The phosphoenolpyruvate carboxykinase (PEPCK) gene promoter contains a glucocorticoid response unit (GRU) that includes three accessory factor-binding sites (AF1, AF2, and AF3), two glucocorticoid receptor-binding sites (GR1 and GR2), and a cAMP response element. All of these elements, and the proteins that bind to them, are required for a complete glucocorticoid response. The PEPCK promoter also contains a retinoic acid response unit (RARU) that consists of two retinoic acid response elements (RARE1 and RARE2) that bind retinoic acid receptor/cis-retinoic acid receptor heterodimers. The sequences of RARE1 and RARE2 coincide with those for AF1 and AF3, respectively. Thus, the PEPCK promoter can mediate different hormone responses through hormone response units that utilize common elements, but that bind different sets of proteins. We reasoned that each response might require a unique structural assembly and therefore tested how various arrangements of the PEPCK promoter affect the actions of either glucocorticoids or retinoic acid.

The activation of the PEPCK gene in response to glucocorticoids requires a specific set of cis-acting elements that must be precisely positioned within the GRU. The distance between AF2 and GR1 is critical for the glucocorticoid response, and since the AF2 factor, HNF3, interacts with GR in vitro, this protein-protein interaction may be important for the glucocorticoid response. By contrast, the distance and orientation requirements of AF1 and AF3 with respect to GR1 are more flexible. In the case of the RARU, although the relative positions of RARE1 and RARE2 are important for the retinoic acid response, more tolerance for distance and stereospecific alignment between RARE1 and RARE2 is allowed. In addition, we show that the GRU and the RARU can act as a module, within a restricted region, in the context of the PEPCK promoter and in limited contexts of a heterologous promoter. These observations suggest that the structural requirements of the GRU and the RARU are different, and this may have important implications for how multiple hormonal signals are integrated through this promoter. (Molecular Endocrinology 12: 1487–1498, 1998)
The proteins that mediate the accessory activities through AF1, AF2, and AF3 have been identified. Both hepatic nuclear factor 4 (HNF4) and chicken ovalbumin upstream promoter transcription factor (COUP-TF) bind the AF1 element and act as accessory factors to the glucocorticoid response (11). Members of the hepatic nuclear factor 3 (HNF3) family and the CCAAT-enhancer binding protein (C/EBP) family bind to AF2, although only binding of HNF3 specifically correlates with the ability of the AF2 element to potentiate the glucocorticoid response (12). COUP-TF also binds to AF3 and acts as the accessory factor through this element (8).

The CRE is important for basal transcription, the cAMP response, and the glucocorticoid response (9, 10, 13). A number of proteins bind the CRE, including CREB, C/EBP family members, and AP1. CREB and GR interact in vitro (9), but this interaction has not been proven to be required for the glucocorticoid response. In fact, recent experiments suggest that C/EBPβ is the accessory factor acting through the CRE (K. Yamada and D. K. Granner, unpublished observation).

AF1, AF2, and AF3, as well as GR1 and GR2 are shown. The sequence of RARE1 contains a direct repeat of imperfect nuclear hormone receptor half-sites separated by 1 bp. The sequence of RARE2 contains a direct repeat of a degenerate RAR/RXR half-site (α) and a consensus half-site (γ) separated by 5 bp. AF3 is coincident with the γ-site. The location of each cis element with respect to the transcription start site is shown above these sequences.
RARE1 and RARE2 each confer a RA response in the context of a heterologous promoter (4, 15). Therefore, both RARE1 and RARE2 are required for the RA response of the PEPCK promoter and, together, they constitute the PEPCK RA response unit (RARU, Ref. 15 and see Fig. 1A). It is noteworthy that RA is required for the proper expression and regulation of the PEPCK gene in transgenic mice, and that the RARU apparently mediates these effects (16, 17). AF2, in addition to its glucocorticoid accessory factor activity, mediates a substantial portion of the inhibitory response of insulin and is a component of an insulin response unit (IRU (18)). The CRE, along with a C/EBPα-binding site in the P3I region of the PEPCK promoter (located between −246/−238), mediates the cAMP response (10, 19). Thus, each hormone response is mediated by a different set of multiple elements, termed hormone response units (HRUs). Furthermore, many of the individual elements participate in more than one HRU and are capable of binding more than one set of protein complexes.

In the case of the glucocorticoid response of the PEPCK gene, the substitution of the AF2 element with certain transcription factor-binding sites replaces accessory activity, but other binding sites are ineffective (12). This suggested to us that specific transcription factors are required for accessory factor activity and that a precise structure of the GRU might be required for the proper induction of the PEPCK gene by glucocorticoids. The interaction between nuclear factors and DNA is an essential event for transcriptional regulation in eukaryotic cells. DNA-binding nuclear factors interact with one another and also with other proteins, termed coactivators, to form complex networks of DNA-protein-protein interactions (20, 21). Thus, the 5′-regulatory regions of genes are often comprised of large nucleoprotein complexes whose correctly assembled structures are required for the proper regulation of the gene. For instance, Thanos and Maniatis (22) have shown that the virus-mediated induction of the human interferon-β (IFNβ) gene requires a specific set of regulatory elements that must be precisely arranged in relation to the helical phasing of the DNA template (22).

If specific DNA elements can bind different trans-acting factors with similar affinity, and these factors are present in approximately equal abundance in the nucleus, what determines the specificity of the hormone response? A structure/function analysis of the PEPCK promoter was initiated in an effort to explore this question. Here we show that there are some similarities, but also some important differences, in the structural requirements for function through the distal component of the GRU and the RARU in the PEPCK promoter (Fig. 1). The activation of the PEPCK gene in response to glucocorticoids requires that a specific set of cis-acting elements be positioned correctly within the GRU. The distance and spatial alignment between AF2 and GR1 is critical for the glucocorticoid response, but more flexibility is allowed for the distance and orientation of AF1 and AF3 with respect to GR1. In contrast, the relative positions of RARE1 and RARE2 to one another are important for the RA response through the RARU, but their specific alignment is less important. In addition, we show that the dGRU and the RARU can act as modules within a restricted region in the context of the PEPCK promoter and in limited contexts in a heterologous promoter. These observations suggest that different sets of promoter structures are required for different hormone responses and have important implications for how multiple hormonal signals are integrated through promoters.

RESULTS

A Complete Glucocorticoid Response Is Dependent on the Order and Position of the Accessory Factor Elements

We tested whether the accessory factor (AF) elements of the GRU are restricted to specific positions in the PEPCK promoter (Fig. 2). Each AF element was switched with another AF element, in a variety of combinations, in the context of the intact PEPCK promoter. The exact spacing between the individual elements was maintained in accordance with the native PEPCK promoter. A 60% reduction of the glucocorticoid response occurred when either AF1 or AF3 was put in the place of AF2 (designated 11G3 and 13G3, respectively, see Fig. 2, lines 2 and 3). The loss of function is equivalent to a 5-bp block mutant in AF2 or a complete deletion of the element (7, 12, 30). While it is possible that the alignment of the substituting element is not optimal, the simplest explanation is that there is no tolerance for a substitution of elements at AF2. By contrast, the substitution of either AF1 for AF3, or AF3 for AF1 (12G2 and 32G3, respectively), resulted in a complete glucocorticoid response (Fig. 2, lines 4 and 5); thus, AF1 and AF3 are interchangeable. This result is consistent with our previous observation that COUP-TF functions as an accessory factor for the glucocorticoid response through both AF1 and AF3 (8, 11). The insertion of AF2 in place of AF1 (22G3) or AF3 (12G2) resulted in a 60% and 40% reduction of the glucocorticoid response, respectively (Fig. 2, lines 6 and 7). This substitution is the functional equivalent of the double-point mutations that completely disable AF1 or AF3 as accessory elements (8, 11). In addition, constructs were made wherein combinations of two of the AF elements were changed. In these constructs (21G3 or 13G2), all three individual AF elements are present in the dGRU, but they are in the wrong sequence. Neither of these constructs supported a complete glucocorticoid response (Fig. 2, lines 8 and 9). These results suggest that a complete glucocorticoid response requires the correct order and position of the AFs.
A Complete RA Response Requires Two Properly Positioned RAREs

We have previously shown that RARE1 and RARE2 are both required for a complete RA response and together constitute the RARU in the PEPCK promoter (Ref. 15 and see Fig. 1). We tested whether the changes made in the constructs described above affected the RA response. The replacement of RARE1 (22G3) or RARE2 (12G2) with AF2 diminished the RA response by about 60% and 40% from the fully induced wild-type value, respectively (compare lines 1, 6, and 7 in Fig. 2). This is the functional equivalent of a deletion or substitution mutation of these elements (4, 15). By contrast, the replacement of RARE1 with RARE2 (32G3), or vice versa (12G1), maintained the RA response (Fig. 2, lines 4 and 5). Thus, RARE1 and RARE2 are interchangeable. The addition of a third RARE at the AF2 site had no effect on the RA response (compare lines 1, 2, and 3 in Fig. 2). This is the functional equivalent of a deletion or substitution mutation of these elements (4, 15). By contrast, the replacement of RARE1 with RARE2 (32G3), or vice versa (12G1), maintained the RA response (compare lines 1, 2, and 3 in Fig. 2). On the other hand, the inverted exchange of RARE1 and AF2 (21G3) or AF2 and RARE2 (13G2) reduced the RA response by 25–50% (Fig. 2, lines 8 and 9) despite the fact that the promoter still contains two RAREs. Together, these data suggest that AF2 occupies a position within the PEPCK promoter that is incapable of supporting an RARE or, alternatively, that the distance between RARE1 and RARE2 is important for the RA response (see below). Thus, while each RARE contributes about 50% of the RA response, these elements may not be completely independent.

The Precise Distance between AF2 and GR1 Is Critical for a Complete Glucocorticoid Response

Since a specific combination and arrangement of AF elements is required for a proper glucocorticoid response, we tested whether the helical orientation or precise distance between each AF element of the PEPCK gene promoter affects the glucocorticoid response. Constructs were made wherein 5- or 10-bp insertions, which result in a half- or full-helical turn of DNA, respectively, were placed at strategic locations within the PEPCK promoter. The glucocorticoid response was reduced by about 40% when a half-helical turn (5 bp) was introduced between AF2 and GR1 (Fig. 3, line 2). The glucocorticoid response was also reduced by 40% when an additional 5-bp insertion was placed between AF1 and AF2, which effectively rotates the AF2 element one half-helical turn with respect to the rest of the promoter (Fig. 3, line 2). However, insertion of a 5-bp sequence between AF1 and AF2 did not affect the glucocorticoid response (Fig. 3, line 4), and a 5-bp insertion placed on both sides of the AF3 element had no effect on the glucocorticoid re-
sponse (Fig. 3, line 5). These results suggest that, while the helical alignment of AF1 or AF3 and GR1 is not important, the alignment of AF2 and GR1 is critical for a full glucocorticoid response. In fact, 5-bp insertions on both sides of GR1, which result in a half-helical turn of GR1 with respect to AF2 and AF3, markedly reduced the glucocorticoid response (Fig. 3, line 6). However, an insertion of 10 bp between AF2 and GR1, which maintains the relative positions of the binding sites on the same face of the DNA helix, did not completely restore the glucocorticoid response (Fig. 3, line 7). These results, in the aggregate, suggest that the distance between AF2 and GR1 is critical for a complete glucocorticoid response.

We next inserted a 5- or 10-bp segment between the dGRU and the proximal promoter to determine whether a certain alignment of the dGRU and the proximal promoter, i.e. the NF1 element, the CRE, and the TATA box (Ref. 10 and see Fig. 1), is required for a maximal response to glucocorticoids. As shown in Fig. 3, there was no difference between the wild-type promoter and these two constructs (compare lines 1, 8, and 9 in Fig. 3). These results suggest that the glucocorticoid response is not sensitive to changes of the helical phasing between the dGRU and the proximal promoter.

A Specific Alignment of RARE1 and RARE2 Is Not Critical for a Complete RA Response

Since the GRU and the RARU share two elements (AF1/RARE1 and AF3/RARE2), one might expect that any perturbations in the DNA template that affect the function of the GRU should also affect the RARU. Intriguingly, none of the constructs described in Fig. 3 affected the RA response, so precise positioning of RARE1 and RARE2 is not required within the RARU. In addition, the relative alignment of the RARU and the proximal promoter does not affect the RA response. These experiments demonstrate that the structural requirements of the GRU and the RARU are different.

HNF3 Associates with GR in Vitro

The requirement for a precise distance between AF2 and GR1 for a full glucocorticoid response suggested that a protein-protein interaction between HNF3 and GR may be involved. Pull down experiments were performed to test whether a glutathione S-transferase (GST)-GR fusion protein and 35S-labeled HNF3β interact in vitro. As shown in Fig. 4, left panel, appreciable binding of HNF3β to GST-GR was detected. The converse experiment was also performed wherein 35S-labeled GR coeluted with a His-tagged HNF3α (Fig. 4,
The presence of 0.5 μM dexamethasone in the binding reactions did not significantly alter the extent of binding of HNF3 to the GR (Fig. 4).

The dGRU and RARU Function within a Restricted Region of the PEPCK Promoter

We interpret the substitution and phasing experiments described above to mean that there are specific requirements for the arrangements of the transcription factors bound to the regulatory elements comprising the dGRU or the RARU, and that this assemblage provides a functional unit for specific hormone responses. A test of the dGRU and the RARU as modular units in the PEPCK promoter was conducted using a series of constructs wherein insertions or deletions were made between the dGRU/RARU and the proximal promoter. All of the constructs were designed so that the length of the insertion or deletion was a multiple of 10.5; thus the dGRU/RARU and the TATA box maintain their relative positions on the DNA helix. The insertion of 126 or 252 bp between the dGRU/RARU and the proximal promoter decreased the glucocorticoid and the RA responses by about 50% and 30%, respectively. This suggests that insertions that increase the distance between the dGRU/RARU and the proximal promoter compromise the ability of the promoter to confer a complete glucocorticoid response, and to a lesser extent, a complete RA response (compare lines 1, 2, and 3 in Fig. 5). The deletion of 63 or 126 bp between the dGRU and the proximal promoter did not reduce the glucocorticoid response (Fig. 5, lines 4 and 5). In fact, the glucocorticoid response was increased by 2-fold when 126 bp was deleted, although basal transcription was not affected in any of these constructs (data not shown).

![Fig. 4. The Interaction of HNF3 and GR in Vitro](image)

Experiments were performed to determine whether HNF3 and GR can interact in vitro. In the experiment illustrated in the left panel, GST-GR or GST bound to glutathione-agarose beads was incubated with 35S-labeled HNF3 in the presence or absence of 0.5 μM dexamethasone. The bound proteins were eluted from the beads, subjected to SDS-PAGE, and visualized by autoradiography. The converse experiment was also performed wherein 35S-labeled GR was incubated with His-tagged HNF3 bound to a nickel-chelating Sepharose column. The bound proteins were also separated on SDS-PAGE gel and visualized by autoradiography (right panel). These results are representative of three separate experiments.

![Fig. 5. The Effects of Insertions or Deletions between the dGRU and the Proximal Promoter on the Glucocorticoid or RA Response](image)

A series of constructs were made that contained insertions or deletions between the dGRU and the NF1-binding site in the context of the PEPCK promoter fused to CAT, as described in Materials and Methods. These reporter plasmids (10 μg) were cotransfected with pSV2GR or pRShRARα (5 μg) into H4IIE cells. Cells were incubated for 18 h with or without Dex (0.5 μM) or RA (2 μM), and CAT activity was measured in cell extracts. The data are expressed as the mean ± SEM of the percentage of the wild-type Dex or RA response from at least six independent experiments. The basal expression of CAT activity from these reporter constructs was not significantly different (data not shown).
However, a 189-bp deletion resulted in a reduction of the glucocorticoid response by about 50% (Fig. 5, line 6). By contrast, neither of these deletions affected the RA response. These results demonstrate that the dGRU functions within a restricted region in the context of the PEPCK promoter and may be dependent on other promoter elements not yet defined. By contrast, the RARU is relatively unaffected by its position in the promoter.

The dGRU and RARU Can Function within the Context of a Heterologous Promoter

We tested whether the dGRU/RARU functions as a modular unit in the context of the minimal thymidine kinase (tk) promoter ligated to the chloramphenicol acetyltransferase (CAT) reporter gene (tkCAT). Neither tkCAT alone, nor a construct wherein GR1 is ligated to tkCAT (GR1tk), conferred a glucocorticoid response (Fig. 6A, lines 1 and 2). This result confirms previous observations that GR1 or GR2, either alone or in combination, cannot confer a glucocorticoid response in a heterologous promoter context (27). A construct was made wherein the dGRU/RARU was placed upstream of the tk promoter [dGRU/RARU(−467/−314)tk] to test whether the addition of the three accessory elements help provide a glucocorticoid response in this context. This construct gave a modest, 4.5-fold glucocorticoid response when transfected into H4IIIE cells (Fig. 6A, line 3). These results are in contrast to those of Imai et al. who found that the sequences from −467 to −200 of the PEPCK promoter, placed upstream of the tk promoter, failed to confer a glucocorticoid response (9). These results, along with the results presented in Fig. 5, suggest that the distance between the dGRU and the proximal promoter is a critical parameter for the proper functioning of the dGRU. Alternatively, there may be undefined repressor elements in the −467/−200 construct that are absent in the −467/−321 construct. To test this, a construct was made [dGRU/RARU(−467/−113)tk] wherein the dGRU was placed upstream of the tk promoter so that the distance between the dGRU and the proximal promoter was the same as that in the PEPCK promoter. This construct conferred the full 15-fold glucocorticoid response (Fig. 6A, line 4), a somewhat surprising result since the tk promoter does not contain a CRE (located at −93/−86 in the PEPCK promoter) but it could contain a cis-acting element(s) that substitutes for the CRE in this context. Another possibility is that one or more elements between −200 and −113 contribute to the glucocorticoid response. Alternatively, the distance between the dGRU and the start site, or the position the dGRU occupies in this context, is optimal.

We also compared the ability of the RARU, and the individual RAREs, to confer a RA response in the context of a heterologous promoter. The RA response conferred by tkCAT was compared with two constructs wherein RARE1 or RARE2 was ligated to tkCAT (compare lines 1, 2, and 3 in Fig. 6B). While tkCAT did not provide a response to RA, the AF1(RARE1) construct conferred a 2-fold greater RA response than did the AF3(RARE2) construct. These results are consistent with previous observations, including a 5’-deletion analysis (4, 15). When the entire RARU was placed upstream of the tk promoter, the RA response was not any greater than that conferred by RARE1 alone in the same context (compare lines 2 and 4 in Fig. 6B). However, when the RARU was placed in a position wherein the distance between the RARU and the start site of the tk promoter was identical to that of the PEPCK promoter, there was a 13-fold RA response, which is about 2-fold greater than when the RARU was positioned 200 bp closer to the proximal promoter (compare lines 4 and 5 in Fig. 6B). This result suggests that the RARU is either in an optimal position for transactivation or that there are accessory elements in the sequence between −321 and −113. The data presented in this figure suggest that the dGRU/RARU functions as a modular enhancer unit, and it responds to two different hormones in the context of a minimal
heterologous promoter. In addition, these data, along with those displayed in Fig. 5, suggest that the distance between the dGRU/RARU and the proximal promoter of the PEPCK gene is optimal for these two hormonal responses.

**DISCUSSION**

Transcription-regulatory regions of eukaryotic cell promoters generally consist of clusters of transcription factor-binding sites (31) that can provide a coordinated, specialized response to an environmental challenge. For example, the virus-induced enhancer complex of the IFNβ gene promoter is comprised of a CRE, an NF-κB binding site, and an IFNβ response element (22). These binding sites all act as simple elements when placed in heterologous contexts. In contrast, the IFNβ promoter is exclusively activated by virus infection and not by any of the effectors that act on the isolated elements. Viral induction is achieved by the assembly of a higher-order enhancer complex, termed an enhanceosome, that requires a precise helical phasing relationship between the individual transcription factor-binding sites. The substitution of any transcription factor-binding sites, or a change of the helical phasing between different transcription factor-binding sites, decreases the magnitude of viral induction (22, 32).

Some years ago we showed that the GR-binding sites (GR1 and GR2) in the PEPCK gene promoter are associated with other, functionally essential transcription factor binding sites (7). It now appears that, in their natural context, most (if not all) hormone response elements, including GREs, confer their cognate responses when in association with DNA-binding accessory factors. These accessory factor-binding sites are often located in the proximity of the HRE and together constitute what has been referred to as HRUs (6). In the case of the PEPCK gene, several HRUs have been described in some detail. A common theme is that each of these HRUs contains multifunctional elements that can bind to more than one set of proteins and thereby mediate responses to different signals. Thus, the RARU is composed of RARE1 and RARE2 (that bind RAR/RXR heterodimers), and these are also components of the GRU, which consists of at least six DNA elements. This overlapping set of HRUs, inclusively termed the PEPCK gene metabolic control domain, provides an integrated response of multiple hormonal and metabolic stimuli. For example, regulation of the transcription of the PEPCK gene, which encodes the enzyme critical for gluconeogenesis, is controlled by a metabolic control domain that consists of glucocorticoid, RA, cAMP, and insulin HRUs (6).

We have now demonstrated that the RARU and GRU of the PEPCK gene promoter operate properly only when in an ordered structure. Despite having common elements, the structural requirements for the glucocorticoid and the RA response units are different. The proper positions of AF1, AF2, and AF3 are required for a complete glucocorticoid response (Fig. 2). AF1 and AF3 are interchangeable, which is consistent with the fact that both elements bind COUP-TF, and this orphan nuclear hormone receptor can confer accessory factor activity from both AF elements (8, 11). AF2 cannot substitute for either AF1 or AF3, and, conversely, neither AF1 nor AF3 can substitute for AF2 (Fig. 2 and Ref. 12). A previous study also provided evidence that AF2 is functionally different from AF1 and AF3. Scott et al. (27) replaced GR1 with a high-affinity palindromic GRE in the context of the PEPCK promoter and found that, while AF1 and AF3 are no longer required for a complete glucocorticoid response, AF2 is still required. In addition, Wang et al. (12) found that AF2 activity has a relatively specific requirement for HNF3. Thus, if AF2 is replaced with a binding site for SP1, or is mutated so that C/EBP isoforms (but not HNF3) can bind, there is a reduction in the glucocorticoid response equivalent to the removal of AF2. There is also a relatively specific requirement for the factors that bind to AF1 and AF3 (Fig. 2, and J.-C. Wang and D. K. Granner, unpublished data). This is in contrast to earlier studies which noted that essentially any transcription factor placed in close proximity to a canonical GRE potentiates the glucocorticoid response (33). The requirement for specific trans-acting factors that bind in the proper spatial alignment is reminiscent of the IFNβ example cited above and implies that multiple protein-protein interactions are made in the context of a large nucleoprotein complex whose integrity is essential for a complete glucocorticoid response.

The precise distance between AF2 and GR1 is critical for the glucocorticoid response, since insertions of 5 or 10 bp between the two elements diminish the response. Thus, it is possible that an interaction between HNF3 and GR, which we demonstrate herein, may be important for the glucocorticoid response. A similar situation exists in a number of gene promoters (34–37). The major histocompatibility class (MHC) II human leukocyte antigen-DRα gene (HLA-DRA) provides one example. Vilen et al. demonstrated that a number of upstream regulatory elements, including the Y box, the X box, and the S element, are important for constitutive and IFNγ-inducible expression of the MHC II HLA-DRA gene (35). The precise spacing between the S element and the X box is required for the proper expression of this gene. The authors conclude that two different proteins may interact with the S and X sites and that rigid protein-protein interaction requirements are highly dependent on the interelement distance. With this in mind, it is noteworthy that the spacing between AF2 and GR1 is exactly conserved at 18 bp in the PEPCK gene promoters of rat, mouse, and human. By contrast, the spacing between AF1 or AF3 and GR1 is variable in these species (Ref. 38 and unpublished data by C. Williams, D. K. Granner, and R. Chalkley). In this context, it is noteworthy that 5- and
10-bp insertions between AF1 and GR1, or GR2 and AF3, had no effect on the glucocorticoid response (Fig. 3).

The RARU and the distal component of the GRU are both contained within the same 140-bp segment of the PEPCK promoter (−460 to −320) and share two elements (AF1/RARE1 and AF3/RARE2). Thus, it is interesting that, in contrast to the glucocorticoid response, the RA response is not sensitive to changes in the helical alignment of elements within this segment of DNA (Fig. 3). One possible explanation is that RARE1 and RARE2 both act as independent RA-stimulated enhancers and are not part of a higher ordered structure that requires the proper placement of these elements for a complete RA response. However, an arrangement where RARE1 and RARE2 are placed relatively close together, so that one or the other element occupies the AF2 position in the PEPCK promoter, results in an incomplete RA response (Fig. 2). In addition, the RARU confers a −7-fold RA response in the context of the tkCAT promoter, but a 13-fold RA response when an additional 200 bp of the PEPCK promoter are added, suggesting that this sequence contains accessory elements that are necessary for the RA response (Fig. 5). Thus, while the RAREs are not completely autonomous and require a higher ordered structure to function properly, the actual structural requirements of the RARU differ from those of the GRU.

The protein-DNA interactions between hormone receptor and cognate DNA element, and between accessory factors and their DNA elements, described here and elsewhere, are probably not sufficient to explain how discrimination between glucocorticoid and RA signals is provided at the PEPCK gene promoter. One or more of the several coactivators known to be involved in the action of members of the steroid hormone receptor superfamily could provide the required selectivity. This class of proteins includes SRC-1 and family members, TIF1 and TIF2/GRIP1, as well as CREB-binding protein (CBP) and its homolog, p300 (39–43). Accordingly, the ligand, either glucocorticoid or RA, by binding to and activating its cognate receptor, could initiate a series of protein-DNA and protein-protein interactions that result in the assembly of different, complex nucleoprotein structures that activate transcription of the PEPCK gene. Korzus et al. demonstrated that RAR-mediated transcription requires CBP/p300, SRC-1, p/CAF, and p/CIP (44–46), while signal transducer and activator of transcription-1 (STAT-1)-mediated transcription requires CBP/p300 and p/CIP. In the PEPCK promoter, there may also be different sets of cofactors assembled in a ligand-specific manner that may thus specify either the glucocorticoid or RA response.

Specificity of the hormone response in the PEPCK gene can be explained by assuming that specific enhancosomes are assembled in response to each ligand, but the fact remains that the GRU and RARU share DNA elements that have the capacity to bind different transcription factors. With the exception of the RAR, these factors (COUP-TF, HNF4, RXR) are in abundance in H4IE cell nuclei, and all bind with approximately the same affinity to the same contact points in the AF1/RARE1 and AF3/RARE2 elements. Hall et al. (11) demonstrated that RAR overexpression, in the absence of RA, had no effect on the glucocorticoid response and suggested that RAR/RXR heterodimers, while capable of binding AF1 and AF3, are not accessory factors for this response. However, the possibility that ligand-bound RAR/RXR may act as an accessory factor for the glucocorticoid response has not been excluded. In this regard, it is interesting to speculate that the integration of multiple hormone responses may proceed through hybrid HRUs that contain portions of each individual HRU. Accordingly, the overall regulatory structure, the metabolic control domain, would consist of a number of DNA elements, DNA-binding proteins, and protein-binding coregulatory proteins from which various combinations are assembled in response to different signals. This general mechanism would provide the selectivity and specificity, the different degrees of repression, additivity, or synergism, and for the dominance of one response over another, which are required of genes that encode proteins involved in complex processes such as gluconeogenesis.

**MATERIALS AND METHODS**

**Plasmid Construction**

The construction of the plasmid pPL32, which contains the PEPCK promoter from −457 to +69 relative to the transcription start site ligated to the CAT reporter gene, has been described (23). Site-directed mutations of pPL32, including AF1(+5)AF2, AF2(+5)GR1, (+5)AF2(+5), 11G3, 22G3, and 21G3 were made using the PCR megaprimer method. Other plasmids, including p32G3, 12G1, 12G2, 13G3, 13G2, AF2(+5)GR1, AF3(+5)P4, AF3(+5)P4, (+5)AF3(+5), AF2(+5)GR1, GR1(+5)GR2, dGRU/RARU(−63)NF1, dGRU/RARU(−128)NF1, and dGRU/RARU(−189)NF1 that were used for substitution, spacing, and deletion experiments, were constructed using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Some of the constructs were made by inserting a 5-bp (5′-TGCAAGAG-3′) or a 10-bp (5′-TGCATGAGAG-3′) sequence (24, 25) into various sites within the PEPCK promoter and were designated with a (+5) or (+10). The sequences of the oligonucleotides used in this study are shown in Table 1.

The mutations of pPL32 that had DNA fragments inserted between the dGRU and the distal boundary of the basal promoter, demarcated at 5′-end of the NF1 element, termed dGRU/RARU(+126)NF1 and dGRU/RARU(+252)NF1, were created in three steps. First, DNA fragments of 126 or 252 bp were generated by PCR using the plasmid pBR322 as a template and the primers (+126/+252)a and (+126)b, or the primers (+126/+252)a and (+252)b, respectively. The primer (+126/+252)a contains a sequence that generates HindIII and XbaI sites after amplification, and the primers (+126)b and (+252)b each contain sequences that generate a single SalI site. Second, the PCR products were digested with HindIII and SalI, and the resulting fragments of 126 or 252 bp were then subcloned into the HindIII-SalI sites of pPL33,
which contains the PEPCK promoter from −310 to +69 ligated to CAT, to generate the plasmids pPL33(+126) and pPL33(+252), respectively. Finally, an XbaI fragment that contains the dGRU was amplified from pPL32 by PCR using the primers dGRUa and dGRUb, which generates an XbaI fragment that subclones into the tkCAT. The plasmid tkCAT contains the tk promoter from −252 to +113 was constructed in a similar manner by amplifying the sequences of pPL32 between −252 and −113 by PCR using the primers dGRU (−252 to −113)a and dGRU (−252 to −113)b that generate a HindIII and a BamHI site, respectively. The HindIII and BamHI fragments were then subcloned into the HindIII and BamHI sites of tkCAT. The plasmids GR1tk, AF1(RARE1)tk, and AF3(RARE2)tk have been previously described (4, 15, 27).

The cDNA that encodes rat HNF3b was a gift from Dr. Robert Costa (University of Illinois, Chicago). The plasmid dGRU/RARU (−467 to −113)tk was constructed in a similar manner by amplifying the sequences of pPL32 between −467 and −314 by PCR using the primers dGRU (−467 to −314)a and dGRU (−467 to −314)b that generate a HindIII and a BamHI site, respectively. The HindIII and BamHI fragments were then subcloned into the HindIII and BamHI sites of tkCAT. The plasmids GR1tk, AF1(RARE1)tk, and AF3(RARE2)tk have been previously described (4, 15, 27). The sequences for AF1, GR1, and AF3 are included in Fig. 1.

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The sequences for AF1, GR1, and AF3 are included in Fig. 1.
synthesis of labeled HNF3α protein. To generate constructs for the overexpression of a GST-fusion protein in Escherichia coli, the full-length cDNA that encodes GR was amplified by PCR using the primers GRf and GRb, which generate an EcoRI and a SalI site, respectively. The PCR product was digested with EcoRI and SalI and subcloned into the pGEX-5X-1 plasmid (Pharmacia, Piscataway, NJ). The resulting plasmid was named GST-GR. The expression vector that encodes rat HNF3α, with an amino-terminal histidine tag, was a gift from Dr. Kenneth Zaret (Brown University, Providence, RI).

The DNA sequence of all constructs was verified byideoxy sequencing. All oligonucleotides were produced on a Perceptive Biosystems Expedite 8909 DNA synthesizer located in the Vanderbilt University Diabetes Research and Training Center.

**Transient Transfection**

The maintenance and transfection of H4IE cells and the measurement of CAT activity have been described previously (14, 23, 28). The mammalian expression vectors, pSV2GR and pRShRAR, were provided by Keith Yamamoto (University of California, San Francisco) and Ronald Evans (Salk Institute, San Diego, CA), respectively.

**GST-Fusion Protein Pull-Down Assay**

The GST-GR fusion proteins were expressed in TOPP3 cells (Stratagene) by induction with 0.4 mM isopropyl-β-D-thiogalactopyranoside at 30°C for 4 h. Cell pellets were lysed and sonicated, after which an extract of soluble protein was prepared by centrifugation. An extract containing the GST fusion protein was mixed with glutathione-Sepharose (Sigma Chemical Co., St. Louis, MO) and incubated at 4°C. One microgram of various supercoiled DNA plasmids was transcribed in vitro then translated in the presence of [35S]methionine (ICN Pharmaceuticals, Inc., Costa Mesa, CA) in the TNT-coupled reticulocyte lysate system (Promega, Madison, WI). GST-GR fusion proteins were bound to glutathione-agarose beads in GST binding/washing buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 100 mM EDTA, 1% Triton X-100/1 mM phenylmethylsulfonyl fluoride, and 10 mM dithiothreitol) and were mixed with 10 µl of a reticulocyte lysate that contained in vitro-translated proteins. This was incubated at 25°C for 1 h in the presence or absence of 0.5 mM dexamethasone. The formed complex was washed three times with 500 µl of the binding/washing buffer and then once with a buffer that contains 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 5 mM EDTA. Bound proteins were eluted by boiling in 50 µl of 2× SDS loading buffer and analyzed by 10% SDS-PAGE.

**His-Tagged Fusion Protein Pull-Down Assay**

The expression and purification of rat His-tagged HNF3α have been described previously (29). The His-tagged protein was incubated with a nickel-chelating Sepharose slurry (Novagen, Madison, WI) at 4°C. The formed His-tagged HNF3α-Sepharose complex was then washed twice with binding buffer (20 mM Tris-HCl pH 7.9, 5 mM imidazole, 0.5 mM NaCl) and once with washing buffer (20 mM Tris-HCl, pH 7.9, 60 mM imidazole, 0.5 mM NaCl). Ten microliters of [35S]methionine-labeled protein and 200 µl of binding buffer were then added to the complex, and the protein-binding reaction was carried out for 1 h at 25°C in the presence or absence of 0.5 mM dexamethasone. The mixture was washed three times with binding buffer and then once with washing buffer. The resin was suspended in 2× SDS loading buffer and analyzed by 10% SDS-PAGE.

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