The Binding Site for *Xenopus* Glucocorticoid Receptor Accessory Factor and a Single Adjacent Half-GRE Form an Independent Glucocorticoid Response Unit†

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ABSTRACT: In *Xenopus laevis*, transcription of the γ-fibrinogen subunit gene is activated by glucocorticoids. Hormone induction is regulated by three glucocorticoid response element (GRE) half-sites and an additional DNA sequence which binds a novel hepatocyte nuclear protein, *Xenopus* glucocorticoid receptor accessory factor (XGRAF). The XGRAF binding site (GAGTTAA) is located directly upstream of the most distal half-GRE. The proximity of the binding sites for XGRAF and the glucocorticoid receptor (GR) led to the hypothesis that these two sites form a glucocorticoid response unit (GRU). By transfecting DNA into primary hepatocytes, we showed that this GRU confers hormone responsiveness in the absence of other half-GREs. The XGRAF binding site enhances function of the half-GRE without itself responding to glucocorticoids. The GRU retains efficacy in other locations relative to the γ-fibrinogen gene promoter, further increases transcription when present in multiple copies, and activates a heterologous promoter. Despite the contiguity of the XGRAF binding site and half-GRE, the two sites can be occupied simultaneously in vitro. The binding characteristics correlate with function since mutations that disrupt concurrent XGRAF and GR binding also impair transcription. This novel GRU represents a new regulatory mechanism that may be applicable to other glucocorticoid responsive genes that lack a full GRE.

The adrenal steroid hormones, glucocorticoids, play a vital role in many diverse physiological functions including control of metabolic, immune, inflammatory, and stress-related processes (*1*). In the classical mechanism of action (*2*, *3*), the hormone binds to the intracellular glucocorticoid receptor (GR)[1] protein in target cells. The hormone-bound receptor then interacts as a homodimer with specific DNA sequences, called glucocorticoid response elements (GREs), and stimulates transcription of nearby genes. A consensus sequence for the GRE has been defined as GGTACAnnnTGTTCT (*4*), with the downstream half (TGTTCT) being the most important since initial contact by the GR monomer occurs at this site (*5*).

In addition to one or more GRE(s), the transcriptional regulatory regions of glucocorticoid-inducible genes often contain DNA sites called accessory elements that amplify hormone responsiveness (*6*). Many proteins that had been identified previously as independent transcriptional regulators bind to accessory elements (*7*−*11*). When acting on such elements, these proteins are defined as accessory factors, which have the characteristics of being bound to the DNA and increasing fold hormonal induction (*6*). Although conventional GREs, often in conjunction with accessory elements, mediate glucocorticoid responsiveness of many genes, GR can modulate gene transcription in other settings as well. Both inhibitory and stimulatory activities have been observed, and a variety of nonclassical mechanisms have been proposed to account for these effects.

Negative regulation of transcription by GR occurs in a myriad of genes (*12*). GR can bind to negative GREs that have some similarity to positive GREs (*4*). In these cases, GR may either interfere with the function of other DNA-bound activators (*13*) or obstruct binding of general or stimulatory transcription factors (*14*, *15*). Negative regulation is also mediated by mechanisms that do not require direct GR·DNA contact. For example, transcriptional induction can

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[1] Abbreviations: Ap-1, activator protein-1; bp, base pair(s); β-Gal, β-galactosidase; CMV, cytomegalovirus; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GRU, glucocorticoid response unit; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HNF-1, hepatocyte nuclear factor-1; NF-κB, nuclear factor that binds site B of the κ immunoglobulin enhancer; MMTV, mouse mammary tumor virus; NF-1, nuclear factor-1; Oct, octamer transcription factor; PCR, polymerase chain reaction; pLL, pLucLink 2.0; SEM, standard error of the mean; Sp1, promoter-specific transcription factor; Stat-5, signal transducer and activator of transcription-5; T7X556, fragment of rat GR comprising amino acids 407−556; XGRAF, *Xenopus* glucocorticoid receptor accessory factor; XGRAF-BS, *Xenopus* glucocorticoid receptor accessory factor binding site.

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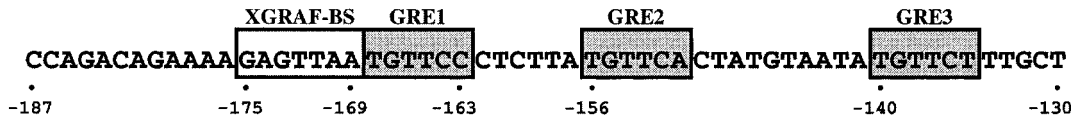

FIGURE 1: The DNA sequences that mediate glucocorticoid induction of the *Xenopus* γ-fibrinogen subunit gene lie between positions -175 and -135 of the 5′-flanking region. The DNA sequence of the sense strand from -187 to -130 relative to the transcription start site at $+1$ is shown. The XGRAF-BS is denoted by a white box while the GRE half-sites, GRE1, GRE2, and GRE3, are denoted by gray boxes.

be inhibited by association between GR and other transcription factors, such as Ap-1 (activator protein-1) (*16*−*18*), NF-κB (nuclear factor that binds site B of the κ immunoglobulin enhancer) (*19*), and Oct (octamer transcription factor)-1 (*20*). Through these interactions, GR may interfere with DNA binding of the other protein, or the DNA-bound transcription factor may tether GR to the DNA.

In addition to the repressive effects, GR can stimulate gene expression by a number of nonclassical mechanisms that involve communication between GR and other transcription factors. In the β-casein gene, Stat-5 (signal transducer and activator of transcription-5) binds to its specific recognition element, and transcriptional induction is augmented by GR, without requiring a GRE (*21*). GR is tethered to the DNA through a protein−protein interaction with Stat-5 (*22*). Thus, GR acts essentially as a coactivator, since it amplifies the activity of a DNA-bound transcription factor without itself contacting the DNA (*23*). A second type of mechanism is GR-mediated delivery of a transcriptional activator to its specific DNA-binding site. An example of this mode of regulation occurs with the hormone-inducible promoter from the mouse mammary tumor virus (MMTV), in which GR and Oct-2 interact in solution to promote the binding of Oct-2 to a site downstream from several GREs (*24*). Contact between the two proteins is not necessarily maintained after they are bound to their respective sites on the DNA. A third scenario is GR-induced chromatin remodeling that potentiates the binding of another stimulatory transcription factor. The MMTV regulatory region is a well-studied case of this type of regulation (*25*). Glucocorticoid treatment facilitates binding of NF-1 (nuclear factor-1) to a site that is inaccessible prior to hormonal activation, due to the arrangement of nucleosomes (*26*).

Positive activity of GR in conjunction with an unrelated transcription factor also occurs in the γ-fibrinogen subunit gene in the liver of the frog *Xenopus laevis* (*27*, *28*). In this gene, glucocorticoid responsiveness is mediated by three half-GREs and by a novel DNA sequence, GAGTTAA (see Figure 1). This sequence binds in vitro to the liver nuclear protein, *Xenopus* glucocorticoid receptor accessory factor (XGRAF) (*27*). The role of XGRAF in hormonal activation of the γ-fibrinogen gene was established by a detailed point mutational analysis of the recognition site, which showed an excellent correlation between nucleotides essential for XGRAF binding and those important for glucocorticoid induction of transcription (*28*). The XGRAF recognition sequence had not previously been defined as a protein binding site, suggesting that XGRAF is a newly discovered transcription factor (*28*).

A distinctive feature of the XGRAF binding site is its adjacency to a half-GRE, replacing the position where a second molecule of GR would usually bind to a conventional full GRE. The proximity of the XGRAF binding site and half-GRE suggests that the two sites may by themselves form a composite glucocorticoid response element (*13*), also called a glucocorticoid response unit (GRU) (*6*). Here, we show that the contiguous XGRAF and GR binding sites do function independently of additional half-GREs or other elements in the γ gene DNA. In addition, the juxtaposition of the two binding sites does not inhibit concurrent binding of XGRAF and GR to the DNA in vitro.

EXPERIMENTAL PROCEDURES

Construction of Transfection Vectors by Linker Scanning Mutagenesis. A series of clustered point mutations was introduced into the 5′-flanking region of the γ gene by linker scanning mutagenesis, which involves the generation of two separate libraries of 5′- and 3′-deleted sequences (*29*). The final constructs contain the γ sequence from -187 to $+41$ (*27*), with *Kpn*I and *Hin*dIII restriction enzyme sites added at the 5′ and 3′ ends, respectively. Nucleotide -187 is the final base of the *Kpn*I site. The sequence TTACTAGTAA, which introduces a *Spe*I restriction enzyme cleavage site, replaces blocks of 10 nucleotides at various positions (see Figure 5B). These constructs were originally assembled in pBluescript SK- (Stratagene) and were ligated into the *Kpn*I site and *Hin*dIII site of the luciferase expression vector pLucLink 2.0 (pLL) (*30*).

The internal deletion constructs GRU(γ-118), GRU(γ-104), and GRU(γ-93) were made exactly as described above for the linker scanning mutants, except that pairs of 5′- and 3′-deleted sequences were chosen such that nucleotides -156 to -119, -156 to -105, and -156 to -94, respectively, were removed and replaced by a 9-bp *Spe*I linker (TACTAGTAA) (see Figure 3A).

The correct structure of all the linker scanning constructs, as well as of all the constructs described in the next section, was confirmed by determination of the nucleotide sequence.

Construction of Transfection Vectors by the Polymerase Chain Reaction. All other plasmids were made by the polymerase chain reaction (PCR) with *Pfu* polymerase (Stratagene) (*27*), using DNA templates located within pLL, a downstream primer consisting of the antisense sequence of the pLL vector from 127 to 108, and appropriate upstream primers as indicated below. Each γ construct terminates at $+41$ and has an added *Hin*dIII site at the 3′ end. Each Bβ construct ends at $+40$ and contains a 3′ adapter with the sequence TCGGATCCGCGGCCGCAAGCTT. The final DNA inserts were cloned into the *Kpn*I site and *Hin*dIII site of pLL. See Table 1 for the wild-type and mutant sequences and the 5′ linkers of the constructs described below.

For the mutants shown in Figure 2, the parent plasmid was γ-187, which contains the wild-type γ sequence from -187 to $+41$ (*27*). The constructs (γ-187)mutG2 and (γ-187)mutG3 are mutated in GRE2 and GRE3, respectively, as shown in Table 1. These mutations are the same as those previously referred to as mut J and mut L (*27*) and are

Table 1: Sequences of Mutations within the γ-Fibrinogen Gene Upstream DNA

Name	DNA Sequence [a]
Wild type	$^{-187}$ CCAGACAGAAAA**GAGTTAA**TGTTCCCTCTTATGTTCACTATGTAATATGTTCTTTGCT $^{-130}$
mutG2 [b,c]	$^{-187}$ CCAGACAGAAAA**GAGTTAA**TGTTCCCTCTTATtTaCtagtaaTAATATGTTCTTTGCT $^{-130}$
mutG3 [c]	$^{-187}$ CCAGACAGAAAA**GAGTTAA**TGTTCCCTCTTATGTTCACTATGTAATATtactagTaaT $^{-130}$
mutG2G3 [b,c]	$^{-187}$ CCAGACAGAAAA**GAGTTAA**TGTTCCCTCTTATtTaCtagAaaTAATATtactagTaaT $^{-130}$
mutXG2G3 [b,d]	$^{-187}$ CCAGACAGAAAAGAGcgcgTGTTCCCTCTTATtTaCtagAaaTAATATtactagTaaT $^{-130}$
GRU [e]	$^{-187}$ CCAGACAGAAAA**GAGTTAA**TGTTCCCTCTTA $^{-157}$
GRUmutX [e]	$^{-187}$ CCAGACAGAAAAGAGcgcgTGTTCCCTCTTA $^{-157}$
GRUmutG1 [e]	$^{-187}$ CCAGACAGAAAA**GAGTTAA**atgTCCCTCTTA $^{-157}$

[a] The sense strand is shown, and mutated bases are denoted by lower case letters. The XGRAF-BS is in bold, and the GRE half-sites are underlined. [b] Note that position −148 is T in mutG2 and is A in mutG2G3 and in mutXG2G3 (see Experimental Procedures). [c] In the 5′ *Kpn*I site, nucleotides −186 and −187 are the final two bases of the restriction enzyme recognition sequence. [d] An adapter (GGTACCCCGTCGAC), containing *Kpn*I and *Sal*I sites, is present on the 5′ end of the γ sequence. Nucleotide −187 is the final base of the restriction enzyme recognition sequence. [e] The 5′ adapter of all constructs with GRU in the name consists of either the *Kpn*I site described for the linker scanning mutants (see Experimental Procedures) or the sequence shown in footnote *d*. No significant differences in basal or hormone-induced transcription were observed with these two adapter sequences (data not shown).

equivalent to the 10-bp *Spe*I linker substitutions in the linker scanning mutants described above. The (γ-187)mutG2G3 construct was assembled by inserting a PCR product (containing an *Xba*I site at the 5′ end and mutG3 in the γ sequence from −145 to +41) into *Spe*I/*Hin*dIII-cut (γ-187)-mutG2 vector. The (γ-187)mutXG2G3 plasmid construct was made using (γ-187)mutG2G3 as a PCR template and an upstream primer with a mutation in the XGRAF-BS.

The GRUmutX(γ-104) and GRUmutG1(γ-104) constructs shown in Figure 3B were made using GRU(γ-104) as a template in a PCR with upstream oligonucleotides incorporating a mutation within the XGRAF-BS or GRE1 as appropriate (see Table 1). The GRU-GRU(γ-104) in Figure 4 was made by digestion of the GRU(γ-104) construct with *Spe*I and *Hin*dIII, leaving a pLL vector containing the GRU (bases −187 to −157). Then PCR methods were used to generate the GRU(γ-104) sequence possessing a linker with an *Xba*I site at the 5′ end. This fragment was digested with *Xba*I and *Hin*dIII and ligated into the vector. The GRUmutX-GRU(γ-104) was prepared in an identical manner except that GRUmutX(γ-104) served as the *Spe*I/*Hin*dIII-cut recipient vector. The 11-bp linker between pairs of GRUs is TAC-TAGATTTG.

To construct the γ/Bβ fusion vectors shown in Figure 6, a PCR was used to generate DNA fragments with a *Spe*I site at the 5′ end plus a Bβ sequence either from −132 to +40 or from −104 to +40, using cloned Bβ sequences as the template (*31*). These DNA fragments were ligated into *Spe*I/*Hin*dIII-cut pLL vector containing the GRU (bases −187 to −157), described in the preceding paragraph, to generate the GRU(Bβ-132) and GRU(Bβ-104) plasmids. The GRUmutX(Bβ-104) was made using GRU(Bβ-104) as the template in a PCR and an upstream primer containing a mutated XGRAF-BS. Construction of Bβ-136 was described previously (*31*).

Transfection of Primary Hepatocytes. Plasmid DNA for the transfection procedure was propagated in DH5α *Escherichia coli* (Bethesda Research Laboratories), amplified by 220 μg/mL chloramphenicol treatment (*32*), and extracted by alkaline lysis on ice in the presence of 100 μg/mL RNase A (*33*). To the cleared lysate was added one-third volume of 4 M NaCl and 40 mM Tris-HCl, pH 7.5, and the DNA was purified by anion-exchange chromatography (Qiagen) and cesium chloride gradient sedimentation (*27*).

Primary hepatocytes were isolated from the livers of estrogen-treated adult female *X. laevis* by collagenase perfusion and metrizamide gradient centrifugation (*31*). Primary cells were transfected by electroporation using 50 μg of luciferase reporter DNA and 10 μg of pCMVβ-Gal control plasmid (*28*). To examine hormonal induction, three independent transfections for every construct were performed in each experiment. Cells from a single transfection were divided for maintenance in culture in the absence or presence of 10^{-7} M dexamethasone and 10^{-9} M triiodothyronine. After 44−48 h, the cells underwent lysis in nonionic detergent as described (*31*) except the Barth's washes and scraping were omitted.

Luciferase and β-Galactosidase Reporter Assays. The cell lysates were analyzed for luciferase reporter gene activity using a luminometer as detailed previously (*27*). The β-galactosidase (β-Gal) activity was measured in triplicate from each sample with a Galactolight kit (Tropix) as described (*27*). In some experiments, the triplicate aliquots were prepared by combining 11 μL of lysate, 11 μL of lysis buffer, and 220 μL of diluted galacton substrate, from which three 75-μL samples were assayed.

Luciferase activity was normalized to β-Gal activity in each sample. The fold hormonal induction for each independent transfection was expressed as normalized luciferase activity in hormone-treated cells divided by normalized luciferase activity in untreated cells. For each construct, the mean fold hormonal induction was calculated from the three independent transfections in each experiment. Then, the hormonal induction for each test construct was expressed as a percentage of that of a control construct as follows: fold induction (% of control) = [(fold induction of test construct − 1)/(fold induction of control construct − 1)] × 100. The value 1 was subtracted from each fold induction to account for the baseline, representing no hormone response (*34*). These percentages from three to five experiments were

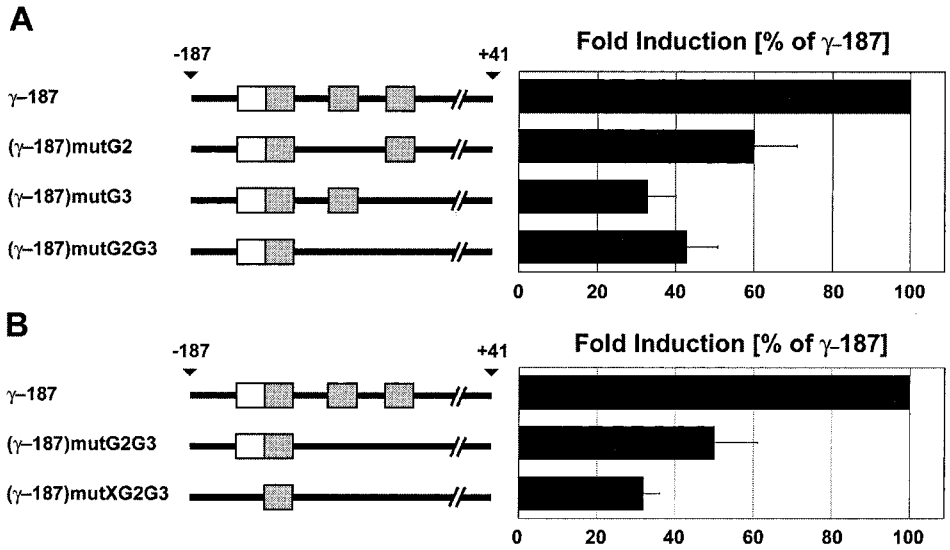

FIGURE 2: The XGRAF-BS enhances glucocorticoid-induced transcription by GRE1. The diagrammatic schemes of the constructs containing the *Xenopus* γ-fibrinogen gene upstream DNA show the presence of the XGRAF-BS (white box) and the GRE half-sites, GRE1, GRE2, and GRE3 (gray boxes) (see Figure 1). See Table 1 for the wild-type and mutant sequences. Constructs were transfected into *Xenopus* primary hepatocytes, and glucocorticoid responsiveness was determined as described in Experimental Procedures. The fold hormonal induction of each construct is given as a percentage of the γ-187 control containing the wild-type sequence from −187 to +41. The induction of each test construct was significantly different from that of the control using Dunnett's test ($P < 0.05$). (A) The data are expressed as the average of five separate experiments ± SEM. The fold induction of γ-187 was 4.5. (B) The data are expressed as the average of four separate experiments ± SEM. The fold induction of γ-187 was 3.3. The two test constructs were significantly different from each other using Student's *t* test ($P < 0.05$).

averaged, and the standard error of the mean (SEM) was calculated. For statistical analyses involving comparison of multiple experimental groups to a single control group (Figures 2−4 and 6A), raw data for fold hormonal induction in each independent transfection were examined using Dunnett's test (*35*). For statistical analyses involving comparison of one sample group to a control group (Figures 2B and 6B), raw data for fold hormonal induction in each independent transfection were examined using Student's *t* test (*35*).

Preparation of Nuclear Extract. Nuclear extract was prepared from *Xenopus* primary hepatocytes maintained in culture for 4 days as previously described (*27*, *28*) except that the extract was in buffer C^+, not diluted with buffer D. Final protein concentration in the extract was determined by the Bradford assay (Bio-Rad) (*36*).

Preparation of DNA Probes and Gel Mobility Shift Assays. Oligonucleotides from the DNA Core Facility at the University of Missouri−Columbia were purified on a denaturing 16% polyacrylamide gel and annealed to produce double-stranded DNA probes (see Figure 7B) (*27*). The mutations introduced into the mutXGRAF-BS probe destroy XGRAF binding (*27*), and the mutations introduced into the mutGRE probe eliminate any resemblance to the consensus GRE (*4*). The probe DNA was labeled at the 5′ end with [γ-^{32}P]ATP as described (*37*).

The GR fragment, T7X556, comprises amino acids 407−556 of the rat GR, which includes the DNA-binding domain (*38*). Purified T7X556 was a kind gift from Dr. Keith Yamamoto. The conditions for the gel shift assay were modified from those described previously (*27*). The standard 14-μL reaction contained 1.8 μL of *Xenopus* liver nuclear extract (3.4 μg of total protein/μL), 5 ng of T7X556, 0.1 μg of poly(dI·dC), and 3 μL of 5 × binding buffer (100 mM HEPES−KOH, pH 7.9, 250 mM KCl, 5 mM Na-EDTA, pH 8.0, 50% glycerol, 0.5% NP40, 5 mM dithiothreitol). Reactions were incubated for 15 min on ice, 1 μL of radioactively labeled probe (0.2 ng, 13 000−20 000 cpm) was added, and incubation was continued for 30 min on ice. The products were resolved on a native 5% polyacrylamide gel run at 350 V for 4 h at 4 °C (*27*). The DNA was detected by phosphorimaging with ImageQuant 3.3 software (Molecular Dynamics, Inc.).

RESULTS

The XGRAF-BS and GRE1 Retain Hormone Responsiveness in the Absence of Additional GREs. Transfection of DNA fragments into *Xenopus* primary hepatocytes previously showed that the glucocorticoid responsive region of the *Xenopus* γ-fibrinogen subunit gene is contained within bases −187 to +41, relative to the start site of transcription at +1 (*27*). As indicated in Figure 1, this region has three GRE half-sites, designated GRE1, GRE2, and GRE3, as well as a binding site for XGRAF. Deletion or mutation of any one of these sites markedly decreases hormonal induction of the gene promoter (*27*). Interestingly, the XGRAF binding site (XGRAF-BS) at nucleotides −175 to −169 lies directly upstream of the most distal GRE half-site (GRE1) at bases −168 to −163. The proximity of the XGRAF-BS and GRE1 suggested that these two sites act as an independent composite element to mediate the glucocorticoid response.

To examine the functional ability of this element, we analyzed the hormone responsiveness of XGRAF-BS and GRE1 in the absence of GRE2 and GRE3. As explained in Experimental Procedures, hormonal stimulation is expressed as a percentage of the control instead of fold induction to facilitate comparison between experiments. As observed previously (*27*), mutation of either GRE2 or GRE3, in the constructs (γ-187)mutG2 and (γ-187)mutG3, respectively, decreased the hormone response (Figure 2A). The (γ-187)-

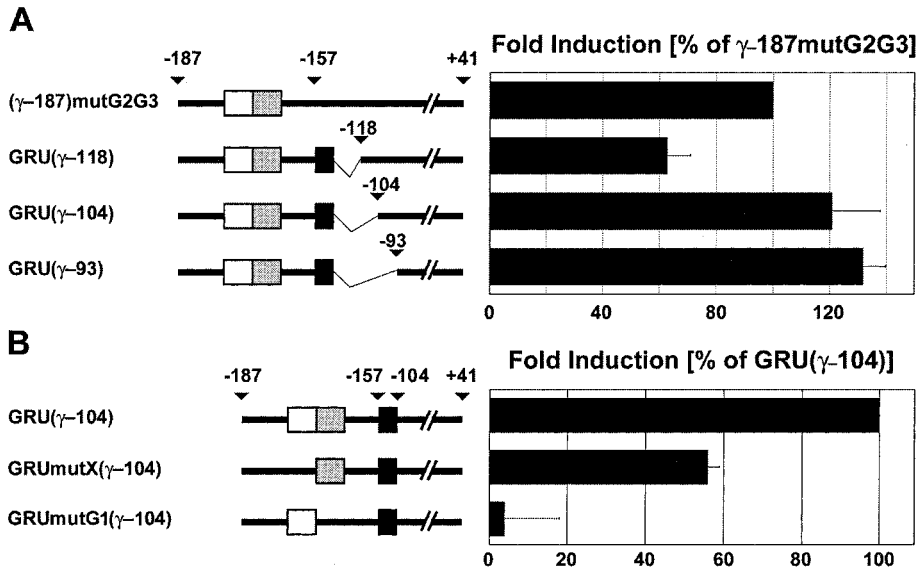

FIGURE 3: The GRU functions at different locations relative to the γ-fibrinogen gene promoter and requires both the XGRAF-BS and GRE1 for full activity. The diagrammatic schemes denote the presence of the XGRAF-BS (white box), the GRE1 half-site (gray box), and a nine base pair linker (black box) with the sequence TACTAGTAA. See Table 1 for the wild-type and mutant sequences. Constructs were transfected into *Xenopus* primary hepatocytes, and glucocorticoid responsiveness was determined as described in Experimental Procedures. The fold hormonal induction of each construct is given as a percentage of the control construct in each panel. (A) The data are expressed as the average of four separate experiments ± SEM. The (γ-187)mutG2G3 control was induced 2.3-fold. (B) The data are expressed as the average of three separate experiments ± SEM. The GRU(γ-104) control was induced 2.4-fold. The induction of each test construct was significantly different from that of the control ($P < 0.05$).

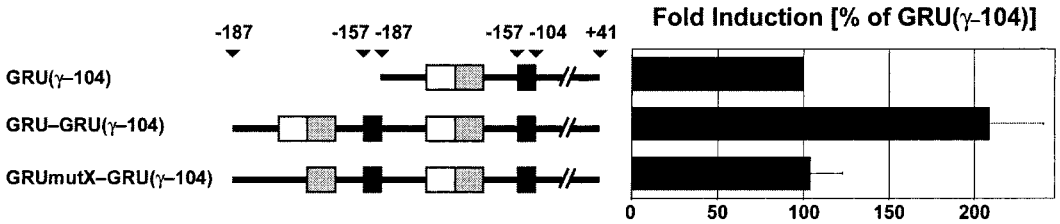

FIGURE 4: Multiple copies of the GRU enhance the hormone response. The diagrammatic scheme denotes the presence of the XGRAF-BS (white box), the GRE1 half-site (gray box), and either an eleven (TACTAGATTTG) or a nine (TACTAGTAA) base pair linker (black boxes) at the distal and proximal locations, respectively. See Table 1 for the wild-type and mutant sequences. Constructs were transfected into *Xenopus* primary hepatocytes, and glucocorticoid responsiveness was determined as described in Experimental Procedures. Values represent the fold hormonal induction of each construct as a percentage of the GRU(γ-104) control, which was induced 2.1-fold. The data are expressed as the average of three separate experiments ± SEM. The induction of GRU-GRU(γ-104) was significantly different from that of the control ($P < 0.05$).

mutG2G3 construct, with both GRE2 and GRE3 mutated, also exhibited a lower hormone induction than the γ-187 control (Figure 2A). However, it is significant that (γ-187)-mutG2G3, containing only the XGRAF-BS and GRE1, retained glucocorticoid induction similar to that observed following mutation of either GRE2 or GRE3 alone (Figure 2A). To determine whether the XGRAF-BS contributed to the remaining hormone response, this site was mutated in (γ-187)mutXG2G3. In this construct, the glucocorticoid induction was further diminished in comparison to (γ-187)-mutG2G3 (Figure 2B). Therefore, the XGRAF-BS enhances the function of GRE1 even when no other GRE half-sites are present. Conversely, we have shown previously that mutation of GRE1 renders the γ gene promoter essentially nonresponsive to hormone (see also Figure 3B) (*27*).

The GRU Formed by the XGRAF-BS and GRE1 Acts at Different Locations Relative to the γ-Fibrinogen Gene Transcription Start Site. One of the hallmarks of a transcriptional enhancer is its ability to function in alternate locations (*39*, *40*). In the constructs GRU(γ-118), GRU(γ-104), and GRU(γ-93), internal nucleotides were deleted such that GRE2 and GRE3 were eliminated, and the GRU, consisting of the bases from −187 to −157, was moved closer to the transcription start site (Figure 3A). In all cases, the constructs retained glucocorticoid responsiveness (Figure 3A), demonstrating that the GRU functions at several different positions upstream of the γ-fibrinogen gene promoter.

Both the XGRAF-BS and GRE1 Are Key Components of the GRU. The individual contribution of the XGRAF-BS or GRE1 to the hormone response was examined using the GRU(γ-104) construct. First, the XGRAF-BS was mutated to generate the construct GRUmutX(γ-104). In this case, hormonal induction was reduced but was not completely eliminated (Figure 3B). In contrast, when GRE1 was mutated in the GRUmutG1(γ-104) construct, the promoter failed to respond to glucocorticoids (Figure 3B). Therefore, GRE1 is an essential component of the GRU while the XGRAF-BS augments the hormonal induction when GRE1 is present. The enhancement by the XGRAF-BS in this context correlates with that observed in (γ-187)mutG2G3, in which the

GRU is in the native position (compare Figure 3B to Figure 2B).

Multiple Copies of the GRU Enhance the Hormone Response. As an additional demonstration that the XGRAF-BS is a critical part of the GRU, two GRUs were arrayed in tandem. The presence of two copies of the −187 to −157 region of the γ upstream DNA, in the GRU-GRU(γ-104) construct, approximately doubled the glucocorticoid induction compared to the GRU(γ-104) control (Figure 4). To ensure that the increased hormone response was not due simply to the presence of an additional GRE1, the 5′-most XGRAF-BS was mutated in the construct GRUmutX-GRU-(γ-104). This construct displayed a hormone induction essentially the same as the GRU(γ-104) control (Figure 4), indicating that the additional GRU absolutely depends on the XGRAF-BS to retain hormone responsiveness. The failure of a single half-GRE to increase glucocorticoid induction significantly above that of the GRU is consistent with the results for constructs (γ-187)mutG2 and (γ-187)-mutG3 compared to (γ-187)mutG2G3 (Figure 2A).

The GRU Acts on a Promoter Other Than That of the γ-Fibrinogen Gene. If the γ GRU is an independent enhancer, it should bestow glucocorticoid responsiveness on a different gene promoter. This hypothesis was tested by transferring the GRU to the *Xenopus* Bβ-fibrinogen gene 5′-flanking region. This gene was chosen because its activity in *Xenopus* hepatocytes has been analyzed in detail (*31*) and the sequence of the DNA upstream of the Bβ gene is not similar to that of the γ gene, even though the fibrinogen genes are evolutionarily related (*41*, *42*). Unlike the γ-fibrinogen gene, in which glucocorticoid induction is controlled by the XGRAF-BS and three GRE half-sites, the sole requirement for hormone responsiveness of the Bβ gene is a single full GRE from −162 to −148 relative to the transcription start site (*31*). In addition, an HNF-1 (hepatocyte nuclear factor-1) binding site located from −132 to −120 elevates basal expression of the Bβ gene (*31*). The sequences involved in basal expression of the γ-fibrinogen gene were analyzed by linker scanning mutagenesis (Figure 5). Out of 12 block mutations within the upstream sequence to position −187, only 3 substantially reduced basal transcription below 40% of the wild-type control (Figure 5A). Two of these mutants, LS(−37 to −28) and LS(−25 to −18), include or are close to the putative TATA box at −31 to −27. The third mutant, LS(−108 to −99), disrupts the sequence $^{-104}$CCTCCC^{-99}, which on the antisense strand has a strong match to the core GGGCGG of the consensus binding site for the promoter-specific transcription factor Sp1 (*43*). These results are consistent with findings for mammalian fibrinogen genes, in which basal transcription is regulated by HNF-1 for the Bβ gene (*44*, *45*) and by Sp1 for the γ gene (*46*). With the exception of one half-GRE, no transcriptional regulatory elements have been found in common between the *Xenopus* Bβ and γ genes. Hence, the Bβ 5′-flanking DNA is an excellent context in which to test the γ GRU.

The γ GRU was introduced into the Bβ gene upstream DNA context by linking the γ sequence from −187 to −157 to the Bβ sequence from −132 to +40, generating the construct GRU(Bβ-132) (Figure 6A). In this construct, the γ GRE1 occupies the same position, relative to the transcription start site, that the downstream half of the full GRE does in the native Bβ gene context. GRU(Bβ-132) responded to

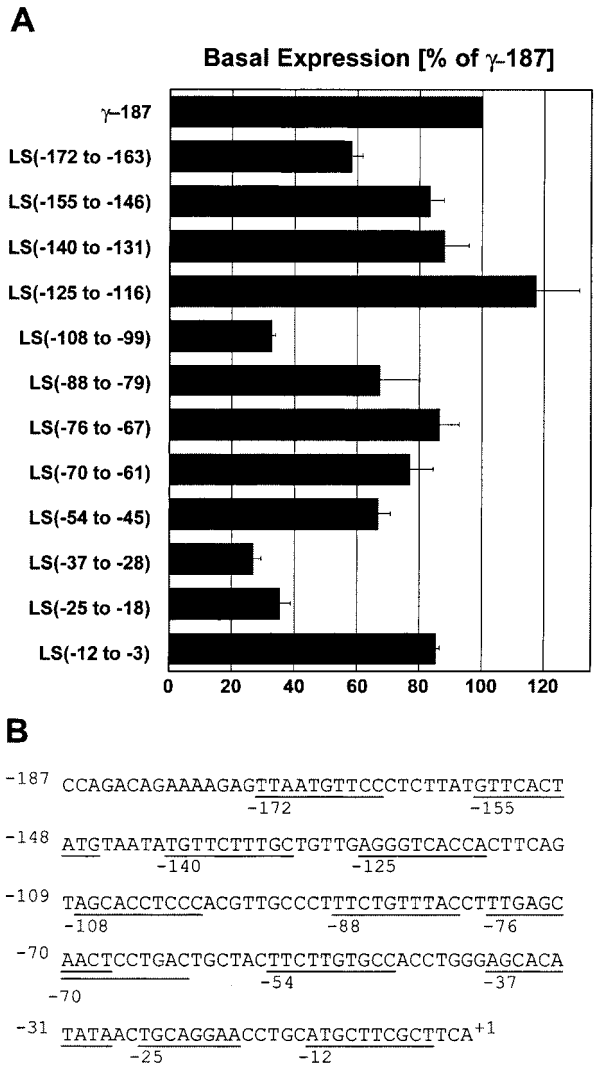

FIGURE 5: Three mutants with substitutions in the γ-fibrinogen gene upstream region have reduced basal promoter activity. (A) The constructs contain the γ gene sequence from −187 to +41. Blocks of 8−10 nucleotides were mutated as described in Experimental Procedures. The construct names on the left indicate the nucleotides replaced by the linker. Basal expression was determined by luciferase activity/β-galactosidase activity in transfected cells maintained in the absence of dexamethasone. The values were normalized as a percentage of the γ-187 control. For each construct, the data are expressed as the average of two to six separate experiments ± SEM, each containing one to four replicates. (B) The wild-type sequence of the γ-fibrinogen gene 5′-flanking DNA is shown. Underlining indicates the nucleotides replaced by the linker sequence (TTACTAGTAA) in each mutant. The linker in the LS(−25 to −18) construct is 8 bp (TTACTAGT).

hormone, although at a lower level than GRU(γ-104), which contains the GRU in the γ-fibrinogen context (Figure 6A). In comparison, a construct containing only the Bβ sequence from −136 to +40, with no GRE, exhibited no hormone induction (Figure 6A).

To ascertain whether the γ GRU retained its position independence in the Bβ context, it was moved closer to the transcription start site to form GRU(Bβ-104) (Figure 6B). GRU(Bβ-104) was induced similarly to GRU(γ-104), indicating that the GRU can function at different locations relative to the Bβ gene transcription start site (Figure 6B). The XGRAF-BS was shown to have a role in the hormone induction of the Bβ promoter by mutating the site to form

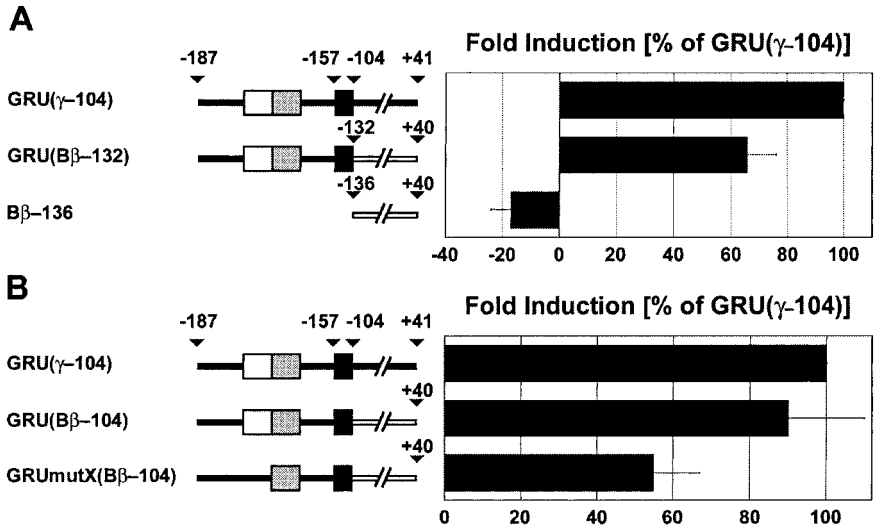

FIGURE 6: The γ GRU is functional in the context of the Bβ-fibrinogen gene upstream DNA. The diagrammatic scheme denotes the presence of the XGRAF-BS (white box), the GRE1 half-site (gray box), and a nine base pair linker, TACTAGTAA (black box). Black bars designate the γ gene 5′-flanking DNA, and white bars designate the Bβ gene 5′-flanking DNA. See Table 1 for the wild-type and mutant sequences. Constructs were transfected into *Xenopus* primary hepatocytes, and glucocorticoid responsiveness was determined as described in Experimental Procedures. Values represent the fold hormonal induction of each construct as a percentage of the GRU(γ-104) control. The data in each panel are expressed as the average of three separate experiments ± SEM. (A) The control was induced 2.0-fold. The induction of each test construct was significantly different from that of the control ($P < 0.05$). (B) The control was induced 2.1-fold. The difference in induction of GRUmutX(Bβ-104) and GRU(Bβ-104) was statistically significant at the level of $P < 0.09$.

GRUmutX(Bβ-104). This construct exhibited decreased function in relation to GRU(Bβ-104) (Figure 6B). Thus, the contribution of the XGRAF-BS to glucocorticoid responsiveness in the Bβ context is similar to that in the γ context (compare Figure 6B to Figures 2B and 3B).

The XGRAF-BS and GRE1 Are Required for Simultaneous Binding of XGRAF and GR to the DNA. The close proximity of the XGRAF and GR binding sites raised the question of whether both proteins could bind to the DNA concurrently. In a gel mobility shift assay, XGRAF bound as a single major band to the wild-type γ sequence (Figure 7, lane 8). Previous work clearly established that XGRAF binding is due to interaction with the GAGTTAA sequence immediately upstream of GRE1 (*28*). The capacity of GR to interact with the γ DNA was demonstrated with a purified fragment of rat GR encompassing the DNA-binding domain, which bound primarily as a monomer (Figure 7, lane 7). When both proteins were present, XGRAF·DNA was reduced in intensity while readily detectable amounts of GR·DNA and a more slowly migrating band designated XGRAF·GR·DNA were observed (Figure 7, lane 9). To establish that this new complex contains both XGRAF and GR, the gel shift assay was carried out with probes in which each site had been mutated individually. When the DNA probe contained a mutated XGRAF-BS, GR bound normally (Figure 7, lane 1), but no binding of XGRAF by itself was detectable (Figure 7, lane 2), and formation of the larger complex was not observed (Figure 7, lane 3). Therefore, XGRAF is a component of the proposed XGRAF·GR·DNA complex. With a DNA probe lacking the GRE1, GR binding was substantially reduced (Figure 7, lane 4) although not completely eliminated, perhaps because of nonspecific binding due to the large amount of purified GR. XGRAF alone bound well to this probe as expected (Figure 7, lane 5), but no XGRAF·GR·DNA complex was observed when both XGRAF and GR were present (Figure 7, lane 6). These results show that XGRAF and the GR fragment are capable of binding to the DNA simultaneously in vitro and that the binding sites for both proteins must be present to form the XGRAF·GR·DNA complex.

DISCUSSION

Formation of an Independent Composite GRE from an XGRAF Binding Site and a Half-GRE. Glucocorticoid induction of the *Xenopus* γ-fibrinogen subunit gene is regulated by DNA sequences between 187 and 157 bases upstream from the transcription start site. Two half-GREs located closer to the promoter also increase hormonal activation, but the −187 to −157 region can function in the absence of these additional half-GREs. This region encompasses a GRU that is a composite of two regulatory elements, the half-GRE1 and a contiguous binding site for the accessory factor, XGRAF. GRE1 is required for hormonal responsiveness, whereas the XGRAF-BS amplifies the induction but does not, itself, mediate glucocorticoid activation of transcription. Thus, XGRAF satisfies the criteria for an accessory factor since it contacts the DNA and enhances the glucocorticoid response. However, in contrast to other known accessory factors, XGRAF binds to the DNA directly upstream from the GR binding site, occluding the position where a second monomer of GR would normally bind to form a GR dimer.

The fact that GRE1 and the adjacent XGRAF-BS form an independent composite element was established using several criteria. First, the other half-GREs (GRE2 and GRE3) are not required. Second, the GRU functions in multiple locations relative to the start site of transcription and when varying amounts of the γ gene DNA are removed. Third, two copies of the composite element are more effective than one, and the increased activity is not due simply to the additional half-GRE. Finally, the GRU can activate a heterologous promoter. This GRU is the first description of an autonomous glucocorticoid-responsive gene regulatory

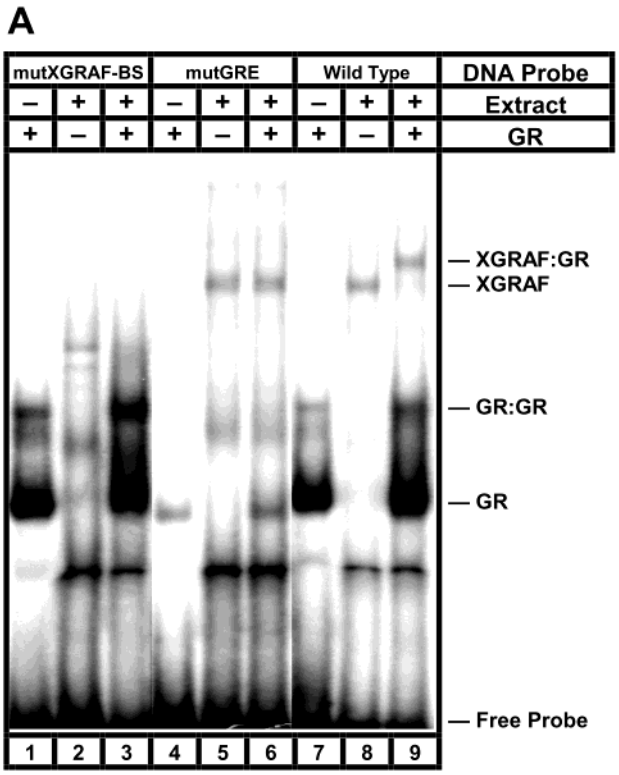

FIGURE 7: The presence of both the XGRAF-BS and GRE1 is required to form the XGRAF·GR·DNA complex. (A) The gel mobility shift assay was carried out as described in Experimental Procedures with a DNA probe containing either the wild-type γ-(−189 to −157) sequence (lanes 7−9), the γ(−189 to −157) sequence with a mutated XGRAF-BS (lanes 1−3), or the γ(−189 to −157) sequence with a mutated GRE1 (lanes 4−6). The proteins binding to the probes are denoted to the right of their respective bands. The relative positions of the GR monomer and dimer bound to the DNA were established previously (*27*). (B) The sense strand of each probe is shown, and bases mutated from the wild-type γ sequence are denoted by lower case letters. The XGRAF-BS is in bold, and the GRE half-site is underlined.

sequence that consists of an accessory element directly adjoining a single half-GRE. Functional half-GREs have been found in several genes and apparently act by a variety of mechanisms, often conferring a modest hormonal induction similar to that observed here (*47*−*50*). The system described in this paper presents a new mode of GR action that may be applicable to other hormone-inducible genes containing half-GREs in the 5′-flanking sequence. Elements in these regulatory regions may interact with proteins homologous to XGRAF or with as yet unidentified transcription factors.

Comparison of the γ Gene GRU to Nonclasssical Regulatory Elements in Other Glucocorticoid Regulated Genes. The identification of the role of XGRAF in glucocorticoid activation of the γ-fibrinogen gene clearly shows that this gene is not controlled by the classical mechanism of homodimeric binding of GR to a conventional full GRE. As described in our introductory remarks, several nonclassical mechanisms of glucocorticoid activation of transcription have been found for other genes. However, many features of the hormonal control of the γ gene are incompatible with these previously described mechanisms. For example, in the β-casein gene, GR is tethered to the DNA via a protein−protein interaction with the DNA-bound transcription factor Stat-5, with no requirement for a GRE (*21*). In contrast, we have shown that both XGRAF-BS and GRE1 play a role in the induction of the γ gene. Furthermore, formation of the XGRAF·GR·DNA complex in vitro is dependent on the DNA having binding sites for both proteins.

Another nonconventional mechanism is the formation of a protein−protein complex between GR and the transcription factor Oct-2 in solution (*24*). This interaction enhances the binding of Oct-2 to its specific DNA-binding site downstream from four GR binding sites in the MMTV regulatory region. Thus, the relationship between the Oct-2 binding site and the multiple GREs is entirely different from the contiguous XGRAF binding site and single half-GRE in the γ gene GRU. Although GR could enhance XGRAF binding to the DNA, as found for Oct-2, it is unlikely that other aspects of the mechanisms are the same since the arrangements of the protein binding sites are so dissimilar.

Finally, a third nonclassical mechanism has been described in which GR binding to the MMTV regulatory region causes rearrangement of nucleosomes and enhancement of binding of NF-1 (*25*, *26*). Although we have not studied XGRAF and GR binding and function with a chromatin template, the γ gene upstream region has significant differences from the MMTV system. First, unlike the XGRAF-BS, the NF-1 site is not located directly upstream from a half-GRE. Second, although GR promotes NF-1 interaction with a chromatin template, binding of these two proteins is mutually inhibitory on naked DNA (*51*). In contrast, XGRAF can bind simultaneously with a purified GR fragment to the γ gene DNA in vitro. This concurrent binding requires intact binding sites for both proteins, which corresponds with the requirement for both elements to achieve full glucocorticoid responsiveness in vivo.

Models for the Interaction between XGRAF and GR. The juxtaposition of the XGRAF-BS and half-GRE in the γ gene DNA is unlike the arrangement of accessory factor and GR binding sites reported for any other genes. We originally proposed four possible scenarios to explain the binding of XGRAF and GR to their contiguous binding sites (*27*). One possibility was that GR bound to the DNA as a dimer, as it normally would to a full GRE, and XGRAF simultaneously occupied its overlapping site. This arrangement would have required XGRAF to bind in the minor groove of the DNA since GR binds in the major groove. However, we showed that XGRAF also binds in the major groove (*28*), thus disfavoring this model. Another proposal was that the proteins do not occupy their sites at the same time but instead bind sequentially. Although this mechanism could operate in vivo, it is not necessary to invoke such an explanation since XGRAF and the GR fragment are capable of binding to the DNA concurrently in the gel shift assay. Furthermore, our recent results show an excellent correlation between in vitro data using truncated forms of GR and

physiological data with full-length GR (B. Morin, G. R. Woodcock, L. A. Nichols, and L. J. Holland, manuscript in preparation). The other two proposed models involve simultaneous binding of XGRAF and GR to the DNA. In one case XGRAF would interact with a monomer of GR to form a trimeric XGRAF·GR·DNA complex. In the other case, XGRAF would bind to its specific site, and GR would bind to the DNA as a dimer. However, one of the monomers would be tethered rather than contacting the DNA, due to displacement by XGRAF. Either of these latter two models represents a previously undescribed mechanism of transcriptional activation by glucocorticoids. Regulation of the γ-fibrinogen subunit gene has broad implications for other hormone inducible genes. In particular, genes with half-GREs may use accessory factors in a similar manner to achieve an appropriate physiological response to glucocorticoids.

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