Identification of a cAMP Regulatory Region in the Gene for Rat Cytosolic Phosphoenolpyruvate Carboxykinase (GTP)

USE OF CHIMERIC GENES TRANSFECTED INTO HEPATOMA CELLS

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Cytosolic phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) is a key regulatory enzyme in hepatic gluconeogenesis. The rate of synthesis of PEPCK increases 8-fold within 90 min after cAMP treatment (3), an effect which is paralleled by an equally rapid change in the sequence abundance of mRNA coding for this enzyme (4, 5). Within 20 min, cAMP stimulates the rate of transcription of this gene 7-fold (6). The hepatic PEPCK gene is thus a good model system to study the mechanism of cAMP regulation of gene transcription. Recently, the gene for cytosolic PEPCK from the rat has been isolated in a 7.0-kb BamHI genomic restriction fragment (7). The 5'- and 3'-ends of the PEPCK gene have been identified and the gene shown to contain eight introns. The 5'-end of the gene was located by in vitro transcription of a restriction fragment from the extreme 5'-end of the gene, and this fragment contains the consensus sequences normally required for transcription by RNA polymerase II (7). The entire BamHI fragment was transfected into COS and HeLa cells. These transfected genes directed the production of transcripts of the same size as mRNA from endogenous rat liver genes (8). Also, chimeric genes containing the coding sequences of several selectable structural genes expressed from the putative PEPCK promoter region were introduced into L-cells (8) and confirmed the location of the PEPCK promoter. In order to identify cAMP responsive regions which control the expression of the PEPCK gene, we have constructed a chimeric gene containing the transcriptional initiation site and 547 bp of 5'-flanking sequence of the PEPCK gene ligated to the coding sequence of the gene for herpes simplex virus thymidine kinase. This chimeric gene was transfected into thymidine kinase-deficient rat hepatoma cells. Transfectants were selected in HAT medium (9), and the ability of cAMP to stimulate TK enzyme activity and TK-specific mRNA was determined. We found that expression of the chimeric gene was regulated by cAMP, whereas the intact TK gene was unaffected by cAMP treatment.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were purchased from Bethesda Research Laboratories, Boehringer Mannheim, or New England Bio-

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Adenosine 3':5'-monophosphate controls a variety of cellular processes in prokaryotic and eukaryotic organisms, including the expression of specific genes. In prokaryotes, cAMP directly regulates gene transcription by binding to a cAMP receptor protein (1). This complex binds to specific DNA sequences and either increases or decreases transcription of the associated gene (2). In eukaryotes, however, the mechanisms by which cAMP regulates gene expression is not well understood.

Cytosolic phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) from the rat is a key regulatory enzyme in hepatic gluconeogenesis. The rate of synthesis of PEPCK increases 8-fold within 90 min after cAMP treatment (3), an effect which is paralleled by an equally rapid change in the sequence abundance of mRNA coding for this enzyme (4, 5). Within 20 min, cAMP stimulates the rate of transcription of this gene 7-fold (6). The hepatic PEPCK gene is thus a good model system to study the mechanism of cAMP regulation of gene transcription. Recently, the gene for cytosolic PEPCK from the rat has been isolated in a 7.0-kb BamHI genomic restriction fragment (7). The 5'- and 3'-ends of the PEPCK gene have been identified and the gene shown to contain eight introns. The 5'-end of the gene was located by in vitro transcription of a restriction fragment from the extreme 5'-end of the gene, and this fragment contains the consensus sequences normally required for transcription by RNA polymerase II (7). The entire BamHI fragment was transfected into COS and HeLa cells. These transfected genes directed the production of transcripts of the same size as mRNA from endogenous rat liver genes (8). Also, chimeric genes containing the coding sequences of several selectable structural genes expressed from the putative PEPCK promoter region were introduced into L-cells (8) and confirmed the location of the PEPCK promoter. In order to identify cAMP responsive regions which control the expression of the PEPCK gene, we have constructed a chimeric gene containing the transcriptional initiation site and 547 bp of 5'-flanking sequence of the PEPCK gene ligated to the coding sequence of the gene for herpes simplex virus thymidine kinase. This chimeric gene was transfected into thymidine kinase-deficient rat hepatoma cells. Transfectants were selected in HAT medium (9), and the ability of cAMP to stimulate TK enzyme activity and TK-specific mRNA was determined. We found that expression of the chimeric gene was regulated by cAMP, whereas the intact TK gene was unaffected by cAMP treatment.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were purchased from Bethesda Research Laboratories, Boehringer Mannheim, or New England Bio-
labeled DNA, RNA, DNA polymerase, and polynucleotide kinase were purchased from Boehringer Mannheim and DNase I was from Worthington. Guanidium thiocyanate was obtained from Fluka A.G. and Kodak. Bt-CAMP and salmon testis DNA were from Sigma. \(\alpha\)-dCTP (400-600 Ci/mmol) and \(\alpha\)-dATP (7000 Ci/mmol) were purchased from New England Nuclear or Amersham-Searle. Nitrocellulose filters from Schleicher & Schuell, and all media and sera from Boehringer Mannheim. Formamide (MCB Biochemicals, Norwood, OH) was deionized before use with Bio-Rad Ag 50-X8 resin. All buffers and salts were purchased from Fisher or Sigma and were of the highest purity available. Cell culture medium and sera were from Gibco or Flow Laboratories. pPCl was a generous gift from R. Flavell (Biogen Research Corp.) (11); isolation, subcloning, and restriction mapping of BH1.2 and B7.0 have been previously described (7).

**General Methods**—Plasmid DNA was prepared by the alkaline lysis procedure of Birnboim and Doly (40) and further purified by CsCl gradient centrifugation. *Escherichia coli* strain HB101 was transformed with DNA by the calcium chloride procedure of Norgard et al. (41). DNA digestion by restriction enzymes was carried out using conditions recommended by Maniatis et al. (42). DNA sequencing was performed by chemical cleavage (43) or by the dideoxynucleotide chain-termination method (44). S1 nuclease mapping was performed by the method of Berk and Sharp (17) as modified by Weaver and Weissmann (18), using the \(^3^P\) 5'-end-labeled probes described in the text.

**Plasmid Construction**—pOPF and BH1.2 were each digested with BamHI and BglII. BamHI-BglII-digested pOPF, which contained the TK gene devoid of its promoter, was then treated with CIAP or with CIAP and T4 DNA ligase at room temperature for 6 h. The ligation mixture was then used to transform *E. coli* strain HB101, and transformants were selected by their resistance to ampicillin. Plasmids were then isolated from ampicillin-resistant bacteria and further characterized by restriction mapping and Maxam-Gilbert sequencing.

Since digestion of DNA with BamHI and BglII results in compatible ends (5'-GATC-3'), we were able to select clones which had the PEPCB promoter fragment ligated to the TK gene in the correct orientation relative to transcription (pPCl-K1) or in the opposite orientation with respect to transcription (pPCl-K2). In pPCl-K2, the BamHI site in pOPF ligated to the BamHI site in the 620-bp promoter fragment, and the BglII sites of each molecule were also ligated. In pPCl-K1, the BamHI and BglII sites were reconstituted, as confirmed by restriction digestion. In pPCl-K6, the BamHI sites in each fragment were ligated to the BglII sites, forming a hybrid BamHI-BglII site (indicated by BB* in Fig. 2) which cannot be digested by either BamHI or BglII. This construction was selected because use as a control in our transfection studies, since the PEPCB promoter is incorporated into the plasmid in an orientation opposite to the direction of transcription (Fig. 2).

**Cell Culture Procedures and DNA Transfection**—FTO-2B cells were grown in a medium containing equal parts of Dulbecco's modified Eagle's minimal essential medium and Ham's F-12 medium with 10% fetal bovine serum (DB/H) in a humidified incubator at 37 °C and 5% CO\(_2\). Cells were grown to confluence at 90% and were used for the experiments. Cells were trypsinized and counted in a hemocytometer. Cell extracts were prepared by sonication in 10 mM Tris-HCl buffer (pH 8.0), containing 10 mM KCl, 2 mM MgCl\(_2\), 10 mM Tris-Cl (pH 8.0), 1 mM ATP, 10 mM NaF, 50 mM \(\epsilon\)-aminoacproic acid, and 10 mM \(\beta\)-mercaptoethanol. Cell extracts were centrifuged at 12,000 \(\times\) g for 10 min, and the supernatant fraction was used for enzyme assays. PEPCB activity was measured by the method of Ballard and Hanson (46), and TK as described by Mayo et al. (26).

**Preparation of Cellular DNA and RNA**—Cellular DNA was prepared by the method of Bliin and Stafford (48), except that after RNase digestion, the DNA was ethanol-precipitated, spooled twice, resuspended in 10 mM Tris (pH 8.0), 1 mM EDTA, total cellular RNA was prepared by extracting cells with 4 M guanidinium thiocyanate (49) and purifying the RNA on CsCl cushions (50).

**DNA Blot Hybridization**—Ten \(\mu\)g of DNA from various cell lines were digested for 2 h at 37 °C with 40 units of EcoRI. The DNA was separated by electrophoresis on 0.8% agarose gels and transferred to nitrocellulose as described by Southern (16). Filters were baked, prehybridized at 44 °C for at least 4 h in 50% formamide, 2 \(\times\) DIGEST buffer (10 \(\times\) DIGEST buffer is 0.1 M PIPES (pH 6.4), 4 mM NaCl, 0.01 M EDTA), 100 \(\mu\)g/ml denatured salmon testis DNA, and 0.5% SDS. The same solution was used for both hybridization and for prehybridization, except that \(3^P\)-labeled probe was added for hybridization (10 \(\times\) cpmp/mg \(\alpha\)-probe). DNA probes were \(3^P\)-labeled by nick translation (51), and hybridizations were carried out at 44 °C for 40-48 h. Unbound probe was washed from the nitrocellulose by washing once at room temperature in 2 X SSC, 0.1% SDS, followed by three washes in 0.1 X SSC, 0.1% SDS at 55 °C. Filters were exposed to XAR film (Kodak) overnight at -70 °C with intensifying screens.

**RESULTS**

**Characterization of the PEPCB Promoter Fragment**—Fig. 1A shows the 7-kb BamHI fragment which contains the entire PEPCB gene, as well as the 5'-3' orientation of the gene. At the 5'-end of this segment of the gene there is a BamHI-BglII fragment, 620 bp in length (Fig. 1B), containing the PEPCB transcriptional start site, 547 bp of 5'-flanking sequence, and 73 bp of the first exon. The nucleotide sequence of the PEPCB promoter fragment has been determined (Fig. 1C). We have also localized the start site for transcription of the PEPCB gene (nucleotide +1) by S1 nuclease mapping (Fig. 1D). When a Ncol-BglII fragment (Fig. 1B) was labeled at the BglII site, hybridized to RNA from kidney or hepatoma cells, and digested with S1 nuclease, a series of fragments between 70 and 75 nucleotides in length were protected. The most intense band was at 73 nucleotides. It is not clear from these experiments whether these fragments result from S1 nuclease digestion artifacts or they represent CAP site microheterogeneity.

Using the 73-nucleotide fragment as the start site, the PEPCB mRNA CAP site was precisely mapped (nucleotide +1 in Fig. 1C) by comparing the migration of the fragment protected from S1 nuclease digestion with Maxam-Gilbert sequencing reactions of the same labeled fragment. Upstream of this start site, between -33 and -21, an AT-rich sequence corresponds to the canonical "TATA" homology (10) that was identified. The 73-nucleotide fragment noted after S1 nuclease digestion has increased sequence abundance after Bt-CAMP or glucocorticoid treatment in both the kidney and liver (Fig. 1D), as one would predict from the known effect of these agents on PEPCB mRNA levels (8).

**Construction of a Chimeric PEPCB-TK Gene**—To determine whether sequences at the 5'-end of the PEPCB gene were responsible for transcriptional regulation by CAMP, we constructed a chimeric gene consisting of the 620-bp BamHI-BglII restriction fragment of the 5'-end of the PEPCB gene described above (Fig. 1) fused to the coding sequences of the TK gene (Fig. 2). The cosmid vector pOPF (11) (Fig. 2) was the source of the TK gene. It contains the entire herpes
The structure of the PEPCK gene and the detailed structure and sequence of the fragment of the gene used to construct chimeric genes. A, the intron-exon structure of the entire PEPCK gene as it is contained in B7.0 is shown. B, the 5'-region between the BamHI and BglII sites is expanded, and a detailed restriction map of the regions presented. This fragment was used to construct the chimeric genes. C, the nucleotide sequence of the coding strand in the region between the BamHI and BglII site is shown. The start site for transcription is indicated, and the TATA homology is boxed. Important restriction sites are indicated, and a sequence homologous to other cAMP-regulated genes is underlined. D, the start site for transcription of the PEPCK gene was identified by S1 nuclease mapping (17, 18). The NcoI-BglII 189-bp fragment was used to map the regions presented. This fragment was used to construct the chimeric genes. If regulatory sequences are present in the 620-bp PEPCK fragment, the regulation of this chimeric gene should be similar to that of the PEPCK gene (i.e. cAMP should increase its rate of transcription). pPCK6B, on the other hand, contains the PEPCK promoter in the opposite orientation relative to the transcription of the TK structural gene. This provides an excellent control to test for promoter function since cells transfected with pPCTK-6B should not make TK mRNA nor survive in HAT medium.

Transfection of Vectors into Rat Hepatoma Cells and Selection of Transfectants in HAT Medium — Since cAMP acutely regulates the gene for cytosolic PEPCK in rat liver, we transfected the vectors containing PEPCK-TK chimeric genes into the TK-deficient rat hepatoma cell line FTO-2B and selected for transfectants in HAT medium. These cells are well-differentiated, having the morphology of normal hepatocytes, and produce a variety of liver-specific proteins, such as albumin, lactate dehydrogenase, alcohol dehydrogenase, tyrosine aminotransferase, and PEPCK. They were derived from FT-1, a TK-deficient hepatoma cell line (15). The regulation and expression of the transfected, chimeric genes were compared with the endogenous PEPCK gene, which served as an internal control in all experiments.

FTO-2B cells were transfected with varying amounts of vector DNA and selected in HAT medium. Cells which were transfected with pOPF produced about 2000 colonies/μg of DNA in HAT medium (Fig. 3). After transfection with pPCTK-6A, about 200 colonies/μg of transfected DNA were selected, or about 10% of the cells selected after transfection with pOPF. In contrast, pPCTK-6B provided negligible protection to transfected cells grown in HAT medium. This is expected since the PEPCK promoter is in an orientation opposite to the direction of transcription of the TK structural gene and indicated that cryptic or SV40 promoters were not responsible for the selection of colonies in HAT medium. The SV40 sequences in these vectors are important for efficient transfection since removal of these sequences from either protected segments were separated by electrophoresis on 8% polyacrylamide-urea gels (43) and exposed to film. The autoradiograph is shown. On the same gel, Maxam-Gilbert sequencing reactions of the same end-labeled fragment were also run as indicated. The nucleotide sequence is written to the top of the sequencing reactions. The sequence of the noncoding strand, as read directly from the gel, is adjacent to the sequencing reactions. The sequence of the coding strand is aligned with the noncoding strand, and the assigned position of the CAP site is indicated as +1. We used 2.5 μg of rat kidney poly(A)* RNA and 25 μg of rat hepatoma cell total RNA in each lane. DEX, dexamethasone.
Fig. 2. Construction of the PEPCK-TK chimeric genes. pOPF was digested with BamHI and BglII to remove the TK promoter sequences, and was then CIAP-treated to prevent religation. The PEPCK promoter fragment, containing the start site and 547 bp of 5'-flanking sequence, was gel-purified after digesting BH1.2 with BamHI and BglII. The BamHI and BglII digestion leaves compatible ends (5'-GATC-3') so that the PEPCK promoter fragment could be ligated in front of the promoterless TK gene in either orientation. pPCTK-6A, containing the PEPCK promoter in the proper orientation relative to TK transcription, was easily identified because the BamHI and BglII sites were reconstituted upon ligation. pPCTK-6B, on the other hand, contained the PEPCK promoter in the opposite orientation relative to TK transcription and could be selected because the BamHI and the BglII sites were not reconstituted (indicated by BB*). HSV, herpes simplex virus.
by the presence of Bt_cAMP, but in cells transfected with plasmids or 
the chimeric PEPCK-TK gene was designated PTK. Clonal cell lines 
gained three-digit designations (i.e. TK-A4A, PTK-A6B). The parent 
FTO-2B cells had low TK activity, which we assume to be background 
levels. In cell lines transfected with pOPF (TK cells), TK activity was 
not altered by Bt_cAMP treatment. In 9 of 10 clonal cell lines 
transfected with pPCTK-6A (PTK cells), TK activity was stimulated 
by Bt_cAMP, with the levels of induction varying from 1.6- to 6.1-fold.

Simultaneous treatment of the cloned cells, transfected 
with pPCTK-6A, with Bt_cAMP and theophylline plus 
minimalin, a specific RNA polymerase II inhibitor, resulted in 
a complete inhibition of the CAMP-mediated induction of TK 
in any of the cell lines tested (Table I). It seems probable, therefore, 
that the major effect of cAMP on the expression of 
the chimeric PEPCK-TK gene is at the transcriptional level.

Since the endogenous PEPCK gene in the parent cell line 
is sensitive to Bt_cAMP induction, we assayed PEPCK activity 
in extracts from cells treated with and without Bt_cAMP 
and theophylline as described above. This treatment caused 
an induction of PEPCK activity in all cell lines tested (Table

| Table I |

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Thymidine kinase</th>
<th>PEPCK</th>
</tr>
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<tbody>
<tr>
<td>Control*</td>
<td>cAMP*</td>
<td>cAMP plus α-amaminatin*</td>
</tr>
<tr>
<td>-----------</td>
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<td>-------------------------</td>
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<tr>
<td>FTO-2B</td>
<td>0.0058 0.0061 (1.0) 0.0051 (1.0)</td>
<td>10.6 38.9 (3.7)</td>
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<tr>
<td>TK-A4A</td>
<td>0.038 0.047 (1.3) 0.040 (1.1)</td>
<td>25.0 50.8 (3.6)</td>
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<tr>
<td>TK-P3B</td>
<td>0.020 0.019 (0.9)</td>
<td></td>
</tr>
<tr>
<td>PTK-A6B</td>
<td>0.073 0.440 (6.1) 0.220 (2.9)</td>
<td>42.7 113.7 (2.7)</td>
</tr>
<tr>
<td>PTK-A7A</td>
<td>0.120 0.230 (1.9) 0.170 (1.3)</td>
<td>36.9 66.2 (2.8)</td>
</tr>
<tr>
<td>PTK-H5A</td>
<td>0.024 0.064 (2.6) 0.056 (1.4)</td>
<td>27.4 60.3 (2.2)</td>
</tr>
<tr>
<td>PTK-A4A</td>
<td>0.384 0.160 (1.9) 0.055 (0.7)</td>
<td>25.1 65.6 (2.6)</td>
</tr>
<tr>
<td>PTK-H4A</td>
<td>0.013 0.038 (2.8) 0.013 (1.0)</td>
<td>28.8 73.2 (2.5)</td>
</tr>
<tr>
<td>PTK-A3A</td>
<td>0.120 0.220 (1.8)</td>
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<td>PTK-H5A</td>
<td>0.110 0.200 (1.9)</td>
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<tr>
<td>PTK-A8A</td>
<td>0.071 0.063 (0.9)</td>
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</table>

*Control cells were incubated for 16 h in serum-free DB/H. 
†cAMP treated cells were incubated for 16 h in serum-free DB/H 
plus 0.5 mM Bt_cAMP plus 1 mM theophylline. 
α-Amanatin-treated cells were incubated for 16 h in serum-free 
DB/H plus 0.5 mM Bt_cAMP plus 1 mM theophylline plus 0.5 μg/μl 
α-amaminatin.

**A. Wynshaw-Boris, T. G. Logo, E. E. K. Fournier, and R. W. Hanson, unpublished observations.**
TK Genes Are Integrated into Hepatoma Cells Transfected with pOPF and pPCTK-6A—The difference in TK activity in the various clonal cell lines may reflect differences in the number of integrated PEPPCK-TK genes or rearrangements of these genes in the genome of the host cell. To test these possibilities, we isolated DNA from various clones for DNA blot analysis. Fig. 5A shows that EcoRI digestion of DNA from all clones yielded a major band of 5.2 kb when hybridized to our PEPCk cDNA clone. This is predicted from the restriction map of the PEPCk gene (7) and indicates that the restriction digestions were complete.

We also used a 2.6-kb EcoRI fragment of pOPF, which contains the entire TK gene, to probe EcoRI restriction digests of clonal DNA (Fig. 5B). FTO-2B cells contained no DNA which hybridizes to this fragment. All the transfected cell lines, however, contained one or more EcoRI fragments homologous to this probe. The 2.6-kb restriction fragment (indicated by the arrow) that appears in all transfected cells is the same size as the intact TK gene or the chimeric PEPCk-TK gene (see Fig. 2) and is the major fragment homologous to the TK Gene in all clones. Some cell lines have no additional EcoRI fragments homologous to this probe. The 2.6-kb restriction fragment may result from integration events which occur at various places within the vectors, causing a loss of EcoRI restriction sites. They appear to consist mainly of pBR327 sequences, since these additional bands hybridize strongly to a pBR327 hybridization probe, the same 70–75-nucleotide fragments of the above probe. Thus, all cell lines produce PEPCk-specific mRNA which initiates at the correct transcriptional start site and is sensitive to Bt2cAMP stimulation.

To independently determine the start site of transcription of the integrated PEPCk-TK chimeric genes, a 920-bp BamHI-AvaI fragment from the chimeric gene was 5′-end-labeled at the AvaI site and used as a hybridization probe in S1 nuclease mapping experiments (Fig. 5B). Both TK and PEPCk-TK mRNA will protect the AvaI-labeled fragment from S1 nuclease digestion. TK mRNA will protect a 300-nucleotide fragment, while PEPCk-TK mRNA will protect a 372-nucleotide fragment. A 372-nucleotide fragment was protected when RNA from cells transfected with pPCTK-6A (PTK-A4B, PTK-A7A, PTK-A6B) was hybridized to this labeled fragment, as predicted, and the amount of this protected fragment was increased using RNA from cells treated with Bt2cAMP and theophylline. RNA from FTO-2B cells did not protect any labeled fragments. RNA from TK-A4A cells protected a 300-nucleotide fragment which was not sensitive to Bt2cAMP treatment.

The number of copies of the chimeric gene present in the various clonal cell lines (Fig. 5B) had no relationship to either the basal TK activity or its inducibility by Bt2cAMP (Table I). For example, clone PTK-A7A had the highest basal TK activity of cell lines we have studied, yet this line contained the lowest number of PEPCk-TK genes. The gene(s) incorporated into this clone were also stimulated by Bt2cAMP. Clone PTK-A6B had a low basal TK activity which was highly inducible by Bt2cAMP, but contained multiple copies of the chimeric gene. Thus, gene dosage effects alone cannot explain the differences in either the basal level of TK or its induction by Bt2cAMP in the various cell lines.

**TK mRNA Is Induced by Bt2cAMP in Clones Transfected with pPCTK-6A—**The mRNA from several cell lines was analyzed by S1 nuclease mapping (17, 18) to determine whether the mRNA from both the endogenous PEPCk gene and from the transfected, chimeric genes is increased by Bt2cAMP and whether the PEPCk-TK gene uses the normal PEPCk transcriptional start site. When the NcoI-BglII fragment (see Fig. 1), 5′-end-labeled at the BglII site, was used as a hybridization probe, the same 70–75-nucleotide fragments were protected by RNA from all cell lines examined (Fig. 6A). In all cell lines, the amount of protection of these fragments was increased using RNA from cells treated with Bt2cAMP and theophylline. These fragments are protected in cells containing only PEPCk mRNA (FTO-2B), PEPCk and TK mRNA (TK-A4A), and PEPCk and PEPCk-TK mRNA (PTK-A4B, PTK-A7A, PTK-A6B). In PTK cell lines, both PEPCk and PEPCk-TK mRNA will protect 70–75-nucleotide fragments of the above probe. Thus, all cell lines produce PEPCk-specific mRNA which initiates at the correct transcriptional start site and is sensitive to Bt2cAMP stimulation.

The PEPCk-TK mRNA produced in these cell lines was also analyzed by RNA blot hybridization. Using as a probe a 2.6-kb EcoRI fragment from pOPF (see Fig. 1) we noted a 1.3-kb TK-specific mRNA produced in three cell lines transfected with pPCTK-6A. Accumulation of this mRNA was induced 4–6-fold by Bt2cAMP. No TK mRNA was detected in FTO-2B cells, and only low levels in TK-A4A cells (transfected with pOPF). The sequence abundance of this TK mRNA in TK-A4A cells was unaltered by Bt2cAMP (data not shown). Thus, transcripts from the PEPCk-TK chimeric genes use the PEPCk promoter and transcriptional start site, and the activity of the promoter is increased by Bt2cAMP treatment.
Theophylline treatment of the chimeric gene PEPCK-TK results in a significant reduction of TK expression, indicating that this gene is sensitive to cAMP-mediated induction. The PEPCK gene contains a sequence between nucleotides -262 and -247 which is strikingly similar to sequences present in the 5'-flanking regions of the rat tyrosine aminotransferase gene (19), the rat prolactin gene (20), and the gene for the common α subunit of the bovine pituitary glycoprotein hormones (21). These sequences are also similar to the consensus sequences found in all prokaryotic genes regulated by cAMP (2). It is interesting that the cAMP-mediated induction of transcription might be conserved in prokaryotes and higher eukaryotes, although we do not know whether this sequence homology has functional significance.

**TK Expression from the Chimeric PEPCK-TK Gene**—The survival rate in HAT medium of cells transfected with the PEPCK-TK fusion gene (pPCTK-6A) was only 5–10% of the rate for cells transfected with the intact TK gene (pOPF). We observed the same result in mouse L-cells transfected with the chimeric genes. The replacement of the TK promoter with the human metallothionein II gene promoter resulted in a similar decrease in transfection efficiency (22). The reasons for the decreased survival of cells transfected with the chimeric gene are not clear. It is possible that pOPF is integrated more efficiently than pPCTK-6A, or that the 620-bp BamHI fragment of the TK promoter fragment inhibits transfection. In support of this, we observed that the insertion of the entire PEPCK gene into pOPF also decreased the number of colonies selected in HAT medium to about 5–10% of the number selected after transfection of pPCTK-6A. It is possible that the CAMP-mediated induction of transcription might be conserved in prokaryotes and higher eukaryotes, although we do not know whether this sequence homology has functional significance.

**FIG. 7. Sequence homologies in the 5'-flanking regions of genes regulated by cAMP.** The sequences were aligned with the cAMP-responsive sequences in a eukaryotic gene. We have identified a 620-bp region at the 5'-end of the PEPCK-TK gene, which when fused to a TK gene devoid of its promoter, brings the expression of TK under the control of Bt2cAMP. A series of cell lines was isolated which contained either intact TK genes or chimeric PEPCK-TK genes stably integrated into their chromosomal DNA. The expression of the PEPCK-TK chimeric genes was sensitive to Bt2cAMP induction at the level of enzyme activity and mRNA, while the expression of the intact TK gene was unaffected by Bt2cAMP treatment. It also appears that the major effect of Bt2cAMP on the chimeric genes is on transcription, since α-amanitin blocked or significantly reduced the Bt2cAMP-mediated induction of TK in these cells.

Since the PEPCK gene is normally expressed and regulated in rat liver, we have used FTO-2B rat hepatoma cells as host for our transfection studies. This allows us to compare both the basal level of expression and the hormonal regulation of the endogenous PEPCK gene with that of the transfected chimeric genes. The endogenous gene produces intact PEPCK mRNA and protein, while the chimeric gene produces a chimeric mRNA and TK protein, so that the expression of the two genes can be distinguished. Both genes were regulated by cAMP, and the levels of enzyme activity and mRNA were increased in parallel. These cells are TK-deficient, allowing the selection of TK-expressing transfectants in HAT medium. Since we used chimeric genes utilizing the TK structural gene, we were able to directly select cells that expressed TK from the PEPCK promoter.

The sequence of the 620-bp BamHI-BglII fragment of the PEPCK gene was compared with sequences in prokaryotic and eukaryotic genes which are known to be regulated by cAMP (Fig. 7). The PEPCK gene contains a sequence between nucleotides -262 and -247 which is strikingly similar to sequences present in the 5'-flanking regions of the rat tyrosine aminotransferase gene (19), the rat prolactin gene (20), and the gene for the common α subunit of the bovine pituitary glycoprotein hormones (21). These sequences are also similar to the consensus sequences found in all prokaryotic genes regulated by cAMP (2). It is interesting that the cAMP-mediated induction of transcription might be conserved in prokaryotes and higher eukaryotes, although we do not know whether this sequence homology has functional significance.

**FIG. 6. S1 nuclease mapping the 5'-end of the PEPCK-TK chimeric mRNA.** One hundred μg of total RNA from the cell lines as indicated above each lane, treated with or without Bt2cAMP and theophylline, were hybridized to one of two probes 5'-end-labeled with 32P, digested with S1 nuclease, and exposed to film. A, as a probe, we used the same 189-bp NcoI-BglII fragment, 5'-end-labeled at the BglII site which was used to S1 nuclease map the 5'-end of the PEPCK gene. RNA originating at the PEPCK CAP site protected a series of fragments 70–75 nucleotides in length. B, a 920-bp BamHI-AvoI fragment of the PEPCK-TK chimeric gene 5'-end-labeled at the AvoI site was used to map the 5'-end of PEPCK-TK chimeric mRNA. This BamHI-AvoI fragment contains the 620-bp PEPPCK promoter fragment and 300 bp of the TK gene. RNA originating at the PEPCK CAP site of the PEPCK-TK chimeric gene protects a 372-nucleotide fragment of DNA. TK mRNA protects a 300-nucleotide fragment of DNA.

**DISCUSSION**

**cAMP Responsive Sequences in the Rat PEPCK Gene**—The results of this study provide the first evidence for cAMP-responsive sequences in a eukaryotic gene. We have identified a 620-bp region at the 5'-end of the PEPCK gene which, when fused to a TK gene devoid of its promoter, brings the expression of TK under the control of Bt2cAMP. A series of cell lines was isolated which contained either intact TK genes or chimeric PEPCK-TK genes stably integrated into their chromosomal DNA. The expression of the PEPCK-TK chimeric genes was sensitive to Bt2cAMP induction at the level of enzyme activity and mRNA, while the expression of the intact TK gene was unaffected by Bt2cAMP treatment. It also appears that the major effect of Bt2cAMP on the chimeric genes is on transcription, since α-amanitin blocked or significantly reduced the Bt2cAMP-mediated induction of TK in these cells.

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**TK Expression from the Chimeric PEPCK-TK Gene**—The survival rate in HAT medium of cells transfected with the PEPCK-TK fusion gene (pPCTK-6A) was only 5–10% of the rate for cells transfected with the intact TK gene (pOPF). We observed the same result in mouse L-cells transfected with the chimeric genes. The replacement of the TK promoter with the human metallothionein II gene promoter resulted in a similar decrease in transfection efficiency (22). The reasons for the decreased survival of cells transfected with the chimeric gene are not clear. It is possible that pOPF is integrated more efficiently than pPCTK-6A, or that the 620-bp BamHI fragment of the TK promoter fragment inhibits transfection. In support of this, we observed that the insertion of the entire PEPCK gene into pOPF also decreased the number of colonies selected in HAT medium to about 10–20% of the number selected after transfection of pOPF. The existence of such "poison" sequences in pBR322 has been observed (23, 24), and it is possible that they also exist in cellular genes. It is also possible that we have constructed a gene which has an unstable, chimeric mRNA. This is unlikely, since the basal TK activity in cells
transfected with the chimeric PEPCK-TK gene is equal to or higher than the basal TK activity in cells transfected with the intact TK gene.

Besides being higher in cells transfected with pPCTK-6A than with pOPF, basal TK activity in these cells is higher than TK activity observed in a variety of other cells transfected with intact or chimeric TK genes. Chandler et al. (25) showed that the intact TK gene transfected into XC-cells produced TK which had an activity of 0.0026 milliunit/mg of protein, which is approximately 10% of the activity we observed in cells transfected with pOPF (0.013–0.038 milliunit/mg of protein). The higher TK activity noted in our cell lines could be due to the presence of the SV40 enhancer sequence in our vectors. Mayo et al. (26) transfected intact TK genes or mouse metallothionein-TK chimeric genes into mouse L-cells and noted that TK activity after transfection with the intact TK gene (0.006 milliunit/mg of protein) was 20–50% of the activity we have observed in cells transfected with pOPF. Also, the TK activity of cells transfected with their metallothionein-TK chimeric genes (0.019–0.025 milliunit/mg of protein basal activity and 0.037–0.069 milliunit/mg protein after cadmium induction) is less than or equal to the activities that we observed in cells transfected with PEPCK-TK chimeric genes. Since the PEPCK gene is normally transcribed at a high rate in rat liver (S), its promoter may contribute to the high TK activities noted in these experiments.

When human growth hormone-TK chimeric genes were transfected into mouse L-cells, two TK mRNA species were produced, one of 1.3 kb, the other 0.9 kb (27). The 0.9-kb transcript initiates within the TK gene and is only rarely observed when intact TK genes are transfected into a variety of cells (28). This transcript appears to be produced when genes with relatively weak promoters are fused to the TK structural gene (27) or when the TK promoter is deleted (28). This 0.9-kb mRNA species was not transcribed in cells after transfection with PEPCK chimeric gene, indicating a strong transcriptional activity of the PEPCK promoter.

The basal activity of the chimeric genes after transfection does not appear to be related either to the number of integrated genes nor to their arrangement in genomic DNA. Similar results have been found by other investigators (26, 27, 29). The higher-molecular-weight bands in some of our cell lines are often found when stable transfecants are selected after using a calcium phosphate precipitate of DNA to transfect cells (26, 30, 31). The incorporation of multiple genes often results in some of the DNA being incorporated as fragments of different lengths. However, the majority of genes in all cell lines maintain their original structure, as evidenced by the strong hybridization signal of the 2.6-kb EcoRI restriction fragment to our specific TK probe. This fragment size is predicted from the restriction map of the transfected plasmid containing the TK structural gene. Also, we have observed BtCAMP regulation in cells containing one or multiple gene copies, and neither the activities nor inducibility can be predicted from the number or arrangement of genes. These results indicate that gene copy number and the rearrangement of the transfected genes in the host cell are not important for the CAMP regulation of transfected chimeric genes.

cAMP Regulation of Eukaryotic Gene Transcription—The transcription of several genes is regulated by CAMP. The rat prolactin gene in pituitary cells (32) and the rat tyrosine aminotransferase gene in liver (19) are transcriptionally stimulated by CAMP. Other genes respond to CAMP by changes in the concentration of their levels of mRNA. Accumulation of mRNA for lactate dehydrogenase is stimulated by CAMP in C6 astrocytoma cells (33). Also, CAMP increases the levels of mRNA coding for alkaline phosphatase in mouse L-cells (34) and tyrosine hydroxylase in pheochromocytoma cells (35), but decreases the levels of mRNA for hepatic m-enzyme in the chicken hepatocytes (36). While transcription rates were not measured in many of these studies, it seems probable that in most cases, CAMP alters gene transcription.

The mechanism by which CAMP modifies gene transcription in eukaryotes is not well understood. We have shown that the PEPCK gene has a specific sequence which is involved in BtCAMP induction of gene transcription. It is possible that CAMP acts by altering the phosphorylation of nuclear proteins which change the levels of transcription of specific genes. Murdoch et al. (37) have shown that after BtCAMP treatment, the phosphorylation of specific nuclear proteins in GH3 cells is correlated with changes in transcription rates of the prolactin gene. It is also possible that CAMP changes gene transcription by binding to a receptor protein which directly interacts with specific DNA sequences in a responsive gene. This interaction either increases or decreases the affinity of the protein for the specific DNA sequences, causing a change in the transcription rate of the gene. This is the mechanism by which prokaryotic genes are regulated by CAMP (2) and glucocorticoids regulate the transcription of mouse mammary tumor virus (25, 38). Further studies are required to clearly delineate the intermediate steps in CAMP stimulation of PEPCK gene transcription.

A variety of inducible genes have been studied by transfection of intact or chimeric genes into responsive cells (for review, see Ref. 39), and the sequences which respond to the inducing agents have been mapped. In all cases, the inducible sequences have been localized to the 5'-end of the corresponding genes. We have also localized the CAMP responsive sequences of the PEPCK gene to its 5'-end and are in the process of carefully mapping the location of these CAMP responsive sequences.

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