J. Biochem. 121, 711-716 (1997)

Nucleotide Sequence of the Promoter Region of Chicken Cytosolic Phosphoenolpyruvate Carboxykinase Gene

Atsushi Sato,*,[1] Hiroaki Takahashi,* Kazuhiko Konishi,* Toshiyuki Suzuki,† and Hideo Kochi†

Information Science Major, Faculty of Liberal Arts, Tohoku-Gakuin University, Tenjinsawa, Izumi-ku, Sendai 981-31; and †Department of Biochemistry 1, Fukushima Medical School, Hikarigaoka, Fukushima 960

Received for publication, November 20, 1996

The nucleotide sequence of the promoter region and its flanking regions which span −1855 to +2083 in the chicken cytosolic phosphoenolpyruvate carboxykinase gene was determined. The transcription initiation site was located at 119 nucleotides downstream of the previously reported chicken kidney transcription initiation site of this gene. The nucleotide sequences of exons 1, 2, and 3 were highly homologous to the corresponding exons of the rat gene. Homology of the sequence −1 to −500 to that of rat gene was 52% and most of the hormone-responsive sequences in rat gene, such as the glucocorticoid-responsive region, were not conserved in the chicken gene, in accord with the species-specific responsiveness to starvation. In contrast, in the region of −1 to −300, some sequence motifs conserved both in the chicken and rat genes were found at essentially the same positions in the promoters. Such sequence motifs included a cAMP-responsive element (CRE), a nuclear factor-1 (NF-1/CTF)-binding site, and a hepatocyte nuclear factor-1 (HNF-1)-binding site. Transient expression of the reporter luciferase gene ligated to the 3′ end of this chicken sequence (−1855 to +7) was observed in a primary culture of chick hepatocytes when dibutyryl cyclic AMP was added to the culture medium.

Key words: chicken liver, cytosolic phosphoenolpyruvate carboxykinase, nucleotide sequence analysis, promoter, transient expression.

Phosphoenolpyruvate carboxykinase (GTP) [EC 4.1.1.32] (PEPCK) is a gluconeogenic enzyme which generates phosphoenolpyruvate (PEP) from oxaloacetate (*1, 2*). The operation of this enzyme is quite different depending on animal species (*3, 4*), stage of development in a given species (*5, 6*), organ (*7*), and subcellular localization (*8*). In chicken, cytosolic PEPCK is dominant in the kidney, but not evident in the liver even during prolonged starvation (*9*). The requirement for hepatic gluconeogenesis in chicken is therefore met by the mitochondrial isozyme, which exists in great quantity irrespective of nutritional condition (*10*). In spite of the lack of response of chicken liver cytosolic PEPCK to starvation, administration of dibutyryl cAMP (Bt_2cAMP), isoproterenol, glucagon, or epinephrine to chicken induces the enzyme (*10-12*). A permissive role of glucocorticoids in this induction has also been reported (*11*). The roles of these agents are essentially the same as their roles in rat liver (*11*). Since it is known that the regulation of transcription is the major target of these agents, comparison of the nucleotide sequence of the promoter region of the chicken cytosolic PEPCK gene with that of the rat gene should provide clues to understand the mechanisms of species- and organ-specific expression of this gene.

In this study, a genomic library of chicken hepatic DNA was screened with a probe prepared from cDNA of chicken cytosolic PEPCK and the nucleotide sequence, including the promoter region, in the gene was determined. Responsiveness of the promoter region to cyclic AMP was assessed with a constructed reporter plasmid transfected into chick hepatocytes by electroporation.

MATERIALS AND METHODS

Screening and Nucleotide Sequence Analysis of PEPCK Gene—A genomic DNA library of adult chicken liver (CL1004D; CLONTEC, USA) was screened with a digoxigenin-labeled 1 kb *Eco*RI fragment of LGTPEP9, a cDNA clone of cytosolic PEPCK prepared with chicken hepatic mRNA (Fig. 1). Insert DNA of positive clones was digested with *Hin*dIII, subcloned in pBluescript SK$^+$ (Toyobo) with XL1-Blue as a host, and then digested with *Sal*I and *Kpn*I to prepare deletion mutants using a reaction kit (Kilo-Sequence Deletion Kit; Takara). The resulting cloned plasmids were obtained by the alkaline lysis method (*13*) and further purified by precipitation with polyethyleneglycol (*14*). Sample plasmids were sequenced by a cycle sequencing procedure using a DNA sequencer (373A; Applied Biosystems, USA) with dyed universal primers supplied by the manufacturer. Recording and analysis of the sequence were performed using PC/GENE (IntelliGenetics, USA).

Analysis of Transcription Initiation Site—The transcrip-

[1] To whom correspondence should be addressed. Phone: +81-22-375-1111 (Ext. 275), Fax: +81-22-375-4040, e-mail: asato@izcc.tohoku-gakuin.ac.jp

Abbreviations: PEPCK, phosphoenolpyruvate carboxykinase; Bt_2cAMP, dibutyryl cyclic AMP; RACE, rapid amplification of cDNA ends.

tion initiation site was analyzed by sequencing the 5′-RACE product which was obtained with anchor primer and anchor DNA (supplied with 5′-AmpliFINDER RACE kit; CLONTECH, USA), in addition to poly(A)$^+$ RNA and ordinary primers (see below). Poly(A)$^+$ RNA as a template was prepared from chicken liver as described (*11*). Primers for reverse transcription (5′AGAGCTTTGAAGTACTAATCACAAT3′) and for PCR (5′CAGGATGTTGAGTCGGATGGGTG3′) were complementary to sequences +284/+260 and +78/+61 in Fig. 2, respectively. The 5′ pentanucleotide (5′CAGGA3′) in the PCR primer and Aminolink-2 (Applied Biosystems) at its 5′ end were added to prepare fluorescence-dye labeled sequence primer as recommended by Applied Biosystems.

Transient Expression of Reporter Plasmid in Chicken Hepatocytes—One of the pBluescript subclone deletion mutants prepared for the sequence study was used as a source of the promoter sequence. This plasmid, namely pPEP910, carried nucleotides +155 to −1855 of the PEPCK gene and a unique *Eco*47III site at +7. PicaGene basic vector (PGV-B, Toyo Ink, Tokyo) was digested with *Sal*I and *Hin*dIII to obtain a 2,697 bp fragment which carried the luciferase gene with the splicing region and a poly A signal on its 3′ end. The protruding end of this fragment was filled in with Klenow fragment and the fragment was inserted at the *Eco*47III site of pPEP910. pPEPLU, the resulting plasmid with correct orientation of the insert, was propagated with XL1-Blue as a host.

The method developed for rat (*15, 16*) was adopted to prepare chick hepatocytes using starved 4-day-old female chick, except that *in situ* infusion of Hanks solution (Ca^{2+} and Mg^{2+}-free) followed by collagenase-containing Hanks solution into the liver was carried out *via* the pancreaticoduodenal vein. The isolated and purified hepatocytes were suspended in culture medium (Williams E) supplemented with 5% (v/v) calf serum, antibiotics (penicillin G, 100 U/ml; streptomycin, 100 μg/ml; and fungizone, 0.25 μg/ml), and hormone (either 2 nM insulin or 1 μM dexamethasone). A 1.0 ml aliquot of the cell suspension (5×10^6 cells) in an electroporation chamber was mixed with reporter plasmid in 10 μl of TE (pH 7.8) at 0°C and electroporation was carried out at 625 V/cm and 330 μFD at 0°C using a Cell-Porator (BRL, USA). After electroporation, the cell suspension was incubated at 0°C for 5 min and then mixed with 7 ml of culture medium in 2 culture dishes (5 cmφ, Nunc, Denmark) and cultured under 5% CO_2 at 37°C. Transient expression was measured after 48 h of incubation following the directions of the manufacturer (PicaGene, Toyo Ink). Luciferase activity was measured using a chemiluminescence analyzer OX-70 (Tohoku Denshi Sangyo, Sendai). Protein concentration of cell lysates was measured with Protein Assay (Bio-Rad, USA).

Other Chemicals and Reagents—Restriction endonucleases were the products of either Toyobo or Takara. All other chemicals and reagents used in this study were of the highest grade available (Wako).

RESULTS

Screening of about 9×10^5 plaques yielded five positive clones. One of the clones, named LEPEP1, was further propagated to prepare a 3.9 kb *Hin*dIII fragment (Fig. 1), which was then subcloned in pBluescript SK$^+$ in the forward and reverse directions. Deletion mutants of this clone were sequenced. Exons were determined by comparison with the cDNA sequence and are boxed in Fig. 2. The only alternative assignment for the exons is that the G at +534 and +1932 may be the 5′ terminal nucleotides of intron 2 and exon 3, respectively. But, such an assignment breaks the GT/AG rule for exon/intron junctions. Validity of the sequences of the exons in Fig. 2 was confirmed by complete agreement with the result of cDNA sequencing (data not shown).

Analysis of the 5′ end of the transcript by sequencing the RACE reaction product showed that the anchor DNA sequence followed the sequence 5′...GGAGCGCTCGTN3′ which is complementary to the sequence +2/+12 in Fig. 2, except for N. A base could not be assigned at N since four bases were equally detected at this position. This phenomenon can be expected when a small amount of cDNA failed to be ligated to anchor DNA and therefore the extension reaction in PCR was terminated at this position. The only possible 5′ terminal sequence of the primary transcript is therefore 5′AACGAGCGCTCC..3′. The presence of more than one transcription initiation site is unlikely since the succession of peaks terminated after a burst of peaks at the position corresponding to the 5′ end of the anchor primer. Thus, the transcription initiation site of the chicken cytosolic PEPCK gene for liver is located at 119 nucleotides downstream of the previously reported site for the kidney

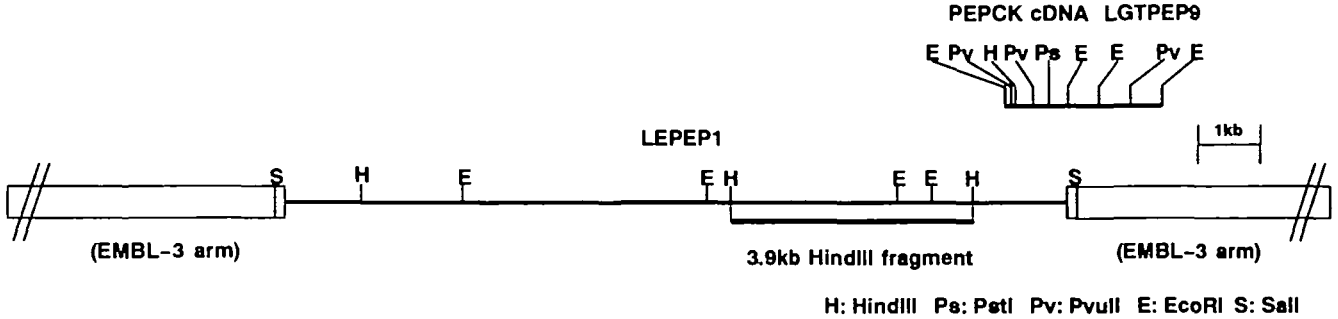

Fig. 1. **Structure of LEPEP1.** The library was screened with 5′ *Eco*RI fragment of LGTPEP9 as a probe to clone LEPEP1, which carries the promoter region of chicken cytosolic PEPCK gene. To sequence the deletion mutants of *Hin*dIII fragment of LEPEP1, at least two overlapping sequence data were obtained for each direction. Any disagreement among the data for different directions was corrected by close examination of the output charts of the sequencer. Then the same procedure was repeated with other data of reversed sequences which covered the same region. Such refinements were made at 71 positions in the sequence of 3,938 bp length.

gene (*17*). Northern blotting also gave a single band for mRNA of chicken cytosolic PEPCK.

A putative TATA box is found at −33, where the sequence 5′TATTTAA3′ starts. The same sequence is also present in rat PEPCK promoter (*18*).

Alignment of the partial sequences −500/−1 of chicken and rat PEPCK genes (*18*) revealed that 286 nucleotides (53%) are identical when 24 (rat) and 30 (chicken) insertions of gaps, which correspond to 45 nucleotides in total length, are allowed, to maximize identity. This alignment showed that, besides the TATA-like sequence (5′TATTTAA3′), sequences homologous to NF-1/CTF and HNF-1 (*19*) binding site consensus sequences, as well as to the cAMP-responsive element (CRE) consensus sequence, are located at essentially the same positions in both chicken and rat PEPCK promoters (Table I). Some other sequences in the chicken gene (5′ACACCTCA3′ at −426/−419, 5′GCAGCCA3′ at −402/−396, and 5′AGCCTCCA3′ at −75/−68) are also found in the rat gene at essentially the same locations (*18*), but no obvious homology to known sequence motifs has been identified so far.

Sequences homologous to the glucocorticoid-responsive element (GRE) consensus sequence [(G/T) (G/T)TAC (A/C)NNNTGT (T/C)CT] were found scattered in the chicken

```
-1855  AAGCTTTTAT TCAGACTTTA ACTGTGCTGC AATATGATCA TTTTCTGGCC ACGTCTTTCC TTTTTTCCCC
-1785  CCTTCAAACT GAAGCACTGC TGTTTTACTC TCAGTTGATT ATTTCTATGT ATAATGACTT TTCTGGGACC
-1715  ACCAGCAAGT ACTGTGATTA TTGCTGTATT CCTCTGGAAA GAAAAGAAGT CTTCTCGGTA TTTCAGAAGA
-1645  ATGTGCCACG TTTTATTTTT ACTGAAGTAC TTCATGACAT ATTTTAACAC GAAAACATTT GGGCAAAATA
-1575  TTTCTGCTCA CAAACACCAC TGGCATTATC ATATTGGCTC TGATTTTACC TCTGCTTACA GCAGAGCCCC
-1505  TTCTGAAGCA ATCTGCAGCA CCTCTTATCC TAACAGATTT TTGTTTTCCT TTTCGGGAAT GTTTGGCCTT
-1435  GCTGTGCAAC AAGGGCAGCC CCTCTTACTT CACTATTTTT TTTCCCCTTT GCATTTACAA CACACAATAA
-1365  AAGGCTTTGC AGGAGAGATA GAGGATGCGG TCCCAGGGGA AGCATTCCCA TGGCCACTGA CCACTGTGCT
-1295  CGTGAGGTTC AGCCCCGCAG TGCCTGCAGC TCAGAGTGCA GCTCGGTGCT GCTCTGGCAG ACCCAGCTCT
-1225  TTGGGGCTTG CAGCTCTGCT GTCTGCGTCG GTGAGAGCCT TGCAGGAAAC ACCTCACTGA CTGCACGAGC
-1155  CTGAGCAGGC GGTGGGTTAA GAATATCCTT TGCATCAATT TTATTGCTTT AAGCAACCCC CAGAAACTTT
-1085  TCTTGGATTT CCAAATTCAC CATTCTGTGA GCTATTATTG ACAATATCTT ACATGATGGT AGAACCCCCA
-1015  CTGATATCAA AAATGCCAGA ATTTAGTATT GCTGGGACTG AATGGAAGAG GAGTGCAATA AATTGGCACA
 -945  GGGGTTATTC TTACACTTAT CTTTGTCTGA AATATATTCA ATCCTCACAC ACCGACTGCA CAGGCAAAAC
 -875  GACACAGAGA TCAGCCTCTG TTTGTGGGCA TGATGGTCTA AAATGGAGTG GCAGAAGGCA AAGAGCTAGC
 -805  AGATGGGCAG AGCCAGGAGC AGTAAGGGGA GACCTAATTT AAGCACAGGT TAATGTTTTA TTGCACCCAC
 -735  AGGATGCCAA GTAAAATAAC AAATAGCTTT TTTGGGGGGT TAGAGGGGTA ACTGCCGAGG CAGTTGGTGA
 -665  CCTGCCTCTT CTCCAAGTTG TAGAGATAGA TAATAATTGC TCAGCTTGCA TGAGCCCATA GTGAGCCATA
 -595  AGCAGAAATT CTCCAGTGCC AAAGGAAGCT CAGCATTTAG ATTTACATTA CGTGCCTTTC TTAGAATCAC
 -525  ACAGGCAAAA ACAAGACCTC CAGGCAGGAG CATACCAAGG TTCTTCAGGC CCATCAGTGC TCAGCACTGA
 -455  ACTTTATCCG CAGACCAGGC AGAGCAAACA CACCTCAGCC GAGCCCCGGC TTCGCAGCCA ACCTGCGAGC
 -385  ATCAGCTTGC TGTGCCAGCT GCTGGGTTGC GCCAGCTCCC TGTTCCAGGT TGTACCCAAC CTTTTCCACA
 -315  CACACAGACG TTGTGACGCT TTTGGTTTCA AAGCTAAACT GCATAAAAGT GTGGTTAAAC TTCATCGAAG
 -245  TTTTGTAACT GCTTCAAACA GCAAACCCGG GGACTAAGAA CCACCGCTTT CAGCATTCAT TAATCAGAGC
                                                  AFP-BoxI   HNF-1
 -175  TGTTAAATGA TTACCTCGCC AGGGCTGTTG ACAACTCCGC ACGATGACAG TGTGTCAGTG GCAGCGTCCC
         HNF-1                                                          NF-1/CTF
 -105  AAAACACAAC CAGTGGTGAT GTAAAGGACA AGCCTCCACC ACCTCACCCG GTGGGATGGA CACCACAGAC
                                          +1
  -35  AATATTTAAG GAAGGAGGAC GCCACCTCAG AAACCAACGA GCGCTCCAAA GCAAGTGAAA CACCATCAGC
         TATA-box
  +36  TGAAAGGGAG CCAAATCCCC ACTGACACCC ATCCGACTCA ACAGTAAGTA CAATGCTTTC TGTGTATTTT
 +106  TTTCCAGCTT GAGATTAGCA GTGATTAAAA TTCTTTCTGT TGACTTTAGG CAGAGGTATA AAATTAAGCC
 +176  TACTACAAAT TCTGTTCTTG CATTATGCTT CATTAATGGC AGAATACCAT AGCATGCTAA TAAACACAAT
 +246  ATATTTGATT TTAGATTGTG ATTAGTACTT CAAAGCTCTC AACAAGACCC ACTGCAGCTG CAGTAATGCC
 +316  CCCAGAGCTG AAAACTGAGG TCAACATCAT GTCTAAGGTT ATCCAGGGGG ACTTGGAGAG CCTGCCTCCA
 +386  CAAGTGAGGG AGTTCATTGA AAGCAATGCC AAGCTGTGCC AGCCTGAGAG CATTCATATC TGCGATGGCT
 +456  CAGAAGAAGA AAACAAAAAA ATTCTGGACA TCATGGTGGA GCAAGGCATG ATCAAGAAGC TGAGCAAGTA
 +526  TGACAACTGG TGAGTAGCAG AAAAGGGGGT CAAGGAGTGT CTCAGAATAG CAATGTGCAT TCTCTGTCCC
 +596  GTTTACATTT AAAGCTGTTC ATAACCTTCA ATATTGCTTT CCATGAACAT TTTGGCAGTG CAGTTTACAC
 +666  AATTGAAATA ATGGAGAACA GCAAACTTTT CCTGTAAAAA TTAACATAAA GCGCAGGAAT ATCAGCAGTA
 +736  CATTGTCTCT ATTTATTAGT TACACAGATC ATGGAACTGA AATAATGTCA CGAGACTTAT GTAACTAATC
 +806  ATTAGGAATG GTTGAATATT ATTTAGCAAA TACATCCTTT GAAGAATTCT ACTATTTTAG TCAATTAGTT
 +876  TATTCGTCAA ATACTTTTTG TGGCATTCAA ATAGATCCGT GGGTGTTCAA ATGTTATTCA GTAGAGGCAT
 +946  TATTCACATC ATGCCGATGA GCTTTGACTG TAATGTATTC AGCAAATACT TTTTTCAAAT ATTTCGTTGT
+1016  CTCTCCCAAC GAACCCAACA TGAAAGGAGA ATATTAACAA CAAGTGCATG CTAAGGAATT AAGTGCCAAC
+1086  CAAGACATCT TCATGGAAGA AATCTGCAAA GACTTTTGAA TAACTAAGTG GAGGAATTTG GCAATCTGTT
+1156  TGCAGACTAT TTGGGAGAGA AAAGGGCAAG ATTCACCAGA TAAATTATTC ATTGTTAATT ATATGCTTAG
+1226  CTGTAGTGAT GATGGTTATG ACAGCCCAGC TAGAACATTT TTATACTCAT GGAAAATTTC TATGCACAGA
+1296  GGTGAAAATG TAAAACAATC CTTGCTATTG TAAATAAATG GCAAAAAATT TAGAGGGGAA AAAAGAATTC
+1366  TGTTTAGTAA ACTCCTTCAC TGTTTAAGGC ATGCTTATAC TCAGTATTTC TTTTGCGATG CCTCTGTCTT
+1436  AGAAGGACAA TGCCAATCTA CAAGGCTGGA TTCTCATCCT GCAGGCATTT TGACCAACAC GTAACGTACT
+1506  TGCTTGGCAT CCACTTTGCT TGAGGCACCA CTCTGTGGAT CCCACGCAAA ACAACTGTAT ACTGATGAGA
+1576  AGGGGAATTA AACAAGATTC TTAACATTTC CCTCAAAGTC AGCAGCAGAA AATAAAGCCG AGACAAGAGG
+1646  CAAAGGAACA TTAACTTGTA ACCAGAATTT CCCAAGCCGT ATAATTTGTT ATTCATACTG CAAAGTTCTT
+1716  GAAGCAATTT GTATGAGAGA ATATATCTGA CTAATTTTCG AAATTTGTCT CAGTTATATT GGCTCTGTCA
+1786  CTTCAGTTTT AGCCTTATAA AACGTTGTAC CCATTTTGCT AAAGAGTTCC TAGCAGAAGG GTGAACAAAG
+1856  TGGGCGAAAA GTGTCACATT GATCTTTTTT TTCTTTAATA ATTAATATTG ATTTTCCCGA TTTACACTGA
+1926  AATGCAGCTG GTTGGCTCTC ACTAACCCAA GAGATGTAGC AAGAATCGAG AGCAAAACCG TCATTATTAC
+1996  TCAAGAACAG AGAGATACCA TTCCAATCCC TAAAACTGGA AGTAGCCAGC TGGGTCGCTG GATGTCAGAA
+2066  GAGGATTTTG AGAAAGCT
```

Fig. 2. **Partial nucleotide sequence of chicken cytosolic PEPCK gene.** The nucleotide sequence of the promoter and its flanking regions of cytosolic PEPCK gene of chicken is given. Motifs for DNA binding factors (underlined), a putative TATA box, and exons (boxed) are indicated. The nucleotide sequence of the exons coincides with that of cDNA for hepatic PEPCK mRNA. The length of exon 3 in the chicken cytosolic PEPCK gene is 182 bp, since sequence analysis of the nucleotide fragment which succeeds the 3′ terminus of this figure indicated the presence of 31 nucleotides before intron 3 (the sequence data will be published elsewhere). At the transcription initiation sites, C precedes A and this combination is most frequently observed with chicken promoters: among 41 chicken promoters fond in GenBank, the nucleotide at the transcription initiation site was A in 59% and the preceding nucleotide was C in 70%. In contrast to the ubiquitous sequence for the transcription initiation site, 5′TATTTAA3′ is unique in its three succeeding T and no other chicken promoter registered in GenBank has such a sequence within the TATA box. The TATA-like sequence commonly found in chicken promoters are 5′TATAAA3′ (35%) and 5′ATAAATA3′ (14%), based on examination of 37 promoters. * Reported putative TATA box (−166/−163) and transcription initiation site (−119) in kidney (*17*).

gene. One of the sequences was 5′TTTACATTACGTGCC3′ (−554/−540) with 75% homology. It is of interest that similar sequences were found in intron 1 and intron 2, for instance, at +82/+96, +179/+193, +1683/+1697, and +1811/+1825. The glucocorticoid-responsive region in rat PEPCK gene (−455/−344) contained four successive glucocorticoid receptor and accessory factor binding regions (*20*). But, no homologous sequence was found within the same area in the chicken promoter and, instead, some were found in other areas. For instance, the sequences 5′GTGGGATGGACACCACA3′ (−55/−39) and 5′CAGGCAGGAGCATA3′ (−505/−492) were homologous to the sequences 5′GTGGGAGTGACACCTCA3′ (−437/−421) and 5′CAGCCAGCAGCATA3′ (−373/−360) in the rat glucocorticoid-responsive region, respectively. The homology of those sequences amounted to 82 and 86%, respectively.

A highly homologous sequence to the rat IRS (insulin responsive sequence, 5′TGGTGTTTTGACAAC3′) (*21*) is located at −155/−141 (5′AGGGCTGTTGACAAC3′).

The sequence 5′CTTTCAGCAT3′ (−199/−190) is homologous to the most conserved sequence in the Box I region of the liver specific AFP enhancers (5′ CTTTGAGCAA3′) (*22, 23*) with 80% homology. Interestingly, no homologous sequence was found in the promoter region of rat PEPCK gene.

As for the region 3′ to the transcription initiation site, the second intron in the chicken PEPCK gene (1,398 bp) was much longer than that of the rat gene (371 bp). Despite this difference, the length from the putative translation initiation site (+311 in Fig. 2) to the 3′ terminus in the second exon (224 bp) and the length of the third exon (182 bp) are the same as those of the rat gene. Homology of the amino acid sequences of the chicken and rat PEPCK is also evident. However, we found some differences from the previously reported sequence data (*24*), which were obtained with cDNA for renal mRNA instead of hepatic mRNA, *i.e.*, +279 (deleted), +314 (G), +346 (C), and +1960 (C). The reason for these differences is not clear since no difference was found from cDNA for hepatic mRNA (data not shown).

To study species-specific (avian *vs.* mammal) as well as organ-specific (liver *vs.* kidney) gene regulation mechanisms of this gene, we next tested whether the promoter region studied here could function in a constructed reporter gene. The reporter plasmid was prepared and the function of the inserted promoter was examined with cultured hepatocytes. Prior to electroporation of the reporter plas-

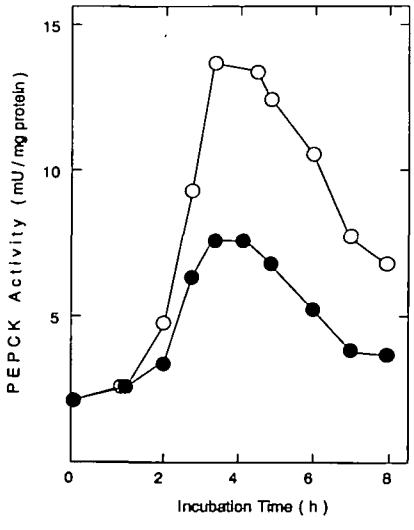

Fig. 3. **Induction of PEPCK in chick hepatocytes in culture.** Hepatocytes were prepared and plated as described in the text. After culture for 17 h, the medium was changed to one without dexamethasone and culture was continued for another 48 h. Agents were added to the medium at the following final concentrations: 0.1 mM Bt_2cAMP (●), or 0.1 mM Bt_2cAMP and 0.09 nM dexamethasone (○).

TABLE II. **Function of sequence −1855/+7 as measured in terms of expression of reporter luciferase gene.** Electroporation of chick hepatocytes to transfect reporter plasmid (pPEPLU) or PicaGene basic vector (PGV-B) as a control plasmid at the concentrations given in the table was conducted. Cells were cultured in the presence of 1 μM dexamethasone. After incubation for 48 h, 0.05 volume of 10 mM Bt_2cAMP, 10 mM theophylline in PBS was added to the culture medium and cells were further cultured for 3 h before preparation of cell lysates. Chemiluminescence was measured for 10 s and the net counts were corrected for the respective total protein concentrations after subtraction of background counts. Luciferase activities are expressed as arbitrary units/μg of protein in lysate. Numbers of samples for each experiment are given in parentheses. N.D., not determined; SD, standard deviation.

Plasmid (μg/ml)		Luciferase activity ± SD	
		0.5 mM Bt_2cAMP	Control
PGV-B	40.0	2.2±0.8(3)	N.D.
pPEPLU	0.0	2.1±0.5(7)	N.D.
	10.6	3.4±0.9(3)	2.0±0.4(3)
	26.7	4.7±0.1(2)	N.D.
	53.2	8.2±0.5(3)	4.1±0.4(3)
	106.4	1.4±0.8(3)	2.2±1.0(3)

TABLE I. **Homologous sequences in promoter regions of chicken and rat PEPCK genes.** Nucleotide sequences of promoter regions (−1 to −500) in rat and chicken PEPCK genes were aligned to find sequences conserved between them. In this table, only pairs of sequence fragments for which putative functions could be assigned are given.

Sequence	Location	Homologous motif
AGCATTCATTAAT	Chicken −194 to −182	HNF-1 site: GTTAATNATTAAC
AACATTCATTAAC	Rat −199 to −187	
GTTAAATGATTACC	Chicken −174 to −163	HNF-1 site: GTTAATNATTAAC
GTTCAATCATTATC	Rat −178 to −167	
TGGCAGCGTCCCAAA	Chicken −117 to −103	NF-1/CTF site: TGG[C/T]NNNNNNCCAAA
TGGCTATGATCCAAA	Rat −118 to −104	
TGATGTAA	Chicken −89 to −82	CRE: T[G/T]ACGTCA
TTACGTCA	Rat −86 to −93	
TATTTAA	Chicken −33 to −27	TATA-box: TATAA
TATTTAA	Rat −31 to −25	

mid, cultured cells were examined for their ability to respond to Bt_2cAMP. Figure 3 shows the time course of induced endogenous PEPCK activity measured as described previously (*11*). This time course closely resembles that observed with intact chick. In *in vivo* studies, PEPCK activity was the highest at 3 to 4 h after injection of either glucagon or epinephrine (*11*). It is therefore likely that cultured hepatocytes mimic the intact liver. A permissive effect of glucocorticoid on PEPCK induction has also been reported (*12*). Table II summarizes the results of electroporation and induction studies in which cells were incubated with Bt_2cAMP (0.5 mM) for 3 h in the presence of dexamethasone (1 μM). cAMP-dependent induction of luciferase is clear. When pPEPLU was transfected at 53.2 μg/ml, some rise in luciferase activity was observed without added cAMP. In this case, transfected plasmids could have responded to a low level of endogenous cAMP but further study to confirm this possibility was not carried out since the rise was only marginal.

DISCUSSION

Hanson *et al.* have reported that chicken cytosolic PEPCK gene appeared as a single copy in the genome and was expressed in both kidney and liver. They also located its transcription initiation site in kidney and determined a partial nucleotide sequence of the gene (*17*) which corresponds to the sequence $-365/+302$ in Fig. 2. However, the identity of the reported sequence to that of renal cDNA (*24*) is low (94.6% for 240 bp) while the entire nucleotide sequences of cDNA for renal and hepatic PEPCK mRNA agree well (99.5%), except for their unique 3′ or 5′ short terminal sequences, respectively. This casts doubt on the reliability of the nucleotide sequence of Hanson *et al.* (*17*). In order to understand the molecular basis of organ- and species-specific regulation of chicken cytosolic PEPCK gene, knowledge of the nucleotide sequence of the chicken gene for comparison with that of the rat gene was essential. In this study, we screened a genomic library with cDNA of hepatic cytosolic PEPCK mRNA as a probe and determined a sequence of 3938 nucleotides which covered the promoter region. The identity of the sequence to the 5′ terminal 601 nucleotides of renal cDNA is 99.0%. The transcription initiation site in chicken liver, which is distinct from the site reported for kidney, was also located. Furthermore, the function of the promoter region was examined by transient expression of a reporter gene in cultured hepatocytes to confirm the hormone-responsiveness of this gene.

Expression characteristics of cytosolic PEPCK genes in rat and chicken share several features in common (*1, 11*). Among them is the involvement of cAMP and glucocorticoid as the most potent inducing factors. Another feature is organ specificity: the liver and kidney are the major sites of expression. Apart from those similarities, species specificities in expression of this gene have been observed. One such phenomenon is termination of hepatic expression of the chicken gene at hatching (*5*), in contrast to abrupt expression of the rat gene after birth (*1*). This different usage of this gene between chicken and rat should be related to the differences in both the nucleotide sequence of the gene and organ-specific *trans*-acting factors. In fact, the sequence motifs which are presumably essential for hepatic expression of the gene are highly conserved in both sequence and position, while other motifs such as glucocorticoid-responsive sequences are not. The rat gene carries a CRE consensus sequence [5′T (G/T)ACGTCA3′ at $-93/-86$] which forms the core sequence in CRE-1 (*18*) while, in the chicken, homology to the consensus sequence is low (75%, see Table I). This may be related to the observation that the rat PEPCK gene is maintained in the activated state for a longer period of time (*25*) than the chicken gene (*11*). It would be of interest to replace this sequence with that of rat CRE. The presence of the sequence homologous to the insulin-responsive sequence (IRS, *21*) is interesting, since an inhibitory effect of insulin on PEPCK induction has never been observed with chicken hepatocytes (A. Sato *et al.*, to be published).

The motifs listed in Table I are conserved not only in their nucleotide sequences, but also in their positions. This suggests that these sequence elements function when they are located at the appropriate positions. On the basis of the TFD database (*26*), 52% of CRE in various genes are found at $-50/-100$ in their respective promoters. On the other hand, GRE are relatively free in terms of their locations, as would be expected for ordinary enhancer elements. As mentioned previously, cAMP is the basic inducer of the PEPCK gene, while glucocorticoid functions to enhance the induction.

The transcription initiation site was distinct from that reported for kidney (*17*), in which the transcription initiation site and putative TATA box are located at -119 and at $-166/-163$ respectively (Fig. 2). Since the role of the kidney in gluconeogenesis in chicken is well established (*9*), this difference in transcription initiation site ought to be related to the organ specificity for gluconeogenesis in chicken. Comparison of *trans*-acting factors in kidney and liver would be of interest in this respect.

The transient expression of the reporter luciferase gene confirmed the cAMP-dependent responsiveness of this gene. The reason for the use of primary cultures of chick hepatocytes instead of established cell lines is that most of the latter respond to hormones differently. For example, in the absence of externally added cAMP, glucocorticoid alone can induce PEPCK in FTO-2B (*27*) and H4IIE (*20*), the hepatoma cell lines most frequently used to study PEPCK induction. This has not been observed with rat hepatocytes, and since we intend to examine developmental differentiation of hepatic transacting factor(s) in chick embryo and chick, there was nothing to be gained by using these cell lines. Our results here show the presence of a cAMP-responsive promoter at sequence at $-1855/+7$, though the presence or absence of either all or part of the enhancer element(s) in the same sequence was not clarified. The transcription initiation site which is actually used in this transfection experiment also remains to be identified. Further work is in progress and the results will be published elsewhere.

REFERENCES

1. Tilghman, S.M., Hanson, R.W., and Ballard, F.J. (1976) Hormonal regulation of phosphoenolpyruvate carboxykinase (GTP) in mammalian tissues in *Gluconeogenesis: Its Regulation in Mammalian Species* (Hanson, R.W. and Mehlman, M.A., eds.) pp. 47-91, John Wiley & Sons, New York
2. Scrutton, M.C. and Utter, M.F. (1968) The regulation of glycoly-

sis and gluconeogenesis in animal tissues. *Annu. Rev. Biochem.* **37**, 249-302
3. Nordlie, R.C. and Lardy, H.A. (1963) Mammalian liver phosphoenolpyruvate carboxykinase activities. *J. Biol. Chem.* **238**, 2259-2263
4. Soling, H.-D. and Kleineke, J. (1976) Species dependent regulation of hepatic gluconeogenesis in higher animals in *Gluconeogenesis: Its Regulation in Mammalian Species* (Hanson, R.W. and Mehlman, M.A., eds.) pp. 369-462, John Wiley & Sons, New York
5. Savon, S., Hakimi, P., and Hanson, R.W. (1993) Expression of the genes for the mitochondrial and cytosolic forms of phosphoenolpyruvate carboxykinase in avian liver during development. *Biol. Neonate* **64**, 62-68
6. Ballard, F.J. and Hanson, R.W. (1967) Phosphoenolpyruvate carboxykinase and pyruvate carboxylase in developing rat liver. *Biochem. J.* **104**, 866-871
7. Iynedjian, P.B., Ballard, J., and Hanson, R.W. (1975) The regulation of phosphoenolpyruvate carboxykinase (GTP) synthesis in rat kidney cortex. *J. Biol. Chem.* **250**, 5596-5603
8. Ogata, K., Watford, M., Brady, L.J., and Hanson, R.W. (1982) Mitochondrial phosphoenolpyruvate carboxykinase (GTP) and the regulation of gluconeogenesis and ketogenesis in avian liver. *J. Biol. Chem.* **257**, 5385-5391
9. Watford, M., Hod, Y., Chiao, Y.-B., Utter, M.F., and Hanson, R.W. (1981) The unique role of the kidney in gluconeogenesis in the chicken. *J. Biol. Chem.* **256**, 10023-10027
10. Kochi, H., Serizawa, K., and Kikuchi, G. (1980) On the nature of three forms of phosphoenolpyruvate carboxykinase occurring in cytosol of chicken liver. *J. Biochem.* **88**, 895-904
11. Sato, A., Suzuki, T., and Kochi, H. (1990) Regulation of hormonal induction of chicken liver cytosol-specific phosphoenolpyruvate carboxykinase. *J. Biochem.* **108**, 720-725
12. Sato, A., Suzuki, T., and Kochi, H. (1986) Purification and characterization of cytosol-specific phosphoenolpyruvate carboxykinase from chicken liver. *J. Biochem.* **100**, 671-678
13. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, pp. 1.21-1.28, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
14. *ibid* pp. 1.40-1.41
15. Seglen, P.O. (1976) Preparation of isolated rat liver cells. *Methods Cell Biol.* **13**, 29-83
16. Tanaka, K., Sato, M., Tomita, Y., and Ichihara, A. (1978) Biochemical studies on liver functions in primary cultured hepatocytes of adult rats. *J. Biochem.* **84**, 937-946
17. Hod, Y., Yoo-Warren, H., and Hanson, R.W. (1984) The gene encoding the cytosolic form of phosphoenolpyruvate carboxykinase (GTP) from the chicken. *J. Biol. Chem.* **259**, 15609-15614
18. Beale, E.G., Chrapkiewicz, N.B., Scoble, H.A., Metz, R.J., Quick, D.P., Noble, R.L., Donelson, J.E., Biemann, K., and Granner, D.K. (1985) Rat hepatic cytosolic phosphoenolpyruvate carboxykinase (GTP). *J. Biol. Chem.* **260**, 10748-10760
19. Roesler, W.J., Vandenbark, G.R., and Hanson, R.W. (1989) Identification of multiple protein binding domains in the promoter-regulatory region of the phosphoenolpyruvate carboxykinase (GTP) gene. *J. Biol. Chem.* **264**, 9657-9664
20. Imai, E., Stromstedt, P.-E., Quinn, P.G., Carlsted-Duke, J., Gustafsson, J.-A., and Granner, D.K. (1990) Characterization of a complex glucocorticoid response unit in the phosphoenolpyruvate carboxykinase gene. *Mol. Cell. Biol.* **10**, 4712-4719
21. O'Brien, R.M., Lucas, P.C., Forest, C.D., Magnuson, M.A., and Granner, D.K. (1990) Identification of a sequence in the PEPCK gene that mediates a negative effect of insulin on transcription. *Science* **249**, 533-537
22. Godbout, R., Ingram, R.S., and Tilghman, S.M. (1988) Multiple regulatory elements in the intergenic region between the α-fetoprotein and albumin genes. *Mol. Cell. Biol.* **8**, 1169-1178
23. Locker, J. and Buzard, G. (1990) A dictionary of transcription control sequences. *DNA Sequence-J. DNA Sequencing and Mapping* **1**, 3-11
24. Cook, J.S., Weldon, S.L., Garcia-Ruiz, J.P., Hod, Y., and Hanson, R.W. (1986) Nucleotide sequence of the mRNA encoding the cytosolic form of phosphoenolpyruvate carboxykinase (GTP) from the chicken. *Proc. Natl. Acad. Sci. USA* **83**, 7583-7587
25. Christ, B., Nath, A., and Jungermann, K. (1990) Mechanism of the inhibition by insulin of the glucagon-dependent activation of the phosphoenolpyruvate carboxykinase gene in rat hepatocyte cultures. *Biol. Chem. Hoppe-Seyler* **371**, 395-402
26. Ghosh, D. (1990) A relational database of transcription factors. *Nucleic Acids Res.* **18**, 1749-1756
27. Wynshaw-Boris, A., Lungo, T.G., Short, J.M., Fournier, R.E.K., and Hanson, R.W. (1984) Identification of a cAMP regulatory region in the gene for rat cytosolic phosphoenolpyruvate carboxykinase. *J. Biol. Chem.* **259**, 12161-12169