0.5–2.0% agarose gel in 40 mM Tris/2 mM EDTA/20 mM sodium acetate, pH 8.1. Electrophoresis was at 2.5 V/cm for 16 hr.

**Southern Blotting.** DNA from rat livers, from hepatoma cell lines Fao and H5, and from buffalo rat liver cells was isolated by standard procedures (15), followed by a final purification with equilibrium CsCl gradient ultracentrifugation. The DNA obtained by this technique was digested with a 3-fold excess of *Bam*HI, *Sph* I, or *Sst* I and was analyzed by electrophoresis on a 0.8% agarose gel as described above. The gels were denatured, neutralized, and transferred to Gene Screen membrane as described by the manufacturer. The digested DNA on the membrane then was hybridized to nick-translated pPCK10 at a final concentration of $1 \times 10^6$ cpm per ml of hybridization buffer (16).

**Electron Microscopy.** The *P-enol*pyruvate carboxykinase genomic clone λPCK1 was used for R-loop analysis by hybridization with poly(A)$^+$ mRNA isolated from the livers of diabetic rats. To form RNA·DNA duplexes, phage DNA (50 μg/ml) was denatured in 0.3 M NaOH for 15 min at 37°C and hybridized with mRNA (100 μg/ml) in 80% formamide/0.15 M NaCl/0.1 M Tricine, pH 8.0/10 mM EDTA, followed by incubation at 52°C for 2–3 hr as described by Kiousses *et al.* (17). The hybrids were spread for electron microscopy by a modification of the method of Kleinschmidt (18). Grids were rotary-shadowed with platinum and palladium (80/20) and were examined with a JEM 100C electron microscope. The length of the single- and double-stranded DNA was determined by using phage φX174 viral and pBR322 DNAs as standard length markers.

**DNA Sequence Determination.** Fragments labeled at the 5′ end were prepared and their sequences were determined by the procedure of Maxam and Gilbert (19).

***In Vitro* RNA Synthesis.** HeLa cell extracts were prepared by the method of Manley *et al.* (20) with logarithmic-phase cells at 5–8 × $10^6$ cells per ml. Each transcription reaction contained, in 25 μl, 10 mM Hepes (pH 7.9), 60 mM KCl, 7.5 mM $MgCl_2$, 2 mM dithiothreitol, 4 mM phosphocreatine, 20 μM [$\alpha$-$^{32}$P]UTP (5 μCi per reaction) and 100 μM each of CTP, GTP, and ATP. Transcription was initiated by the addition of 0.10–0.15 μg of HeLa cell extract protein, and the entire reaction mixture was incubated at 30°C for 60 min. The RNA was extracted with chloroform/phenol, precipitated in ethanol, and then subjected to electrophoresis on 2% agarose gel containing 5 mM methylmercuric hydroxide at 135 mA for 4 hr. Autoradiograms were obtained by exposure of the dried gel to X-Omat AR film (Kodak) at −70°C with an intensifying screen.

## RESULTS

**Isolation of a λ Recombinant Phage Containing *P-enol*pyruvate Carboxykinase Sequence.** Using as a probe pPCK2, a 600-bp cDNA clone previously shown (8) to correspond to the 3′ end of the *P-enol*pyruvate carboxykinase gene, we screened ≈200,000 phage and selected several plaques containing sequences that hybridized with our cDNA clone. DNA from these phage were digested with *Eco*RI, and four internal *Eco*RI fragments (2.0, 2.3, 4.7, and 5.4 kb) were identified (the 2.0- and 2.4-kb fragments do not have naturally occurring *Eco*RI sites) (Fig. 1). The total length of the inserted rat genomic DNA was 14.4 kb. Southern blotting of an *Eco*RI digest of these phage with either pPCK2 or pPCK10 as probes confirmed that the 5.4- and 2.0-kb fragments are part of the *P-enol*pyruvate carboxykinase gene. This clone was designated λPCK1 and contains 7.4 kb of 5′ flanking DNA, 6 kb of gene, and 1 kb of 3′ flanking DNA. The 5.4-kb *Eco*RI fragment hybridized with pPCK10 and not with pPCK2, whereas the 2.0-kb fragment hybridized with both probes (see Fig. 2). This orients the two fragments so that the 5.4-kb fragment is 5′ to the 2.0-kb piece. These two *Eco*RI fragments also contained several restriction sites in positions similar to those in pPCK10, further confirming its identity and position. As shown in Fig. 2, this cluster of restriction sites further indicates that at least 1.1 kb of the 3′ end of the *P-enol*pyruvate carboxykinase gene is colinear with its cDNA.

**Restriction Endonuclease Mapping.** Restriction endonuclease analysis of λPCK1 with 12 restriction enzymes is shown in Fig. 1. In order to characterize the gene for *P-enol*pyruvate carboxykinase in further detail, *Eco*RI and *Bam*HI restriction fragments were subcloned into their corresponding sites in pBR322, and each subclone was analyzed individually. Subsequently, the entire *P-enol*pyruvate carboxykinase gene and its 5′ flanking region were shown to be contained within the 7.0-kb *Bam*HI fragment adjacent to the left arm of the phage λ vector by R-loop mapping.

**R-Loop Mapping.** In order to further analyze the organization of the *P-enol*pyruvate carboxykinase gene, the positions

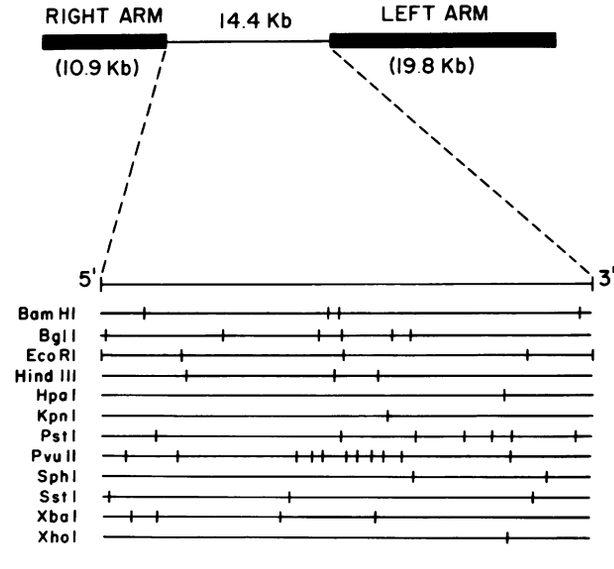


FIG. 1. Restriction endonuclease map of the rat genomic fragment containing the *P-enol*pyruvate carboxykinase gene. Restriction enzyme sites were determined by a combination of single, partial, and double digests as outlined.

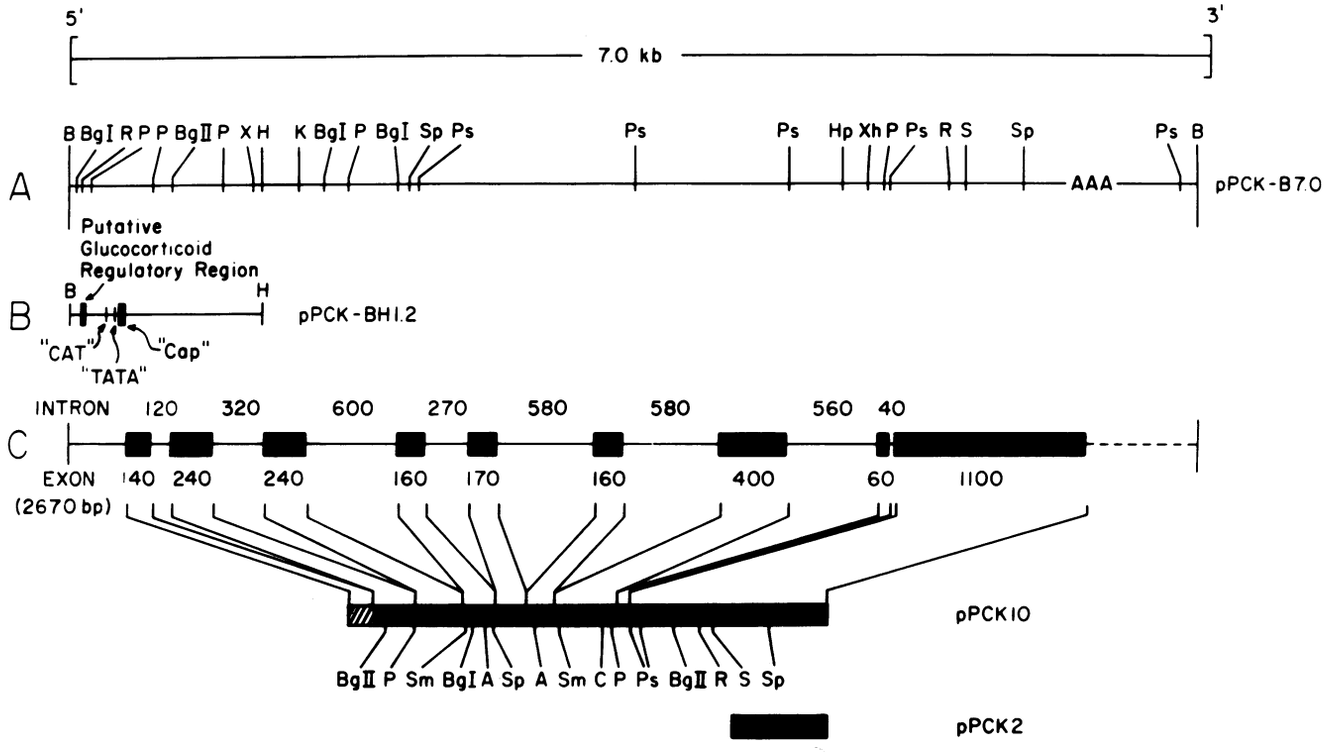


FIG. 2. Diagram of the structure of the gene for cytosolic *P-enol*pyruvate carboxykinase from the rat. (*A*) A detailed restriction map of the subclone pPCK-B7.0 which contains the *P-enol*pyruvate carboxykinase gene and 3′ and 5′ flanking sequences. (*B*) Positions of putative regulatory sequences in genomic subclone pPCK-BH1.2. See Table 1 for nucleotide sequence for each of these control areas. (*C*) The top line shows the structure of the *P-enol*pyruvate carboxykinase gene showing exons (■) and introns (connecting lines). Numbers indicate the average number of nucleotides in each exon or intron averaged from 17 separate analyses. In the middle and bottom lines, the solid bars represent the 2.6-kb cDNA (pPCK10) and the 0.6-kb cDNA (pPCK2), which have been aligned relative to the gene, while the hatched segment is an area of cDNA for which no restriction sites have been identified in the genomic DNA. Specific restriction sites in pPCK10 are indicated. R, *Eco*RI; B, *Bam*HI; BgI, *Bgl* I; BgII, *Bgl* II; P, *Pvu* II; Ps, *Pst* I; X, *Xba* I; H, *Hin*dIII; K, *Kpn* I; C, *Cla* I; S, *Sst* I; Sp, *Sph* I; Hp, *Hpa* I; Sm, *Sma* I; Xh, *Xho* I; A, *Ava* I.

and sizes of introns and exons in the gene were examined by electron microscopy of DNA·RNA hybrids. Intact λPCK1 was hybridized with poly(A)$^+$ mRNA isolated from the livers of diabetic rats in which *P-enol*pyruvate carboxykinase mRNA is ≈2% of the total hepatic mRNA. R-loop analysis showed that the gene for *P-enol*pyruvate carboxykinase contains eight introns varying in size from 600 to 40 bp (Fig. 3). We also carried out R-loop analysis with the pPCK-B7.0, a subclone of the original λPCK1, which had the same eight-intron pattern (data not shown). The 3′ end of the *P-enol*pyruvate carboxykinase gene is visible in the R-loop pattern shown in Fig. 3, with a possible poly(A) tail indicated by the arrow. R-loop mapping with subcloned restriction fragments of λPCK1 containing the 3′ end of the gene confirmed the orientation of the gene (data not shown). The 5′ end of the gene is located close to the *Bgl* II site found ≈625 bp from the 5′ end of pPCK-B7.0 (see Fig. 2*A*). From our electron microscopy studies, we also conclude that the gene for the enzyme is ≈6.0 kb in length.

To eliminate any possibility that the 5′ end of the *P-enol*pyruvate carboxykinase gene extended beyond the *Bam*HI site in the 7-kb subcloned fragment, two *Eco*RI fragments, 2.3 and 4.7 kb in length, that mapped 5′ to the 7-kb *Bam*HI fragment were nick-translated and hybridized to *P-enol*pyruvate carboxykinase mRNA by RNA blotting. Only the 7-kb *Bam*HI fragment hybridized with enzyme mRNA (data not shown), suggesting that the gene for *P-enol*pyruvate carboxykinase is contained within pPCK-B7.0 and does not extend into the adjacent 5′ sequence of the *Eco*RI fragment.

**Southern Blotting.** An analysis of the rat *P-enol*pyruvate carboxykinase gene by Southern blotting is shown in Fig. 4. DNAs isolated from rat liver, from cells derived from a Buffalo rat hepatoma line, and from two different derivatives of Reuber H-35 hepatoma cells (Fao and H5) were digested with *Bam*HI for Southern blotting. All were found to contain a single 7-kb fragment that hybridized with [$^{32}$P]pPCK10 (Fig. 4*A*), as predicted from Fig. 1. This indicates that the gene for *P-enol*pyruvate carboxykinase is either unique or that it is part of a multigene family in which each member is contained fortuitously in 7-kb *Bam*HI fragments. In order to exclude the latter possibility, DNA from rat liver was digested with *Sst* I, (Fig. 4*B*, lane 5), with *Sph* I (Fig. 4*B*, lane 6) or with *Eco*RI, *Pst* I, or *Pvu* II (data not shown), transferred to nitrocellulose, and probed with [$^{32}$P]pPCK10. In each case, the only fragments hybridizing to our cDNA probe were predicted from the restriction map shown in Fig. 1 with the one exception of *Sph* I sites. This variation could be due to the restriction site polymorphism with the gene. The results of these experiments indicate that the gene for *P-enol*pyruvate carboxykinase is unique and probably present as a single copy.

**Analysis of the 5′ Control Region of the *P-enol*pyruvate Carboxykinase Gene.** Because the gene for *P-enol*pyruvate carboxykinase appeared to be completely contained within the 7-kb *Bam*HI fragment, the most 5′ segment of this fragment, contained in a 1.2-kb *Bam*HI/*Hin*dIII fragment, was subcloned into the corresponding *Bam*HI/*Hin*dIII sites in pBR322 (pPCK-BH1.2) (see Fig. 2 *A* and *B*) for further study. R-loop mapping had predicted that the transcriptional initiation site was contained within this fragment, and its position was confirmed and mapped more accurately with an *in vitro* transcription assay. First, in order to test the specificity of our assay, we demonstrated the synthesis of a 600-nucleotide transcript from the *Sma* I digest of the late promoter of adenovirus 2 (Ad2) DNA (Fig. 5, lane 1) and showed that its transcription was sensitive

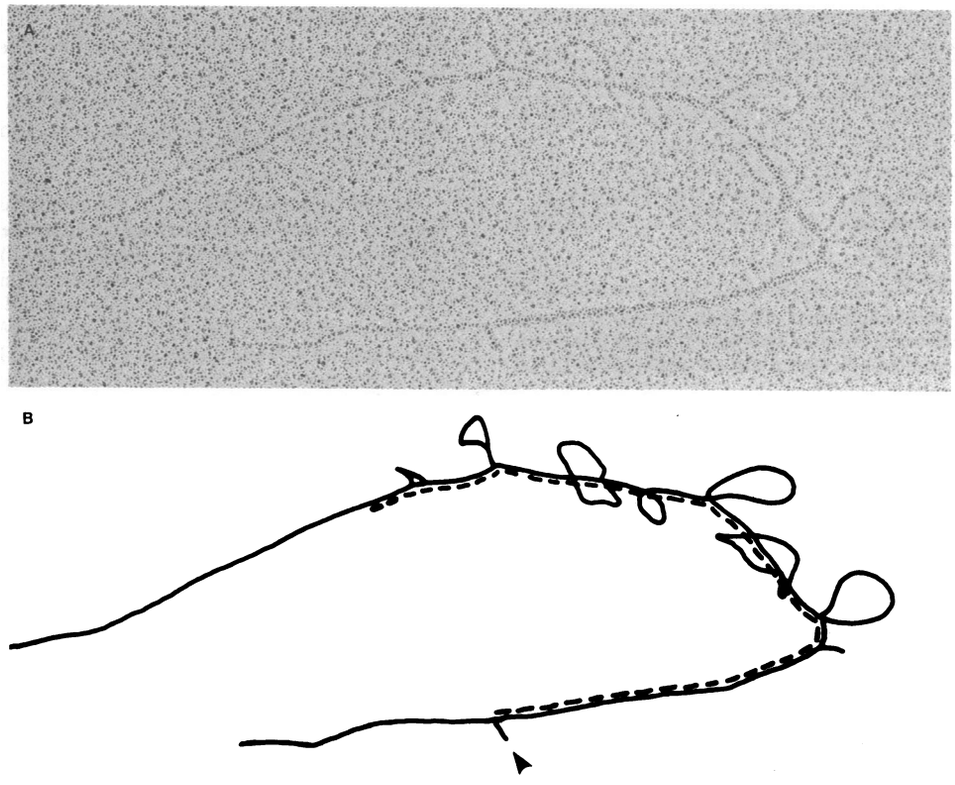


FIG. 3. Electron microscopic analysis of recombinant phage λPCK1. (*A*) Heteroduplex formed between λPCK1 DNA and *P-enol*pyruvate carboxykinase mRNA. (*B*) Diagrammatic representation. ——, λPCK1; ----, *P-enol*pyruvate carboxykinase mRNA. Arrowhead identifies the 3′ end of the gene.

to α-amanitin (Fig. 5, lane 2). When pPCK-BH1.2 was digested with *Hin*dIII and then transcribed, an RNA of ≈730-nucleotide length was formed (Fig. 5, lanes 3 and 5). When the plasmid was digested with *Eco*RI, which cuts pBR322 at a point ≈30 bp 3′ to the *Hin*dIII site, a slightly larger transcript of 760 nucleotides was synthesized (Fig. 5, lane 7). These transcripts were shown to be sensitive to α-amanitin (1 μg/ml), indicating that the added pPCK-BH1.2 was transcribed by RNA polymerase II. The 730- and 760-nucleotide transcripts position the 5′ end of the *P-enol*pyruvate carboxykinase mRNA at a point ≈450 bp 3′ from the *Bam*HI site and very close to the site predicted by R-loop mapping. These findings indicate that the promoter for the *P-enol*pyruvate carboxykinase gene is contained within the 1.2-kb 5′ segment of pPCK-B7.0. Nucleotide sequence of ≈800 bases on one strand of pPCK-BH1.2 indicates the presence of several areas of sequence homology with regulatory regions reported to be present in the promoters of other

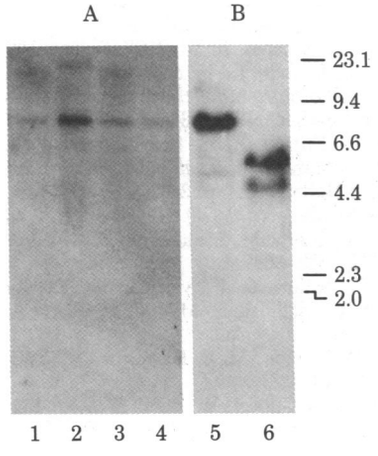


FIG. 4. Southern blots of rat liver DNA and rat hepatoma DNA that had been digested with restriction enzymes and hybridized to pPCK10. (*A*) Rat liver DNA (lane 1), rat Fao hepatoma DNA (lane 2), rat H5 hepatoma DNA (lane 3), and Buffalo rat liver DNA (lane 4) were digested (≈3.5 μg each) with *Bam*HI. (*B*) Rat liver DNA (7.0 μg) was digested with *Sst* I (lane 5) or *Sph* I (lane 6). After digestion with these restriction enzymes, the fragments of DNA were separated by electrophoresis on 0.8% agarose gels, transferred to nitrocellulose filters, and hybridized with nick-translated pPCK10 as described. Sizes are shown in kb, indicating the positions of the genomic fragments relative to phage λ DNA digested with *Hin*dIII.

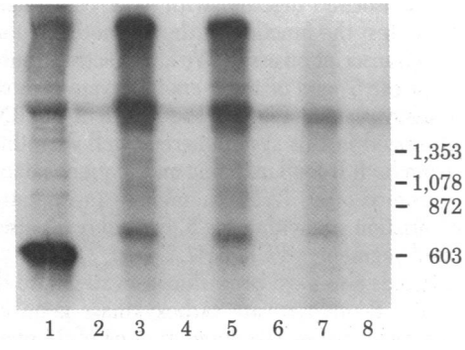


FIG. 5. Transcription of pPCK-BH 1.2 in HeLa cell extracts. Lanes: 1 and 2, transcription products of 0.8 μg of *Sma* I-digested pSmaF, Ad2 DNA; 3–6, 0.8 μg of *Hin*dIII-digested pPCK-BH1.2 DNA; 7 and 8, *Eco*RI-digested pPCK-BH1.2 DNA. In lanes 2, 4, 6, and 8, the transcription was carried out in the presence of α-amanitin (1 μg/ml). *In vitro* transcription was carried out by the procedure of Manley *et al.*, and the RNA was separated by electrophoresis on 2% agarose gels containing methylmercuric hydroxide. Molecular weight markers are phage φX174 viral DNA digested with *Hae*III.

Table 1. Putative regulatory sequences in the control region of the *P-enol*pyruvate carboxykinase gene

| Base number | Specific area | Nucleotide sequence |
|---|---|---|
| +1 to +12 | Cap site | 5′ A-C-T-G-T-G-C-T-A-G-G-T 3′ |
| −39 to −32 | "TATA box" | T-A-A-T-A-A-T-A |
| −84 to −77 | "CAT box" | G-A-C-T-C-A-A-C-T |
| −230 to −207 | Putative glucocorticoid regulatory region | A-A-A-T-G-T-G-C-A-G-C-C<br>A-G-C-A-G-C-A-T-A-T-G-A |

The specific sequences are numbered relative to the most 5′ base in the cap site, which is arbitrarily assigned position +1. This data is drawn from an 800-bp sequence of a single strand at the 5′ end of pPCK-BH1.2 (see Fig. 2*B*).

RNA polymerase II-transcribed genes (see Table 1). We also noted a sequence that is virtually identical to the putative glucocorticoid regulatory domain found in the 5′ control region of the human proopiomelonocortin (21).

## DISCUSSION

We demonstrate in this report that the gene of *P-enol*pyruvate carboxykinase is ≈6 kb in length and contains eight introns and nine exons. The largest exon is a 1.1-kb segment at the 3′ end of the gene that is colinear with a cDNA clone containing the same region. We mapped by electron microscopy the positions of each of the introns and identified the transcription start site both by electron microscopy and by an *in vitro* transcription assay, using a subclone of the 5′ end of the gene with its flanking sequences. Southern blot analysis of genomic DNA from rat liver and liver-derived cell lines indicates that the *P-enol*pyruvate carboxykinase gene is unique and entirely located on a 7-kb *Bam*HI fragment.

Some of us have shown (8, 9) that nuclei isolated from the livers of rats injected with dibutyryl cAMP contain eight distinct species of RNA that hybridize with our cDNA probe. The largest of these putative *P-enol*pyruvate carboxykinase mRNA precursors was estimated to be 6.4 kb in length, about the size of the *P-enol*pyruvate carboxykinase gene (see Fig. 2), suggesting that this large nuclear precursor is the primary transcript of the gene. Of the eight nuclear RNA species, three are smaller than the 2.8-kb length of the mature, cytosolic *P-enol*-pyruvate carboxykinase mRNA (8, 9).

Because the complete gene for cytosolic *P-enol*pyruvate carboxykinase was isolated together with its 5′ control region, it was possible to test the function of the promoter region by using an *in vitro* transcription assay. We found active transcription of a portion of the 5′ end of the *P-enol*pyruvate carboxykinase gene in our assay system (Fig. 5). The length of the RNA transcripts synthesized in this system agrees well with the length predicted from both R-loop mapping and sequence data, which show the presence of a "TATA" homology and putative transcriptional initiation site within the 5′ control region isolated in our subcloned segment, pPCK-BH1.2.

Glucocorticoids also have been shown to stimulate the transcription of the *P-enol*pyruvate carboxykinase gene in nuclei isolated from rat liver (9). We noted an area of 24 nucleotides at position −207 to −230 which contains ≈80% homology to a putative glucocorticoid regulatory region at position −480 of the human proopiomelonocortin gene (21). This same sequence has been shown by Cachet *et al.* (21) to share homology with sequences from the rat growth hormone gene (22, 23) and the mouse mammary tumor virus long terminal repeat (24). Also, the sequence A-A-A-T-G-T, which is located at position −230 in the 5′ flanking region of the *P-enol*pyruvate carboxykinase gene, is entirely conserved at position −80 in the rat, bovine, and human growth hormone genes (22). The functional significance of these homologies is not as yet known. However, in a recent review, Davidson *et al.* (25) discuss a possible role for these sequences in controlling the expression of their respective genes.

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