

The Glucose-6-Phosphatase Catalytic Subunit Gene Promoter Contains Both Positive and Negative Glucocorticoid Response Elements

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Glucose-6-phosphatase catalyzes the final step in the gluconeogenic and glycolytic pathways. Glucocorticoids stimulate glucose-6-phosphatase catalytic subunit (G6Pase) gene transcription and studies performed in H4IIE hepatoma cells demonstrate the presence of a glucocorticoid response unit (GRU) in the proximal G6Pase promoter. *In vitro* deoxyribonuclease I footprinting analyses show that the glucocorticoid receptor binds to three glucocorticoid response elements (GREs) in the –231 to –129 promoter region and transfection results indicate all three contribute to glucocorticoid induction of G6Pase gene transcription. Furthermore, binding sites for hepatocyte nuclear factor-1 and -4, CRE binding factors, and FKHR (FOXO1a) are required for the full glucocorticoid response. Chromatin immunoprecipitation assays

show that dexamethasone treatment stimulates glucocorticoid receptor and FKHR binding to the endogenous G6Pase promoter. Surprisingly, although glucocorticoids stimulate G6Pase gene transcription, deoxyribonuclease I footprinting and transfection analyses demonstrate the presence of a negative GRE and an associated negative accessory factor element in the –271 to –225 promoter region, which inhibit the glucocorticoid response. This appears to be the first report of a promoter that contains both positive and negative GREs, which function within the same cellular environment. We hypothesize that targeted signaling to the negative accessory element within the GRU may provide tight regulation of the glucocorticoid stimulation. (*Molecular Endocrinology* 19: 3001–3022, 2005)

GLUCOSE-6-PHOSPHATASE catalyzes the final step in both the gluconeogenic and glycolytic pathways, namely the hydrolysis of glucose-6-phosphate (G6P) to glucose and inorganic phosphate. Enzymatic activity is primarily detected in the liver and kidney (1, 2), but it is also found in the small intestine (3), pancreatic islets (4), and brain (5). Glucose-6-phosphatase is located in the endoplasmic reticulum

membrane and it is thought to exist as a multicomponent enzyme system, which is comprised of a catalytic subunit as well as individual transporters for G6P, glucose, and inorganic phosphate (1, 2). However, only the glucose-6-phosphatase catalytic subunit (G6Pase) (6) and G6P transporter (7) have been identified to date.

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Abbreviations: CAT, Chloramphenicol acetyltransferase; CBP, CREB-binding protein; C/EBP, CCAAT/enhancer binding protein; ChIP, chromatin immunoprecipitation; CRE, cAMP response element; CREB, CRE binding protein; poly(dI-dC), poly(deoxyinosine-deoxycytosine); DNase, deoxyribonuclease; DTT, dithiothreitol; G6P, glucose-6-phosphate; G6Pase, glucose-6-phosphatase catalytic subunit; GR, glucocorticoid receptor; GRE, glucocorticoid response elements; GRU, glucocorticoid response unit; GST, glutathione-S-transferase; HNF, hepatocyte nuclear factor; IRS, insulin response sequence; MMTV, mouse mammary tumor virus; nGRE, negative GRE; PEPCK, phosphoenolpyruvate carboxykinase; SDM, site-directed mutation; SRC, steroid receptor coactivator; TAT, tyrosine aminotransferase; TK, thymidine kinase.

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G6Pase gene expression is regulated by a number of hormones and metabolites, including insulin, glucagon, glucose, and glucocorticoids (see Ref. 8 for individual references). Glucocorticoids induce G6Pase mRNA expression in rat primary hepatocytes (9), as well as in rat H4IIE hepatoma cells (10, 11) and studies in the rat FAO (12) and H4IIE hepatoma cell lines (10, 13, 14) have shown that this effect is manifest, at least in part, at the transcriptional level.

Glucocorticoids are ligands for the glucocorticoid receptor (GR), a member of the nuclear hormone receptor superfamily (15). The non-ligand-bound GR is sequestered in the cytoplasm, but glucocorticoid binding induces GR translocation to the nucleus, where the receptor can either directly or indirectly bind gene promoters to regulate gene transcription (16). The GR stimulates gene transcription primarily through the recruitment of cofactor complexes and chromatin remodeling complexes (17–20). Glucocorticoid induc-

tion of transcription has been studied extensively for a number of promoters, including those of the mouse mammary tumor virus (MMTV), tyrosine aminotransferase (TAT), and phosphoenolpyruvate carboxylase (PEPCK). The MMTV (21–23), TAT (24, 25), and PEPCK (26) promoters contain multiple glucocorticoid response elements (GREs), which are DNA elements that directly bind GR. The GRE consensus sequence, (T/G)GTACANNNTGTTCT, consists of two hexamer half-sites, which are each recognized by a single subunit of a GR homodimer (27). The bases highlighted in bold have been shown to be most critical for GR activation of gene transcription through the GRE (28, 29).

Glucocorticoids use a common mechanism to regulate transcription of these genes, which involves the coordinated action of GR and multiple DNA-bound transcription factors. These factors, known as accessory factors, contribute to the glucocorticoid response by stabilizing GR binding and/or recruiting coregulators to the gene promoter (30, 31). In addition, some accessory factors are expressed in a tissue-specific manner, which enables them to provide cell type specificity to the glucocorticoid response (32). Together, the binding elements for GR and its accessory factors form a glucocorticoid response unit (GRU) (30). Accessory factors are essential for full glucocorticoid induction of MMTV, TAT, and PEPCK transcription because GR binding alone is insufficient. Nuclear factor-1 (33, 34), octamer transcription factors (35, 36), and Ets transcription factors (37) contribute to glucocorticoid induction of MMTV transcription. CCAAT/enhancer binding protein (C/EBP) (38), Ets transcription factors (39), and hepatocyte nuclear factor-3 (HNF-3) (38, 40) enhance glucocorticoid stimulation of TAT gene transcription. Finally, HNF-3 (41–43), hepatocyte nuclear factor-4 (HNF-4) (42, 44, 45), chicken ovalbumin upstream transcription factor (44), and C/EBP (46) contribute to glucocorticoid induction of PEPCK gene transcription.

For some genes, glucocorticoids can either activate or repress gene transcription, depending on the expression levels of individual transcription factors and the availability and activity of cofactors that interact with GR and its accessory factors (47). For instance, glucocorticoids activate PEPCK gene transcription in the liver and kidney, but repress PEPCK gene transcription in adipose tissue (48–51). The regulation of PEPCK gene transcription by glucocorticoids is therefore one example of a gene in which the hormone response is dependent on the cellular environment in which the gene is expressed. However, glucocorticoids specifically inhibit the expression of multiple genes, including those encoding proopiomelanocortin, osteocalcin, and prolactin (see Ref. 16 for references). Each of these gene promoters contain negative GREs (nGREs), which are DNA elements that directly bind GR and mediate an inhibitory effect of glucocorticoids on gene transcription. The nGREs are related to the well-defined GRE described above, but

they often do not closely match the consensus sequence (27, 52, 53).

The goal of this study was to determine which binding elements in the proximal G6Pase promoter play a role in glucocorticoid induction of G6Pase gene transcription. Surprisingly, the results reveal that the G6Pase GRU contains both positive and negative GREs as well as both positive and negative accessory factor elements. We believe this is the first example of a promoter that contains both positive and negative GREs, which both function within the same cellular environment.

RESULTS

The Proximal G6Pase Promoter Contains a GRU

To determine which regions in the proximal G6Pase promoter contain binding elements involved in glucocorticoid induction of gene transcription, a series of 5'-truncated G6Pase-luciferase fusion genes ranging from –231 to –35 were generated. These fusion genes were transfected into the rat H4IIE hepatoma cell line and the ability of dexamethasone, a synthetic glucocorticoid, to stimulate expression of each was assessed. There were significant decreases in dexamethasone-stimulated fusion gene expression when the promoter was truncated from –231 to –198, from –198 to –158, and from –158 to –129 (Fig. 1). These results indicate there are multiple elements downstream of –231 that contribute to dexamethasone induction of G6Pase-luciferase fusion gene expression.

Identification of Three Glucocorticoid Response Elements (GREs) in the Proximal G6Pase Promoter

Although dexamethasone-stimulated G6Pase-luciferase fusion gene expression was lost when the promoter was truncated from –231 to –129 (Fig. 1), it was not clear whether this region contained GREs. This was because glucocorticoids can regulate gene transcription by multiple mechanisms, including those that involve direct GR binding to a gene promoter and those that involve GR tethering to a gene promoter via interaction with a DNA-bound transcription factor (16). To determine whether GR directly binds the –231 to –129 promoter region, *in vitro* deoxyribonuclease (DNase) I footprinting analyses were performed with both the sense and antisense strands of the G6Pase promoter (Fig. 2A). Increasing concentrations of the DNA binding domain of GR (GR-DBD), which contains the dimerization domain, were incubated with ³²P-radiolabeled G6Pase promoter fragments and the samples were subjected to DNase I digestion. The GR-DBD protected multiple nucleotides from DNase I digestion, indicating GR directly binds the proximal G6Pase promoter (Fig. 2A). Results from the sense and antisense strand analyses were consistent in that

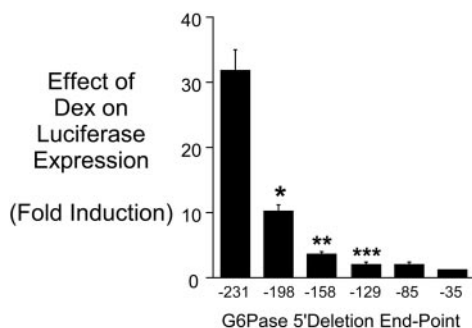


Fig. 1. The Proximal G6Pase Promoter Contains a GRU

H4IIE cells were transiently transfected, as described in *Materials and Methods*, with a series of 5'-truncated G6Pase-luciferase fusion genes (15 μ g), and expression vectors encoding *Renilla* luciferase (0.15 μ g) and GR (5.0 μ g). After transfection, cells were incubated for 18–24 h in serum-free medium in the presence or absence of 500 nM dexamethasone. The cells were then harvested and luciferase and protein assays were performed as described in *Materials and Methods*. Results are presented as the ratio of firefly luciferase activity, corrected for the protein concentration in the cell lysate, in dexamethasone-treated vs. control cells (expressed as fold induction). Results represent the mean \pm SEM of three to six experiments, in which each sample was assayed in duplicate. *, $P < 0.05$ vs. -231; **, $P < 0.05$ vs. -198; ***, $P < 0.05$ vs. -158. There was a statistically significant decrease ($28 \pm 9\%$; $n = 3$) in basal G6Pase-luciferase fusion gene expression when the promoter was truncated from -231 to -35. However, basal expression did not change when the -231 to -85 G6Pase promoter region was deleted (data not shown).

they both suggest the presence of three separate GREs, designated GRE A, GRE B, and GRE C (Fig. 2, A and B). The predicted locations of the GREs are based on the DNase I footprinting data as well as comparison with the consensus GRE sequence (Fig. 2B). GRE A is located between -197 and -183; it matches the GRE consensus sequence at 8/12 total bases and 3/5 critical bases. GRE B is located between -180 and -166; it matches the GRE consensus sequence at eight of 12 total bases and three of five critical bases. GRE C is located between -156 and -142; it matches the GRE consensus sequence at 9/12 total bases and 2/5 critical bases.

To determine whether GRE A, GRE B, and GRE C play a role in the regulation of G6Pase gene transcription by glucocorticoids, G6Pase-luciferase fusion genes were constructed which contain mutations in each element individually (Fig. 2C). The mutations in GRE A and B were designed so as to leave intact two insulin response sequences (IRs) that overlap these elements (54). These fusion genes were transfected into H4IIE cells and the ability of dexamethasone to stimulate expression of each was assessed. There was a significant decrease in dexamethasone induction of fusion gene expression when GRE A, GRE B, and GRE C were mutated individually (Fig. 2D). These results are consistent with the DNase I footprinting

analyses (Fig. 2, A and B) and further suggest that GRE A, GRE B, and GRE C are each functional GREs.

The HNF-1, HNF-4, CRE 1, and CRE 2 Binding Elements Are Required for Full Glucocorticoid Induction of G6Pase-Luciferase Fusion Gene Expression

Binding sites for HNF-1 (55, 56) and HNF-4 (57) have previously been identified in the proximal G6Pase promoter. In addition, this region of the promoter contains two binding sites for the cAMP response element (CRE) binding protein (CREB), designated CRE 1 and CRE 2 (55, 58, 59). It has recently been shown that CRE 1 also binds members of the C/EBP transcription factor family (59). Interestingly, all of these factors have been shown to play a role in the glucocorticoid-induced transcription of other genes (see introductory text and Ref. 60). To assess the ability of these elements to contribute to dexamethasone induction of G6Pase expression, G6Pase-luciferase fusion genes containing mutations in each of these binding elements were constructed (Fig. 3A). Importantly, the introduced mutations have been shown to abrogate transcription factor binding [Refs. 8, 56, and 59; and Boustead, J. N., and Richard M. O'Brien, unpublished data). These fusion genes were transfected into H4IIE cells and the ability of dexamethasone to stimulate expression of each was assessed. Mutation of the HNF-1, HNF-4, CRE 1, and CRE 2 elements individually resulted in a significant decrease in dexamethasone induction, when compared with that of the -231 wild-type (WT) fusion gene (Fig. 3B). These results indicate that the HNF-1, HNF-4, CRE 1, and CRE 2 binding sites in the G6Pase promoter act as accessory factor elements for glucocorticoid induction of G6Pase gene transcription. The involvement of HNF-1 and CRE 1 in glucocorticoid stimulation of G6Pase gene transcription are consistent with previously published studies on the human G6Pase promoter (10, 58).

Identification of Multiple FKHR and HNF-3 β Binding Elements in the Proximal G6Pase Promoter

Binding sites for the forkhead transcription factors FKHR (54, 61, 62) and HNF-3 (55) have also been previously identified in the proximal G6Pase promoter. Moreover, as with HNF-1, HNF-4, CREB, and C/EBP, these factors have also been shown to play a role in the glucocorticoid-induced transcription of other genes (see introductory text and Refs. 63 and 64). However, before assessing whether FKHR and/or HNF-3 play accessory factor roles in glucocorticoid-stimulated G6Pase gene transcription, binding elements for these factors in the proximal G6Pase promoter were studied more extensively. This was because sequence analyses suggested that these fac-

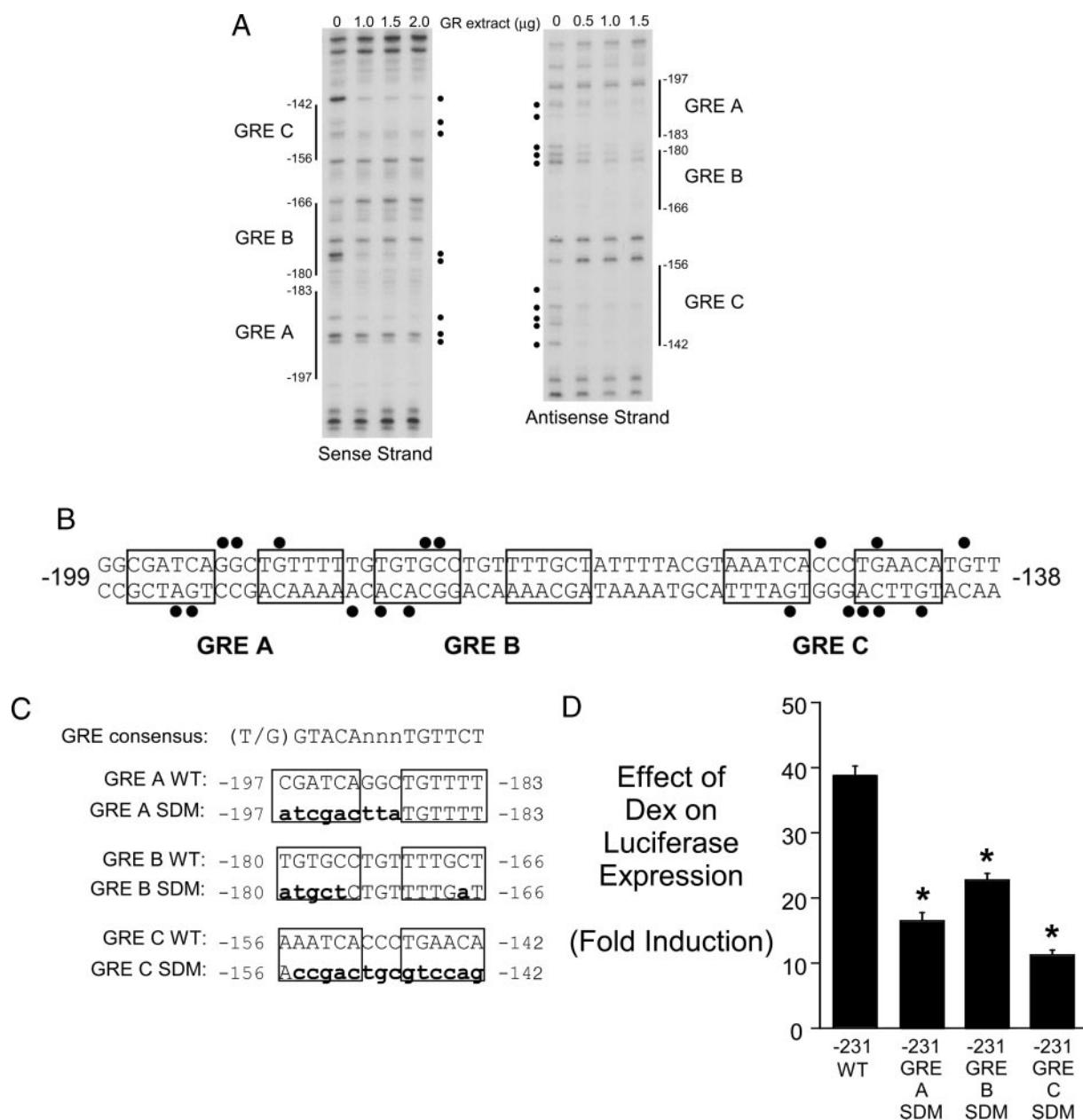


Fig. 2. Identification of Three GREs in the Proximal G6Pase Promoter

A, End-labeled sense and antisense fragments of the G6Pase promoter were incubated with 0–2.0 μg of GR-DBD protein extract and an *in vitro* DNase I footprinting analysis was performed as described in *Materials and Methods*. Representative experiments, which indicate the nucleotide positions of the predicted GREs, are shown. B, Sequence of the G6Pase promoter with summary of *in vitro* DNase I footprinting results. ●, Nucleotides protected from DNase I digestion by GR. C, The GRE consensus sequence (27), the WT GRE A, GRE B, and GRE C sequences in the G6Pase promoter, and the SDMs introduced into each GRE, indicated in *bold lowercase letters*, are shown. ●, Nucleotides protected from DNase I digestion by GR. D, H4IIE cells were transiently cotransfected, as described in *Materials and Methods*, with various G6Pase-luciferase fusion genes (15 μg) and expression vectors encoding *Renilla* luciferase (0.15 μg) and GR (5.0 μg). The G6Pase-luciferase fusion genes incorporated either the WT promoter sequence, located between –231 and +66 (–231 WT), or contained the same promoter fragment with site-directed mutations in the indicated elements. After transfection, the cells were incubated for 18–24 h in serum-free medium in the presence or absence of 500 nM dexamethasone. The cells were then harvested and luciferase and protein assays were performed as described in *Materials and Methods*. Results are presented as the ratio of firefly luciferase activity, corrected for the protein concentration in the cell lysate, in dexamethasone-treated vs. control cells (expressed as fold induction). Results represent the mean \pm SEM of three to seven experiments, in which each sample was assayed in duplicate. *, $P < 0.05$ vs. –231 WT. There was a statistically significant decrease in basal G6Pase-luciferase fusion gene expression when GRE A ($33 \pm 11\%$; $n = 4$) or GRE B ($49 \pm 3\%$; $n = 3$) was mutated.

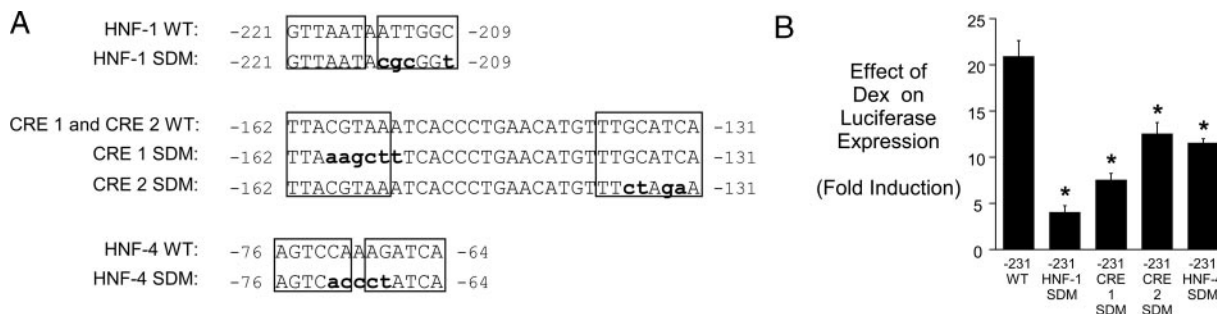


Fig. 3. The HNF-1, CRE 1, CRE 2, and HNF-4 Binding Elements Are Required for Full Glucocorticoid Induction of G6Pase-Luciferase Fusion Gene Expression

A, The WT HNF-1, CRE 1, CRE 2, and HNF-4 sequences in the G6Pase promoter and the SDMs introduced into each element, indicated in *bold lowercase letters*, are shown. B, H4IIE cells were transiently cotransfected, as described in *Materials and Methods*, with various G6Pase-luciferase fusion genes (15 μ g) and expression vectors encoding *Renilla* luciferase (0.15 μ g) and GR (5.0 μ g). The G6Pase-luciferase fusion genes incorporated either the WT promoter sequence, located between -231 and $+66$ (-231 WT), or contained the same promoter fragment with site-directed mutations in the indicated elements. After transfection, cells were incubated for 18–24 h in serum-free medium in the presence or absence of 500 nM dexamethasone (Dex). The cells were then harvested and luciferase and protein assays were performed as described in *Materials and Methods*. Results are presented as the ratio of firefly luciferase activity, corrected for the protein concentration in the cell lysate, in dexamethasone-treated vs. control cells (expressed as fold induction). Results represent the mean \pm SEM of three to 15 experiments, in which each sample was assayed in duplicate. *, $P < 0.05$ vs. -231 WT. There was a statistically significant decrease in basal G6Pase-luciferase fusion gene expression when the HNF-1 ($40 \pm 4\%$; $n = 3$), CRE 1 ($18 \pm 3\%$; $n = 6$) or CRE 2 ($23 \pm 6\%$; $n = 6$) element was mutated.

tors may bind additional elements other than those previously identified. To directly determine whether additional FKHR and HNF-3 binding sites exist in the proximal G6Pase promoter, *in vitro* DNase I footprinting analyses were performed. Increasing concentrations of His-FKHR (Fig. 4A) or GST-HNF-3 β (Fig. 4B) were incubated with 32 P-radiolabeled G6Pase promoter fragments and the samples were subjected to DNase I digestion.

FKHR protected multiple nucleotides from DNase I digestion (Fig. 4A) and results from the sense and antisense strand analyses were consistent in that they both suggest the presence of four different FKHR binding elements (Fig. 4, A and C). The predicted locations of the FKHR binding sites are based on the DNase I footprinting data as well as comparison with the consensus FKHR sequence (Fig. 4C). We have previously shown that FKHR binds two IRSs in the G6Pase promoter, designated IRS 1 and IRS 2 (54). IRS 1 is located between -188 and -183 , whereas IRS 2 is located between -174 and -168 . Both elements match the core FKHR consensus sequence at 6/6 bases (65, 66). As expected, FKHR binding to these elements was detected using the DNase I footprinting assay (Fig. 4, A and C). The G6Pase promoter also contains a third IRS, designated IRS 3, which is located between -166 and -160 . However, extensive analysis of this element indicates that it does not bind FKHR (54). FKHR does contact one nucleotide in the IRS 3 binding element (Fig. 4, A and C). However, this is likely due to the limited resolution of the DNase I footprinting assay because IRS 2 is adjacent to IRS 3. Interestingly, the DNase I footprinting analyses also

reveal two FKHR binding elements downstream of IRS 2, designated FKHR B and FKHR C (Fig. 4, A and C). FKHR B is located between -141 and -135 and it matches the core FKHR consensus element at 6/7 bases, whereas FKHR C is located between -110 and -104 and it matches the core FKHR consensus element at four of seven bases.

Although three different HNF-3 isoforms, HNF-3 α , β , and γ , have been identified (67–69), HNF-3 β (FOXA2) was the predominant isoform that bound the G6Pase promoter in gel retardation analyses using H4IIE nuclear extract (data not shown). Therefore, HNF-3 β was the isoform used in the footprinting (Fig. 4B) and overexpression (Fig. 5) analyses in this study. HNF-3 β protected multiple nucleotides from DNase I digestion (Fig. 4B), and results from the sense and antisense strand studies were consistent in that they both suggest the presence of two HNF-3 β binding elements, designated HNF-3 B and HNF-3 C (Fig. 4, B and C). When 10-fold more HNF-3 β was used in the footprinting analysis an additional HNF-3 β binding site, designated HNF-3 A, was detected on the antisense strand (Fig. 4, B and C), although no additional binding elements were apparent on the sense strand (data not shown). This result suggests that HNF-3 β binds the HNF-3 A motif with a low affinity. The predicted location of each HNF-3 binding site is based on the DNase I footprinting data as well as comparison with the consensus HNF-3 sequence (Fig. 4C). HNF-3 A overlaps IRS 1 and IRS 2, is located between -185 and -174 , and matches the HNF-3 consensus sequence at eight of 12 bases (70). The HNF-3 B and HNF-3 C binding elements are downstream of IRS 2.

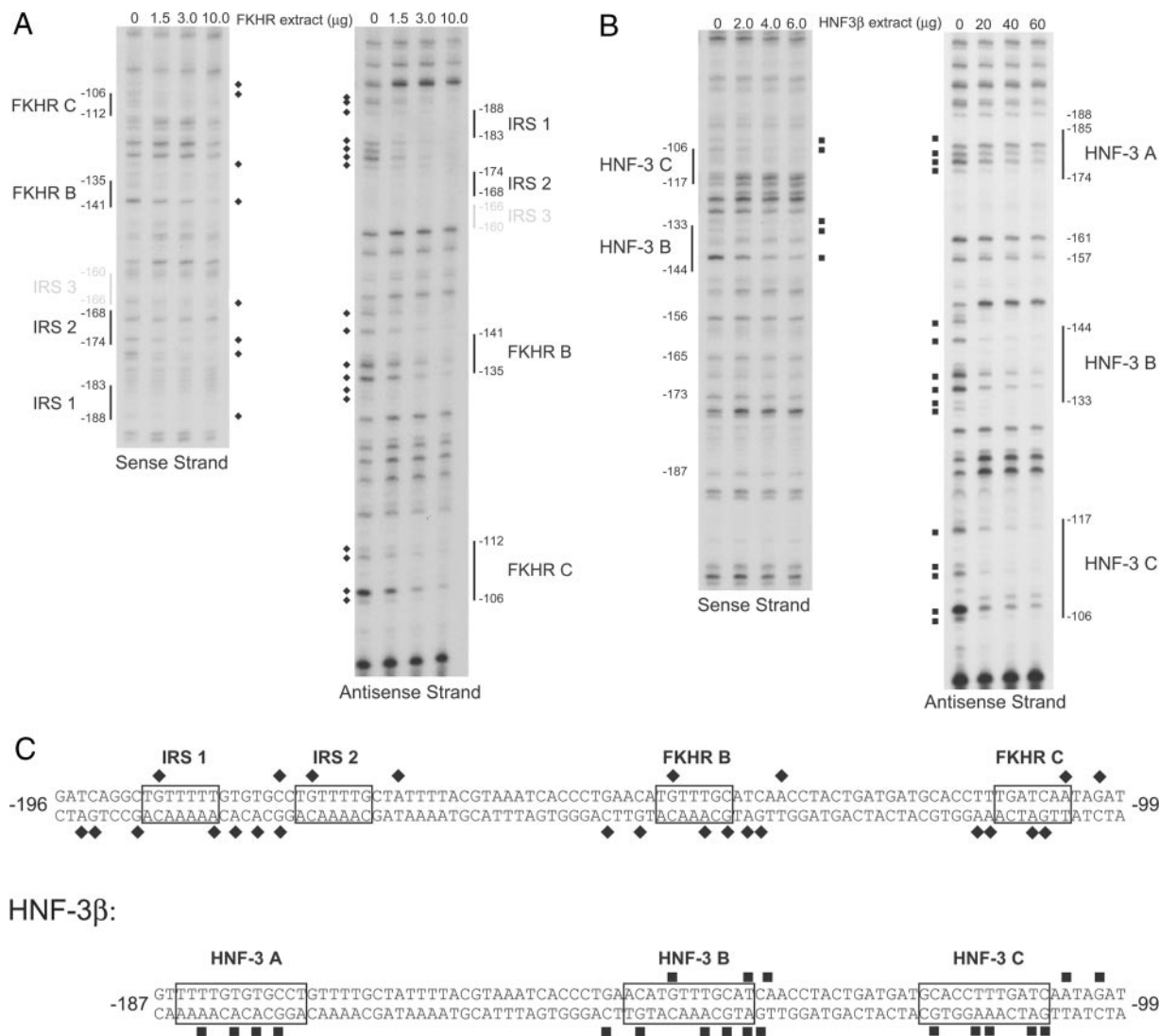


Fig. 4. Identification of FKHR and HNF-3 β Binding Elements in the Proximal G6Pase Promoter

A, End-labeled sense and antisense fragments of the G6Pase promoter were incubated with 0–10 μg of His-FKHR protein extract and an *in vitro* DNase I footprinting analysis was performed as described in *Materials and Methods*. Representative experiments, which indicate the nucleotide positions of the predicted FKHR binding elements, are shown. IRS 3, which does not bind FKHR (54), is indicated in gray. \blacklozenge , Nucleotides protected from DNase I digestion by His-FKHR. B, End-labeled sense and antisense fragments of the G6Pase promoter were incubated with 0–6.0 μg (sense) or 0–60 μg (antisense) of GST-HNF-3 β protein extract and an *in vitro* DNase I footprinting analysis was performed as described in *Materials and Methods*. Representative experiments, which indicate the nucleotide positions of the predicted HNF-3 β binding elements, are shown. \blacksquare , Nucleotides protected from DNase I digestion by GST-HNF-3 β . C, Sequence of the G6Pase promoter with summary of *in vitro* DNase I footprinting results. Nucleotides protected from DNase I digestion by His-FKHR (\blacklozenge) or GST-HNF-3 β (\blacksquare).

HNF-3 B is located between –144 and –133, whereas HNF-3 C is located between –117 and –106. Both match the HNF-3 consensus sequence at eight of 12 bases.

FKHR and HNF-3 β Stimulate G6Pase-Luciferase Fusion Gene Transcription through Multiple Elements

A number of the FKHR and HNF-3 β binding elements that were detected using DNase I footprinting analysis

(Fig. 4) have not been previously characterized. Therefore, although FKHR and HNF-3 β bind these sites in the G6Pase promoter *in vitro* (Fig. 4), it was important to determine whether FKHR and/or HNF-3 β can bind these elements *in situ*. A comparison of the location of the four FKHR and three HNF-3 β binding sites in the proximal G6Pase promoter indicates that the HNF-3 A element lies between and partially overlaps IRS 1 and IRS 2 (Fig. 5A). In contrast, the HNF-3 B/FKHR B and HNF-3 C/FKHR C elements overlap completely (Fig.

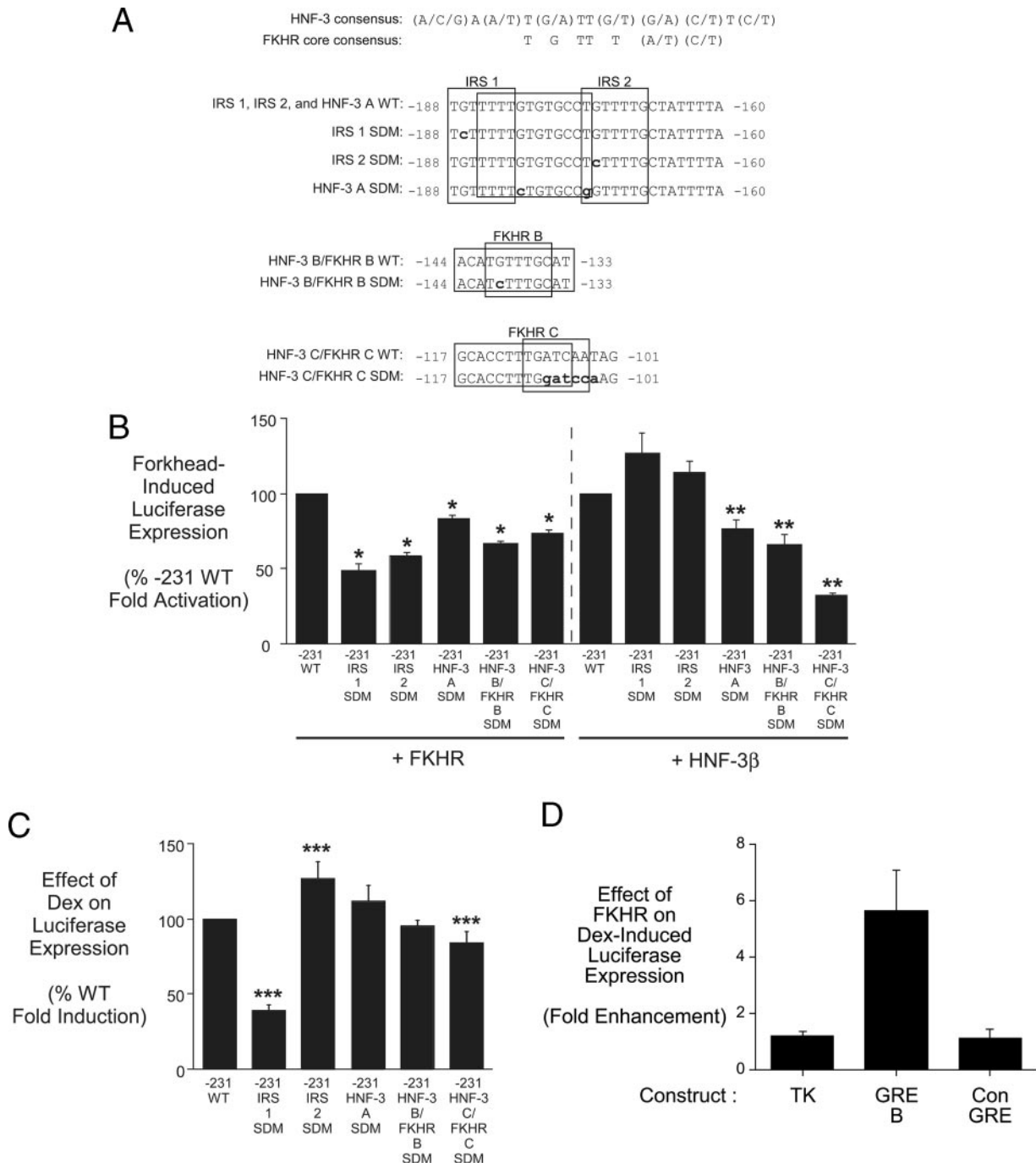


Fig. 5. FKHR and HNF-3 β Are Required for Full Glucocorticoid Activation of G6Pase-Luciferase Fusion Gene Expression

A, The consensus binding elements for HNF-3 β (70) and FKHR (65, 66), the WT IRS 1, IRS 2, HNF-3 A, HNF-3 B/FKHR B, and HNF-3 C/FKHR C sequences in the G6Pase promoter, and SDMs introduced into each element, indicated in *bold lowercase letters*, are shown. B, H4IIE cells were transiently cotransfected, as described in *Materials and Methods*, with various G6Pase-luciferase fusion genes (15 μ g) and expression vectors encoding *Renilla* luciferase (0.15 μ g) and either pcDNA3 (1.0 μ g), FKHR-pcDNA3 (1.0 μ g), or HNF-3 β -pcDNA3 (1.0 μ g). The G6Pase-luciferase fusion genes incorporated either the WT promoter sequence, located between -231 and +66 (-231 WT), or contained the same promoter fragment with site-directed mutations in the indicated elements. After transfection, cells were incubated for 18–24 h in serum-free medium. The cells were then harvested and luciferase assays were performed as described in *Materials and Methods*. Results are presented as the ratio of firefly luciferase activity, corrected for *Renilla* luciferase activity in the cell lysate, in FKHR/HNF-3 β -stimulated vs. control cells (expressed as a percentage of the fold induction obtained with the -231 WT G6Pase-luciferase fusion gene, which ranged from ~2- to 6-fold). Results represent the mean \pm SEM of three to five experiments in which each sample was assayed in quadruplicate. *, $P < 0.05$ vs. -231 WT + FKHR; **, $P < 0.05$ vs. -231 WT + HNF-3 β . C, H4IIE cells were transiently cotransfected, as described in *Materials and Methods*, with the same plasmids as described above and expression vectors encoding *Renilla* luciferase (0.15 μ g). (Legend continues on next page.)

5A). Therefore, for the mutational analyses of these binding elements, IRS 1, IRS 2, and HNF3 A were mutated individually, whereas HNF-3 B/FKHR B and HNF-3 C/FKHR C were mutated together (Fig. 5A). Importantly, previous studies have shown that the point mutations introduced into the IRSs completely disrupt FKHR binding (54). Also, because the HNF-3 A element overlaps both GRE A and GRE B and the HNF-3 B element overlaps both GRE C and CRE 2, only limited mutation of these elements was possible.

To determine whether FKHR and/or HNF-3 β can stimulate G6Pase-luciferase fusion gene expression through each of the identified forkhead binding elements, H4IIE cells were cotransfected with either the WT or one of the mutated fusion genes, and an expression vector encoding either FKHR or HNF-3 β . There was a significant decrease in FKHR-stimulated fusion gene expression when IRS 1, IRS 2, HNF-3 A, HNF-3 B/FKHR B, and HNF-3 C/FKHR C were mutated individually (Fig. 5B). The FKHR overexpression studies were consistent with the DNase I footprinting data, which indicated that FKHR can bind IRS 1, IRS 2, FKHR B, and FKHR C *in vitro* (Fig. 4, A and C). The one exception is that FKHR activation decreased when HNF-3 A was mutated, but this may be due to mutation of the 5' end of the core IRS 2 sequence in the HNF-3 A-mutated fusion gene (Fig. 5A).

Similarly, there was a significant decrease in HNF-3 β activation of the –231 G6Pase-luciferase fusion gene when HNF-3 A, HNF-3 B, and HNF-3 C were mutated individually (Fig. 5B). However, there was no significant change in HNF-3 β activation of the –231 G6Pase-luciferase fusion gene when IRS 1 and IRS 2 were mutated individually (Fig. 5B), which is consistent with the observation that the mutations in these elements lie outside the HNF-3 A motif (Fig. 5A). These results are also consistent with the DNase I footprinting data, which indicated that HNF-3 β can bind HNF-3 A, HNF-3 B, and HNF-3 C *in vitro* (Fig. 4, B and C).

FKHR Binding to IRS 1 Is Probably Required for Full Glucocorticoid Induction of G6Pase-Luciferase Fusion Gene Expression

To assess the ability of the FKHR and HNF-3 β binding elements to contribute to dexamethasone induction of G6Pase-luciferase fusion gene expression, the fusion genes containing mutations in each of these elements (Fig. 5A) were transfected into H4IIE cells and the ability of dexamethasone to stimulate expression of each was determined. When IRS 2, HNF-3 A, and HNF-3 B/FKHR B were mutated individually, there was either no change or an increase in dexamethasone-stimulated fusion gene expression compared with that of the –231 WT fusion gene (Fig. 5C). These results suggest that the factors that bind IRS 2, HNF-3 A, and HNF-3 B/FKHR B do not contribute to glucocorticoid activation of G6Pase-luciferase fusion gene expression. In contrast, when IRS 1 was mutated there was a significant decrease in dexamethasone-stimulated fusion gene expression compared with that of the –231 WT fusion gene (Fig. 5C). This suggests that FKHR binding to IRS 1 contributes to dexamethasone-stimulated G6Pase-luciferase fusion gene expression. However, the IRS 1 motif completely overlaps with the 3' half site of GRE A (Figs. 2B and 4B). Therefore, although only a single base has been mutated in IRS 1, we cannot exclude the possibility that the decrease in dexamethasone induction seen upon mutation of IRS 1 is due to reduced GR binding. To further assess the potential for FKHR to serve as an accessory factor, we investigated the ability of FKHR to enhance dexamethasone-stimulated fusion gene expression in a heterologous context. An oligonucleotide representing the G6Pase promoter sequence from –188 to –160 (Table 1) was ligated into a heterologous thymidine kinase (TK)-luciferase vector. This oligonucleotide includes the IRS 1 element, the adjacent GRE B motif, but only the 3' half site of GRE A. Expression of the resulting fusion gene was not induced by glucocorti-

μ g) and GR (5.0 μ g). After transfection, cells were incubated for 18–24 h in serum-free medium in the presence or absence of 500 nM dexamethasone. The cells were then harvested and luciferase and protein assays were performed as described in *Materials and Methods*. Results are presented as the ratio of firefly luciferase activity, corrected for the protein concentration in the cell lysate, in dexamethasone-treated vs. control cells (expressed as a percentage of the fold induction obtained with the –231 WT G6Pase-luciferase fusion gene, which ranged from approximately 20- 50-fold (data not shown). Results represent the mean \pm SEM of six to 12 experiments, in which each sample was assayed in duplicate. ***, $P < 0.05$ vs. –231 WT. There was a significant decrease in basal fusion gene expression when IRS 1 ($29 \pm 2\%$; $n = 4$), HNF-3 B/FKHR B ($18 \pm 7\%$; $n = 3$), or HNF-3 C/FKHR C ($42 \pm 3\%$; $n = 4$) was mutated. Furthermore, there was a significant increase in basal fusion gene expression when IRS 2 ($12 \pm 8\%$; $n = 3$) or HNF-3 A ($19 \pm 4\%$; $n = 5$) was mutated. D, H4IIE cells were transiently cotransfected, as described in *Materials and Methods*, with various TK-luciferase fusion genes (15 μ g) and expression vectors encoding *Renilla* luciferase (0.15 μ g), GR (5.0 μ g) and either pcDNA3 (1.0 μ g) or FKHR-pcDNA3 (1.0 μ g). The fusion gene plasmids represented either the basic TK-luciferase vector or constructs in which two copies of oligonucleotides representing a consensus GRE or GRE B (Table 1) had been ligated into the *HindIII* site of the vector. After transfection, cells were incubated for 18–24 h in serum-free medium in the presence or absence of 500 nM dexamethasone. The cells were then harvested and luciferase assays were performed as described in *Materials and Methods*. The fold induction of firefly luciferase activity, corrected for the protein concentration in the cell lysate, in dexamethasone-treated vs. control cells, was calculated. The results represent the ratio of this fold induction in FKHR-pcDNA3 vs. pcDNA3 transfected cells (expressed as fold enhancement) and are the mean \pm SEM of three to four experiments in which each sample was assayed in duplicate.

coids (1.14 ± 0.12 ; $n = 4$) and overexpression of FKHR had no effect alone (1.14 ± 0.09 ; $n = 4$). However, after overexpression of FKHR, glucocorticoids now induced fusion gene expression (Fig. 5D). In contrast, luciferase expression directed by the TK vector was not induced by glucocorticoids (1.14 ± 0.05 ; $n = 3$), overexpression of FKHR had no effect alone (0.97 ± 0.01 ; $n = 4$), and after overexpression of FKHR glucocorticoids still failed to induce fusion gene expression (Fig. 5D). When an oligonucleotide representing the consensus GRE sequence (Table 1) was ligated into a heterologous TK-luciferase vector, expression of the resulting fusion gene was markedly induced by glucocorticoids (42.53 ± 10.55 ; $n = 4$), but overexpression of FKHR had no effect alone (1.21 ± 0.19 ; $n = 4$). In addition, the glucocorticoid-stimulated expression of this fusion gene was not enhanced by overexpression of FKHR (Fig. 5D). These results suggest that FKHR binding to IRS 1 has the potential to contribute to dexamethasone-stimulated G6Pase-luciferase fusion gene expression. However, this result cannot exclude the possibility that, in the context of the G6Pase promoter, binding of GR to GRE A precludes binding of FKHR to IRS 1.

There was also a significant decrease in dexamethasone-stimulated fusion gene expression, compared with that of the -231 WT fusion gene, when the HNF-3 C/FKHR C element was mutated (Fig. 5C). However, the effect of this mutation was relatively minor (Fig. 5C). In addition, the HNF-3 C/FKHR C binding element overlaps a previously identified HNF-6 binding site (71), so we cannot exclude the possibility that the decrease in dexamethasone induction seen upon mutation of the HNF-3 C/FKHR C element is due to reduced HNF-6 binding. Interestingly, overexpression of HNF-6 inhibits glucocorticoid-stimulated PEPCK gene expression (72), but it does not affect glucocorticoid activation of G6Pase fusion gene expression (Streeper, R.S., and Richard M. O'Brien, unpublished data). This suggests that FKHR and/or HNF-3 β , rather than HNF-6, contribute to glucocorticoid activation of G6Pase gene transcription through this element.

The Regulation of Promoter Occupancy Supports the Role of FKHR as an Accessory Factor in Glucocorticoid Stimulation of Endogenous G6Pase Gene Transcription

The heterologous TK fusion gene studies described above (Fig. 5D) suggest that FKHR binding to IRS 1 has the potential to contribute to dexamethasone-stimulated G6Pase-luciferase fusion gene expression. But as an alternative approach to investigate the potential accessory factor role of FKHR chromatin immunoprecipitation (ChIP) assays were performed. If FKHR is an accessory factor for glucocorticoid stimulation of G6Pase gene transcription, its promoter occupancy would be predicted to increase in the presence of dexamethasone because assembly of a GRU involves recruitment of GR and its accessory factors to the promoter (32).

To determine whether there is an increase in GR and FKHR binding to the endogenous G6Pase promoter upon glucocorticoid treatment, fragmented chromatin from formaldehyde-cross-linked H4IIE cells, which were treated in the absence and presence of dexamethasone, was subjected to immunoprecipitation with a GR or FKHR antibody. The presence of the G6Pase promoter in the immunoprecipitates was then analyzed by PCR using primers representing the proximal G6Pase promoter sequence. Figure 6A shows results of a representative ChIP assay visualized by agarose gel electrophoresis, and Fig. 6B shows quantitated results from three independent real-time PCR analyses. The results indicate there is a significant increase in GR and FKHR binding to the endogenous G6Pase promoter upon treatment of the cells with dexamethasone. To test the specificity of the antibody-chromatin interactions, the immunoprecipitates were also analyzed for the presence of exon 5 of the G6Pase gene using PCR primers that represent G6Pase exon 5 coding sequence (Fig. 6C). Approximately 10 kb of genomic DNA separates the rat G6Pase promoter and exon 5 (12), so given that the average chromatin fragment size is approximately 500 bp in this assay, immunoprecipitates of GR and FKHR bound to the proximal G6Pase promoter should not include exon 5 genomic sequence. Figure 6C shows

Table 1. Oligonucleotides Used in Gel Retardation Assays

Position	Sequence
Consensus GRE	AGCT GGTACA AACT TGTTCT AGCT
G6P 188/160 GRE B	TGTTTTT TGTG CCCTGTT TTTGCT ATTTTA
G6P 244/212 GRE D	GCACT GTCAAGCAGTGTG CCCAAGTTAATAATT
G6P 271/231 WT	AAGGACCAGGAAGGAGGGCAGCCTCTAGCACTGTCAAGCAG
G6P 271/231 MUT1	AAGGACCAGGA cttctt GCAGCCTCTAGCACTGTCAAGCAG
G6P 271/231 MUT2	AAGGACCAGGAAGGAGGGCAGCCTCTA ta ca gt TCAAGCAG

All nucleotide positions are negative and are numbered relative to the transcription start site at +1. The GRE half sites are shown in *bold*. Non-wild-type sequence is shown in *lowercase letters*. MUT, Mutant. The first three oligonucleotides were synthesized with *Hind*III-compatible ends (AGCT), the second three with *Bam*HI-compatible ends (GATC).

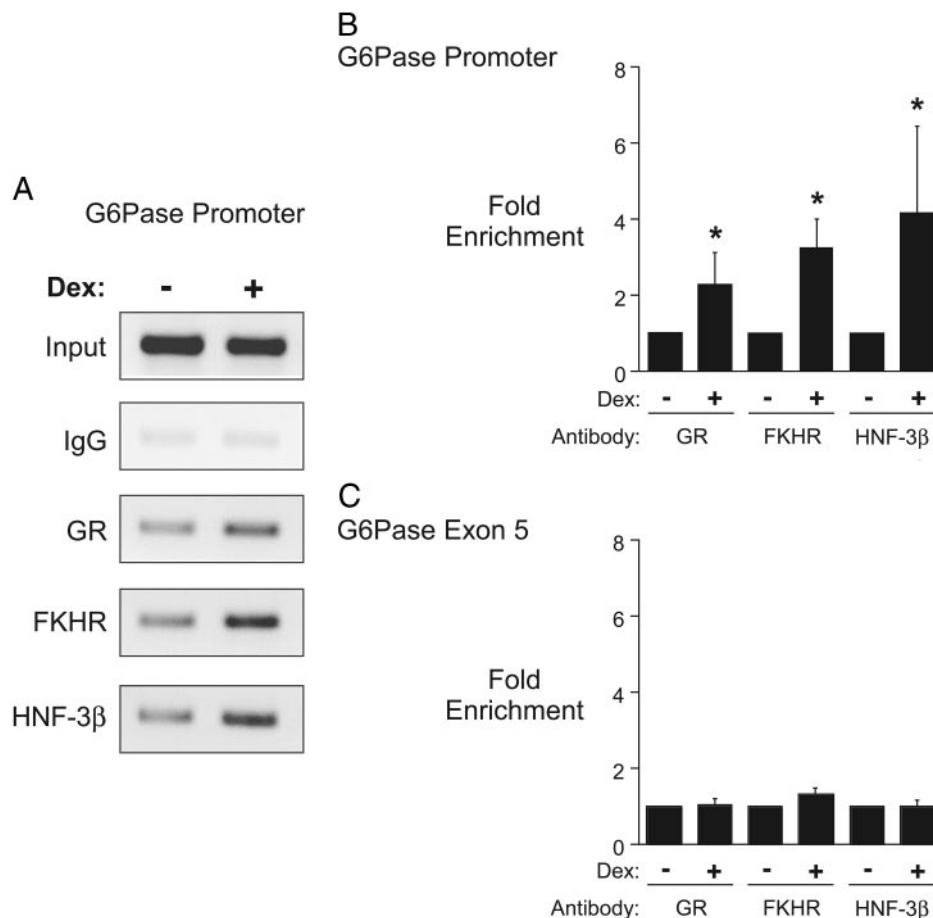


Fig. 6. The Regulation of FKHR and HNF-3 β Promoter Occupancy Supports Their Role as Accessory Factors in Glucocorticoid Stimulation of Endogenous G6Pase Gene Transcription

GR, FKHR, and HNF-3 β binding to the endogenous G6Pase promoter were analyzed *in situ* in control or dexamethasone-treated (for 60 min) H4IIE cells using the ChIP assay, as described in *Materials and Methods*. Chromatin from formaldehyde-treated H4IIE cells was immunoprecipitated using an anti-GR, anti-FKHR, or anti-HNF-3 β antibody. An anti-IgG antibody was used as a negative control. The presence of the G6Pase promoter in the chromatin preparation before immunoprecipitation (Input) and in the immunoprecipitates was then assayed using PCR, as described in *Materials and Methods*. A, Samples were separated on an agarose gel and a representative result is shown. B, The presence of the G6Pase promoter and (C) G6Pase exon 5 in the immunoprecipitates was assayed using real-time PCR analysis, as described in *Materials and Methods*. Quantitated results from three independent experiments, in which each sample was assayed in duplicate, are shown. Results are presented as the fold enrichment over the IgG control in the presence and absence of dexamethasone, normalized to the value obtained with the corresponding dexamethasone control. The fold enrichment was variable (see panel B), which may reflect the variable fold induction of G6Pase gene transcription by dexamethasone (~20- to 50-fold; Fig. 5). Therefore, for statistical analysis, the data were normalized to the value obtained with the corresponding + dexamethasone sample. *, $P < 0.05$ vs. corresponding antibody fold enrichment – dexamethasone.

that the background levels of GR and FKHR binding to exon 5 were not regulated by dexamethasone, which suggests that the increase in GR and FKHR binding seen at the G6Pase promoter upon dexamethasone treatment is specific. This result is consistent with a role for FKHR as an accessory factor for the glucocorticoid response.

The ChIP assay also revealed that there is a significant increase in HNF-3 β binding to the endogenous G6Pase promoter upon treatment of the cells with dexamethasone (Fig. 6, A and B) whereas the background level of HNF-3 β binding to exon 5 was not regulated (Fig. 6C). This result suggests a potential

role for HNF-3 β as an accessory factor even though mutation of the three HNF-3 binding sites had a minimal effect on the glucocorticoid response (Fig. 5C).

The G6Pase Promoter Contains a nGRE

To determine whether promoter elements upstream of –231 contribute to glucocorticoid regulation of G6Pase gene transcription, a series of 5'-truncated G6Pase-luciferase fusion genes ranging from –484 to –231 were generated. These fusion genes were transfected into H4IIE cells, and the ability of dexamethasone to stimulate expression of each was assessed.

Surprisingly, there were significant increases in dexamethasone-stimulated fusion gene expression when the promoter was deleted from –271 to –252 and from –252 to –231 (Fig. 7A). This result suggests there are multiple factors that bind the promoter region between –271 and –231 that inhibit glucocorticoid stimulation of G6Pase-luciferase fusion gene expression.

To ascertain whether the G6Pase promoter region between –271 and –231 contains an nGRE, *in vitro* DNase I footprinting analyses were performed over both the sense and antisense strands of the G6Pase promoter (Fig. 7B). Increasing concentrations of GR-DBD were incubated with ³²P-radiolabeled G6Pase promoter fragments and the samples were subjected to DNase I digestion. The GR-DBD protected multiple nucleotides between –252 and –231 from DNase I digestion, indicating that GR directly binds this region of the G6Pase promoter (Fig. 7B). Results from the sense and antisense strand analyses were consistent in that they both suggest the presence of one nGRE, designated nGRE D (Fig. 7, B and C). The predicted location of nGRE D is based on the DNase I footprinting data as well as comparison with the consensus GRE sequence (Fig. 7C). nGRE D is located between –239 and –225 and it matches the GRE consensus sequence at 6/12 total bases and four of five critical bases. Importantly, in order for a GRE to be functional, both half sites must be intact (29). Therefore, the increase in dexamethasone induction seen when the promoter was truncated from –252 to –231 (Fig. 7A), can be attributed to the partial deletion of nGRE D.

GR-DBD binding to the nGRE D element was analyzed further using the gel retardation assay. As a positive control, we first labeled an oligonucleotide containing a consensus GRE motif (Table 1). When this labeled oligonucleotide was incubated with 10 ng purified GR-DBD two protein-DNA complexes were detected (Fig. 7D). Based on previous work from Chalepakis *et al.* (73), we interpret this result to indicate that the slower migrating complex represents the binding of a GR-DBD dimer, whereas the faster migrating complex represents the binding of a GR-DBD monomer. In contrast, when labeled oligonucleotides representing the WT G6Pase promoter sequence from –244 to –212 (Table 1 and Fig. 7C), that encompasses nGRE D, or the WT G6Pase promoter sequence from –188 to –160 that encompasses GRE B (Table 1 and Fig. 2C), were incubated with 10 ng purified GR-DBD only binding of the GR-DBD monomer was detected (Fig. 7D, *left panel*). However, when incubated with 1000 ng purified GR-DBD both the labeled nGRE D and the GRE B probes bound the GR-DBD dimer, although binding of the monomer still predominated (Fig. 7D, *right panel*). These results are consistent with the DNase I footprinting analyses that initially defined nGRE D and GRE B (Figs. 2A and 7B). The low affinity of GR-DBD dimer binding to these elements is also consistent with the fact that their sequences do not perfectly match that of the consensus element (Table 1).

Competition experiments, in which a varying molar excess of unlabeled DNA was included with the labeled consensus GRE probe, were used to compare the relative affinity of GR-DBD binding to the consensus GRE, nGRE D and GRE B. The results demonstrate that oligonucleotides representing either nGRE D or GRE B, but not an HNF-1 binding site, can compete with the labeled consensus GRE probe for binding of the GR-DBD (Fig. 7E). But as expected, the unlabeled consensus GRE oligonucleotide competed with the labeled consensus GRE probe for binding of the GR-DBD at much lower concentrations (Fig. 7E).

To compare the ability of the consensus GRE, nGRE D and GRE B to mediate an effect of dexamethasone in a heterologous context, the same oligonucleotides as used in the gel retardation experiments were ligated into the *Hind*III site of a heterologous TK-luciferase vector. Dexamethasone had almost no effect on luciferase expression directed by the TK-luciferase vector alone, whereas the consensus GRE was able to confer a strong glucocorticoid response (Fig. 7F). Consistent with the low affinity of GR-DBD dimer binding to the nGRE (Fig. 7D), this element was only able to mediate a weak glucocorticoid response when multimerized (Fig. 7F). And in the context of the heterologous TK-luciferase vector, it mediated a stimulatory glucocorticoid response, rather than the negative glucocorticoid response seen in the context of the G6Pase promoter (Fig. 7F). GRE B, which binds the GR-DBD dimer with a slightly lower affinity than nGRE D (Fig. 7E), failed to mediate a glucocorticoid response even when multimerized (Fig. 7F). These results are consistent with the idea that accessory factors are required to stabilize GR binding to GRE B in the G6Pase promoter to promote a robust glucocorticoid response. In addition, these results show that the negative effect of glucocorticoids mediated through nGRE D is determined by the context of the G6Pase promoter rather than being an inherent feature of GR binding to this element.

The G6Pase Promoter Also Contains a Negative Accessory Factor Element

There was also a significant increase in dexamethasone-stimulated fusion gene transcription when the promoter was deleted from –271 to –252 (Fig. 7A), suggesting that there is at least one additional binding element, in addition to nGRE D, that contributes to the inhibition of glucocorticoid-stimulated G6Pase-luciferase fusion gene expression. To begin to elucidate which element(s) in this region of the promoter contributes to the inhibition of the dexamethasone response, two fusion genes, designated –271 SDM (site-directed mutation) 1 and –271 SDM 2, were generated. Each fusion gene contains a 6-bp block mutation in sequences that are 100% conserved between the human, mouse, and rat promoter in the –271/–252 region (Figs. 8A and 9). These fusion genes were transfected into H4IIE cells and the ability of dexa-

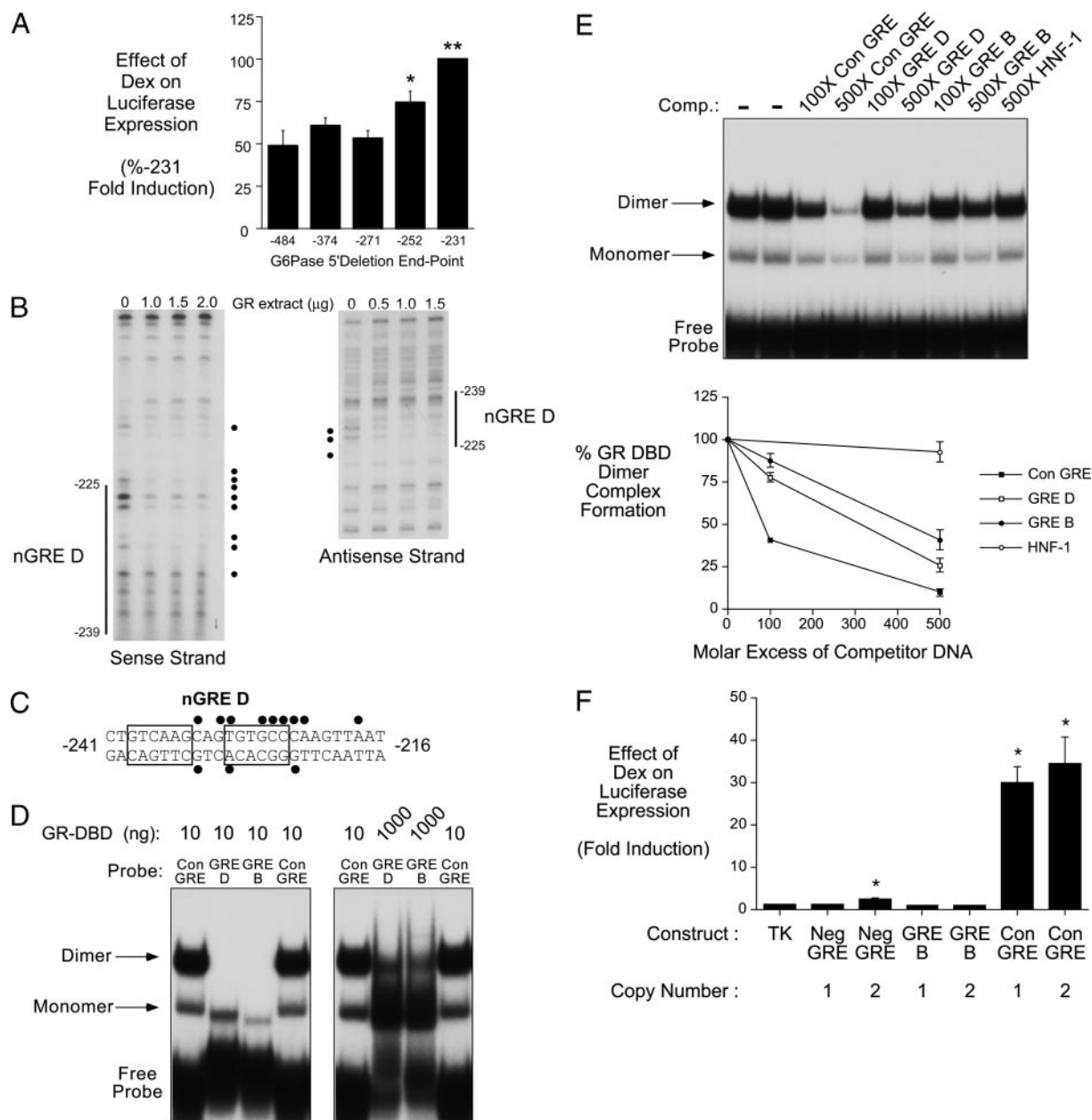


Fig. 7. The G6Pase Promoter Contains a nGRE

A, H4IIE cells were transiently transfected, as described in *Materials and Methods*, with a series of 5'-truncated G6Pase-luciferase fusion genes, and expression vectors encoding *Renilla* luciferase (0.15 μg) and GR (5.0 μg). After transfection, cells were incubated for 18–24 h in serum-free medium in the presence or absence of 500 nM dexamethasone. The cells were then harvested and luciferase and protein assays were performed as described in *Materials and Methods*. Results are presented as the ratio of firefly luciferase activity, corrected for the protein concentration in the cell lysate, in dexamethasone-treated vs. control cells (expressed as % –231-fold induction, which ranged from ~20- to 40-fold). Results represent the mean ± SEM of three to seven experiments, in which each sample was assayed in duplicate. *, $P < 0.05$ vs. –271; **, $P < 0.05$ vs. –252. There was a statistically significant decrease in basal G6Pase-luciferase fusion gene expression when the promoter was truncated from –484 to –374 ($37 \pm 0.3\%$; $n = 3$), from –484 to –271 ($72 \pm 1\%$; $n = 3$), from –271 to –252 ($8 \pm 2\%$; $n = 4$), and from –484 to –231 ($66 \pm 2\%$; $n = 3$). B, End-labeled sense and antisense fragments of the G6Pase promoter were incubated with 0–2.0 μg of GR-DBD protein extract and an *in vitro* DNase I footprinting analysis was performed as described in *Materials and Methods*. Representative experiments, which indicate the nucleotide position of the predicted nGRE, are shown. ●, Nucleotides protected from DNase I digestion by the GR. C, Sequence of the G6Pase promoter with summary of *in vitro* DNase I footprinting results. ●, Nucleotides protected from DNase I digestion by the GR. D, Oligonucleotide representing a consensus GRE, nGRE D and GRE B (Table 1) were labeled to a similar specific activity and incubated with purified GR-DBD (10 or 1000 ng). Protein binding was then analyzed as described in *Materials and Methods*. Representative autoradiographs are shown. E, The labeled consensus GRE oligonucleotide probe (Table 1) was incubated in the absence (–) or presence of the indicated molar excess of the unlabeled

methasone to stimulate expression of each was determined. Although dexamethasone-induced -271 SDM 2 fusion gene expression was not different from that of the -271 WT fusion gene, dexamethasone-induced -271 SDM 1 fusion gene expression was significantly greater than that of the -271 WT fusion gene (Fig. 8B). This suggests that a binding element that contributes to the inhibition of the dexamethasone response was inactivated by the SDM 1 mutation, or in other words, that a G6Pase promoter element that encompasses or overlaps the sequence between -260 and -255 binds a protein that plays a negative accessory factor role in the regulation of G6Pase-luciferase fusion gene expression.

We next analyzed protein binding to this element using the gel retardation assay. When a labeled oligonucleotide representing the WT G6Pase promoter sequence from -271 to -231 (Table 1), which encompasses the negative accessory factor element, was incubated with nuclear extract prepared from H4IIE cells, 8 protein-DNA complexes were detected (Fig. 8C). Competition experiments, in which a 100-fold molar excess of unlabeled DNA was included with the labeled probe, were used to correlate protein binding with the effect of mutations on the activity of the negative accessory factor. The WT $-271/-231$ oligonucleotide competed effectively for the formation of six of these protein-DNA complexes, designated 1, 2, 3, 5, 6, and 7 (Fig. 8C), indicating that complexes 4 and 8 must represent nonspecific protein-DNA interactions. By contrast, an oligonucleotide, designated $-271/-231$ MUT 1, which contains a mutation identical to that described in the -271 SDM 1 fusion gene (Fig. 8A), only competed effectively for the formation of complex 3 (Fig. 8C). An oligonucleotide, designated $-271/-231$ MUT 2, that contains a mutation identical to that described in the -271 SDM 2 fusion gene (Fig. 8A), competed effectively for the formation of complexes 1, 5, 6, and 7 (Fig. 8C). Taken together, these data indicate that complexes 1, 5, 6, and 7 (Fig. 8C) represent specific protein-DNA interactions and that their formation correlates with the activity of the negative accessory factor. In contrast, complex 2 represents a protein-DNA interaction with an element that is disrupted by both the MUT 1 and MUT 2 mutations,

whereas complex 3 represents a protein-DNA interaction with an element that is disrupted selectively by the MUT 2 mutation. Further analysis will be required to determine whether one or all of the four complexes associated with the MUT1 region represent the binding of the negative accessory factor.

DISCUSSION

The G6Pase GRU

The results presented above demonstrate that glucocorticoid induction of mouse G6Pase gene transcription requires the coordinated action of four GREs and multiple accessory factor elements. An alignment of the rat, mouse, and human sequences of the proximal G6Pase promoter is shown in Fig. 9, and the DNA binding elements that are involved in glucocorticoid regulation of G6Pase gene transcription are indicated. Importantly, this region of the G6Pase promoter is highly conserved between species. The G6Pase GRU is particularly complex because it contains both elements that contribute to and elements that inhibit glucocorticoid stimulation of gene transcription. The GRU contains three positive GREs (GRE A, GRE B, and GRE C) (Fig. 2) and multiple positive accessory factor elements, including binding sites for HNF-1, HNF-4, CRE binding proteins and FKHR (Figs. 3–5). Interestingly, the G6Pase promoter also contains one negative GRE (nGRE D) (Fig. 7, A–C) and one negative accessory factor element, which binds several unidentified factors (Figs. 7A and 8, A–C).

Multiple Promoter Elements Contribute to Glucocorticoid Activation of G6Pase Gene Transcription

DNase I footprinting studies demonstrate that GR binds all three positive GREs *in vitro* (Fig. 2A) and mutational analyses support the involvement of all three elements in glucocorticoid-stimulated G6Pase-luciferase fusion gene expression (Fig. 2D). Based on sequence analysis, a previous report predicted the presence of GRE B and GRE C, but only GRE B was

consensus GRE, nGRE D, or GRE B oligonucleotide competitors before the addition of purified GR-DBD (10 ng). Protein binding was then analyzed as described in *Materials and Methods*. Protein binding was quantified by using a Packard Instant Imager to count ^{32}P associated with the retarded GR-DBD dimer complex. The *upper panel* shows a representative autoradiograph, whereas the *lower panel* shows the mean data \pm SEM of three to four experiments. The HNF-1 oligonucleotide has been previously described and represents the HNF-1 binding site in the mouse β -fibrinogen gene (56). F, H4IIE cells were transiently transfected, as described in *Materials and Methods*, with various TK-luciferase fusion genes (15 μg) and expression vectors encoding *Renilla* luciferase (0.15 μg) and GR (5.0 μg). The fusion gene plasmids represented either the basic TK-luciferase vector or constructs in which one or two copies of oligonucleotides representing the indicated GRE sequences, as shown in Table 1, had been ligated into the *HindIII* site of the TK vector. After transfection, cells were incubated for 18–24 h in serum-free medium in the presence or absence of 500 nM dexamethasone. The cells were then harvested and luciferase assays were performed as described in *Materials and Methods*. Results are presented as the ratio of firefly luciferase activity, corrected for the protein concentration in the cell lysate, in dexamethasone-treated vs. control cells (expressed as fold induction). Results represent the mean \pm SEM of three to eight experiments in which each sample was assayed in duplicate. *, $P < 0.05$ vs. TK.

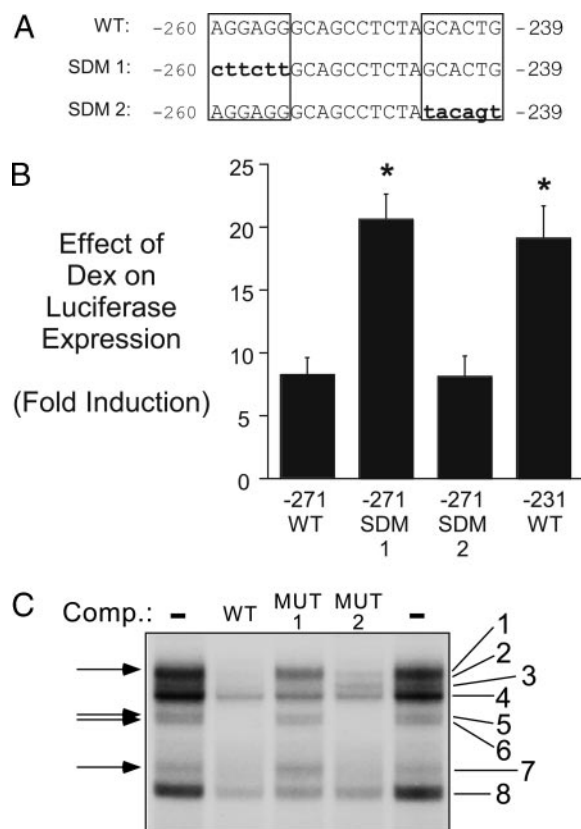


Fig. 8. The G6Pase Promoter Contains a Negative Accessory Factor Element

A, The WT sequence of the G6Pase promoter from -260 to -239 and the SDMs introduced in this region, indicated in *bold lowercase letters*, are shown. B, H4IIE cells were transiently transfected as described in panel A, except the G6Pase luciferase fusion genes incorporate either the WT promoter sequence located between -271 and $+66$ (-271 WT), between -231 and $+66$ (-231 WT), or contain the -271 to $+66$ promoter sequence with mutations in the region between -260 and -239 (-271 SDM 1 and -271 SDM 2). Results are presented as the ratio of firefly luciferase activity, corrected for the protein concentration in the cell lysate, in dexamethasone-treated vs. control cells (expressed as fold induction). Results represent the mean \pm SEM of three to seven experiments, in which each sample was assayed in duplicate. *, $P < 0.05$ vs. -271 WT. The -271 SDM1 mutation was associated with a statistically significant decrease in basal fusion gene expression relative to the -271 WT fusion gene ($16 \pm 2\%$; $n = 3$). C, A labeled oligonucleotide probe representing the WT G6Pase promoter sequence between -271 and -231 was incubated in the absence (–) or presence of a 100-fold molar excess of the unlabeled WT, $-271/-231$ MUT1 or $-271/-231$ MUT2 oligonucleotide competitors (Table 1) before the addition of H4IIE cell nuclear extract. Protein binding was then analyzed as described in *Materials and Methods*. In the representative autoradiograph shown, only the retarded complexes are visible and not the free probe, which was present in excess. The arrows point to complexes 1, 5, 6, and 7, which represent specific protein-DNA interactions whose formation correlates with the activity of the negative accessory factor.

found to contribute to glucocorticoid-stimulated G6Pase gene transcription (10). The mutation that was introduced to disrupt the GRE C element in the previous study would actually be predicted to create a higher affinity GRE than the WT GRE C element because its resulting sequence conformed more closely to the GRE consensus sequence (10). This is likely the reason the authors did not see a decrease in glucocorticoid induction of G6Pase gene transcription upon mutation of GRE C.

The mechanisms by which the positive accessory factors mediate their action at the G6Pase promoter are not known at this time, but previous studies on other genes provide insight into this issue. First, HNF-1 can recruit the general transcription machinery to specific gene promoters and it can also promote chromatin remodeling and the demethylation of individual promoters (74–76). These effects are mediated, in part, through interactions between HNF-1 and the coactivators CREB-binding protein (CBP), p300/CBP-associated factor, steroid receptor coactivator-1 (SRC-1), and receptor-associated coactivator 3 (75). Similarly, HNF-4 can interact with the coactivators CBP, p300, SRC-1, and glucocorticoid receptor-interacting protein-1. One result of such interactions is that HNF-4 can stabilize GR binding to gene promoters (45, 77–79). Finally, CRE binding proteins have been shown to interact with GR and the coactivators CBP and p300 and they can also stabilize GR binding to gene promoters (46, 80–82).

Importantly, this is the first report that an FKHR binding element contributes to glucocorticoid induction of G6Pase gene transcription. However, the involvement of this factor in G6Pase gene transcription is complex because multiple FKHR binding elements are present in the G6Pase promoter. FKHR had previously been shown to bind IRS 1 and IRS 2 (54), but DNase I footprinting and overexpression analyses reveal that FKHR binds two additional elements in the G6Pase promoter, namely FKHR B, and FKHR C (Figs. 4A and 5B). Interestingly, although there are multiple FKHR binding elements in the G6Pase promoter, only the IRS 1 and HNF-3/FKHR C binding sites are required for induction of G6Pase-luciferase fusion gene expression by glucocorticoids, with IRS 1 playing the quantitatively more important role (Fig. 5C). The interpretation of the IRS 1 mutagenesis experiment (Fig. 5C) is complex because this element completely overlaps with the 3' half site of GRE A (Figs. 2B and 4B). However, the results of heterologous fusion gene experiments (Fig. 5D) and ChIP assays (Fig. 6) both support the conclusion that FKHR plays an accessory factor role in the induction of G6Pase gene transcription by glucocorticoids. The mechanism of FKHR accessory factor action at the G6Pase promoter remains to be determined, but FKHR has been shown to interact with the coactivators SRC-1 and CBP (63, 64).

Interestingly, we have previously shown that insulin inhibits basal G6Pase gene transcription by inhibiting FKHR binding (54). The data presented here suggest

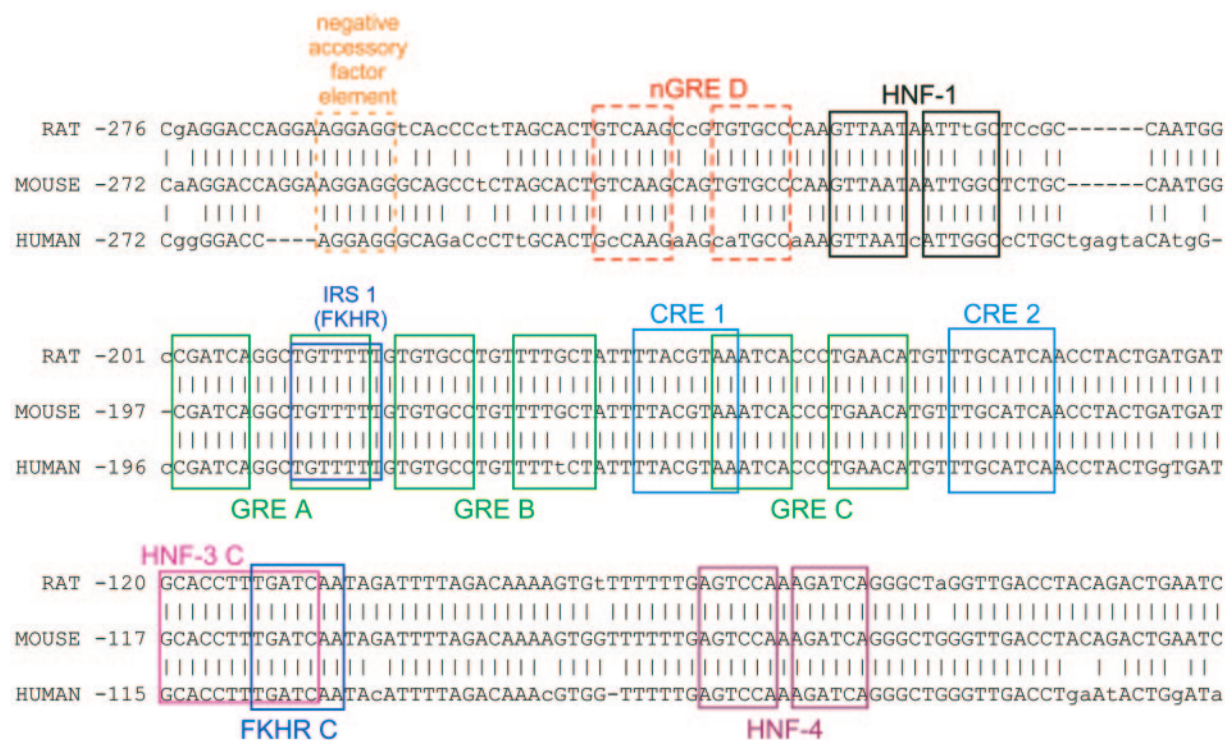


Fig. 9. The G6Pase GRU

Alignment of the rat, mouse, and human proximal G6Pase promoter sequences. The elements that play a role in glucocorticoid regulation of G6Pase gene transcription are indicated.

that the same mechanism could also explain, at least in part, how insulin inhibits glucocorticoid-stimulated G6Pase gene transcription. This conclusion is consistent with a recent report that shows FKHR and FKHL1 play an accessory factor role in glucocorticoid stimulation of pyruvate dehydrogenase kinase-4 (PDK4) gene transcription, and that insulin signaling blocks this induction by inhibiting FKHR and FKHL1 function (64). Similarly, combined glucocorticoid and cAMP treatment has been shown to stimulate FKHR binding to the PEPCK and G6Pase promoters, whereas insulin treatment has been shown to decrease FKHR binding (83).

The data supporting the involvement of HNF-3 β in glucocorticoid-stimulated G6Pase gene transcription are less clear. HNF-3 β binds three elements in the G6Pase promoter, designated HNF-3 A, HNF-3 B, and HNF-3 C (Figs. 4B and 5B). Lin and colleagues (55) previously identified the HNF-3 A and HNF-3 B binding elements in gel retardation studies. In addition to these two elements, HNF-3 C was identified using DNase I footprinting analysis (Fig. 4B). Of these three elements, only the HNF-3 C site was required for full induction of G6Pase-luciferase fusion gene expression by glucocorticoids (Fig. 5C); however, the effect of mutating this element was relatively minor (Fig. 5C). Furthermore, the mutation introduced into this element disrupts not only HNF-3 β , but also FKHR (Fig. 5B) and HNF-6 binding (71). It should be noted that the mutations introduced into the HNF-3 A and HNF-3 B bind-

ing elements had to be limited to avoid disrupting overlapping elements (Fig. 5A). So it could be argued that these mutations were insufficient to disrupt HNF-3 β binding sufficiently to reveal accessory factor activity. However, that conclusion is at odds with the observation that these mutations were sufficient to blunt HNF-3 β -stimulated G6Pase fusion gene expression (Fig. 5B). Despite the absence of convincing mutagenesis data to support an accessory factor role for HNF-3 β in glucocorticoid-stimulated G6Pase gene transcription, the ChIP assay results showed that HNF-3 β binding to the endogenous G6Pase promoter does increase upon glucocorticoid treatment (Fig. 6). In addition, HNF-3 β has been shown to interact with GR *in vitro* and to stabilize GR binding to a low-affinity GRE (45, 84), and experiments with truncated HNF-3 β constructs suggested that it contributes to glucocorticoid stimulation of endogenous G6Pase gene expression in H4IIE cells (43). Further studies will therefore be required to definitively establish whether HNF-3 β acts as an accessory factor for glucocorticoid-stimulated G6Pase gene transcription.

An interesting aspect of the ChIP assay results is that fairly high levels of basal occupancy of the promoter by GR, FKHR, and HNF-3 were detected in H4IIE cells (Fig. 6A). This observation is consistent with the results of transfection experiments that suggest that each factor contributes to basal G6Pase fusion gene expression. Specifically, there were statistically significant decreases in basal expression

when GRE A, GRE B, IRS 1, HNF-3 B/FKHR B, and HNF-3 C/FKHR C were mutated (see legends to Figs. 2 and 5). Similarly, we have previously shown that, in HepG2 cells, insulin inhibits basal G6Pase gene expression by inhibiting binding of FKHR to IRS 1 (54). Both FKHR and HNF-3 reside in the nucleus in the basal state, although insulin can stimulate the nuclear exclusion of both proteins (85, 86), but many studies have shown that GR is found in the cytoplasm in the absence of glucocorticoids. Although the ChIP experiments were performed in serum-free media, it is possible that glucocorticoids present in serum during cell culture had not been fully depleted. Alternatively, it is possible that the complex machinery that regulates the intracellular location of GR (87) is altered in H4IIE cells because Wang *et al.* (88) also detected GR binding to the endogenous PEPCK promoter in the absence of glucocorticoids.

Two Promoter Elements Inhibit Glucocorticoid Activation of G6Pase Gene Transcription

The G6Pase promoter also contains binding elements that inhibit glucocorticoid stimulation of gene transcription (Fig. 7). A negative GRE, nGRE D, was identified in the G6Pase promoter between –239 and –225 (Fig. 7, A and B). To the best of our knowledge, this is the first report of a promoter that contains both positive and negative GREs that function in the same cellular environment. Interestingly, nGRE D only matches the GRE consensus sequence at six of 12 bases, which is consistent with previous findings that nGREs often do not closely match this consensus (see introductory text).

A negative accessory factor element was identified in the G6Pase promoter just upstream of nGRE D (Figs. 7A and 8, A–C). Although the identity of the factor that binds this site is unknown, MatInspector transcription factor binding analysis (89) indicates that the sequence is similar to an Ets factor binding element. Ets factors bind a purine-rich GGA(A/T) core sequence, often in cooperation with other transcription factors, and their activity can be regulated by their phosphorylation status (90, 91). Ets factors have been shown to contribute to glucocorticoid activation of the MMTV and TAT promoters (see introductory text), as well as glucocorticoid repression of matrix metalloproteinase-9 gene expression (92). Based on this sequence analysis and the precedent for their role in glucocorticoid regulation of gene transcription, this suggested that an Ets transcription factor was a primary candidate for the negative accessory factor that binds the G6Pase promoter. Gel retardation assays using the negative accessory factor element as the labeled probe revealed four specific protein-DNA complexes whose formation correlate with negative accessory factor activity (Fig. 8C). There are more than nine subfamilies of Ets transcription factors (91), but expression profiling studies suggest that Ets-2 and PEA3 are the most abundant Ets factors in liver cells

(93). However, antisera to these factors, as well as to Ets-1, that have previously been shown to disrupt binding in gel retardation assays, did not affect the formation of any of the four specific complexes detected (data not shown).

The Potential Role of Positive and Negative Elements in the G6Pase GRU

We hypothesize that the positive and negative elements within the G6Pase GRU provide a mechanism for a graded and tightly regulated response to glucocorticoid induction of gene transcription, rather than a simple all-or-none hormone response. Indeed, there are many examples of glucocorticoid-stimulated promoters that contain binding elements that modulate glucocorticoid induction of gene transcription. For instance, CCAAT displacement protein (94) and the transcription enhancer factor-1 family of transcription factors (95) have been shown to inhibit glucocorticoid-induced MMTV transcription. Similarly, the TAT promoter contains an activator element, a negative element, and a neutralizer element, in addition to the GREs and positive accessory factor elements that mediate glucocorticoid stimulation of TAT gene transcription. These three elements all serve to provide flexibility to the glucocorticoid response (96, 97). Finally, the HNF-6 binding element in the PEPCK promoter inhibits glucocorticoid stimulation of PEPCK gene transcription (72). As for the G6Pase promoter, a key question that remains to be addressed is whether there are signaling pathways that selectively modulate the activity of the positive and negative accessory factors which bind the promoter.

MATERIALS AND METHODS

Materials

[γ -³²P]ATP (>5000 Ci mmol⁻¹) was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Dexamethasone 21-phosphate was purchased from Sigma Chemical Co. (St. Louis, MO), and DNase I was purchased from Roche Diagnostics Corp. (Indianapolis, IN). Specific antisera to GR (sc-1004), FKHR (sc-11350), HNF-3 β , (sc-6554) and rabbit IgG (sc-2027) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Plasmid Construction

The generation of mouse G6Pase-luciferase fusion genes, containing promoter sequence located between –484/+66, –271/+66 and –158/+66, –85/+66 and –35/+66, relative to the transcription start site, in the pGL3 MOD vector has been previously described (57). This vector is based on the pGL3 Basic firefly luciferase vector (Promega, Madison, WI) but contains a modified polylinker (98). Additional G6Pase promoter fragments, containing sequence between –374/+66 and –252/+66, were generated by digestion using *Bbv* II or PCR, respectively, and were also cloned into the pGL3 MOD vector.

G6Pase-chloramphenicol acetyltransferase (CAT) fusion genes containing promoter sequence between –231/+66,

–198/+66 and –129/+66 have been previously described (14, 56). G6Pase-CAT fusion genes containing SDMs in two CRE motifs (–231 CRE1 SDM and –231 CRE 2 SDM), an HNF-1 motif (–231 HNF-1 SDM), and in two insulin response sequence (IRS) motifs (–231 IRS 1 SDM and –231 IRS 2 SDM) have also been previously described (8, 54, 56). These promoter fragments were all subcloned into the pGL3 MOD vector.

A three-step PCR strategy (99) was used to introduce mutations into GREs A, B, and C (Fig. 2), a HNF-4 motif (Fig. 3), and HNF-3 motifs A, B, and C (Fig. 5). The resulting constructs, designated –231 GRE A, B or C SDM, –231 HNF-4 SDM, –231 HNF-3 A SDM, –231 HNF-3 B/FKHR B SDM and –231 HNF-3 C/FKHR C SDM, respectively, were generated within the context of the –231 to +66 G6Pase promoter fragment. Briefly, two complementary PCR primers were designed to mutate nucleotides within each of these motifs. The sequence of the sense strand oligonucleotides were as follows (mutated nucleotides are in *bold lowercase letters*): For –231 GRE A SDM: 5'-TGGCTCTGCCAATG-GatcgacttaGTTTTGTGTGCCTGTTTTG-3'. For –231 GRE B SDM: 5'-ATCAGGCTGTTTTGatgctCTGTTTTGaTATTTT-ACGTAATCA-3'. For –231 GRE C SDM: 5'-TTTGCTATTT-TACGTAc**ccgactgctcag**TGTTTGCATCAACCT-3'. For –231 HNF-4 SDM: 5'-GTGGTTTTTGTAGT**Caccct**ATCAGG-GCTGGGTTG-3'. For –231 HNF-3 A SDM: 5'-GATCAGGC-TGTTTT**c**TGTGCC**g**GTTTTGCTATTTTAC-3'. For –231 HNF-3 B/FKHR B SDM: 5'-CACCCGAACAT**c**TTTGCATC-AAAC-3'. For –231 HNF-3 C/FKHR C SDM: 5'-GATGATG-CACCTTT**gatcca**AGATTTTAGACAAAAGTGG-3'. These sense strand oligonucleotides were used in conjunction with a 3' PCR primer to generate the 3' half of the G6Pase promoter whereas the complementary antisense strand oligonucleotides were used in conjunction with a 5' PCR primer to generate the 5' half of the G6Pase promoter. These 5' and 3' primers were designed to maintain the 5' and 3' junctions of the G6Pase promoter fragment to be the same as those in the WT –231 to +66 G6Pase-luciferase fusion gene construct. The PCR products from these two reactions were then combined and used themselves as both primer and template in a second PCR to generate a small amount of the full-length, mutated G6Pase promoter fragment. Finally, the 5' and 3' PCR primers were then used to amplify this fragment. These promoter fragments were all subcloned into the pGL3 MOD vector.

Two separate mutations were introduced into the –260 to –239 G6Pase promoter region using PCR with the following oligonucleotides as the 5' primers and the –271 to +66 promoter fragment as the template: 5'-CCGCTCGAG(–271)-AAGGACCAGG**acttctt**GCAGCCTCTAGCACT-3' and 5'-CCGCTCGAG(–271)AAGGACCAGGAAGGAGGGCAGCCTC-T**atcag**tTCAAGCAGTGTGCC-3'. The mutated nucleotides are in *bold lowercase letters*, and *XhoI* sites used for cloning purposes are *underlined*. The 5' and 3' primers were designed to conserve the junctions between the G6Pase promoter and luciferase reporter gene to be the same as those in the WT –271 G6Pase-luciferase fusion gene construct. These promoter fragments were subcloned into the pGL3 MOD vector and the resulting constructs were designated –271 SDM 1 and –271 SDM 2. All promoter fragments generated by PCR were completely sequenced to ensure the absence of polymerase errors.

The TK-luciferase plasmid contains the herpes simplex virus thymidine kinase (TK) promoter sequence from –105 to +51 ligated to the luciferase reporter gene and has a unique *HindIII* site in the polylinker at –105. It was generated by subcloning the TK promoter as a *HindIII*-*BglII* fragment from TKCAT (100) into pGL3MOD. Various double-stranded complementary oligonucleotides, representing a consensus GRE, nGRE D, or GRE B (Table 1), were synthesized with *HindIII* compatible ends and were ligated in one or two copies into *HindIII*-cleaved TK-luciferase.

For protein expression in H4IIE cells, vectors encoding the rat glucocorticoid receptor [pRSV-GR; (101)] and human FKHR [pcDNA3-FKHR; (102)] were generously provided by Drs. Keith Yamamoto and Frederic Barr, respectively. A vector encoding full-length rat HNF-3 β was constructed by isolating an *EcoRI* fragment from pCMV-HNF-3 β , a generous gift from Dr. James Darnell (69), and ligating it into pcDNA3 (Invitrogen, Hercules, CA).

For protein expression in bacterial cells, a vector encoding a glutathione-S-transferase (GST)-mouse HNF-3 β fusion protein [pGEX2T-HNF-3 β ; (103)], was generously provided by Dr. David Powell. A vector encoding a histidine-tagged variant of human FKHR (His-FKHR) was constructed by isolating the FKHR open reading frame as a *KpnI*-*XbaI* fragment from pcDNA3-FKHR (102). This fragment was blunt ended using Klenow and ligated into *XhoI* digested, blunt ended pET-15b (Novagen, San Diego, CA).

Recombinant Protein Overexpression and Purification

The expression and purification of the DNA binding domain of GR (GR DBD), a generous gift from Dr. Keith Yamamoto, was as previously described (104). GST-HNF-3 β and His-FKHR proteins were expressed in the Rosetta(DE3) *Escherichia coli* strain (Novagen, San Diego, CA). One liter cultures were grown to OD₆₀₀ = 0.4 at 37 C and were temperature-shifted to 25 C for 30 min. Cultures were then induced with 0.5 mM isopropyl- β -D-thiogalactopyranoside. Four hours after induction, cells were harvested by centrifugation at 8000 \times g for 15 min. Thirty-five milliliters of lysis buffer [20 mM Tris HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA] and lysozyme (100 μ g/ml) was added to each sample and incubated on ice for 15 min. The cells were lysed by sonicating for 2 min (cycles of 5 sec pulse, 5 sec rest). Cell debris was removed by centrifugation at 35,000 \times g. The GST-HNF-3 β fusion protein was purified over glutathione Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ). PBS [140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ (pH 7.3)] was used for column equilibration and wash buffer. The cell lysate was loaded onto the column and washed with four column volumes of PBS. GST-HNF-3 β was then eluted using a three column-volume gradient from PBS to 50 mM Tris (pH 8.0), 10 mM reduced glutathione. His-FKHR was purified using Ni²⁺-nitrilotriacetic acid (Ni²⁺-NTA) (QIAGEN, Valencia, CA). A mixture of 20 mM Tris (pH 8.0), 150 mM NaCl, 5 mM β -mercaptoethanol, and 25 mM imidazole was used for column equilibration and wash buffer. The cell lysate was loaded onto a 20-ml column and washed with four column volumes of wash buffer. His-FKHR protein was then eluted using a seven column-volume linear gradient of imidazole (from 25–300 mM). Purified proteins were dialyzed against 50 mM HEPES (pH 7.8), 20 mM NaCl to exchange the buffer. GST-HNF-3 β and His-FKHR protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA).

In Vitro DNase I Footprinting Analysis

To study protein binding to the proximal region of the G6Pase promoter by DNase I footprinting, the –484 to +66 G6Pase-luciferase fusion gene was used as a template in a PCR with the following primers: For sense strand analysis: 5' to 3'; (–274)TTCAAGGACCAGGAAGGAGG (–255) and (+72)TTGGTACCTCAGGAAGCTGC(+53) and for antisense strand analysis: 5' to 3'; (–406)CTTAAAAGGTCACCTCCGGC(–387) and (–58)CAGCCCTGATCTTTGGACTC(–77). One hundred picomoles of the –274/–255 or the –58/–77 primer was end-labeled with [γ -³²P]ATP to visualize the sense or antisense strand of the promoter, respectively. The probe was generated by PCR amplification using 10 ng of template DNA, 100 pmol of each primer, and the following reaction conditions: 94 C, 30 sec; 54 C (sense strand)/60 C (antisense strand), 30 sec; 72 C, 30 sec for 35 cycles. Eigh-

teen microliters of probe cocktail [20 mM HEPES (pH 7.8), 2 mM dithiothreitol (DTT), 50 mM NaCl, 30 ng/ μ l poly-(deoxyinosine-deoxycytosine) poly(dI-dC)-poly(dI-dC), 50,000 cpm probe/sample] was incubated with 18 μ l of increasing concentrations of recombinant protein. The protein samples were brought to a volume of 18 μ l using the following buffers: GR [10 mM potassium phosphate (pH 7.4), 5 mM DTT, 0.1 mM EDTA, 10 mM sodium molybdate, 10% glycerol], GST-HNF-3 β and His-FKHR [50 mM HEPES (pH 7.8), 20 mM NaCl, 0.2 μ g/ μ l BSA]. 4.0 μ l of diluted (1:1000–1:1250 dilution of original 10 U/ μ l stock) DNase I (DNase I dilution buffer = 20 mM HEPES, 2 mM DTT, 37.5 mM MgCl₂, and 18.75 mM CaCl₂) was added to each sample for 2 min at room temperature. DNase I (125 μ l) stop solution [100 mM Tris (pH 7.4), 15 mM EDTA, 0.4% SDS, 100 mM NaCl, 0.1 μ g/ μ l tRNA] and 20 μ l of proteinase K (20 μ g/ μ l) were then added to each sample and they were incubated for 2 h overnight at 65 C. The DNA was precipitated, resuspended in 8.0 μ l of loading buffer (95% formamide, 0.025% xylene cyanol and bromophenol blue, 18 mM EDTA, 0.025% SDS), and 5.0 μ l of each sample was separated on a 5% polyacrylamide gel. The results were visualized by autoradiography. To determine the identity of the protected nucleotides, G and G+A chemical sequencing reactions (105, 106) were performed using the labeled promoter fragment and DNA fragments were separated on the polyacrylamide gel alongside the DNase I-treated samples.

Cell Culture, Transient Transfection, and Luciferase Assay

Rat H4IIE hepatoma cells were grown in DMEM containing 2.5% (vol/vol) fetal calf serum and 2.5% (vol/vol) newborn calf serum. Cells were transiently transfected in suspension with the plasmids indicated in the figure legends using the calcium phosphate-DNA coprecipitation method as previously described (107, 108). Luciferase assays were performed using the Promega Dual-Luciferase Reporter Assay System according to the manufacturer's instructions. For comparisons of basal gene expression and forkhead-stimulated gene expression, firefly luciferase activity directed by the various fusion gene constructs was expressed relative to SV40-*Renilla* luciferase activity in the same sample. Because dexamethasone inhibits SV40-*Renilla* expression about 2-fold in H4IIE cells (data not shown), for comparisons of the effect of dexamethasone on fusion gene expression, firefly luciferase activity from control, and dexamethasone-treated cells was corrected for the protein concentration in the cell lysate, as measured by the Pierce BCA assay (Rockford, IL). Each construct was analyzed in duplicate or in quadruplicate in multiple transfections, as specified in the figure legends, using at least three independent plasmid preparations.

ChIP Assay and Real-Time PCR Analysis

PCR amplification of the rat G6Pase promoter fragment, visualized by gel electrophoresis in Fig. 6A, was performed as previously described (54). Real-time PCR analyses, shown in Fig. 6, B and C, were performed using a Bio-Rad iCycler (Hercules, CA). Real-time PCR primers were designed to amplify the rat G6Pase promoter [5' to 3'; (–257)CACCCC-TTAGCACTGTCAAGCCGTGTG(–231) and (–39)GGATTCA-GTCTGTAGGTCAACCTAGCCC(–66)] and exon 5 (5' to 3'; (+686)AATGCCAGCCTCAAGAAATATTTCTC (+712) and (+861)AGGCTGGCAAAGGGTGTGGTGTCAATG (+835)]. The rat G6Pase promoter and exon 5 fragments were amplified using the Bio-Rad iQ SYBR Green Supermix (Hercules, CA) and the following reaction conditions: 95 C, 30 sec; 60 C, 30 sec; 72 C, 30 sec for 40 cycles. Standard curve analyses were performed for each set of samples to determine the efficiencies of the promoter and exon 5 PCRs, which ranged from 87–95% efficiency. The fold enrichment

over the IgG negative control was determined using the C_T method (109) and the following calculation: fold enrichment = [1 + (PCR efficiency)(0.01)]ⁿ; where n = (C_T IgG antibody) – (C_T experimental antibody), and C_T = threshold cycle.

Gel Retardation Assay

Labeled Probes. Oligonucleotides representing the sense and antisense strands of a consensus GRE, nGRE D, GRE B and the G6Pase promoter sequence from –271 to –231 were synthesized with *Hind*III or *Bam*HI compatible ends (Table 1), gel purified, annealed and then labeled with [α -³²P]deoxy-ATP using the Klenow fragment of *Escherichia coli* DNA polymerase I to a specific activity of approximately 2.5 μ Ci/pmol.

Nuclear Extract Preparation. H4IIE nuclear extracts were prepared exactly as previously described (110).

GR-DBD Binding Assay. Labeled GRE oligonucleotides (~7 fmol, ~30,000 cpm) were incubated with purified GR-DBD (10–1000 ng) (104) in a final reaction volume of 20 μ l containing 20 mM HEPES (pH 7.8), 50 mM NaCl, 0.38 mM spermidine, 0.08 mM spermine, 0.1 mM EDTA, 1 mM EGTA, 2 mM DTT, 12.5% glycerol (vol/vol), 250 ng of poly(dI-dC)-poly(dI-dC) and 100 ng BSA. After incubation for 10 min at room temperature and then 10 min on ice, the reactants were loaded onto a 6% polyacrylamide gel containing 0.25 \times TBE (22.5 mM Tris base, 22.5 mM boric acid, 0.5 mM EDTA) and 2.5% (vol/vol) glycerol. Samples were electrophoresed at 4 C for 150 min in 0.25 \times TBE. After electrophoresis, the gels were dried, exposed to Kodak XAR5 film (Eastman Kodak, Rochester, NY), and binding was analyzed by autoradiography.

Negative Accessory Factor Binding Assay. When the G6Pase –271/–231 oligonucleotide was used as the labeled probe, the binding conditions were identical with those described for GR-DBD except that H4IIE nuclear extract (5 μ g) was used, BSA was omitted and poly(dI-dC)-poly(dI-dC) was increased to 0.5 μ g. In addition, after incubation for 10 min at room temperature, the reactants were loaded onto a 6% polyacrylamide gel containing 1 \times TGE (25 mM Tris Base, 190 mM glycine, 1 mM EDTA) and 2.5% (vol/vol) glycerol. Samples were electrophoresed at room temperature for 90 min in 1 \times TGE.

Competition Experiments. For competition experiments (Figs. 7E and 8C), the indicated unlabeled double-stranded oligonucleotides (100- to 500-fold molar excess) were mixed with the labeled oligomer before addition of nuclear extract or GR-DBD. Binding was then analyzed by acrylamide gel electrophoresis as described above. Data were quantitated through the use of a Packard Instant Imager.

Statistical Analysis

The transfection data and ChIP assay data were analyzed for differences from the control values, as specified in the figure legends. Statistical comparisons were calculated using an unpaired Student's *t* test. The level of significance was *P* < 0.05 (two-sided test).

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