MOLECULAR AND CELLULAR BIOLOGY, Feb. 1990, p. 480–485
0270-7306/90/020480-06$02.00/0

Vol. 10, No. 2

In Vitro Analysis of Promoter Elements Regulating Transcription of the Phosphoenolpyruvate Carboxykinase (GTP) Gene

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A cell-free system for the study of transcription from the promoter of the phosphoenolpyruvate carboxykinase (GTP) gene by using nuclear extracts from rat tissues was developed. The level of basal transcription from the phosphoenolpyruvate carboxykinase (PEPCK) promoter between −490 and +73 was highest when extracts from liver nuclei, rather than kidney, spleen, and HeLa nuclear extracts, were used. A series of 5′ deletions and block mutations were also tested for their effects on basal transcription in vitro. The promoter truncated to −355 had the highest rate of basal transcription, while subsequent deletion to −277 markedly decreased the rate of transcription. Further deletion of the promoter to −134 resulted in a twofold increase in the basal level of transcription compared with that of the promoter deleted to −277. However, subsequent deletion of the NF-1–CCAAT-binding transcription factor binding site or the proximal cyclic AMP (cAMP) regulatory element caused a decrease in basal transcription. Block mutations were inserted into nine specific protein-binding regions of the PEPCK promoter previously shown to be of functional significance or to bind nuclear proteins. Mutation of the TATA box resulted in a 94% decrease in the level of transcription noted with the intact promoter, while sequence substitutions within the proximal cAMP regulatory element decreased the transcription rate to 25%. The addition of the catalytic subunit of cAMP-dependent protein kinase to the in vitro system stimulated transcription from the intact promoter or from a promoter deletion to −109. However, a promoter deletion to −68, which removes the proximal cAMP regulatory element, was unresponsive to added protein kinase catalytic subunit. These findings indicate that the PEPCK promoter between −490 and +73 contains sequences responsive to hormonal and tissue-specific factors in nuclei from rat tissues. The sensitivity of this in vitro transcription system closely mimics the processes regulating PEPCK transcription in rat tissues and should make it ideal for testing the function of purified transcription factors.

The gene for the cytosolic form of phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) (PEPCK) is expressed primarily in the liver and kidneys and is transcriptionally regulated by several hormones (7, 12, 15). Glucocorticoids, thyroid hormones, and glucagon (acting through cyclic AMP [cAMP]) stimulate PEPCK mRNA synthesis in liver, while insulin inhibits both basal and hormone-stimulated levels of transcription. Thus, the PEPCK gene provides an excellent model for studying the mechanisms of hormone-regulated and tissue-specific transcription.

Early studies of the PEPCK gene with a series of 5′ deletion mutations of the promoter region showed that approximately 500 base pairs (bp) of the promoter immediately 5′ to the start site of transcription was required for hormonal and tissue-specific expression (18, 23). DNase I footprinting of this region of the PEPCK promoter indicated the presence of eight protein-binding domains, several of which have significant homology to sequences known to influence the transcription of other genes (22). For example, a site located at −121 to −99 bp from the transcription start site shows a high degree of homology to the NF-1–CCAAT-binding transcription factor (CTF) consensus sequence and will bind NF-1 protein purified from HeLa cells. Other elements include a cAMP regulatory element (CRE) and putative glucocorticoid regulatory elements (1, 23). However, despite the sequence homologies and protein binding data, the functions of various PEPCK promoter elements can be determined only by measuring their effects on transcription.

We have developed a functional assay, based on the in vitro transcription system described by Gorski et al. (6), for measuring transcription from the PEPCK promoter. This system uses nuclear extracts from rat liver as the source of transcription factors. Since PEPCK is normally expressed and regulated in the liver, the system provides a biochemically relevant system for measuring PEPCK transcription.

We report that the PEPCK promoter is efficiently and accurately transcribed in vitro by using this system with nuclear extracts from rat liver. Transcription from the PEPCK promoter was accurately initiated at the in vivo CAP site and was sensitive to alpha-amanitin. Tissue-specific expression of the PEPCK gene was faithfully mimicked in vitro by testing nuclear extracts prepared from various tissues and HeLa cells. Using this system, we measured transcription from a series of 5′ deletion mutations and a series of block mutations through each of the eight protected regions of the PEPCK promoter. We also showed that the addition of the catalytic subunit of cAMP-dependent protein kinase (PK-A) to nuclear extracts increases transcription from the PEPCK promoter. These results suggest that in addition to aspects of basal transcription, some features of hormonal and tissue-specific PEPCK expression might be elucidated by using this in vitro system.

MATERIALS AND METHODS

Materials. DNA-modifying enzymes, avian myeloblastosis virus reverse transcriptase, and deoxynucleoside triphos-

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phates were from Boehringer Mannheim Biochemicals. Ribonucleotides were obtained from Pharmacia LKB Biotechnology, Inc. [γ-^{32}P]ATP (6,000 Ci/mmol) was purchased from Dupont, NEN Research Products, and RNasin was from Promega Biotec. Yeast tRNA was obtained from Sigma Chemical Co. Susan Taylor (University of California, San Diego) generously provided purified catalytic subunit from cAMP-dependent PK-A. Oligonucleotide primers were chemically synthesized with DNA synthesizer (model 380A; Applied Biosystems).

DNA templates. All DNA manipulations followed procedures described by Maniatis et al. (17). The plasmid pML(C2AT), which contains the adenovirus major late promoter (AdMLP) linked to a 380-bp guanosine-free cassette, was the generous gift of Robert G. Roeder (Rockefeller University, New York, N.Y.) Deletion mutations of the 5′ end of the PEPCK promoter were prepared from the 1,200-bp *Bam*HI-*Hin*dIII fragment of the PEPCK gene, as previously described (23). The series of deletions from −68 to −355 were removed from the vector pΔ5′-PCneo (23) as *Eco*RI-*Bgl*II fragments (the *Bgl*II site is located at +73), and the −490 promoter was removed as an *Xba*I-*Bgl*II fragment from pBH1.2. The promoter fragments were cloned into the appropriate sites of the polyCAT vector, itself derived from pXSV1CAT, from which simian virus 40 promoter and enhancer sequences were removed and replaced with a short multiple cloning site.

A series of block mutations (from 5 to 15 bp) were prepared through each of the PEPCK promoter regions detected by DNase I footprinting (22) and the TATA sequence by oligonucleotide-directed mutagenesis as described by Kunkel (11). The block mutations consisted of nucleotide substitutions 5 bp long in CRE-1 (from −90 to −86) and in CRE-2 (from −143 to −139); 10 bp long in P3 (from −259 to −250); 12 bp long in P1 (from −115 to −104), in P2 (from −190 to −179), and in P6 (from −447 to −436); 13 bp long in the TATA sequence (from −36 to −24) and in P5 (from −285 to −271); and 15 bp long in P4 (from −285 to −271). The mutated sites were confirmed by dideoxy sequencing, and DNase I footprinting confirmed that the introduced mutations effectively disrupted protein binding. These mutations were originally cloned into the polyCAT vector as *Xba*I-*Bgl*II fragments and were subsequently cloned into the plasmid pTZ18R, along with the chloramphenicol-acetyltransferase structural gene. A detailed description of the preparation and analysis of the block mutations will be published in the near future (J. Liu and R. W. Hanson, personal communication).

Preparation of nuclear extracts. Nuclear extracts were prepared essentially as described by Gorski et al. (6). However, we found it necessary to suspend the pellet of nuclear proteins from $(NH_4)_2SO_4$ precipitation in dialysis buffer at 1 ml/600 A_{260} units for liver or kidney or at 1 ml/1,200 A_{260} units for spleen. The concentrations of protein in the nuclear extracts were determined by the method of Kalb and Bernlohr (10) and usually ranged from 7 to 15 mg/ml. Nuclear extracts from HeLa cells were prepared exactly as described by Dignam et al. (5).

In vitro transcription assays. In vitro transcription reactions with nuclear extracts from tissues were carried out by the method of Gorski et al. (6). Reactions with HeLa nuclear extract were carried out by the method of Dignam et al. (5). Primer extension analysis was performed as previously described (9). The oligonucleotide primer for PEPCK transcripts corresponded to the sequence from +51 to +70 nucleotides (5′-GACTCAGAGCGTCTCGCCGG-3′) in the PEPCK gene, and the primer for adenovirus transcripts hybridized to the sequence from +20 to +39 nucleotides (5′-AGGAGAGTAGGGTGGTATA-3′) from the 5′ end of the G-free cassette.

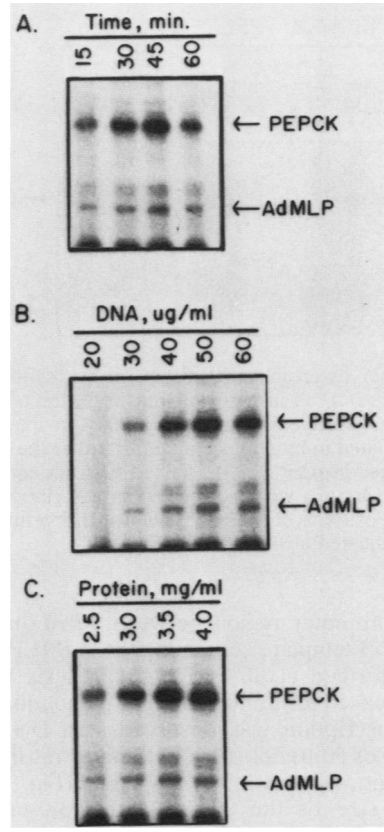

FIG. 1. Primer extension analysis of mRNA synthesized in vitro from PEPCK and AdMLPs. The −490 to +73 region of the PEPCK promoter in pΔ5′-PCCAT was mixed with an equal weight of pML(C2AT) and incubated with rat liver nuclear extract in reactions performed as described in Materials and Methods, except that the reactions were incubated for the times indicated above each lane (A), the total concentration of DNA in each reaction was as shown above each lane (B), and the concentration of rat liver nuclear protein in each reaction was as indicated above each lane (C). The positions of the PEPCK and AdMLP primer extension products are indicated at the right.

RESULTS

Basic parameters of PEPCK transcription in vitro. Chimeric genes containing regions of the PEPCK promoter linked to the chloramphenicol-acetyltransferase structural gene contained in circular DNA templates were added to a transcription assay system containing rat liver nuclear extracts, and the RNA synthesized was quantitated by primer extension analysis. Plasmid pML(C_2AT) containing the AdMLP was included in reactions to demonstrate the transcriptional competency of nuclear extracts and to correct for variations in reaction conditions. To ensure that the results were not artifacts of a particular preparation of nuclear extract or template DNA, each experiment was repeated with at least two different nuclear extracts and template preparations. The variation between experiments was less than 10%. With this system, we detected transcription from

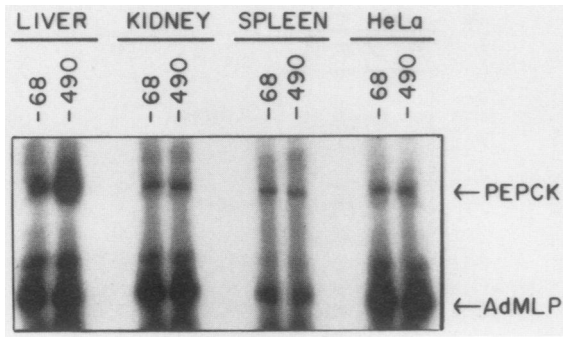

FIG. 2. Primer extension analysis of mRNA synthesized from the PEPCK and AdMLPs in vitro with nuclear extracts from various sources. Nuclear extracts from rat liver, kidney, or spleen and HeLa cells were incubated in reactions containing either the −68 or −490 PEPCK promoter in pΔ5′-PCCAT mixed with an equal weight of pML(C2AT). Reactions were performed as described in Materials and Methods. Positions of PEPCK and AdMLP primer extension products are indicated at the right.

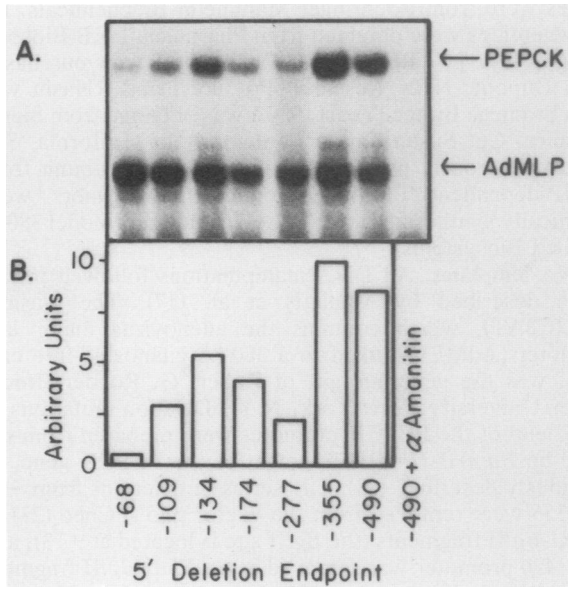

FIG. 3. In vitro transcription from 5′ deletion mutations of the PEPCK promoter. (A) Primer extension analysis of mRNA synthesized in reactions performed with rat liver nuclear extract as described in Materials and Methods. Each reaction contained 50 μg of DNA per ml [composed of equal amounts (by weight) of pML(C2AT) and pΔ5′-PCCAT containing the PEPCK promoter truncated at the 5′ endpoint indicated below the figure]. The reaction in lane 8 was the same as that in lane 7 but contained 2 μg of alpha-amanitin per ml. The positions of PEPCK and AdMLP primer extension products are indicated at the right. (B) Relative levels of transcription from the PEPCK promoter deletions as determined by densitometry of the data shown in panel A. The levels were corrected for differences in transcription from the AdMLP.

the PEPCK promoter region between −490 and +73 and from the control template containing the AdMLP (Fig. 1). In reactions performed at 30°C, RNA synthesis from either promoter increased for up to 45 min of incubation (Fig. 1A). Optimum transcription was observed at a DNA template concentration of 50 μg/ml (Fig. 1B) and at rat liver nuclear protein concentrations of 3.5 to 4.0 mg/ml (Fig. 1C). On the basis of the size of the primer extension products (70 nucleotides for PEPCK and 50 nucleotides for AdMLP), we conclude that the transcripts were properly initiated at the in vivo CAP site. Transcription was inhibited by 2 μg of alpha-amanitin per ml, indicating that the RNA synthesis was done by RNA polymerase II (see Fig. 3, lane 8).

We examined the tissue-specific transcription pattern of the PEPCK promoter to learn if it would be amenable to in vitro transcription analysis. Nuclear extracts were prepared from two tissues that express the PEPCK gene (rat liver and kidney) and from two sources in which the gene is not expressed (rat spleen and HeLa cells). All the extracts were transcriptionally competent, as evidenced by transcription from the AdMLP (Fig. 2). Both the intact PEPCK promoter and the promoter truncated at −68 were transcribed in the extracts tested. However, the level of PEPCK gene transcription was higher in the presence of liver nuclear extracts than in the presence of nuclear extracts prepared from kidney, spleen, or HeLa cells. Moreover, the level of transcription from the intact PEPCK promoter was higher than transcription from the −68 deletion when nuclear protein from rat liver was used, whereas approximately equal levels of transcription from the two promoter regions were observed with extracts from other sources. Nuclei from kidney, spleen, and HeLa cells apparently lack a factor present in liver nuclei that is required for higher levels of transcription from the intact PEPCK promoter, since mixing an equal volume of liver nuclear extract with any of the other extracts resulted in a level of transcription comparable to that noted with liver extract alone (data not shown). Thus, with the exception of proteins isolated from kidney nuclei, the differential transcription of the 490-bp promoter region in these extracts mimics the in vivo expression of PEPCK in the tissues from which they were prepared.

The influence of promoter elements on PEPCK gene transcription was examined with a series of 5′ deletion mutations of the promoter (Fig. 3). Levels of transcription were determined by densitometry of autoradiograms of primer extension products and were corrected for differences in the transcription of the internal control template containing the AdMLP. The level of transcription from the intact PEPCK promoter (−490) was approximately 17-fold higher than that observed with the −68 deletion, which contains the TATA sequence alone. A slightly higher level of transcription was observed when the promoter was truncated at −355; this level was approximately 20% higher than the level of transcription from the intact promoter. The level of gene transcription was markedly lower for the PEPCK promoter deleted to −277, which was only 27% of the intact promoter. Interestingly, rather than decreasing transcription further, deletion of the promoter to −174 increased RNA synthesis over that seen with the deletion at −277, and the −134 deletion directed still higher levels of transcription. The removal of an additional 25 bp from the promoter (−109 deletion) decreased transcription to only 33% of the level noted with the intact promoter. Transcription from the AdMLP varied only slightly among the reactions. These results suggest that elements throughout the 490-bp region of the PEPCK promoter influence the basal rate of gene transcription.

Recent DNase I footprinting analysis of the PEPCK promoter in the presence of rat liver nuclear extracts revealed eight regions (in addition to the TATA sequence) protected from digestion (22) (Fig. 4C). Briefly, the TATA box is located approximately 30 bp upstream of the transcription start site, and two putative CREs (designated

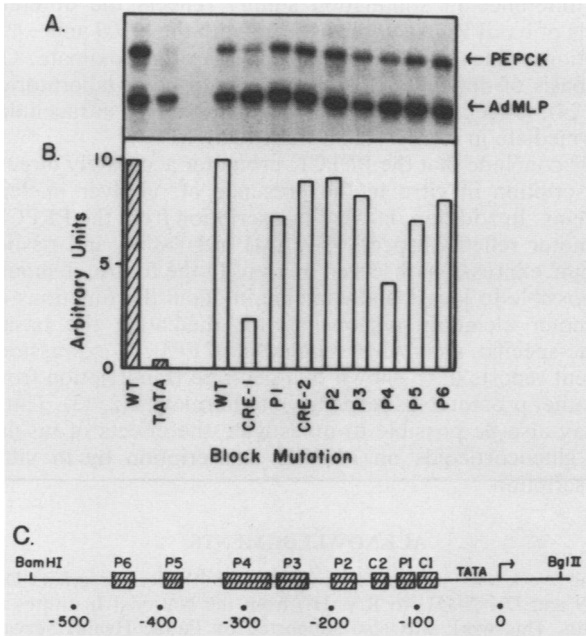

FIG. 4. In vitro transcription from a set of block mutations in the PEPCK promoter. (A) Primer extension analysis of mRNA synthesized in reactions containing rat liver nuclear extract and performed as described in Materials and Methods. Equal amounts (by weight) of each PEPCK template and pML(C2AT) were used in each reaction. The PEPCK promoter mutations were contained in either a pBR322-based plasmid (lanes 1 and 2) or a pTZ-based plasmid (lanes 3 through 11). The site of each block mutation is indicated below the figure. The positions of PEPCK and AdMLP primer extension products are at the right. (B) Relative levels of transcription from the PEPCK promoter block mutations determined by densitometry and corrected for differences from those of the AdMLP. (C) Diagram of the PEPCK promoter from −540 to +73. The approximate locations of protein-binding regions detected by DNase I footprinting with rat liver nuclear extracts are shown (▨). The two putative CREs are labeled C1 and C2, and other sites are labeled P1 through P6. The TATA sequence was detected by footprinting with fractionated extracts.

CRE-1 and CRE-2) are located at −96 to −77 and −155 to −130, respectively. The P1 region at −123 to −99 has significant homology to the NF-1–CTF consensus sequence. Five other protected areas (designated P2 through P6) were also detected in this region of the promoter.

To assess the effects of individual promoter elements on transcription, we prepared a set of block mutations specifically introduced within each of the eight protein-binding domains identified by DNase I footprinting studies (22) and the TATA box of the PEPCK promoter (Fig. 4). Each of the mutations decreased PEPCK gene transcription, but the reductions in RNA synthesis were not equivalent. Mutation of the P2 or CRE-2 elements reduced transcription by only 8 and 10%, respectively, while mutation of P1, P3, P5, or P6 reduced transcription by approximately 17 to 30%. The greatest reductions of PEPCK gene transcription occurred with mutation of the CRE-1, P4, or TATA element. Transcription from the promoter mutated at P4 was 42% of that from the unmutated promoter, and mutation of CRE-1 decreased RNA synthesis to 24% of that from the intact promoter. Only 4% of the transcriptional activity of the intact promoter remained when the TATA sequence was mutated.

Regulation of PEPCK transcription in vitro. Yamamoto et al. have shown that treatment of PC-12 cell nuclear extracts with the catalytic subunit of PK-A stimulates transcription from templates containing the CRE of the somatostatin promoter (24). In a similar fashion, we preincubated rat liver nuclear extracts with the catalytic subunit of PK-A for 5 min prior to measuring transcription. This treatment did not affect transcription from the AdMLP or the −68 PEPCK promoter deletion, which contains only the TATA sequence (Fig. 5). However, transcription from the −109 PEPCK promoter deletion, which contains the TATA box and the proximal CRE, was stimulated ninefold by the catalytic subunit of PK-A. Gene transcription from the intact PEPCK promoter was increased twofold by this treatment, which roughly corresponds to the increase in PEPCK expression following treatment with cAMP noted in transient expression systems (23).

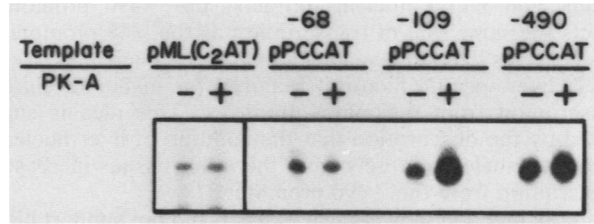

FIG. 5. Effects of treating rat liver nuclear extracts with the catalytic subunit of PK-A on transcription from the PEPCK promoter. Primer extension analysis of mRNA synthesized in reactions containing rat liver nuclear extract is shown. The extract was treated with 20 μM ATP alone (−) or with ATP plus 1 μg of catalytic subunit of PK-A (+) per ml for 5 min before transcription reactions were initiated. Reactions were performed as described in Materials and Methods and contained 50 μg of pML(C2AT) per ml or the −68, −109, or −490 PEPCK promoter deletion in pΔ5′-PCCAT as shown.

DISCUSSION

The PEPCK promoter provides an excellent system for studying the control of gene expression by various metabolic signals. Several studies have shown that 500 bp of the promoter immediately 5′ to the start site of transcription mediates the effects of cAMP, glucocorticoids, insulin, and tissue-specific and developmental regulation (7, 12, 16, 18, 23). Specific promoter sequences, −94 to −77 and −468 to −420, have been shown to confer some regulation by cAMP (1, 21) and glucocorticoids (20), respectively. However, the precise locations of promoter sequences that mediate the effects of insulin, tissue specificity, and developmental regulation have not been identified. Likewise, the functional significance of the promoter elements detected by DNase I footprinting, distal to CRE-1, has not been determined. In this study, we employed the in vitro transcription system developed by Gorski et al. (6) to examine several aspects of PEPCK transcription. This system provides a rapid technique for evaluating gene transcription under a variety of carefully controlled conditions.

As has been shown for the promoters for mouse albumin (6) and rat alpha-1-antitrypsin genes (14), in vitro transcription from the PEPCK promoter occurs in a tissue-specific manner. The −68 PEPCK promoter, which contains the TATA homology alone, is transcribed by the core transcriptional machinery of all the nuclear extracts. In liver nuclear extract, the −490 promoter is transcribed at a higher level than the −68 promoter under the influence of promoter elements distal to the TATA sequence. However, in kidney,

spleen, and HeLa nuclear extracts, the −490 promoter directs the same rate of transcription as the −68 promoter. This suggests that kidney, spleen, and HeLa nuclear extracts lack a liver-specific factor(s) required for higher levels of transcription from the intact promoter. This idea is supported by the observation that the addition of liver nuclear extract to nuclear extracts from the other tissues increases transcription from the −490 promoter.

Interestingly, kidney nuclear extracts did not support high levels of transcription from the −490 promoter region relative to the −68 deletion, since the endogenous PEPCK gene is highly expressed in the kidney. A chimeric PEPCK-bovine growth hormone gene containing the −460 PEPCK promoter region is also highly expressed in the kidneys of transgenic animals (18). Thus, our inability to demonstrate kidney-specific in vitro transcription from the −490 promoter region suggests that a factor(s) required for high levels of transcription in the kidney is lost during the preparation of the nuclear extract.

An important feature of our in vitro transcription analysis was the use of both 5′ deletion mutations and block mutations of the PEPCK promoter. The mutation analysis indicates that several areas of the promoter influence basal transcription, and the levels of RNA synthesis observed with the deletion mutations in vitro closely reflect the levels of transcription observed when these deletions were tested by stable transfection into hepatoma cells in culture (23). We also noted some underlying similarities between the two mutational analyses. For example, deletion of the promoter sequences between −109 and −68 and between −355 and −277 produced significant decreases in transcription. These deletions correspond to block mutations, i.e., CRE-1 and P4, respectively, that also decrease the transcriptional activity of the PEPCK promoter. Likewise, deletions that result in increased transcription, such as −174 to −134, −277 to −174, and −490 to −355, correspond to block mutations P1, P2, P3, P5, and P6, which have minimal effect on PEPCK transcription. The differences between the two assays probably reflect the differences between deletion and block mutations. Significant portions of the promoter are lost when deletions are created, whereas block mutations maintain the overall integrity of the promoter.

Although mutation of most of the promoter elements reduced transcription from 10 to 60%, mutation of CRE-1 reduced transcription by 75%, and only mutation of the TATA sequence resulted in a greater reduction in PEPCK gene transcription. CRE-1 is clearly an important basal transcription element. The role of CRE-1 in basal PEPCK transcription has also been demonstrated by Quinn et al. (21), who showed that mutation of CRE-1 reduced basal transcription in transient transfection studies. Regulation of basal transcription appears to be a common characteristic of CREs and has also been reported for the promoter for the alpha-subunit of the human glycoprotein hormone gene (3, 4, 8).

Evidence also suggests that CRE-1 mediates cAMP stimulation of PEPCK transcription. Although several elements may be involved in cAMP regulation, we showed that CRE-1 specifically mediates enhanced transcription by nuclear extracts treated with the catalytic subunit of PK-A. The CRE-1 element has also been shown to confer cAMP inducibility on a heterologous promoter (1). Interestingly, we found that the promoter region containing CRE-1 and the TATA box (−109 deletion) was stimulated to a greater degree than the intact promoter. Perhaps the activity of CRE-1 is attenuated by distal promoter elements in the intact promoter, or perhaps the difference in stimulation simply reflects the different levels of basal RNA synthesis from both the −109 and −490 deletions, since the stimulated levels are approximate. On the basis of our findings and those from other laboratories (19, 24), it seems likely that PK-A is the direct intracellular intermediate in cAMP signal transduction.

We conclude that the PEPCK promoter accurately directs transcription in vitro in the presence of rat liver nuclear proteins. In addition, in vitro transcription from the PEPCK promoter reflects aspects of cAMP regulation and tissue-specific expression observed in vivo. In the future, it should be possible to locate and examine in detail the functions of promoter elements responsible for mediating the basal, tissue-specific, and cAMP regulation of PEPCK expression. Recent reports have shown that cell-free transcription from the other promoters is responsive to hormones (2, 13). Thus, it may also be possible to investigate the effects of insulin and glucocorticoids on PEPCK transcription by in vitro transcription.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants DK 21859 and DK 24451 (to R.W.H.) from the National Institutes of Health. This work was also supported by Public Health Service Metabolism Training Program grant DK 07319-11 (to D.J.K. and E.A.P.) from the National Institutes of Health and by a Postdoctoral Fellowship from the Medical Research Council of Canada (to W.J.R.).

We thank Jack Thatcher for his technical assistance in preparing nuclear extracts. Finally, we thank David Samols for reading the manuscript and sharing valuable ideas on its content.

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