Characterization of the Phosphoenolpyruvate Carboxykinase (GTP) Promoter-regulatory Region

1. MULTIPLE HORMONE REGULATORY ELEMENTS AND THE EFFECTS OF ENHANCERS*

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Transcription of the gene for cytosolic Phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) (PEPCK) from rat liver is increased by cAMP and glucocorticoids and decreased by insulin. A PEPCK-thymidine kinase (TK) chimeric gene was transfected into FTO-2B rat hepatoma cells, which were TK-deficient. Previous studies showed that a cAMP regulatory element is located at the 5' end of the PEPCK gene. In this report, we demonstrate that the 5' end of the gene also contains a glucocorticoid regulatory element, but not one for insulin. Regions of the PEPCK gene that contain these regulatory elements were attached to the Herpes simplex virus TK structural gene containing its own promoter. The hormone regulatory elements within the 5' flanking region of the PEPCK gene conferred cAMP and glucocorticoid responsiveness on the TK gene after transfection into FTO-2B cells. Like viral enhancer elements, these regulatory elements functioned properly when placed in either orientation at various positions 5' or 3' to TK. The presence of the SV40 enhancer element upstream from the PEPCK-TK gene had little effect on the basal level of expression or hormonal regulation of the chimeric gene.

Hormonal regulation of gene expression is a primary mechanism by which eukaryotic organisms integrate cellular responses to environmental and metabolic conditions. Many of the hormonally regulated genes that have been studied to date are controlled by alterations in the level of transcription. The mechanism of this regulation is best understood for genes controlled by steroid hormones, where a hormone-receptor complex binds to defined regions of these genes (1, 2). However, little is known of the mechanism(s) by which non-steroid hormones regulate gene transcription, or how multiple hormones regulate the transcription of a single gene.

The gene for cytosolic phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) (PEPCK*) from the rat is a good model for studies of the regulation of gene transcription by a variety of hormones. The transcription of the gene for PEPCK is stimulated by cAMP, glucocorticoids, and thyroid hormones and depressed by insulin in the liver (3-5, for review, see Ref. 6). The PEPCK gene is contained within a 7.0-kb BamHI restriction fragment (7), and the start site of transcription has been localized (7, 8). A chimeric gene was constructed which contained the PEPCK transcriptional start site and 548 bp of 5' flanking sequence fused to the structural gene of Herpes simplex virus thymidine kinase (TK). After transfection into FTO-2B rat hepatoma cells (8, 9), the transcription of the PEPCK-TK chimeric gene was regulated by Bt-¿-AMP (8), indicating that the cAMP regulatory element for the PEPCK gene is contained within a 621-bp fragment at the 5' end of the gene.

In this report, we demonstrate the presence of cAMP and glucocorticoid regulatory elements in the immediate 5' flanking region of the PEPCK gene. However, the same 621-bp segment of DNA does not appear to contain an insulin regulatory element. The orientation dependence and distance dependence of the hormone regulatory elements of the PEPCK gene on the TK promoter, as well as the effect of these elements when placed 3' to the TK gene, were determined. We also examined the effect of the SV40 enhancer element on the expression and regulation of the PEPCK-TK chimeric gene.

EXPERIMENTAL PROCEDURES

Materials—Bt-¿-AMP, restriction enzymes, DNA ligase, DNA polymerase, calf intestinal alkaline phosphatase, polynucleotide kinase, and 32P nucleoside were purchased from Boehringer Mannheim. Theophylline and bovine insulin were purchased from Calbiochem-Behring. Dexamethasone was from Sigma and was dissolved and stored in 100% ethanol at -20 °C. [γ-32P]ATP (7000 Ci/mmol) was purchased from ICN or Amersham Corp. Formamide (MBC Biochemical) was deionized before use with Bio-Rad Ag-50-XA resin. All buffers and salts were purchased from Sigma or Fisher and were of the highest purity available. Cell culture medium and sera were from Flow Laboratories or GIBCO. pOPF (10) was a gift from Dr. Richard Flavell (Biogen Research Corp) and pTK-109 (11) was a gift from Dr. Steven McKnight (University of Washington). FTO-2B rat hepatoma cells were previously described (8, 9) and were provided by Dr. Keith Fournier (University of Southern California). These cells are TK-deficient; after transfection with TK-containing plasmids, cells expressing TK can be selected in HAT medium (12).

Plasmid Construction—Digestion with restriction enzymes, transformation of Escherichia coli, and purification of plasmid DNA were carried out as described (8, 13). DNA fragments were isolated by electrophoresis from agarose gels (13). Ligations were carried out in 20-μl reaction mixtures consisting of 20 mM Tris-Cl (pH 7.5), 2 mM MgCl2, 0.5 mM ATP, 2 mM dithiothreitol, and 1 unit of T4 DNA ligase. Vector DNA (200 ng) was incubated with a 5-20-fold molar excess of insert DNA at room temperature for at least 6 h.

The first series of plasmid vectors contained the 621-bp BamHI-BglII fragment of the PEPCK gene (Fig. 1A). This fragment contains

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*The abbreviations used are: PEPCK, phosphoenolpyruvate carboxykinase; TK, thymidine kinase; Bt-¿-AMP, dibutyryl cyclic AMP; HAT, 100 mM hypoxanthine, 4 x 10-4 M aminopterin, 1.6 x 10-5 M thymidine; PIPES, 1,4-piperazinediethanesulfonic acid; kb, kilobase pair; bp, base pair.
Multiple Hormone Regulatory Elements of the PEPCK Gene

**RESULTS**

**Hormonal Regulatory Elements at the 5' End of the PEPCK Gene**—The hormonal regulation of the PEPCK, TK, or PEPCK-TK genes were determined by measuring changes in the levels of specific mRNA by S1 nuclease mapping after transfection of either pOPF or pPCTK-6A into FTO-2B cells (Fig. 2). The endogenous PEPCK gene was used as an internal standard for the hormonal regulation of either the intact TK gene in cells transfected with pOPF or the chimeric PEPCK-TK gene in cells transfected with pPCTK-6A. As expected, the endogenous PEPCK gene was regulated by BtCAMP, dexamethasone, and insulin in transfected cells. PEPCK mRNA was increased 5-10-fold by BtCAMP treatment, an effect which was completely blocked by simultaneous insulin treatment (Table I). Dexamethasone increased the level of PEPCK mRNA by approximately 3-4-fold in these cells.
Effects of Enhancer Elements on TK and PEPCK-TK—In order to determine if the SV40 enhancer elements altered the expression and regulation of either the TK or PEPCK-TK genes, we measured the levels of TK or PEPCK-TK mRNA in pools of cultures of cells transfected with pOPF, pOPF(−SV40), pPCTK-6A, or pPCTK-6A(−SV40) (Fig. 1). In the absence of hormonal stimulation the amount of PEPCK-TK mRNA was similar in cells transfected with either plasmid (Fig. 3). The largest difference in basal levels of expression was a 50% increase when the SV40 enhancer was linked to the PEPCK-TK gene. In this cell line, there were a significant number of HAT-resistant colonies which were TK-positive revertants of FTO-2B cells that could account for the slight difference in expression. In all other cell lines transfected with either plasmid the levels of PEPCK-TK mRNA were nearly identical. Also, the amount of the TK mRNA was comparable in cells transfected with either pOPF or pOPF(−SV40) (Fig. 3). The PEPCK-TK gene was induced by 5–6-fold by Bt2cAMP and about 4-fold by glucocorticoids in cells transfected with pPCTK-6A(−SV40) (Fig. 3), which is virtually identical to the level of induction of the chimeric gene in cells transfected with pPCTK-6A (see Table I). These results demonstrate that the SV40 enhancer element has little effect on the expression and regulation of linked PEPCK-TK genes.

The Effect of Position and Orientation of the Hormone Regulatory Elements of PEPCK on the TK Gene Containing Its Own Promoter—Fragments of the PEPCK promoter-regulatory region which contain sequences responsive to cAMP and dexamethasone were placed at −650 or −109 from the 5′ end or approximately +1800, near the 3′ end of the TK gene (Fig. 1, C and D). These fragments of the PEPCK gene were most effective at conferring hormonal responsiveness to the TK gene (23). RNA was isolated from hormonally treated, HAT-resistant colonies after transformation with these plasmids and quantitated by S1 nuclease mapping. In all plasmids tested, TK mRNA initiated at the proper start site of transcription, 50 bp 5′ to the BglII site within the TK gene (data not shown). To minimize the variation in expression level and hormonal inducibility due to the site of integration of transfected DNA, we pooled 100–200 HAT-resistant colonies as mass cultures for RNA isolation, each of which contained multiple copies of the transfected genes. All of these cells were responsive to Bt2cAMP and dexamethasone, based on changes in the level of PEPCK mRNA transcribed from the endogenous gene (data not shown).

The Effect of Orientation at −650 of TK—The vectors pOPF-BB(+) and pOPF-BB(−) contain the BamHI-BglII fragment in the same and opposite orientation relative to TK transcription, respectively, placed approximately 650 bp 5′ to the TK gene (Fig. 1C). Bt2cAMP increased the level of TK mRNA 4–5-fold in cells transfected with either pOPF-BB(+) or pOPF-BB(−) (Fig. 4). Treatment of these cells with dexamethasone caused about a 4-fold increase in TK mRNA, regardless of the orientation of the BamHI-BglII fragment. TK-specific mRNA from cells transfected with pOPF was not altered by hormonal treatment.

The Effect of Orientation at −109 of TK—The hormonal regulation of the TK gene in pOPF-BB(+) and pOPF-BB(−) suggested that the hormonal regulatory elements in the PEPCK gene acted transcriptionally, since no PEPCK se-
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TABLE I

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Transfected Plasmid</th>
<th>PEPCK mRNA</th>
<th>TK mRNA</th>
<th>PEPCK-TK mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bt2cAMP</td>
<td>Bt2cAMP + insulin</td>
<td>Dex</td>
</tr>
<tr>
<td>TK-1</td>
<td>pOPF</td>
<td>7.6</td>
<td>1.0</td>
<td>4.0</td>
</tr>
<tr>
<td>TK-3B</td>
<td>pOPF</td>
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<td>pOPF</td>
<td>6.0</td>
<td>1.0</td>
<td>2.9</td>
</tr>
<tr>
<td>PTK-1</td>
<td>pPCTK-6A</td>
<td>5.0</td>
<td>1.1</td>
<td>3.2</td>
</tr>
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<td>PTK-2</td>
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<tr>
<td>PTK-3</td>
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<td>9.9</td>
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<tr>
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<td>pPCTK-6A</td>
<td>5.2</td>
<td>1.5</td>
<td>2.7</td>
</tr>
<tr>
<td>PTK-A6B</td>
<td>pPCTK-6A</td>
<td>6.5</td>
<td>1.0</td>
<td>3.7</td>
</tr>
</tbody>
</table>

This fragment of DNA was ligated to the TK gene at -109, in either orientation, to give pTK-109-PP(+) and pTK-109-PP(-) (Fig. 1D).

The amount of TK mRNA was increased by treatment with both Bt2cAMP or dexamethasone in cells transfected with either pTK-109-PP(+) or pTK-109-PP(-) (Fig. 4). Bt2cAMP caused an approximate 4-fold increase in TK mRNA in cells transfected with pTK-109-PP(+), and a 3.6-fold increase in cells transfected with pTK-109-PP(-). Glucocorticoid treatment induced TK-specific mRNA 3-fold in cells transfected with either plasmid. TK-specific mRNA was unaltered by hormonal treatment in cells transfected with pTK-109.

The Effect of Orientation at the 3' End of TK—The PuuII-PuuII fragment of the PEPCK gene (−416 to −61) was inserted in both orientations relative to transcription, into a unique HindIII site located 3′ to the TK gene in pTK-109 to give pTK-109 3′-PP(+) and pTK-109 3′-PP(-) (Fig. 1D). This places the PuuII-PuuII fragment of the PEPCK gene approximately 1.8 kb 3′ to the TK promoter or 4.8 kb 5′ to TK.

The levels of TK mRNA were increased approximately 2-fold by either Bt2cAMP or dexamethasone treatment in cells transfected with pTK-109 3′-PP(+) or pTK-109 3′-PP(-) (Fig. 4). Although the extent of induction of TK mRNA by these hormones was less than noted for cells transfected with constructs containing the PEPCK hormone response elements 5′ to the TK gene, the TK genes in pTK-109 3′-PP(+) and pTK-109 3′-PP(-) were hormonally responsive. Since the TK gene in cells transfected with pTK-109 was insensitive to hormonal stimulation, placing the PuuII-PuuII fragment of PEPCK promoter-regulatory region 3′ to the TK gene conferred hormonal responsiveness to the TK gene. Thus, the hormone regulatory elements at the 5′ end of the PEPCK gene are active from a variety of locations relative to a heterologous promoter.

DISCUSSION

The Hormonal Regulation of the PEPCK Gene—Both Bt2cAMP and glucocorticoid regulatory elements are located in the 5′ flanking region of the gene for PEPCK, within a 350-bp fragment. These elements retained their activity when linked to the TK gene containing its own promoter. The effects of the hormone regulatory elements on TK were

Fig. 3. The effect of the SV40 enhancer sequences on the expression of TK or PEPCK-TK genes. Cells were transfected with pOPF, pOPF(-SV40), pPCTK-6A, or pPCTK-6A(-SV40). HAT-resistant cells were pooled for each plasmid. RNA was isolated from cells after a 4-h treatment with serum-free medium (filled bars), Bt2cAMP (0.5 mM) and theophylline (1 mM) (open bars), or dexamethasone (1 μM) (hatched bars). S1 nuclease mapping was used to determine the levels of TK mRNA in cells transfected with pOPF or pOPF(-SV40) or PEPCK-TK mRNA (in cells transfected with pPCTK-6A or pPCTK-6A(-SV40)). Autoradiographs were scanned densitometrically, and for each treatment the mean ± S.E. was determined.
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Fig. 4. Effect of the PEPCK promoter-regulatory region on hormonal control of the intact TK gene. FTO-2B cells were transfected with the plasmids shown in the left-hand side of the figure. HAT-resistant cells were pooled for each plasmid. RNA was isolated from cells after a 4-h treatment with serum-free medium (filled bars), or medium containing Bt2cAMP (0.5 mM) and theophylline (1 mM) (open bars), or dexamethasone (1 μM) (hatched bars). S1 nuclease mapping was used to determine the levels of mRNA initiating from the TK start site. Autoradiographs were scanned densitometrically, and the values were expressed as densitometric units for RNA from control cells. A representative experiment is shown. It was repeated at least twice for the RNA samples shown, and confirmed in at least two separate mass cultures of transfected cells.

The effects of cAMP and glucocorticoids on the transcription rate of the gene for PEPCK have been demonstrated using isolated hepatic nuclei from intact animals (3) and from cells in culture (4). However, cAMP or glucocorticoids may also alter PEPCK gene expression at a post-transcriptional step, as previously suggested (24, 25). The responsiveness of the chimeric plasmid pTK-109-PP(+) and pTK-109-PP(-) to both Bt2cAMP and glucocorticoids demonstrates that these hormones act transcriptionally in these plasmids, since no PEPCK sequences are represented in TK mRNA. This is an important consideration, since the mouse methallothionein I gene is regulated post-transcriptionally by glucocorticoids when transfected into HeLa and L cells (26).

Since insulin treatment blocks the cAMP- or glucocorticoid-mediated increase in transcription of PEPCK (4), it is interesting that sequences responsive to insulin do not appear to be located within 2.1 kb of 5' flanking sequence of the PEPCK gene, while the cAMP and glucocorticoid regulatory elements are contained within 400 bp of the start site of transcription. The insulin regulatory element could be either further than 2.1 kb 5' to the gene or within the gene and further than 73 bp 3' to the start site of transcription. It is unlikely that the insulin regulatory element is further upstream since there is a transcriptionally active region at this part of the genome with an unknown function, which is not regulated by insulin. The PEPCK gene may contain intragenic sequences involved in the regulation of transcription by insulin, as was shown in the genes for immunoglobulins (27-30), chicken TK (31), and β-globin (32, 33). Alternatively, we may not have found the optional conditions for insulin regulation of the transfected chimeric gene.

The cAMP and glucocorticoid regulatory elements from the PEPCK gene can also regulate a heterologous promoter. There are 109 bp of 5' flanking sequences in pTK-109, including all of the elements required for maximal TK mRNA production in frog oocytes (11). pOPF contains about 650 bp of 5' flanking sequences including sequences upstream of -109 which may be part of the TK promoter. The cAMP and glucocorticoid regulatory elements of PEPCK were effective when linked to either of these intact promoters, suggesting that the hormone regulatory elements were independent of TK promoter functions in these vectors. In support of this, the addition of PEPCK sequences to pOPF or pTK-109 in the plasmids described in this paper had no effect on transfection efficiency and the presence of PEPCK sequences had little effect on basal TK mRNA levels in transfected cells (data not shown). It is possible that these hormone regulatory elements could stimulate TK gene expression more effectively if they were inserted in place of the proper promoter elements in the TK gene. This positional enhancement of inducible response elements was recently demonstrated with the interferon genes (34).

Hormonal Regulatory Elements and Enhancer Elements—Like the PEPCK gene, other inducible genes have been shown to contain regulatory elements which could be placed in a number of locations relative to a gene and retain their activity. The glucocorticoid regulatory elements in the long terminal repeat of mouse mammary tumor virus were the first regulatory elements shown to be active on a heterologous gene from a variety of positions (1, 35). More recently, similar properties were demonstrated for the inducible element in the β-interferon gene (34), the regulatory elements in the human methallothionein IIα gene (36) and mouse methallothionein I gene (37, 38). Positional flexibility is also a property of viral enhancer elements which can markedly affect the expression of linked genes from a variety of locations (39, 40; for a review, see Ref. 41). This common property of positional flexibility has been used to imply a similar mechanism of action between regulatory elements of inducible genes and enhancer elements (1, 34, 35).

2 E. A. Park, A. Wynshaw-Boris, and R. W. Hanson, unpublished observation.
3 D. S. Loose, H. Meisner, J. Cook, and R. W. Hanson, unpublished data.
Hormone regulatory elements and viral enhancer elements have other properties in common. The activity of these elements depends upon the binding of trans-acting factors to DNA sequences. Factors which bind enhancer elements have been identified by competition experiments (42). They are constitutively active and do not appear to depend upon specific cellular treatments. Hormonally regulated gene expression also involves the binding of specific factors to hormonal regulatory elements in target genes (1, 2), but these factors are active only in the presence of hormones. This model is based upon the regulation of gene expression by steroid hormones, and may also extend to peptide hormones. Recently, Yamamoto and co-workers (43) found that the Moloney murine leukemia virus long terminal repeat contains both enhancer elements and glucocorticoid regulatory elements, which have additive effects on linked chloramphenicol acetyltransferase genes after transient expression. It is possible, then, that these elements share a common mechanism of action to modulate gene transcription.

Evidence presented in this paper suggests, however, that hormone regulatory elements and viral enhancers may modulate gene transcription by different mechanisms. The SV40 enhancer element had no effect on the expression or regulation of linked PEPCK-TK genes, which contains hormone response elements. If both elements acted via the same mechanism, the basal level of PEPCK-TK gene expression should be higher in cells transfected with genes linked to the enhancer, since it is constitutively active. The factors which interact with the SV40 enhancer are present in FTO-2B cells, since the transfection efficiency of PEPCK-TK or TK genes linked to the SV40 enhancer is 10-fold greater than the transfection efficiency of unlinked genes in these cells. Similar results were found with TK genes (44) or Harvey sarcoma virus ras genes (45) when stably transfected into cells. These experiments seem contradictory to those using the Moloney murine leukemia virus long terminal repeat (43). It is possible that differences were observed because the PEPCK, TK, or ras genes were stably transfected into cells, while the long terminal repeat activities were assayed after transient expression. Enhancer elements frequently increase gene expression transiently (39, 46, 48–49), conversely, since the Moloney murine leukemia virus long terminal repeat is a complete unit, the enhancer element and hormone response element could be in the proper position necessary for interaction, while the artificially constructed linkage of PEPCK sequences to the enhancer element and hormone response element could not be optimal for interaction.

Positional flexibility is a property which is common to a variety of DNA sequence elements which affect a diverse range of genetic events, in species from bacteria to humans. In bacteria, for instance, the inversion of DNA sequences by site-specific recombination is stimulated by elements which act in an orientation- and distance-independent manner (49, 50). Silencer sequences in yeast decrease the expression of linked genes from a variety of locations (51). Enhancer elements have also been identified in specific cellular genes which appear to play some role in tissue-specific gene expression (27–30). Regulatory elements may simply bind trans-acting factors bi-directionally and from a variety of locations relative to the processes which they affect, but the result of that binding is different for different element-factor combinations. We are presently examining the relationship between hormone-response elements and viral enhancers so that we may better understand their mechanisms of action.

A. Wynshaw-Boris, D. S. Loose, and R. W. Hanson, unpublished data.

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**REFERENCES**


