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Identification of Basal and Cyclic AMP Regulatory Elements in the Promoter of the Phosphoenolpyruvate Carboxykinase Gene

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Promoter elements important for basal and cyclic AMP (cAMP)-regulated expression of the phosphoenolpyruvate carboxykinase (PEPCK) gene have been identified by analysis of a series of PEPCK promoter mutations in transfection experiments. Fusion genes containing wild-type and mutated PEPCK promoter sequences from −600 to +69 base pairs (bp) fused to the coding sequence for chloramphenicol acetyltransferase were studied. Internal deletion mutations that replaced specific bases with a 10-bp linker within the region from −129 bp to −18 bp of the PEPCK promoter were examined. In addition, wild-type and mutated DNA templates were used as probes in DNase I protection experiments to determine sites of protein-DNA interaction. The PEPCK promoter contains a binding site for nuclear factor 1-CAAT. Deletion of the 5′ end of this binding site reduced the size of the DNase I footprint in this region but had no effect on promoter activity. In contrast, deletion or disruption of the 3′ end of this binding site completely eliminated protein binding and reduced promoter activity by 50%. Deletion of core sequences of the cAMP regulatory element (CRE) resulted in loss of cAMP responsiveness and an 85% decrease in basal promoter activity, indicating that the CRE also functions as a basal stimulatory element. Mutation of the core sequence of the CRE resulted in loss of the DNase I footprint over the CRE. Internal deletions flanking the CRE showed no loss of induction by cAMP but did have reduced promoter activity. This delimits the CRE to an 18-bp region between nucleotides −100 and −82. Analysis of mutations that disrupted bases between the CRE and the initiation site identified a basal inhibitory element adjacent to a basal stimulatory element, both located just 3′ of the CRE, as well as a basal stimulatory element coincident with the TATA consensus sequence centered at −27. These data demonstrate that several *cis*-acting elements are located within 130 nucleotides of the initiation site of the PEPCK gene and that the CRE is essential for both basal promoter activity and cAMP-regulated expression of this gene.

Analysis of the expression of fusion genes, in which the coding sequence of a reporter gene is placed under the control of putative regulatory elements of a test gene, has resulted in the identification of various *cis*-acting elements of eucaryotic and viral genes which interact with *trans*-acting factors to control transcription in mammalian cells (8, 28, 30). The two classes of *cis*-acting elements which have been identified are termed promoter and enhancer elements. Promoter elements are characterized by their dependence on position and orientation with respect to the transcription start site (8, 28, 30). These are typified by the CAAT, SP1, and TATA elements. Enhancer elements are characterized by their ability to exert effects relatively independently of distance from and orientation to the transcription start site (41). Enhancers may be constitutively active, as in the cases of the simian virus 40 enhancer (34) and the basal-level enhancers of the human metallothionein IIa gene (16, 40), or they may be regulatable, as exemplified by the metal-regulatory elements of the human metallothionein IIa gene (22, 23) and the steroid hormone response elements (22, 23, 37). Typically, deletion of a regulatable enhancer sequence affects regulated expression but has little or no effect on basal expression of the gene.

The gene encoding phosphoenolpyruvate carboxykinase (PEPCK) is regulated at the transcriptional level by a number of hormones (3, 10, 24, 39). PEPCK gene transcription in H4IIE hepatoma cells is induced by treatment with cyclic AMP (cAMP) or with glucocorticoids (39), and both basal and hormone-induced transcription of PEPCK is inhibited by treatment with insulin (10, 39) or phorbol esters (3). The effects of these agents are detectable within 5 min and in the absence of protein synthesis, which indicates that they act directly on gene transcription, probably by controlling the rate of initiation (39a). A PEPCK-CAT fusion gene that includes promoter sequences between −600 and +69 of the PEPCK gene fused to the bacterial reporter chloramphenicol acetyltransferase (CAT) gene contains *cis*-acting elements that mediate the effects of cAMP, glucocorticoids, and insulin (26). Studies by Short et al. (42) had shown that the PEPCK cAMP regulatory element lies within bases −108 to −62 and has enhancerlike properties.

An understanding of the control of basal expression of PEPCK gene transcription is needed to comprehend how hormones modify this expression. In the present study, a series of internal deletion mutations in the promoter of PEPCK were used to determine which regions of DNA are important for basal and cAMP-regulated expression of PEPCK-CAT fusion genes. Some of these elements contained DNA sequences similar to consensus sequences for known *cis*-acting regulatory elements and DNA-binding proteins. In addition, the binding of *trans*-acting factors to these *cis*-acting elements was examined by DNase I footprint analysis of wild-type and mutant promoters.

MATERIALS AND METHODS

PEPCK promoter internal deletion mutations. The construction of a series of 5′ and 3′ deletion mutations used to prepare the internal deletion mutations tested in this study has been described in detail (37). Briefly, *Sal*I-*Kpn*I fragments were isolated from a series of 5′ deletion mutations by

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restriction digestion and gel electrophoresis and inserted into the same sites in a series of 3′ deletion mutations. This resulted in reconstruction of the entire promoter region of PEPCK from −600 to +69, except for those bases between the 5′ and 3′ endpoints of the deletions used. The missing bases are replaced by the 10-base-pair (bp) linker 5′-GGGTC GACCC-3′, which contains a *Sal*I recognition site. The endpoints of the deletions used were determined by dideoxy sequence analysis (45). The wild-type promoter used for this study was pPL9CAT, which includes all PEPCK promoter sequences from −600 to +69 bp. All DNA manipulations were done by standard techniques (27), and *Escherichia coli* HB101 was transformed by the method of Hanahan (15). Plasmids were isolated by alkaline lysis of *E. coli* (2) and repeated isopycnic centrifugation in CsCl-ethidium bromide gradients to obtain supercoiled plasmid DNA (38).

Cell culture and transfection. CV-1 cells, obtained from the American Tissue Type Culture Collection, were grown in Earle modified Eagle medium containing 10% (vol/vol) serum (5% calf bovine, 3% newborn bovine, and 2% fetal bovine). Cells from a 150-cm^2 flask were harvested by trypsinization, pelleted by centrifugation, and suspended in 2 ml of a calcium phosphate-DNA coprecipitate containing 50 μg of PEPCK-CAT plasmid plus 10 μg of pCH110, an SV2–β-galactosidase plasmid (14). The cells were incubated for 30 min at room temperature and then divided into three 75-cm^2 flasks containing medium supplemented with 1/20 of the volume of $CaCl_2$ and HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline (pH 7.10) that was used to prepare precipitates (4, 21) and 12.5 μM chloroquine (25). Five hours later, the cells were treated with 20% dimethyl sulfoxide for 5 min and then washed and incubated in fresh medium until harvested. Where indicated, 8-(4-chlorophenylthio)-cAMP was added to a concentration of 0.1 mM during the last 8 h. Cells were harvested at 16 and 24 h after dimethyl sulfoxide treatment. Lysates for analysis of reporter enzyme activities were prepared by sonication of the cells followed by centrifugation to remove cellular debris. β-Galactosidase activity was determined as described previously (17). CAT activity was determined under standard conditions as described previously (9) and quantified by scintillation spectrometry. CAT activity was normalized for β-galactosidase activity for each plasmid and then expressed as a percentage of wild-type expression as determined for pPL9 in that experiment.

RNase protection assay. A single-stranded radiolabeled probe was generated by incubation of a plasmid containing a PEPCK-CAT gene fragment (p76FCAT) with SP6 polymerase in the presence of 100 μM ATP, UTP, and CTP plus 100 μCi (~12 μM) of [α-^{32}P]GTP (31). The plasmid p76FCAT was constructed by subcloning the fragment of a PEPCK-CAT fusion gene corresponding to sequences −65 of PEPCK through +150 of CAT into the SP6 expression vector pIBI76. When transcribed by SP6 polymerase, an RNA probe complementary to PEPCK-CAT mRNA is generated. When hybridized to correctly initiated PEPCK-CAT mRNA and digested with RNases A and T_1, a protected hybrid 227 bp in length is generated. Cells were transfected as above in duplicate and harvested after 24 h for the preparation of total RNA. Cells were scraped into a solution of 50 mM sodium acetate containing 1% sodium dodecyl sulfate (SDS), which was added to an equal volume of phenol equilibrated to 65°C, vortexed vigorously, and incubated at 65°C for 15 min. The tubes were inverted once, incubated on ice for 15 min, and inverted again, and the phases were separated by centrifugation. The aqueous phase was then extracted with phenol, phenol-chloroform, and chloroform, and nucleic acids were concentrated by precipitation with ethanol. The RNA was then treated with DNase I (20 μg/ml) for 15 min at room temperature in the presence of RNasin (500 U/ml), extracted with phenol-chloroform, and precipitated with ethanol. DNase I treatment was repeated to remove plasmid DNA contaminants, which contributed to background signals in the assay. For the RNase protection assay, 15 μg of total RNA from transfected cells was incubated with 500,000 cpm of a ^{32}P-labeled complementary RNA probe at 65°C for 18 h in a total volume of 20 μl of hybridization buffer, as described by Melton et al. (31). The hybrids were then digested with RNase A (50 μg/ml) plus RNase T_1 (20 U/ml) for 1 h at 37°C, followed by incubation with 0.1% SDS–80 μg of proteinase K per ml for 15 min at 37°C. Following extraction with phenol and precipitation with ethanol, the samples were dissolved in formamide loading buffer, heated to boiling for 2 min, and analyzed by electrophoresis in a 6% polyacrylamide–7 M urea gel. The products of a Maxam and Gilbert sequencing reaction (29) were run in adjacent lanes to determine sizes.

DNase I footprinting. A rat liver nuclear extract was prepared by a modification of an existing method (11). Nuclei were sedimented through 2.1 M sucrose, washed with 0.1 M NaCl, and then extracted with 0.35 M NaCl. Ammonium sulfate was added to a final saturation of 50%, and the precipitate was collected by centrifugation. The protein pellet was suspended in a small volume of buffer (20 mM Tris hydrochloride [pH 8.0], 0.2 mM EDTA, 10% glycerol, 2 mM β-mercaptoethanol), dialyzed twice against 2 liters of the same buffer, and stored at −70°C until use. For DNase I footprinting experiments, 1.5 to 15 μg of nuclear extract was incubated with 0.2 ng (~10,000 cpm) of an *Mst*II-*Bgl*II (−212 to +69) PEPCK promoter fragment labeled at the 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP plus 2 μg of poly(dI-dC) · poly(dI-dC) in 20 mM Tris hydrochloride (pH 7.5)–5 mM $MgCl_2$–1 mM $CaCl_2$. After 15 min of incubation at room temperature, 2 ng of DNase I was added and incubated for 90 s. DNase I digestion was stopped by the addition of 80 μl of stop buffer (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA, 0.1% SDS, proteinase K [100 μg/ml]). After being incubated for 1h, nucleic acids were precipitated with ethanol, and the samples were analyzed by electrophoresis in a 6% polyacrylamide–7 M urea gel together with the products of Maxam and Gilbert sequencing reactions (29).

RESULTS

Construction of PEPCK-CAT vectors with promoter mutations. The strategy used for constructing internal deletion mutations of the PEPCK promoter in PEPCK-CAT fusion genes is illustrated in Fig. 1. A series of 5′ deletion mutations was constructed by Bal 31 digestion of the PEPCK promoter in PEPCK-CAT fusion vectors (pPLFCAT series). A series of 3′ deletion mutations was constructed in an analogous fashion (pPLGCAT series). The termini of these deletion mutations were determined by sequence analysis, and all were adjacent to a *Sal*I restriction enzyme recognition site. *Sal*I-*Kpn*I fragments of the 5′ deletion series were isolated from appropriate pPLFCAT vectors and inserted in the same sites in the 3′ deletion series (pPLGCAT vectors), resulting in internal deletion mutations of the PEPCK promoter, the pIDCAT series. These mutations maintain all other promoter elements 5′ and 3′ of the deletion, and the deleted bases are replaced by a 10-bp linker which includes a *Sal*I site. Screening of the 5′ and 3′ deletion series focused on the

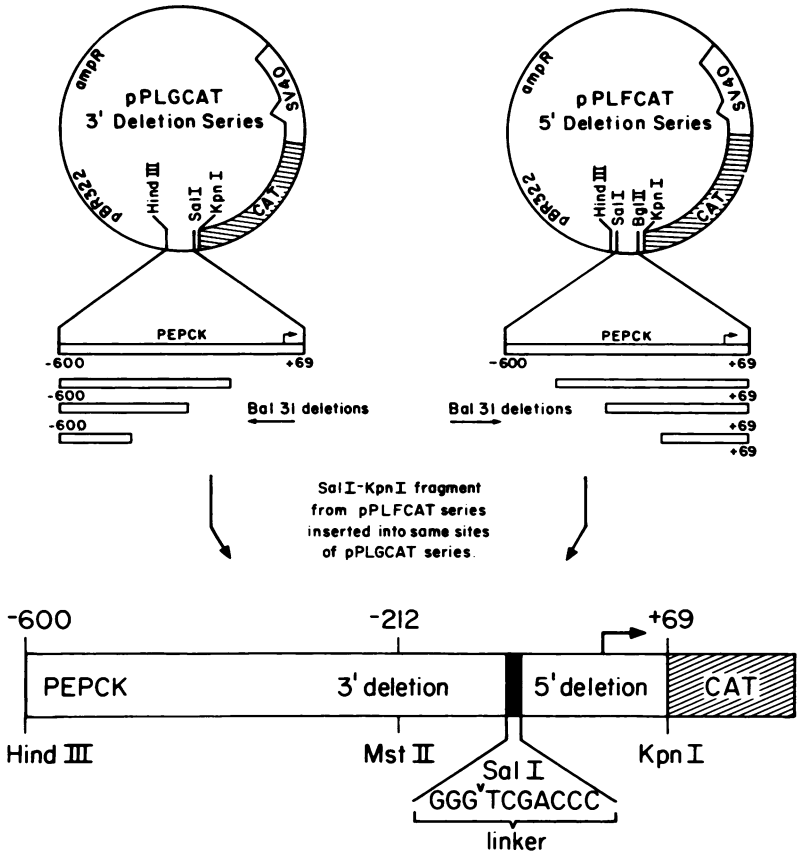

FIG. 1. Construction of PEPCK-CAT promoter internal deletion mutation vectors. A schematic view is depicted of the methods used to construct 5′ and 3′ deletion mutations and the combination of plasmid fragments to generate the internal deletion mutations. Internal deletion mutation vectors reconstitute the entire promoter region of the PEPCK gene from −600 to +69 on either side of the deleted sequences, which are replaced by the 10-bp linker containing a *Sal*I site, as indicated.

promoter region immediately upstream of the transcription start site (−130 to +1), so that a series of mutations containing small, overlapping deletions could be analyzed. Analysis by sequence comparison of the promoter region of the PEPCK gene had shown that this region, located within 130 nucleotides of the start site of transcription, contained the putative recognition sequences for nuclear factor 1 and/or CAAT binding factors (NF1/CAAT), the cAMP regulatory element, and the TATA promoter element.

Because the hormonal responses of the endogenous PEPCK gene are maintained in H4IIE cells, previous studies of PEPCK-CAT fusion genes have used this hepatoma cell line (26, 37, 42). However, these cells cannot be transfected efficiently enough to allow an analysis of basal promoter activity. Therefore, CV-1 cells were used for this study because of their relative efficiency of transfection (~200-fold-greater signal than in H4IIE cells) and their ability to respond to cAMP (5). H4IIE cells exhibited qualitatively similar changes to CV-1 cells when transfected with pID3 and pID4, which showed decreased and increased promoter activity, respectively (data not shown). Cells were cotransfected in suspension with 50 μg of supercoiled PEPCK-CAT plasmid plus 10 μg of a pSV2–β-galactosidase plasmid to correct for differences in transfection efficiency.

Effect of mutation of NF1/CAAT recognition sequences. The PEPCK promoter contains a sequence very similar to the recognition sites for nuclear factor I (NF1), 5′-TGGN$_{6-7}$G CCAA-3′, where N$_{6-7}$ indicates variable nucleotides (12, 20);

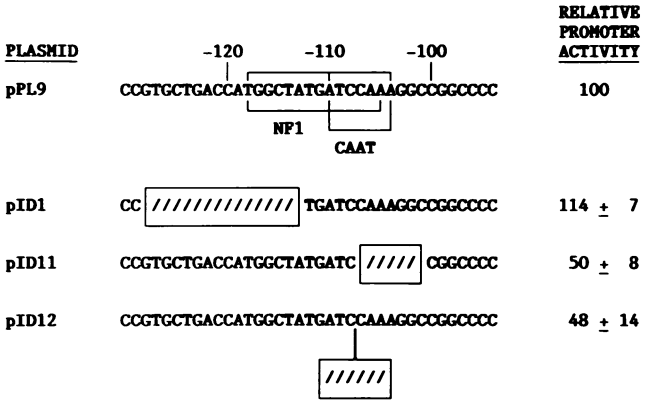

FIG. 2. Effects of mutations of the NF1/CAAT recognition sequence on basal promoter activity. CV-1 cells were cotransfected with the indicated PEPCK-CAT plasmid and the SV2–β-galactosidase plasmid as described in Materials and Methods. The canonical NF1 and CAAT recognition sequences are indicated by the boxed sequences. The hatched bars indicate the deleted sequences in pID1 and pID11 which were replaced by the linker sequence 5′-GGGTCGTACCC-3′ and indicate the site of insertion of this linker sequence in pID12. CAT activity was determined and normalized to the β-galactosidase activity to control for transfection efficiency as described in Materials and Methods. The promoter activity of each plasmid is shown as the mean ± SEM relative to that of the wild-type plasmid, pPL9, determined in three to five independent experiments.

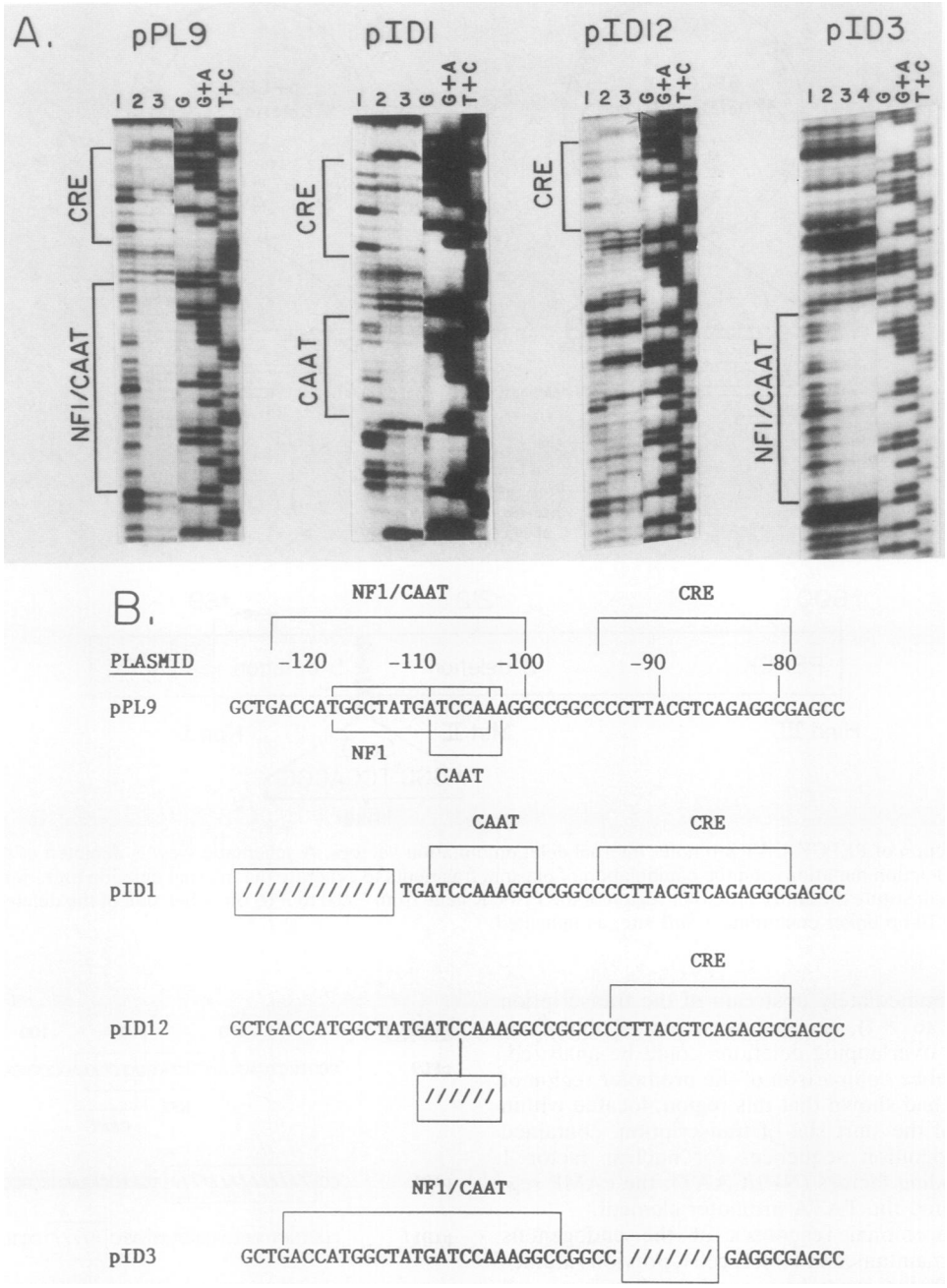

FIG. 3. Effects of mutations in the NF1/CAAT and CRE recognition sequences on DNase I footprint analysis. *Mst*II-*Bgl*II fragments of the wild-type, pPL9, and mutated promoter plasmids were radiolabeled at the 5′ end and incubated with rat liver nuclear extract prior to digestion with DNase I as described in Materials and Methods. (A) The DNase I digestion pattern in the absence and presence of nuclear extracts of wild-type and mutated promoter fragments is shown. In each case, lane 1 shows the digestion pattern in the absence of added protein, whereas lanes 2 to 4 show the pattern in the presence of increasing amounts of nuclear extract (pID1 and pID12: lane 2, 3 μg; lane 3, 15 μg; pID3: lane 2, 1.5 μg; lane 3, 5 μg, lane 4, 7.5 μg of protein). Boundaries of the protected regions were determined from Maxam-Gilbert sequencing reaction products of each DNA probe and are indicated by brackets. (B) Sequences of the wild-type and mutated promoters. Recognition sequences are indicated by the boxed sequences. The footprints indicating areas of protection from DNase I digestion are indicated by brackets above each sequence. The hatched bars indicate bases replaced by the linker sequence 5′-GGGTCGACCC-3′.

CAAT transcription factor (CTF), AGCCAA (20); and the CAAT box-binding protein (CBP), CCAAT (11). We examined promoter mutations in which only the 5′ portion (pID1) or the 3′ end (pID11 and pID12) of the NF1/CAAT site was disrupted in order to determine the role any of these sequences play in PEPCK gene transcription. Deletion of the 5′ terminus of the NF1 site (pID1) had no effect on basal promoter activity (Fig. 2). In contrast, disruption of the 3′ terminus of the NF1/CAAT site by deletion of the consensus sequence (pID11) or by insertion of a 10-bp linker within the consensus sequence (pID12) markedly reduced PEPCK promoter activity (Fig. 2). It should be noted that the linker

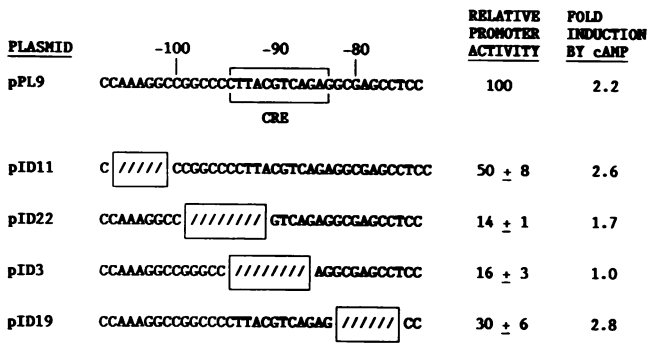

FIG. 4. Effects of mutations in the promoter region near the CRE on basal and cAMP-regulated promoter activity. CV-1 cells were cotransfected with the indicated PEPCK-CAT fusion gene plasmids and the pSV2–β-galactosidase plasmid, and cell portions were treated with 0.1 mM 8-(4-chlorophenylthio)-cAMP for the last 8 h of incubation, as described in Materials and Methods. The data presented are the mean ± SEM, determined for four to six independent experiments, of the promoter activity relative to that of the wild-type plasmid pPL9, and the fold induction by cAMP.

sequence maintains the CCAAA sequence motif homologous to the CBP-binding site in pID12 but not in pID11. Therefore, it seems unlikely that the sequence being utilized is the CCAAT recognition sequence for CBP.

The effects of these mutations on DNA-protein interactions were examined by analyzing the DNase I protection patterns of wild-type (pPL9) and mutated (pID1 and pID12) promoter fragments. As illustrated in Fig. 3, incubation of wild-type promoter fragments with a rat liver nuclear extract resulted in two protected regions, or footprints, centered over the consensus sequences for NF1/CAAT and the cAMP regulatory element (CRE). Disruption of the 5′ end of the consensus sequence for NF1 in pID1 resulted in a reduction in size of the footprint region in the mutated promoter, with protection being limited to the AGCCAA sequence motif. In contrast, disruption of the 3′ end of the consensus sequence by linker insertion in pID12 (or in pID11 [unpublished data]) resulted in a lack of protection of the entire NF1 region. This loss of protection was selective, because the footprint over the CRE was maintained in both NF1/CAAT mutations (pID1 and pID12). Conversely, in the deletion mutation of the CRE (pID3), NF1/CAAT binding was unaffected. Thus, the analysis of DNA-protein interactions corroborated the functional data obtained through transfection analysis which suggest that the ATCCAA sequence motif plays an important role in PEPCK promoter function, whereas the NF1 and CBP recognition sequences do not.

Effect of mutation of CRE sequences. Short et al. (42) provided evidence that the CRE is contained within nucleotides −108 to −62 of the PEPCK gene and, by comparison with sequences of other cAMP-regulated genes, derived the consensus sequence, 5′-CTTACGTCAGAG-3′. Subsequent analysis of other cAMP-responsive genes has verified the existence of similar sequences within functional CREs (5, 6, 33, 43, 44). Internal deletion mutations surrounding this CRE consensus sequence were analyzed for their effects on basal and cAMP-stimulated PEPCK-CAT expression (Fig. 4). The basal expression of two vectors containing mutations which disrupt the CRE consensus sequence, pID22 and pID3, was reduced by 85% from that of the wild-type promoter, pPL9. In addition, these mutations in the CRE resulted in a loss of responsiveness to cAMP. Mutations that disrupted sequences located 5′ of the CRE core (pID11) and 3′ of the CRE core (pID19) maintained cAMP responsiveness and exhibited lesser reductions in basal promoter activity. All other internal deletion mutations examined in this study also maintained cAMP responsiveness (data not shown). These data indicate that the PEPCK CRE is contained within nucleotides −100 to −82 and also that the region containing the CRE is a very potent basal stimulatory element in the PEPCK promoter. The CRE core was protected from DNase I digestion by rat liver nuclear extract (Fig. 3). Footprinting of the CRE was abolished in the internal deletion mutation of the CRE (pID3, Fig. 3), but the NF1/CAAT footprint was maintained, demonstrating that, at least in vitro, nuclear factors bind independently to these regulatory regions.

Determination of mRNA initiation sites of PEPCK-CAT fusion genes. An RNase protection assay was performed to determine the initiation site(s) of mRNAs synthesized from wild-type and mutant PEPCK-CAT fusion genes. Total RNA, harvested from transfected cells, was hybridized with a radiolabeled RNA probe spanning the start site and 65 bp of the promoter region and then digested with RNases A and T_1. As shown in Fig. 5, the majority of mRNA was initiated correctly in cells expressing the wild-type plasmid, pPL9 (lane 1). In cells expressing the CAAT deletion mutation, pID11 (lane 2), the majority of mRNA was also correctly initiated, whereas no mRNA was detectable in cells expressing the CRE mutation, pID3 (lane 3). Minor bands corresponding to initiation at nucleotides −19 and −45 were also detected. No significant bands were observed at a position corresponding to readthrough transcripts, which would appear, due to the length of the probe, to be initiated at −65. In a separate experiment, when mRNA from the internal deletion of the TATA box (pID8) was analyzed, most of the

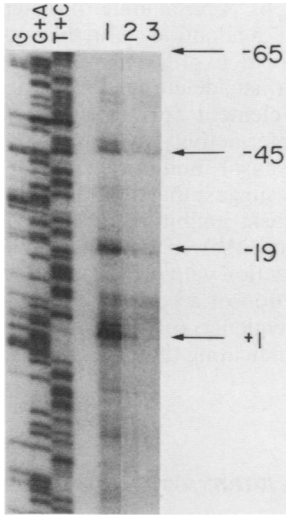

FIG. 5. Determination of mRNA initiation sites of PEPCK-CAT fusion genes. Total RNA was isolated from transfected cells and hybridized to a radiolabeled complementary RNA spanning nucleotides +150 of the *CAT* sequence through nucleotide −65 of the PEPCK promoter sequence. Hybrids were treated with RNases A and T_1 to digest unhybridized RNA and analyzed by electrophoresis in a 6% polyacrylamide–7 M urea gel along with Maxam-Gilbert sequencing reaction products to determine the sizes of protected fragments. Cells were transfected with the following plasmids: lane 1, pPL9 (wild type); lane 2, pID11 (CAAT deletion); lane 3, pID3 (CRE deletion). Arrows indicate the positions of correctly initiated PEPCK-CAT mRNA at +1 and apparently aberrant start sites at −19 and −45. Readthrough transcripts initiated elsewhere in the fusion gene plasmid would have been observed at −65.

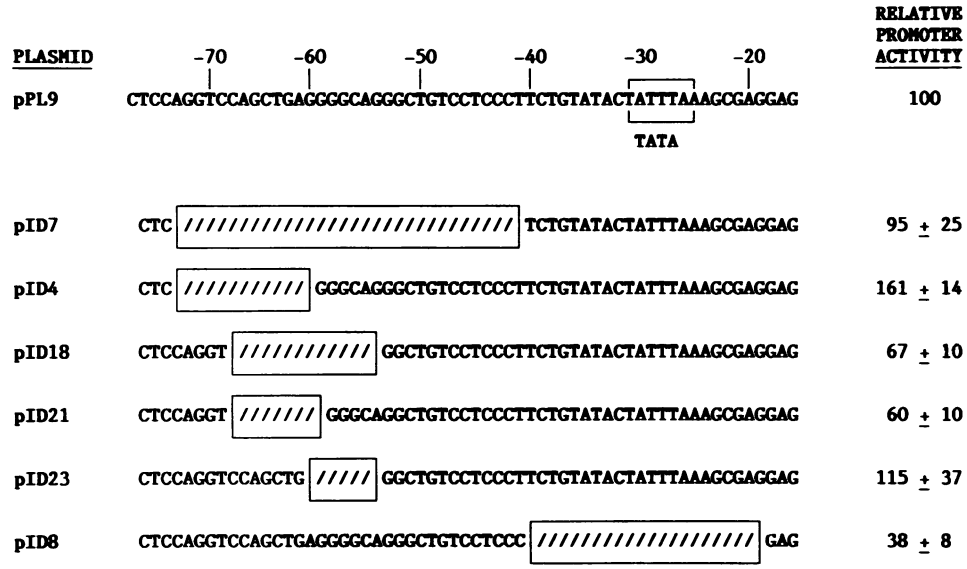

FIG. 6. Effects of mutations in sequences between the CRE and mRNA initiation site on basal promoter activity. CV-1 cells were cotransfected with the indicated PEPCK-CAT plasmids and the SV2–β-galactosidase plasmid as described in Materials and Methods. The data presented represent the mean ± SEM for two to six independent experiments. See Fig. 2 legend for details.

mRNAs were initiated 19 bp or more upstream of the correct start site (data not shown).

Effects of mutation of sequences between the CRE and the mRNA initiation site. Analysis of overlapping internal deletion mutations within the region between the CRE and the mRNA initiation site revealed the existence of additional elements with modest stimulatory and inhibitory effects on transcription (Fig. 6). A basal inhibitory element was present in the vicinity of nucleotide −70, because pID4 showed consistently elevated expression relative to the wild-type promoter. In contrast, deletion of sequences just 3′ of this basal inhibitory element (pID18 and pID21) resulted in diminished promoter activity, whereas deletion of sequence further 3′ of the basal inhibitory element (pID23) had no effect. These data suggest that a basal stimulatory element is adjacent to the basal inhibitory element. No protection of DNA sequence in either of these elements from DNase I digestion by interaction with protein has been observed (data not shown). Deletion of a sequence including homology to the TATA box sequence (pID8, Fig. 6) reduced promoter activity by 60%, indicating that, as expected, the TATA box of the PEPCK promoter is functionally important as a basal stimulatory element. Deletion of sequences between −19 and +69 of the PEPCK promoter had no effect on basal promoter activity (data not shown), indicating that no additional promoter elements were contributed by sequences in the area surrounding the initiation site. A summary of the promoter elements identified in this study is given schematically in Fig. 7.

DISCUSSION

The analysis of the promoter region of the PEPCK gene reported here yielded some unexpected results. This region of the gene contains sequences that are similar to those for the NF1/CTF, CBP, CRE, and TATA elements. Although both the CAAT and TATA sequences found in the promoter appear to be functional, neither is as crucial to basal expression of PEPCK as they are to the expression of some other genes. Unlike most previously described regulatable enhancers, the PEPCK CRE appears to function as both a crucial basal promoter element and a regulatable element, because

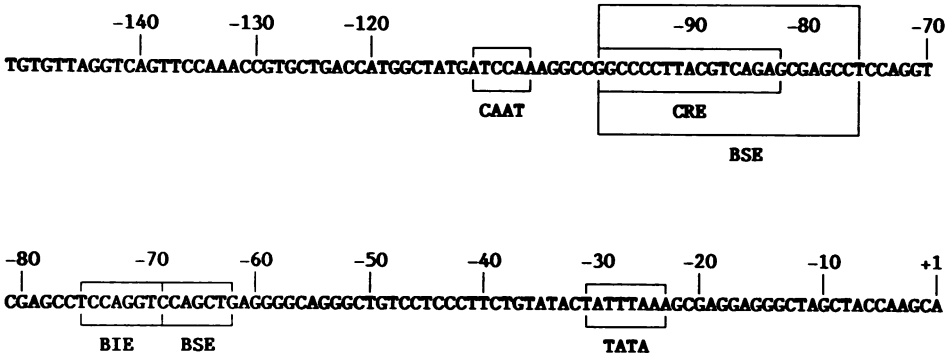

FIG. 7. Promoter elements of the PEPCK gene identified by analysis of internal deletion mutations. The promoter elements identified are summarized in this map of the PEPCK promoter. Abbreviations: BSE, basal stimulatory element; BIE, basal inhibitory element. The CAAT and TATA homologies are also identified and are basal stimulatory elements.

its activity was increased further in the presence of cAMP. Additional promoter elements with offsetting stimulatory and inhibitory effects were located in the region just 3′ of the CRE. Analysis of DNase I footprints showed that factor binding was correlated with functional activity for the CAAT and cAMP regulatory elements and that factors bound independently to these elements.

The NF1/CAAT homology of the PEPCK gene is a potential binding site for a number of DNA-binding proteins that have been characterized previously; CBP (11), NF1/CTF (20), and NFY (7). Binding to the consensus sequence for NF1, 5′-TGGN$_{6-7}$GCCAA-3′, has been studied in detail by saturation mutagenesis (12). It was concluded that both the 5′ and 3′ ends of the recognition sequence are required. The in vitro binding data reported here are in agreement with those from binding studies on NF1. However, the significance of NF1 binding to the PEPCK promoter is not clear, because deletion of the 5′ end of the NF1 site had no effect on promoter activity. In contrast, mutation of the 3′ end of the NF1/CAAT site resulted in loss of footprinting that was accompanied by a loss of basal promoter activity. This is most likely explained by the preservation of the ATCCAA sequence motif in this mutant (pID1) and the appearance of a smaller unique footprint over the CAAT homology that apparently is sufficient to maintain promoter function.

A recent study suggested that a transcription factor recognizing the CAAT box, CTF, was biochemically indistinguishable from NF1 (20). Other recent studies have demonstrated separate binding sites for NF1 and CAAT binding factors (36) and provided evidence for the recognition of CAAT sequences by multiple factors (7). The data reported here suggest that rat liver nuclear extracts contain an NF1-like factor that binds to the wild-type promoter and to the 5′-end NF1-mutated promoter, pID1, over the CAAT sequence, as would be suggested by the results of Jones et al. (i.e., NF1 is identical to CTF [20]). Alternatively, NF1 binding may be of no functional significance to the PEPCK promoter but may mask the binding of a functional transcription factor to the ATCCAA sequence in these in vitro assays. The 3′ portion of the NF1/CAAT-binding site of the PEPCK gene also has the potential to bind another rat liver protein, CBP (11), that is biochemically distinct from NF1. In the linker insertion mutation, pID12, the putative homology to the CBP-binding site is preserved (CCAAA) and yet protein binding was abolished and promoter activity was reduced. Therefore, CBP is probably not involved in PEPCK gene function. In any event, the functional portion of the NF1/CAAT sequence in the PEPCK gene appears to surround the ATCCAA sequence at the 3′ end of this site.

Previous reports have demonstrated the existence of cAMP regulatory elements in the 5′-flanking regions of the genes encoding PEPCK (42), somatostatin (33), proenkephalin (5), vasoactive intestinal peptide (44), and the alpha subunit of chorionic gonadotropin (αCG) (6, 43). The CREs of PEPCK and other genes have the properties of enhancers; they act in a relatively distance- and orientation-independent manner on their own promoters as well as on heterologous promoters (5, 6, 33, 43, 44). However, the core consensus sequence of the somatostatin gene was shown to be insufficient for mediation of cAMP responses (33), which indicates that additional sequence is required. The present study demonstrates that no additional sequence immediately 5′ or 3′ of the CRE core is required for induction of PEPCK-CAT fusion genes. Together with previous evidence on the PEPCK CRE (42), the data in this report demonstrate that the functional CRE of the PEPCK gene is contained within nucleotides −100 to −82 of the promoter. In addition, our data demonstrate that the CRE can operate independently of the CAAT box, although the physical association of these two elements in the promoter is close.

It is clear from the data in this report that the CRE region also functions as a basal promoter element in the PEPCK gene. Deletion of CRE core sequences led to greater reductions in basal promoter activity than any other deletions examined, including those in the CAAT and TATA homologies. Deletion of CRE core sequence also resulted in loss of protection of this area in the footprinting assay. These data suggest that a transcription factor required for basal expression of the PEPCK gene interacts, directly or indirectly, with the CRE core sequence. Because the effects of cAMP on gene transcription appear to be mediated by the catalytic subunit of cAMP-dependent protein kinase (1, 13), it seems likely that phosphorylation of a factor(s) binding to the CRE is involved in cAMP-regulated expression. This is consistent with work reported by Montminy and Bilezikjian (32), who purified a factor that binds to the CRE of the somatostatin gene. Phosphorylation of this factor is enhanced by treatment with cAMP in vivo and by incubation of the partially purified factor in vitro. We detected no difference in the footprinting sensitivity of extracts isolated from control or cAMP-treated hepatoma cells (T. W. Wong and D. K. Granner, unpublished data), as is the case with binding of factor to the somatostatin CRE (32). This suggests that phosphorylation of the factor binding to the CRE modifies its functional activity rather than its gross affinity for specific DNA-binding sites. This is consistent with competition experiments which indicate that cAMP does not appear to alter the affinity of limiting transcription factors for the 5′-flanking sequence, including the CRE, of the αCG gene (19).

The CREs of other cAMP-regulated genes may serve as essential promoter elements as well, but this activity appears to be dependent on the juxtaposition of other promoter elements and on the cell type in which expression is measured. The CRE of the αCG gene consists of a repeated 18-bp sequence, which Deutsch et al. (6) showed dramatically increases the basal level of CAT fusion genes utilizing the αCG promoter but not those utilizing the SV1 promoter (a truncated simian virus 40 promoter lacking the 72-bp enhancer sequences). This effect on basal promoter activity of the αCG gene CRE was restricted to choriocarcinoma cells and required a tandem repeat of the CRE consensus sequence (6). In contrast, Silver et al. (43) found no effect of the 18-bp repeat of the αCG gene on basal promoter activity of a different choriocarcinoma cell line but did observe significant stimulation when longer 5′-flanking sequences of the αCG gene were fused to the SV1 promoter in a CAT fusion gene.

An additional sequence, distinct from the CRE, contained within an oligomer which binds the transcription factor AP-2 is capable of conferring cAMP responsiveness on a heterologous promoter (18). Since the recognition sequence for AP-2 has no homology with any documented consensus sequence for a CRE, these data indicate that cAMP-regulated gene expression may be modulated by several *trans*-acting factors.

The putative basal stimulatory and inhibitory elements identified in the region downstream of the CRE show no homology with consensus sequences for identified *cis*-acting elements. They also show no evidence of DNA-protein interaction, but this is probably due to weak association, impurity of the nuclear extract used for footprinting, or both.

The apparent TATA homology of the PEPCK promoter appears to be functional, since deletion of these sequences reduced basal expression of the fusion gene and resulted in an increased use of upstream initiation sites.

The relative activity of the promoter elements identified for the PEPCK gene is different from that predicted by studies of other eucaryotic genes. Mutation of the CAAT box of PEPCK had a far smaller effect on promoter activity than does mutation of the CAAT sequences of the β-globin (35) or thymidine kinase (31) promoters. Deletion of the PEPCK TATA box reduced expression to the same extent as mutation of the TATA box of the β-globin gene (35) but to a lesser extent than mutation of the TATA boxes of the thymidine kinase (31) and metallothionein (16, 21) genes. In contrast, mutation of CRE sequences drastically reduced PEPCK promoter activity, an effect which has not been extensively studied in other genes. The fact that both basal and cAMP-regulated expression mapped to the same *cis*-acting element in the PEPCK gene strongly suggests that modification of a transcription factor(s) is involved in the regulation of PEPCK by cAMP. Whether the relevant modification is of a transcription factor which binds to the PEPCK CRE directly, or indirectly through protein-protein contacts, remains to be determined.

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LITERATURE CITED

1. **Beebe, S. J., S. R. Koch, D. T. W. Chu, J. D. Corbin, and D. K. Granner.** 1987. Regulation of phosphoenolpyruvate carboxykinase gene transcription in H4IIE hepatoma cells: evidence for a primary role of the catalytic subunit of 3',5'-cyclic adenosine monophosphate-dependent protein kinase. Mol. Endocrinol. **1:** 639–647.
2. **Birnboim, H., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
3. **Chu, D. T. W., and D. K. Granner.** 1986. The effect of phorbol esters and diacylglycerol on expression of the phosphoenolpyruvate carboxykinase (GTP) gene in rat hepatoma H4IIE cells. J. Biol. Chem. **261:**16848–16853.
4. **Chu, G., and P. Sharp.** 1981. SV40 DNA transfection of cells in suspension: analysis of the efficiency of transfection and translation of T-antigen. Gene **13:**197–202.
5. **Comb, M., N. C. Birnberg, A. Seashotz, E. Herbert, and H. M. Goodman.** 1986. A cyclic AMP- and phorbol ester-inducible DNA element. Nature (London) **323:**353–356.
6. **Deutsch, P. J., J. L. Jameson, and J. F. Habener.** 1987. Cyclic AMP responsiveness of human gonadotropin-α gene transcription is directed by a repeated 18-base-pair enhancer: α-promoter receptivity to the enhancer confers cell-preferential expression. J. Biol. Chem. **262:**12169–12174.
7. **Dorn, A., J. Bollekens, A. Staub, C. Benoist, and D. Mathis.** 1987. A multiplicity of CCAAT box-binding proteins. Cell **50:** 863–872.
8. **Dynan, W. S., and R. Tjian.** 1985. Control of eucaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins. Nature (London) **316:**774–778.
9. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
10. **Granner, D., T. Andreone, K. Sasaki, and E. Beale.** 1983. Inhibition of transcription of the phosphoenolpyruvate carboxykinase gene by insulin. Nature (London) **305:**549–551.
11. **Graves, B. J., P. F. Johnson, and S. McKnight.** 1986. Homologous recognition of a promoter domain common to the MSV LTR and HSV tk gene. Cell **44:**565–576.
12. **Gronostajski, R. M.** 1986. Analysis of nuclear factor I binding to DNA using degenerate oligonucleotides. Nucleic Acids Res. **14:** 9117–9132.
13. **Grove, J. R., D. J. Price, H. M. Goodman, and J. Avruch.** 1987. Recombinant fragment of protein kinase inhibitor blocks cyclic AMP-dependent gene transcription. Science **238:**530–533.
14. **Hall, C. V., P. E. Jacob, G. M. Ringold, and F. Lee.** 1983. Expression and regulation of *Escherichia coli lacZ* gene fusions in mammalian cells. J. Mol. Appl. Genet. **2:**101–109.
15. **Hanahan, D.** 1983. Studies on the transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**577–580.
16. **Haslinger, A., and M. Karin.** 1985. Upstream promoter element of the human metallothionein-II_A gene can act like an enhancer element. Proc. Natl. Acad. Sci. USA **82:**8572–8576.
17. **Herbomel, P., B. Bourachot, and M. Yaniv.** 1984. Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. Cell **39:**653–662.
18. **Imagawa, M., R. Chiu, and M. Karin.** 1987. Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. Cell **51:**251–260.
19. **Jameson, J. L., P. J. Deutsch, G. D. Gallagher, R. C. Jaffe, and J. F. Habener.** 1987. *trans*-Acting factors interact with a cyclic AMP response element to modulate expression of the human gonadotropin α gene. Mol. Cell. Biol. **7:**3032–3040.
20. **Jones, K. A., J. T. Kadonaga, P. J. Rosenfeld, T. J. Kelly, and R. Tjian.** 1987. A cellular DNA-binding protein that activates eukaryotic transcription and DNA replication. Cell **48:**79–89.
21. **Jones, P. B. C., D. R. Galeazzi, J. M. Fisher, and J. P. Whitlock, Jr.** 1985. Control of cytochrome P_1-450 gene expression by dioxin. Science **227:**1499–1502.
22. **Karin, M., A. Haslinger, H. Holtgreve, G. Cathala, E. Slater, and J. D. Baxter.** 1984. Activation of a heterologous promoter in response to cadmium and dexamethasone by metallothionein II_a gene by 5' flanking DNA. Cell **36:**371–379.
23. **Karin, M., A. Haslinger, H. Holtgreve, R. Richards, P. Krauter, H. Westphal, and M. Beato.** 1984. Characterization of DNA sequences through which cadmium and glucocorticoid hormones induce human metallothionein II_a gene. Nature (London) **308:**513–519.
24. **Lamers, W. H., R. W. Hanson, and H. M. Meisner.** 1982. cAMP stimulates transcription of the gene for cytosolic phosphoenolpyruvate carboxykinase in rat liver nuclei. Proc. Natl. Acad. Sci. USA **79:**5137–5141.
25. **Luthman, H., and G. Magnuson.** 1983. High-efficiency polyoma DNA transfection of chloroquine-treated cells. Nucleic Acids Res. **11:**1295–1308.
26. **Magnuson, M. A., P. G. Quinn, and D. K. Granner.** 1987. Multihormonal regulation of phosphoenolpyruvate carboxykinase-chloramphenicol acetyltransferase fusion genes: insulin's effects oppose those of cAMP and dexamethasone. J. Biol. Chem. **262:**14917–14920.
27. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Maniatis, T., S. Goodbourn, and J. A. Fischer.** 1987. Regulation of inducible and tissue-specific gene expression. Science **236:** 1237–1245.
29. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65:**499–560.
30. **McKnight, S., and R. Tjian.** 1986. Transcriptional selectivity of viral genes in mammalian cells. Cell **46:**795–805.
31. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient *in vitro* synthesis of

biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.

32. **Montminy, M. R., and L. M. Bilezikjian.** 1987. Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature (London) **328**:175–178.
33. **Montminy, M. R., K. A. Sevarino, J. A. Wagner, G. Mandel, and R. H. Goodman.** 1986. Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. Proc. Natl. Acad. Sci. USA **83**:6682–6686.
34. **Moreau, P., R. A. Hen, B. Wasylyk, R. Everett, M. Gaub, and P. Chambon.** 1981. The SV40 72 base pair repeat has a striking effect on gene expression both in SV40 and other chimeric recombinants. Nucleic Acids Res. **9**:6047–6068.
35. **Myers, R. M., K. Tilly, and T. Maniatis.** 1986. Fine structure analysis of a β-globin promoter. Science **232**:613–618.
36. **Oikarinen, J., A. Hatamochi, and B. de Crombrugghe.** 1987. Separate binding sites for nuclear factor I and a CCAAT DNA binding factor in the mouse α_2(I) collagen promoter. J. Biol. Chem. **262**:11064–11070.
37. **Petersen, D. D., M. A. Magnuson, and D. K. Granner.** 1988. Location and characterization of two widely separated glucocorticoid response elements in the phosphoenolpyruvate carboxykinase gene. Mol. Cell. Biol. **8**:96–104.
38. **Radloff, R., W. Bauer, and J. Vinograd.** 1967. A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc. Natl. Acad. Sci. USA **57**:1514–1521.
39. **Sasaki, K., T. Cripe, S. Koch, T. Andreone, D. Petersen, E. Beale, and D. Granner.** 1984. Multihormonal regulation of the phosphoenolpyruvate carboxykinase gene: the dominant role of insulin. J. Biol. Chem. **259**:15242–15251.

39a. **Sasaki, K., and D. K. Granner.** 1988. Regulation of phosphoenolpyruvate carboxykinase gene transcription by insulin and cAMP; reciprocal actions on initiation and elongation. Proc. Natl. Acad. Sci. USA **85**:2954–2958.

40. **Scholer, H., A. Haslinger, A. Heguy, H. Holtgreve, and M. Karin.** 1986. *In vivo* competition between a metallothionein regulatory element and the SV40 enhancer. Science **232**:76–80.
41. **Serfling, E., M. Jasin, and W. Schaffner.** 1985. Enhancers and eucaryotic gene transcription. Trends Genet. **1**:224–230.
42. **Short, J. M., A. Wynshaw-Boris, H. P. Short, and R. W. Hanson.** 1986. Characterization of the phosphoenolpyruvate carboxykinase (GTP) promoter-regulatory region. II. Identification of cAMP and glucocorticoid regulatory domains. J. Biol. Chem. **261**:9721–9726.
43. **Silver, B. J., J. A. Bokar, J. B. Virgin, E. A. Vallen, A. Milsted, and J. H. Nilson.** 1987. Cyclic AMP regulation of the human glycoprotein hormone α-subunit gene is mediated by an 18-base-pair element. Proc. Natl. Acad. Sci. USA **84**:2198–2202.
44. **Tsukada, T., J. S. Fink, G. Mandel, and R. H. Goodman.** 1987. Identification of a region in the human vasoactive intestinal polypeptide gene responsible for regulation by cAMP. J. Biol. Chem. **262**:8743–8747.
45. **Wallace, R. B., M. J. Johnson, S. V. Suggs, K. Miyoshi, R. Bhatt, and K. Itakura.** 1981. A set of synthetic oligodeoxyribonucleotide primers for DNA sequencing in the plasmid vector pBR322. Gene **16**:21–26.