MOLECULAR AND CELLULAR BIOLOGY, Sept. 1990, p. 4712–4719
0270-7306/90/094712-08$02.00/0

Vol. 10, No. 9

Characterization of a Complex Glucocorticoid Response Unit in the Phosphoenolpyruvate Carboxykinase Gene

ENYU IMAI,[1] PER-ERIC STROMSTEDT,[2] PATRICK G. QUINN,[1]† JAN CARLSTEDT-DUKE,[2] JAN-ÅKE GUSTAFSSON,[2] AND DARYL K. GRANNER[1]*

Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0615[1] and Department of Medical Nutrition, Karolinska Institutet, Huddinge Hospital F60 NOVUM, S-141 86 Huddinge, Sweden[2]

Received 4 April 1990/Accepted 13 June 1990

The minimal DNA sequence required for glucocorticoid induction of the phosphoenolpyruvate carboxykinase (PEPCK) gene in H4IIE rat hepatoma cells was defined. This novel glucocorticoid response unit (GRU) spans about 110 base pairs (bp) and includes two receptor-binding elements plus two accessory factor-binding elements. Purified glucocorticoid receptor bound to two regions (GR1 and GR2) between −395 and −349 bp relative to the transcription start site. Factors in crude rat liver nuclear extract bound to DNA in the regions −455 to −431 and −420 to −403 bp, which are designated accessory factor 1 (AF1) and accessory factor 2 (AF2) elements, respectively. Gel retardation analysis revealed that at least two proteins bound to AF1 and that they were distinct from the protein(s) that bound to AF2. Various combinations of GR1, GR2, AF1, and AF2 were fused to the chloramphenicol acetyltransferase (CAT) reporter gene and cotransfected with a glucocorticoid receptor expression plasmid (pSVGR1) into H4IIE cells to identify the functional GRU. Neither the glucocorticoid receptor binding region nor the accessory factor binding region alone was sufficient to confer glucocorticoid responsiveness. The two components of the glucocorticoid receptor binding region functioned independently, and each accounted for half of the maximal response, provided the accessory factor elements were present. Similarly, deletion of either AF1 or AF2 diminished glucocorticoid induction of the PEPCK gene to approximately half of the maximum. We propose that the complex PEPCK gene GRU provides the stringent regulation required of this critical enzyme in liver.

Cytosolic phosphoenolpyruvate carboxykinase (GTP; EC 4.1.1.32; PEPCK), the rate-limiting gluconeogenic enzyme, catalyzes the conversion of oxaloacetate to phosphoenolpyruvate. The hormones that regulate gluconeogenesis appear to do so by altering the rate of synthesis of PEPCK (13). Although effects of cyclic AMP (cAMP) and glucocorticoids on $mRNA^{PEPCK}$ stability have been reported (10, 21), the predominant control of PEPCK synthesis appears to be accomplished through changes in transcription of the PEPCK gene. cAMP and glucocorticoids increase the transcription of the gene, and insulin is inhibitory (24, 27). The cAMP and glucocorticoid effects are additive, and the insulin effect is dominant both in rat liver and in H4IIE hepatoma cells (24). Exactly the same relationship is seen in the effect that these agents have on gluconeogenesis in hepatoma cells (12).

The glucocorticoid hormone-receptor complex is thought to bind in a site-specific manner to the glucocorticoid response element (GRE), for which the consensus sequence is (T/G)GTACAnnnTGTTCT (1). This consensus sequence directs the specific binding of the glucocorticoid receptor (GR) to DNA in the promoter region of target genes. One molecule of the GR binds to the TGTTCT half-site of the GRE, and a second molecule then binds to the TGTACA half-site in a cooperative manner (31). The GR contacts the DNA through two zinc fingers in the DNA-binding domain (6, 7, 17). Beato (1) postulated that the amino-terminal finger interacts specifically with one half of the GRE, whereas the carboxyl-terminal finger interacts with the DNA helix flanking the GRE consensus sequence. The receptor-DNA interaction presumably enhances transcription by approximating the transactivation domain(s) of the receptor with one or more components of the initiation complex (8, 35).

The GRE is considered to be a transcription enhancer because it functions through heterologous promoters and in a relatively position- and orientation-independent manner (2, 35). A single GRE works when located immediately upstream of the TATA box, but multiple copies appear to be required when the GREs are located at a distance (30). There is considerable variation in the number and location of GREs. The multiple GREs in the long terminal repeat of the murine mammary tumor virus (MMTV) gene are clustered near the transcription initiation site (20). Those in the tyrosine aminotransferase (TAT) gene are also clustered but are located 2.5 kilobases from the start site (11). The tryptophan oxygenase gene GREs are split and are located at a distance from the cap site (4). Multiple GREs enhance receptor binding and increase function. The occupation of one GRE by a receptor dimer enhances binding of another dimer to a second GRE by 100-fold (32). Thus, two tandemly linked GREs confer a synergistic induction by glucocorticoids upon a reporter gene (30).

In addition to apparent receptor-receptor interactions, recent studies suggest the importance of functional interactions between the receptor molecules and other transcription factors (e.g., NF1, SP1, OTF, and the CACCC box-binding protein). The GRE is inactive in the absence of an NF1 site in the wild-type MMTV promoter (3). The GRE in the tryptophan oxygenase gene appears to interact with an adjacent DNA element, similar to the CACCC box that is operative in the β-globin gene promoter, to modulate tran-

* Corresponding author.
† Present address: Department of Cellular and Molecular Physiology, College of Medicine, Pennsylvania State University, Hershey, PA 17036.

scription of this gene (4, 26). Strahle et al. (30), using synthetic combinations of the TAT GRE and various other transcription factor-binding elements, demonstrated a synergistic induction of transcription, although the necessity of such accessory elements has not been shown in the wild-type TAT gene promoter. Synergism was also noted when various factor-binding elements were linked to a consensus GRE in the context of the thymidine kinase promoter (25). In the rat α_1-acid glycoprotein gene, a sequence immediately downstream of the receptor binding site renders glucocorticoid induction sensitive to inhibition of protein synthesis by cycloheximide (14). This site presumably binds a labile transcription factor that interacts with the receptor. These implied heterologous protein-protein interactions (there is no direct evidence for such interactions) may serve a purpose similar to that of the receptor-receptor interaction vis-à-vis the activation of the transcription complex.

Our aim in this study was to define the minimal DNA sequence required for glucocorticoid induction of the PEPCK gene. We used the DNase I protection assay to identify regions of DNA-protein interaction and used the transient expression of chimeric genes consisting of various regions of the 5′ flanking sequence of PEPCK gene DNA fused to a CAT reporter gene for functional analysis. The glucocorticoid regulatory unit (GRU) of the PEPCK gene includes at least 110 base pairs (bp) of DNA and is composed of two GR binding regions and two accessory factor binding regions. All of these elements are required for maximal response of the PEPCK gene to glucocorticoids.

MATERIALS AND METHODS

Plasmid constructions. The construction of a series of 5′ deletion mutations of the PEPCK promoter fused to the chloramphenical acetyltransferase (CAT) gene has been described in detail (22, 23). Oligonucleotides were prepared with an Applied Biosystems 380A DNA synthesizer and purified as recommended by the manufacturer. The annealed complementary strands contained a *Hin*dIII site at the 5′ end, and bases complementary to the *Hin*dIII overlap at the 3′ end. Various combinations of the PEPCK gene DNA sequence, between −467 and −306 relative to the transcription initiation site, were created by inserting different oligonucleotides into the *Hin*dIII sites of plasmids pPL12 and pPL33; these plasmids contain PEPCK promoter regions between −402 to +69 and −306 to +69, respectively. All constructs were sequenced. All ligations and transformations were done by standard techniques (15). Plasmid DNA was prepared by alkaline lysis and was banded twice in CsCl-ethidium bromide isopycnic gradients to purify supercoiled plasmid DNA.

Cell culture and transfection. H4IIE cells were grown to 50 to 60% confluence in Earle modified Eagle medium containing final concentrations (volume/volume) of bovine calf (5%), newborn bovine (3%), and fetal bovine (2%) sera. Then 10 μg each of a PEPCK-CAT plasmid and pSVGR1, a GR expression vector (kindly supplied by K. Yamamoto; 18), and 5 μg of pRSVL-AΔ5′ (5), an internal control for normalizing transfection efficiency, were transfected into 50% confluent H4IIE cells in a 75-cm^2 dish. Transfections were performed by the calcium phosphate coprecipitation method as described previously (22). Five hours after transfection, the cells were shocked with 20% dimethyl sulfoxide for 5 min, washed, and incubated for 24 h in the absence or presence of 500 nM dexamethasone phosphate.

Cotransfection of PEPCK promoter mutants and a GR expression vector. Low intracellular levels of glucocorticoid receptor in H4IIE cells could limit the response to glucocorticoids in simple transient transfection analysis of the PEPCK-CAT gene, as has been reported in the analyses of other genes (33). If so, cotransfection with a GR expression vector would facilitate this analysis. The 5′ promoter fragment containing DNA from −467 to +69 (pPL32) responded with an 11-fold induction of CAT expression, at a concentration of 500 nM of dexamethasone, when cells were cotransfected with pSVGR1 (18), as opposed to a 4-fold induction when they were not cotransfected. The latter value resembled that obtained with this vector in our previous studies (22). On the other hand, plasmids with shorter portions of the PEPCK promoter DNA (pPL12 [−402 to +69] or pPL33 [−306 to +69]) responded to glucocorticoids at a similar, low level (~2-fold) in either method. The basal level of CAT protein was not affected by cotransfection, and there was a less than 20% difference in basal expression among these three constructs (data not shown). Therefore, the increased glucocorticoid induction noted in cells cotransfected with the GR expression vector resulted from a specific enhancement in transcription of the PEPCK-CAT gene under the condition of sufficient GR. The studies described herein were conducted by using cotransfection with the GR expression vector pSVGR1, but it should be stated that qualitatively similar results were obtained in the absence of cotransfection.

CAT assay. The cells were harvested by trypsin digestion and sonicated in 150 μl of 250 mM Tris hydrochloride at pH 7.8. After sonication, the samples were heated for 10 min at 50°C and centrifuged to remove cellular debris. A CAT assay was performed on the supernatant by the method of Nordeen et al. (19). Briefly, 100 μl of cell extract was incubated for 2 h at 37°C in a reaction containing 160 mM Tris hydrochloride (pH 7.8), 6 mM $MgCl_2$, 75 mM KCl, 0.4 mM coenzyme A, 3 mM ATP, 1 mM chloramphenicol, 0.06 U of acetyl coenzyme A synthetase, and 0.34 mM ^{3}H-sodium acetate (0.5 Ci/mmol). The reaction mixture was extracted with benzene and transferred into scintillation vials. The benzene was evaporated, and the ^{3}H-sodium acetate incorporated into chloramphenicol was quantified by scintillation spectroscopy. The CAT activity was corrected for the protein concentration in the lysate. Background activity from a lysate-free blank was subtracted from all assays, and results are expressed as fold induction in the presence versus in the absence of dexamethasone. The no-dexamethasone or basal value was corrected for transfection efficiency by cotransfection with a vector containing a luciferase reporter gene, pRSVL-AΔ5′ (5). Variations in basal activity were no more than 20% between all the constructs used in this study with the exception of pPL1 (−2100), which had basal activity 30% lower than the reference, pPL9 (−600).

DNase I protection assay. Rat liver GR was purified as described elsewhere (34). Crude rat liver nuclear extracts were obtained by the method of Gorski et al. (9). DNA was radiolabeled at the *Xba*I site (−490) of the PEPCK gene (for analysis of binding to the coding strand) or at the *Sal*I site (−319) (for analysis of the template strand). A 5- to 10-fmol sample of the *Xba*I-*Bsu*36I fragment from pPL9 or the *Xba*I-*Sal*I fragment from pPG43 (22) was incubated with 0 to 1,500 fmol of rat liver GR or 0 to 15 μg of crude nuclear extract in a buffer [20 mM Tris hydrochloride (pH 7.5) containing 0.5 μg of poly(dI-dC) · poly(dI-dC), 10 mM dithiothreitol, 50 mM NaCl, 100 μg of insulin per ml, 1 mM EDTA, 2 mM $MgCl_2$, and 20% (vol/vol) glycerol] for 15 min at room temperature. Thereafter, 2 μl of the reactant,

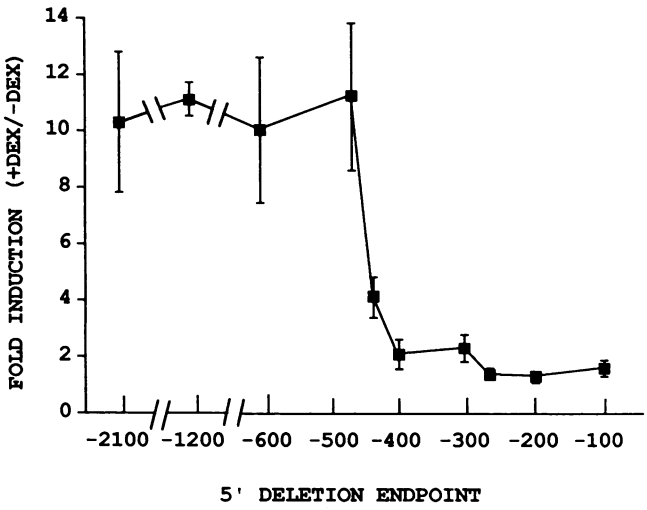

FIG. 1. Analysis of glucocorticoid induction of CAT expression in various 5′ deletion mutations of the PEPCK gene promoter. A series of 5′ deletion mutations of the PEPCK promoter were fused to the CAT reporter gene and were cotransfected with the GR expression vector (pSVGR1) into H4IIE cells. Transfected cells were incubated with or without 500 nM dexamethasone for 24 h in serum-free medium. Cell lysates were prepared and assayed for CAT activity. Basal activity, corrected for transfection efficiency, was 30% lower in the −2100 construct than in the reference construct −467. All others varied <20% from the reference construct. The results are expressed as fold increase over control (see Materials and Methods). Each datum point represents the mean ± standard deviation of at least three independent experiments.

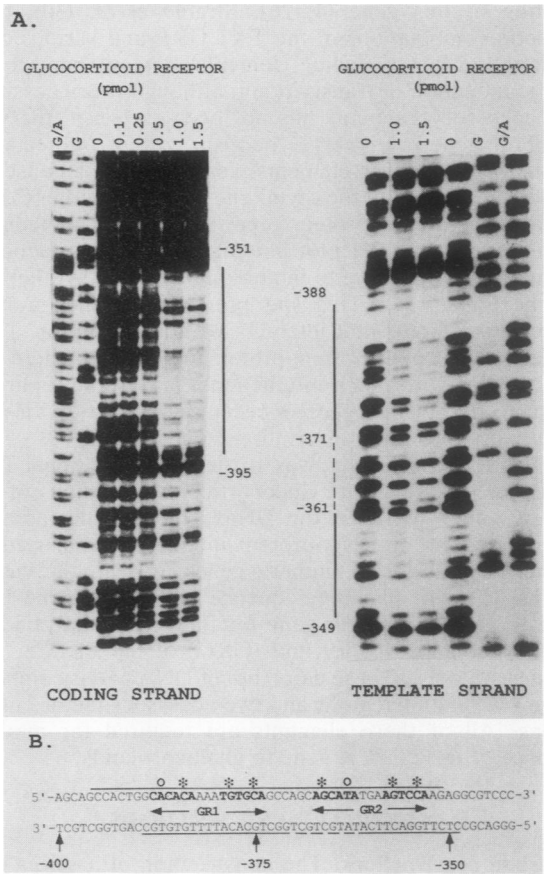

FIG. 2. DNase I protection analysis of specific binding of purified rat liver GR to the PEPCK gene promoter. An end-labeled fragment of the PEPCK gene (from bp −490 to −212) was incubated with 0.1 to 1.5 pmol of purified rat liver GR as described in Materials and Methods. After DNase I digestion, the samples were analyzed on a 6% polyacrylamide–7 M urea gel. The Maxam-Gilbert reactions (G/A and G) are shown in parallel lanes in panel A. Homologies to the GRE consensus sequence are labeled GR1 and GR2 in panel B. Symbols: *, nucleotides present in the PEPCK promoter noted to be most critical in receptor binding to the TAT GRE (29, 31); O, a nucleotide demonstrated to be important for binding in the TAT GRE that is different in the PEPCK promoter.

containing 4 ng of DNase I and 10 mM $MgCl_2$, was added and incubated for 30 s. DNase I digestion was stopped by the addition of 8.4 μl of stop solution (0.7 mg of proteinase K per ml, 0.12 M EDTA, 1% [wt/vol] sodium dodecyl sulfate). After incubation for 1 h, nucleic acids were precipitated with ethanol and the samples were analyzed by electrophoresis in a 6% polyacrylamide–7 M urea gel.

Gel retardation assay. DNA fragments, radiolabeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, were incubated with rat liver nuclear extracts at room temperature for 15 min in the presence of 10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES; pH 7.8) containing 5 mM spermidine, 2.5 mM dithiothreitol, 50 mM NaCl, 20 mM KCl, 0.5 mg of bovine serum albumin per ml, 1 μg of poly(dI-dC) · poly(dI-dC), 10% (vol/vol) glycerol, 0.2% Nonidet P-40, and 2 mM each phosphoserine, phosphothreonine, and phosphotyrosine. Unlabeled competitor DNA fragments (10- to 100-fold molar excess) were added for competition analysis. Samples were loaded onto a 6% acrylamide gel and electrophoresed for 120 min at 150 V in a buffer containing 25 mM Tris, 190 mM glycine, and 1 mM EDTA (28). Gels were dried and exposed to X-ray films.

RESULTS

5′ deletion analysis of the PEPCK gene promoter. A set of 5′ deletion mutations of the PEPCK promoter (Fig. 1) was used to locate the 5′ boundary of the GRU. As mentioned above, there was no significant difference in basal expression between these various 5′ deletion mutations. Plasmids with promoter endpoints at −2100, −1264, −600, and −467 were equally responsive to glucocorticoids (about an 11-fold induction). A prominent decrease in the glucocorticoid response occurred between −467 and −402 (from 11- to 2-fold induction). A second, small diminution in glucocorticoid induction occurred between −306 and −271; no significant glucocorticoid response was obtained when less than −271 bp of the PEPCK 5′ flanking sequence was fused to the CAT gene. The predominant glucocorticoid effect in H4IIE cells appears to require the sequence between −467 and −402.

Interaction of GR with the PEPCK promoter. We performed a DNase I footprinting analysis to precisely identify the DNA sequence(s) that interacts with the GR. Highly purified GR protected the region between −395 and −349 of the coding strand against DNase I digestion (Fig. 2A). This protected region contained two putative GRE core sequences, CACACAnnnTGTGCA (GR1) and AGCATAnn nAGTCCA (GR2) (Fig. 2B). GR1 matches the consensus GRE sequence (GGTACAnnnTGTTCT) at 7 of 12 positions, whereas GR2 matches at 6 of 12. The location of the GR binding sites, well 3′ or proximal to the region demonstrated to contain the 5′ (−467) boundary of the GRU, was surprising. This, plus the observation that a 5′ deletion mutant with

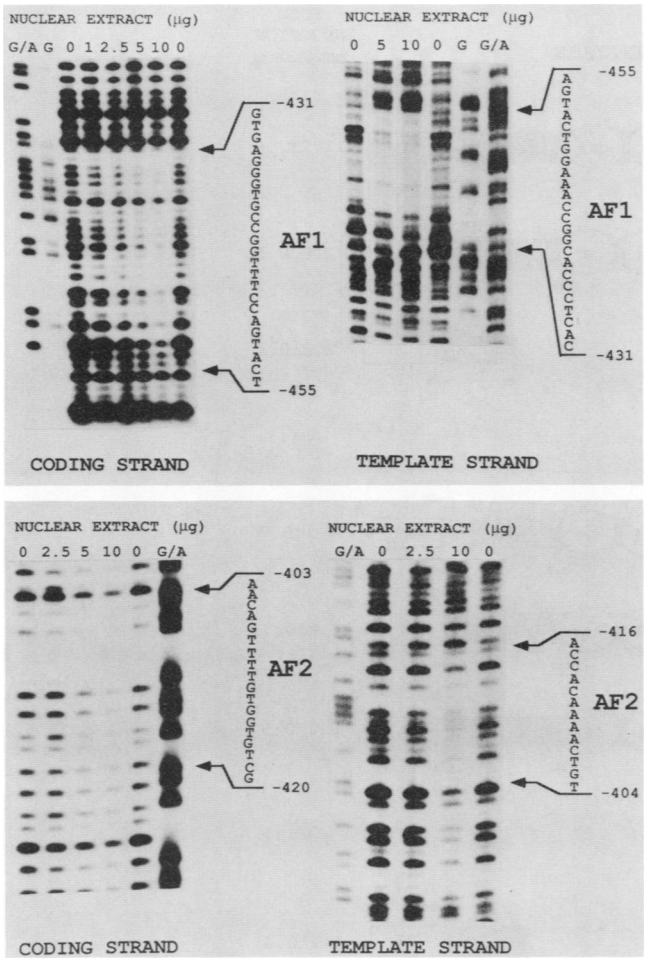

FIG. 3. DNase I protection analysis of the interaction of liver nuclear extracts with the PEPCK gene promoter. Rat liver nuclear extracts were prepared as described in Materials and Methods and used in a DNase I protection assay. The probe of the coding strand of DNA was labeled at the *Xba*I site (−490) and cut at the *Bsu*36 I site (−212). The probe for the template strand (−319 to −490) was obtained by endlabeling at the *Sal*I and *Xba*I sites. DNA (5 to 10 fmol) was incubated with 0 to 10 μg of the rat liver nuclear extract, and then the digestions with DNase I were performed. Samples were analyzed on a 6% acrylamide gel–7 M urea. Two protected regions were observed in the region located between bp −467 and −402 relative to the transcription start site. The protected region at the 5′ site is labeled AF1, and that at the 3′ protection site is labeled AF2.

an endpoint at −402 showed little response to dexamethasone, suggested that other DNA elements, and associated *trans*-acting factors, were an integral component of the GRU.

Physical evidence of accessory DNA elements or factors. The discrepancy between the boundary of the functional GRU and the GR binding sites raised the possibility that accessory elements were required. To ascertain whether accessory factors bind to the region required for glucocorticoid induction, we looked for evidence of protein-DNA interactions in the region between −467 and −403, using crude rat liver nuclear extract in the DNase I footprint assay. Two DNA sequences were protected (Fig. 3). The first region, from −455 to −431, was called accessory factor site 1 (AF1), and the second, from −420 to −403, was called accessory factor site 2 (AF2). There is no DNA sequence homology between AF1 and AF2.

Functional evidence for accessory elements or factors. We characterized the relative functional contributions of the accessory factor binding sites and the GR binding sites by analyzing the regulation of CAT expression in plasmids in which one or more element was deleted. Deletion of both AF1 and AF2 reduced glucocorticoid induction to the low levels characteristics of the promoter lacking the entire GRU (Fig. 4A). The promoter containing the entire GRU (pPL32) gave an 11-fold induction in response to dexamethasone, whereas the promoter lacking either both accessory elements (pPL12) or the entire GRU (pPL33) gave a 2-fold induction (Fig. 4A). This accounts for the original observation that a major loss of response occurred even though the GR binding sites were still intact (the −402 5′ deletion, pPL12; Fig. 4).

The deletion of AF1 caused about a 60% reduction of CAT expression in response to dexamethasone (4.1-fold for pPL10; Fig. 4B). This explains the difference in activity noted between the −467 and −437 5′ deletions (Fig. 1). Similarly, an internal deletion in which AF2 was removed diminished the glucocorticoid inducibility by approximately 50% (4.8-fold for 467−431/pPL12). In this construct, the AF1 site was moved closer to the GR binding sites. To evaluate the effect of spacing between AF1 and the GR binding sites, we tested a plasmid (AF1+15/pPL12) in which the PEPCK DNA segment from −430 to −402 was replaced with pBR322 DNA such that the correct spacing between AF1 and the receptor binding sites was maintained. Plasmid AF1+15/pPL12 responded to dexamethasone with a 5.1-fold induction of CAT protein. Therefore, the diminution of induction noted in 467−431/pPL12 was not due to a spacing artifact.

Plasmids containing AF1 alone (467−431/pPL33), AF2 alone (433-396/pPL33), or AF1 and AF2 together (467−390/pPL33) were no more responsive to dexamethasone than was the construct that lacked the entire GRU (pPL33) (Fig. 4C). These results show that AF1 and AF2 do not themselves function as glucocorticoid-responsive enhancers. However, AF1 and AF2 are both indispensable for full glucocorticoid induction of the PEPCK gene.

Role of the glucocorticoid receptor-binding sites. We next examined the functional role of the two glucocorticoid receptor-binding sites (Fig. 6). Deletion of either GR1 or GR2 resulted in a 50% reduction of the response to dexamethasone (4.3-fold for ΔGR1 and 5.2-fold for ΔGR2). The deletion of both GR1 and GR2 in a construction containing both accessory elements reduced glucocorticoid induction to the low level noted when the entire GRU was absent (compare plasmid 468-390/pL33 with pL33). These results suggest that each GR-binding site functions independently and that each accounts for half of the full response, provided that both accessory factors are present. The GR-binding sites themselves are inert (compare pL12 with pPL33).

Unique proteins bind to the AF1 and AF2 elements. To confirm that different proteins bind to the AF1 and AF2 elements, gel retardation assays were performed with oligonucleotides AF1 (−460 to −430) and AF2 (−420 to −402). Several bands were detected when labeled AF1 was incubated with crude rat liver nuclear extract (Fig. 6A). Unlabeled oligonucleotide AF1 competed for this binding, but AF2 did not. Conversely, binding to labeled AF2 (which showed a much simpler pattern) was displaced by unlabeled AF2 but not by AF1 (Fig. 6B).

The presence of multiple bands suggested that more than

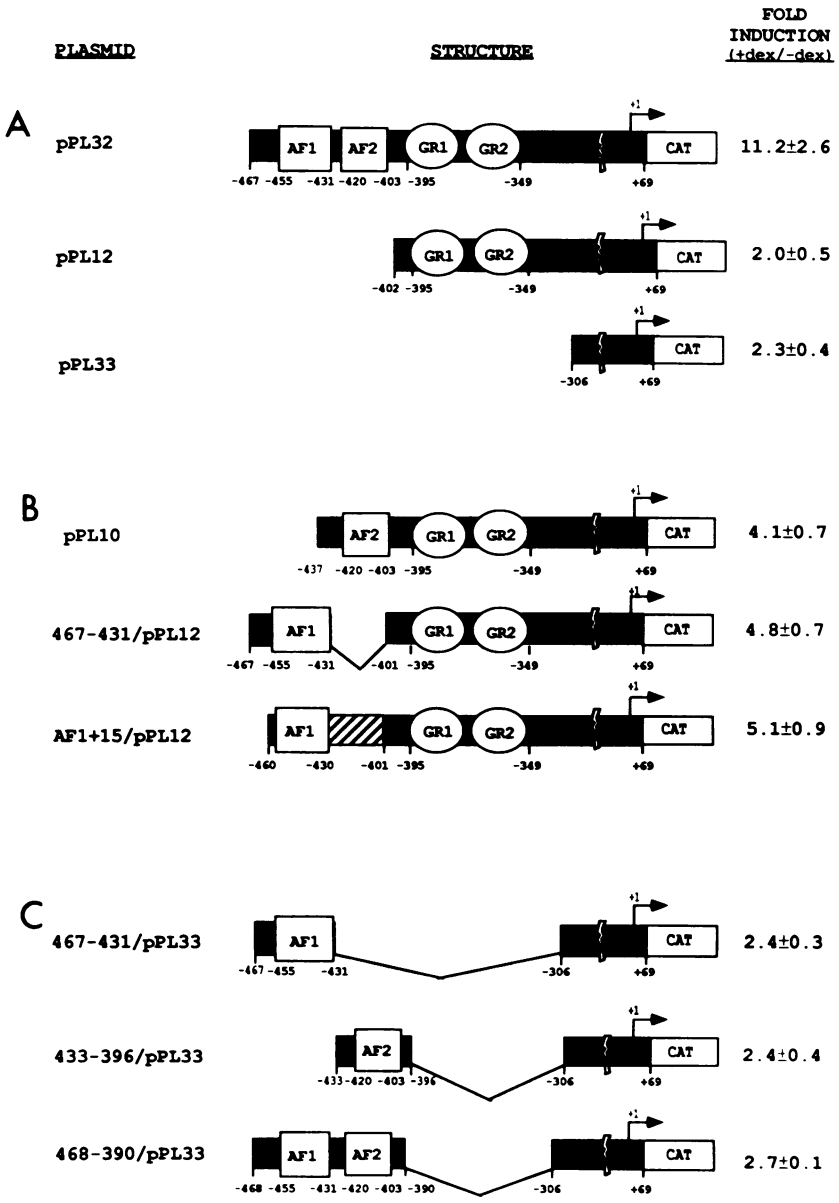

FIG. 4. Functional analysis of the accessory factor sites in the regulation of the PEPCK-CAT fusion genes by dexamethasone. H4IIE cells were cotransfected with the GR expression plasmid pSVGR1 and the various plasmids described in the figure and then were incubated in the presence or absence of 500 nM dexamethasone for 24 h. Cell lysates were then prepared and assayed for CAT activity. AF1 and AF2 represent the DNA sequences between −455 to −431 and −420 to −403, respectively. GR1 and GR2 represent DNA sequences between −389 to −375 and −367 to −353, respectively. The results are expressed as fold induction (mean ± standard deviation) of at least four independent transfections with each construct.

one protein binds to the AF1 region (−460 to −430). To ascertain whether AF1 contained different protein recognition sites, we synthesized two oligonucleotides, AF1-a (−455 to −438) and AF1-b (−441 to −426), and performed a gel retardation assay. When the labeled AF1-a fragment was incubated with crude nuclear extract, only one band was detected (Fig. 6C). This binding was significantly reduced by a 50-fold molar excess of the specific oligonucleotide AF1-a but not by the same amount of the AF1-b oligonucleotide. The reciprocal result was obtained when the AF1-b fragment was used. A band(s) indicating a binding interaction was detected when labeled AF1-b was incubated with the crude nuclear extract, and this was displaced by a 50-fold molar excess of specific fragment but not by the AF1-a fragment (Fig. 6D).

The addition of GR with nuclear extracts resulted in no detectable qualitative or quantitative change in the binding of the accessory factors to AF1 or AF2 (as assessed by gel retardation or DNase I protection assays). Likewise, additions of nuclear extracts known to contain the accessory factor-binding proteins had no discernible effect on the binding of GR to GR1 or GR2 (data not shown).

DISCUSSION

The DNA sequence required for stimulation of PEPCK gene transcription by glucocorticoids is complex. The region

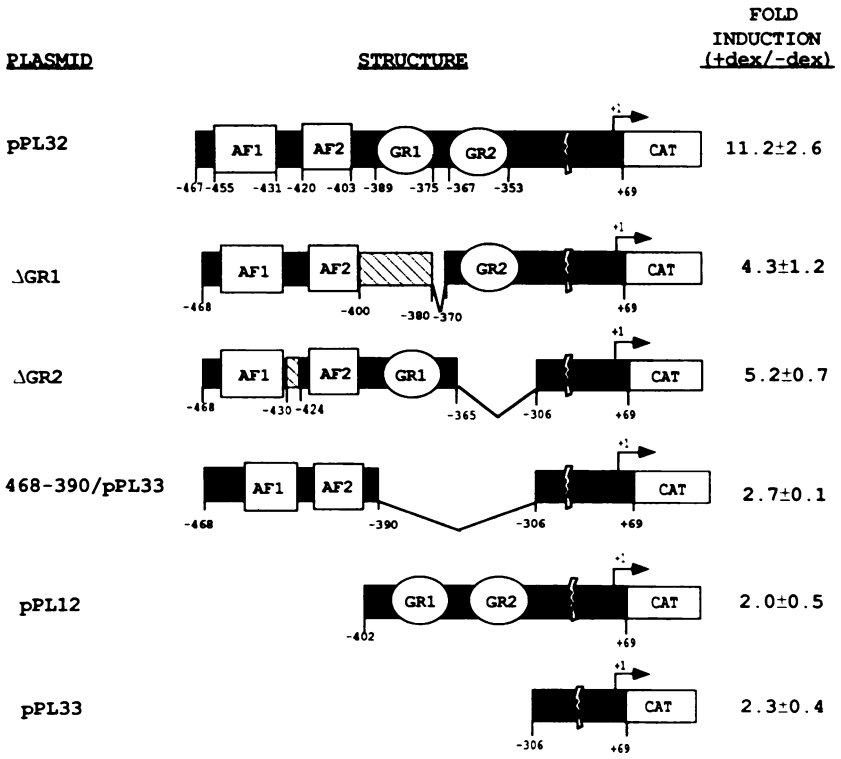

FIG. 5. Functional analysis of GR1 and GR2 in the regulation of the PEPCK-CAT fusion genes by dexamethasone. H4IIE cells were cotransfected with the GR expression plasmid pSVGR1 and the plasmids described in the figure were incubated in the presence or absence of 500 nM dexamethasone for 24 h. GR1, GR2, AF1, and AF2 are described in the legend to Fig. 4. The results are expressed as fold induction over control (mean ± standard deviation) for at least four independent transfections with each construct.

extends over at least 110 bp of the PEPCK promoter, and it consists of binding sites for several different proteins. Two contiguous sites, each of which presumably binds a GR dimer, comprise the 3'-most portion of the region. These are similar to, but not highly homologous with, the typical GRE. In H4IIE cells, these receptor binding sites are themselves functionally inert in the context of the PEPCK promoter, which also differentiates them from the typical GRE. Two rather extended DNA elements, designated AF1 and AF2, are located immediately adjacent to the 5' boundary of the receptor binding sites. AF1 is composed of at least two distinct elements (AF1-a and AF1-b), and AF2 may have multiple components. AF1 contains a sequence similar to a CACCC box (26); however, a construct in which this sequence was mutated still had most of the function of AF1 (data not shown). Although the CACCC box is critical to the function of the tryptophan oxygenase gene GRE and is close to the GRE in several other genes (metallothionein II_A, growth hormone, TAT, lysozyme, MMTV, murine sarcoma virus, and uteroglobin; 26), it is apparently no more than a small component of the accessory elements in the PEPCK gene GRU. The AF2 region contains the sequence 5'-TGTGGTTTT-3', which is identical in eight of nine positions to the sequence 5'-TGTGGTGTT-3', which is a component of the polyomavirus enhancer (16). Otherwise, the accessory factor sites bear no resemblance to known DNA elements.

The AF1 and AF2 regions function independently when fused to a PEPCK promoter that includes two GR binding regions but do not act in the absence of the latter. Although we found no direct evidence of an influence of the accessory factors on the binding of GR to its sites, or vice versa, there is evidence that proteins other than the GR are somehow required for activation of glucocorticoid-inducible genes, as reviewed in the introduction. Given the complexity of this entire element and the fact that the receptor binding sites are themselves, inactive, we have chosen to call this region a GRU rather than a GRE.

Previous analyses of the GRE in the PEPCK gene promoter led to conflicting results. Short and co-workers, using the stable transfection approach, identified two regions, located between −108 and −62 and between −68 and +73, that appeared to be involved (27). In a study from this laboratory, a construct in which the sequence between −76 and −39 was deleted did not affect the glucocorticoid response (21). We still are unable to demonstrate a role of any sequence between −100 and the cap site. Petersen et al. (22), using the transient transfection approach, identified two very different elements, located at ~−1165 and ~−465. Subsequently, we have not been able to demonstrate a functional element at ~−1165, even though there is a strong association of the GR with a DNA sequence in this region. Our present view, which will be described in detail elsewhere (E. Imai et al., unpublished data), is that the GRU described in this report is primarily responsible for the regulation of the PEPCK gene by glucocorticoid hormones.

It is obvious that the entire glucocorticoid response is not conferred by the GRU. Even when this unit is disabled, there remains about a two-fold response to the hormone. A set of 5' deletion mutation experiments showed a small diminution in the response to dexamethasone (from twofold to the control level) between −306 and −271 (Fig. 1). Whether this small effect of dexamethasone in constructs containing the DNA between the GRU and the cap site comes from a cryptic GRU, represents the interaction of the

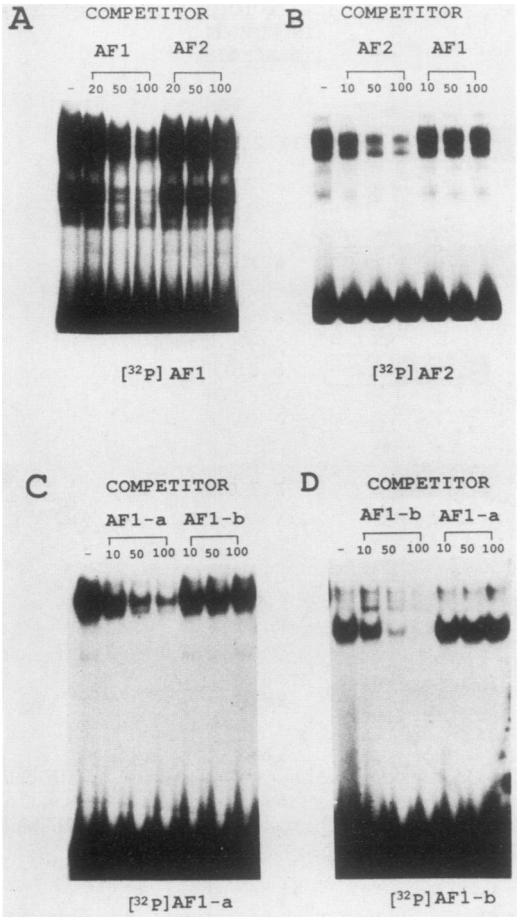

FIG. 6. Analysis of the interaction of accessory factors with AF1 and AF2. A gel retardation assay was performed as described in Materials and Methods. The DNA fragments used were as follows: (A) 8 fmol of DNA fragment −460 to −430 (AF1); (B) 5 fmol of DNA fragment −420 to −402 (AF2); (C) 18 fmol of DNA fragment −454 to −438 (AF1-a); (D) 20 fmol of DNA fragment −441 to −426 (AF1-b). Each was end labeled and incubated with 4 μg of rat liver nuclear extract with or without competitor DNA. The molar excess of competitor DNA is indicated at the top of each panel. Samples were subjected to electrophoresis through 6% native acrylamide gels as described in Materials and Methods.

hormone with a heterologous receptor, or is a nonspecific effect of the glucocorticoid-receptor complex acting through some other element remains at issue.

The central issue in this analysis is, Why is the PEPCK gene GRU so complex? One mechanistic possibility is that the glucocorticoid receptor binds weakly to the DNA in this promoter and therefore requires the interaction of other factors in order to achieve the binding specificity and affinity required for transactivation. The receptor binding elements certainly have the poorest homology with the consensus GRE of any such element yet reported, but this lack of homology may be more apparent than real. In recent analyses of the TAT GRE, 4 of the 12 conserved nucleotides were shown to be especially important for GR binding and function (29, 31). Three of four of these critical nucleotides are present in both GR1 and GR2 (Fig. 2B). We did not directly determine relative binding affinities, but comparable footprints of the MMTV GRE were obtained by using significantly less GR. The purpose of the accessory factor sites in the PEPCK gene GRU could be to enhance binding of the receptor, although in vitro assays with the AF1 and AF2 elements have so far failed to show any apparent interactions with the GR sites. A variation of this view would be that the accessory factor-receptor complex is necessary for transactivation at a distance, as implied in the studies of Strale et al. (30).

A more interesting possibility is that several of the positive (cAMP and glucocorticoids) and negative (insulin and phorbol esters) mechanisms involved in PEPCK gene regulation are mediated through a common DNA sequence. Insulin, for example, could induce or modify a transcription factor which would then bind to a site that precludes the binding of the GR to its site(s). The role of the accessory elements would be to direct binding of one or more factors that prevent the binding of the insulin response factor. This would thereby allow the glucocorticoid receptor to bind, and in this scenario the accessory factors would have no direct involvement with the GR in transactivation. According to this model, one would predict that an insulin response element must be located within, or overlap, the GRU. Also, in cells in which the PEPCK gene is not affected by insulin, perhaps because of the absence of the insulin response factor, the GR would work directly through its binding sites without the requirement of the accessory elements and associated factors. These possibilities can be tested directly.

Given the central role that PEPCK plays in gluconeogenesis, it is not surprising that the regulation of this gene involves positively and negatively acting hormones, each of which may work through complex combinations of DNA elements and *trans*-acting factors. Therein lies the probable answer to the question posed above. As glucocorticoids were named for their ability to promote gluconeogenesis, one could argue that the PEPCK gene GRU is not unusual but rather is the standard.

ACKNOWLEDGMENTS

We thank Keith Yamamoto for helpful comments and Deborah Caplenor for preparing the manuscript.

This work was supported by Public Health Service grant DK35107 from the National Institutes of Health, the Vanderbilt Diabetes Research and Training Center (grant DK20593), and the Swedish Medical Research Council (grant 2819).

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