Flanking Sequence Composition Differentially Affects the Binding and Functional Characteristics of Glucocorticoid Receptor Homo- and Heterodimers†

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ABSTRACT: The core binding sites for a multitude of transcription factors have been identified and characterized, but these sequences cannot fully account for the nuances of cell-specific and gene-specific control of gene transcription. Many factors may contribute to the precise responsiveness of a gene to a particular transcriptional regulatory protein, including the nucleotides in the proximity of the core binding site for that protein. Here, we examine two flanking sequences bordering a site in the γ-fibrinogen gene regulatory region that binds a heterodimer of the *Xenopus* glucocorticoid receptor accessory factor (XGRAF) and the glucocorticoid receptor (GR). Mutation of the upstream flank results in a decrease in the level of XGRAF binding but little change in hormone induction. However, alteration of the downstream flank adjacent to the GR binding site causes a decrease in levels of both GR monomer binding and hormone induction. Conversion of the XGRAF−GR binding site to a full glucocorticoid response element (GRE) alters the role of the flanking sequences. A full GRE in this position requires the wild-type upstream flank to bind GR homodimer and induce transcription to maximal levels. In contrast, mutation of the downstream flank is not detrimental to either the binding or the function of the GR dimer. Thus, flanking sequence composition and dimer partner combine to influence GR function, underscoring the complexities involved in the identification of authentic transcription factor response elements.

The dynamic interaction of transcription factors with DNA regulates gene expression, conferring temporal and cell-specific control in response to a wide range of intracellular and extracellular cues. However, these protein−DNA interactions are not always easily predicted, as the exact sequence of a binding site can alter its function in unexpected ways. For instance, particular sequences may influence the recruitment of cofactors by altering the conformation of the bound transcription factor via specific interactions with individual nucleotides (*1*−*3*). If a site binds transcription factors as a dimer, both the spacing and the orientation of the two half-sites affect how the site behaves (*4*, *5*). Also, not all functional binding sites are a good match to the consensus sequence compiled from known binding sites for a particular factor (*6*).

In addition, the interactions between transcription factors and DNA do not necessarily rely solely on the sequence of the actual binding site but may be influenced by other DNA elements. Sequences flanking the binding site can affect response element utilization by altering the protein conformation of a factor bound to the DNA (*7*). Nearby sequences may bind transcription factors of their own that change the functionality of an unrelated site (*8*−*10*). Distal DNA sequences can silence transcription from a known site in a cell-specific manner (*11*). A more complex example is a factor−DNA interaction that recruits a corepressor to affect a transcription factor bound at a distal site (*12*). The location of the binding site relative to basal regulatory elements is sometimes important, as demonstrated by the positional dependence of a hormone response element in relation to the TATA box of a gene promoter (*13*). A single transcription factor binding site is also capable of affecting multiple genes simultaneously, sometimes over a considerable distance (*14*).

Here, we examine interplay between two distinct determinants, flanking sequence and dimerization partner, that influence the binding and function of the glucocorticoid receptor (GR).[1] The *Xenopus* γ-fibrinogen gene upstream regulatory region contains a binding site for a heterodimer of *Xenopus* glucocorticoid receptor accessory factor (XGRAF) and GR (*8*), which is essential for maximal hormone induction (*15*−*17*). Individually, XGRAF and GR interact with DNA to form single distinct bands in a gel mobility shift assay. Both XGRAF binding and GR binding are highly specific for their respective sites, as confirmed by competition experiments using mutated DNA competitors (*8*, *15*, *16*). The combined XGRAF−GR heterodimer binding site is readily converted to a GR homodimer binding site by a single point mutation (*15*). Taking advantage of this property here, we used transfections and quantitative gel mobility shift assays

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[1] Abbreviations: β-gal, β-galactosidase; CMV, cytomegalovirus; GR, glucocorticoid receptor; GRE, glucocorticoid response element; LP, large probe; PCR, polymerase chain reaction; pLL, pLucLink2.0; SEM, standard error of the mean; SP, small probe; T7X556, fragment of rat GR consisting of amino acids 407−556; wt, wild type; XGRAF, *Xenopus* glucocorticoid receptor accessory factor.

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

Name	DNA Sequence [a]
Wild type [b,d]	$^{-187}$ CCAGACAGAAAA**GAGTTAA**TGTTCCCTCTTA $^{-157}$
MutU*D [c]	$^{-187}$ CtAcACtGtAAA**GAGTTAA**TGTTCCgcaaac $^{-157}$
MutU*XD [c]	$^{-187}$ CtAcACtGtAAAGAGcgcgTGTTCCgcaaac $^{-157}$
MutU*GD [c]	$^{-187}$ CtAcACtGtAAA**GAGTTAA**TacTaCgcaaac $^{-157}$
MutUD [c,e]	$^{-187}$ attccatatcAA**GAGTTAA**TGTTCCgcaaac $^{-157}$
MutU [c,e]	$^{-187}$ attccatatcAA**GAGTTAA**TGTTCCCTCTTA $^{-157}$
MutD [b,d]	$^{-187}$ CCAGACAGAAAA**GAGTTAA**TGTTCCgcaaac $^{-157}$
GRE [b,d]	$^{-187}$ CCAGACAGAAAAGAcTTAATGTTCCCTCTTA $^{-157}$
GREmutUD [c,e]	$^{-187}$ attccatatcAAGAcTTAATGTTCCgcaaac $^{-157}$
GREmutU [c,e]	$^{-187}$ attccatatcAAGAcTTAATGTTCCCTCTTA $^{-157}$
GREmutD [b,d]	$^{-187}$ CCAGACAGAAAAGAcTTAATGTTCCgcaaac $^{-157}$

[a] The sense strand is shown, and mutated bases are denoted with lowercase letters. The XGRAF binding site is in bold, and the GRE half-sites are underlined. [b] The *Kpn*I site at the 5′ junction with the vector includes γ-fibrinogen bases -187 and -186 as the final two bases of the enzyme recognition sequence. [c] Constructs have five additional bases (TCCAC) between the 5′ *Kpn*I junction and the sequence shown. [d] Probes for gel mobility shift assays had two additional bases; bases -189 and -188 were present as CT. [e] Probes for gel mobility shift assays had two additional bases; bases -189 and -188 were present as Cc.

to determine the effects of mutating individual flanking sequences on the abilities of XGRAF−GR and GR−GR species to stimulate transcription.

MATERIALS AND METHODS

Construction of Transfection Vectors. Plasmid constructs were assembled in the pLucLink2.0 (pLL) luciferase reporter vector (*18*). DNA inserts were prepared by PCR using *Pfu* polymerase (Stratagene), templates containing Bβ-fibrinogen sequence, and appropriate primers to introduce the desired γ-fibrinogen gene sequences. All plasmid constructs included *Xenopus* Bβ-fibrinogen sequence from position -141 to $+40$ relative to the transcription start site (*19*). The Bβ-sequence ended in a 3′ adapter sequence with a *Hin*dIII restriction site as described previously (*17*). The appropriate wild-type or mutated γ-fibrinogen sequence (see Table 1) was 5′ of, and directly adjacent to, the Bβ-sequence within each construct. A *Kpn*I site was placed 5′ to the γ-sequence for ligation into pLL. The PCR inserts were digested with *Kpn*I and *Hin*dIII and ligated into the multiple cloning site of pLL. The resulting constructs were sequenced (DNA Core Facility, University of Missouri), and DNA for transfections was prepared as previously described (*17*).

Transfection of Primary Hepatocytes. Primary hepatocytes from estrogen-treated adult female *Xenopus laevis* (Nasco) were isolated as previously described (*19*). For some experiments, albino frogs were used, which obviated the requirement of a Metrizamide gradient for removal of melanocytes from the hepatocyte fraction. There were no detectable differences in transfection results between the two types of frog.

Purified cells were transfected by electroporation with 50 μg of luciferase reporter DNA and 10 μg of pCMVβ-gal as a control (*15*). Hormone induction was examined using three or four independent transfections for each construct in a given experiment. Cells from each transfection were divided into two wells on a 24-well Primaria plate (Falcon) and incubated in the absence or presence of 10^{-7} M dexamethasone and 10^{-9} M triiodothyronine for 44−48 h. Cells were then harvested as described previously (*17*).

Luciferase and β-Gal Reporter Assays. Cell lysates were analyzed for luciferase reporter gene activity as described previously (*19*) except 700 μM coenzyme A was added to the assay buffer (*20*). β-Galactosidase (β-gal) activity was measured with a Galactostar kit (Applied Biosystems, Tropix). Briefly, 11 μL of cell lysate, 11 μL of lysis buffer, and 300 μL of diluted Galacton substrate were combined and incubated at room temperature for 30 min. Following incubation, light output was measured for 5 s in a luminometer (Monolight 2010).

Luciferase activity was normalized to β-gal activity for each sample. The fold hormone induction was expressed as normalized luciferase activity in dexamethasone-treated cells divided by normalized luciferase activity in untreated cells. The mean fold hormonal induction was calculated from the independent transfections of each construct in an experiment.

In the figures, the hormonal induction for each test construct was expressed as a percentage of the reference construct as follows: fold induction (% of reference) = [(fold induction of experimental construct − fold induction of Bβ-136)/(fold induction of reference construct − fold induction of Bβ-136)] × 100. The value for Bβ-136 represents a noninduced baseline from a construct previously shown to be unresponsive to glucocorticoids (*19*). The percentages from 6−10 experiments were averaged, and the standard error of the mean (SEM) was calculated.

Gel Mobility Shift Assays. For all gel mobility shift assays, gel-purified oligonucleotides (Integrated DNA Technologies) were annealed (*19*) to produce double-stranded DNA probes (see Table 1). Probes were labeled at the 5′ end with [γ-^{32}P]-ATP as described previously (*21*) except some reactions were stopped with 25 mM Na-EDTA (pH 8) and mixtures processed through MicroBiospin6 columns (Bio-Rad) per the manufacturer's instructions.

To examine binding of XGRAF to DNA, we utilized a dual-probe procedure described by La Baer and Yamamoto (*22*). A small probe (33-mer) (Table 1) was mixed with a large probe (98-mer) containing the XGRAF binding site. This large probe contained pLL vector sequence, γ-fibrinogen sequence from position -187 to -115, and an *Mfe*I restriction site on the 3′ end (*15*). The small and large probes were mixed in equimolar ratios (0.2 ng of small to 0.6 ng of large) and combined in a reaction mix described previously (*17*), except 2 μg of poly(dI·dC) was present in each reaction mixture. *Xenopus* liver nuclear extract was present at a level of 10−20 μg per reaction mixture as the source of XGRAF (*8*). Reactions were run on a 5% polyacrylamide gel at 350 V for 5.75 h (*17*). The bottom fourth of the gel was composed of a 15% polyacrylamide plug to retard migration of free probe, allowing better resolution of protein−DNA complexes while keeping the free probe on the gel.

Formation of the XGRAF−GR−DNA complex was performed as described previously (*17*), except each reaction mixture contained 10 μg of *Xenopus* liver nuclear extract as the source of XGRAF. Since endogenous GR is generally undetectable in crude nuclear extract via a gel shift assay, it is routine to use exogenously synthesized GR to examine GR binding (*23*−*25*). Therefore, the reaction mixtures

contained the DNA-binding domain fragment of rat GR, T7X556 (a kind gift from K. Yamamoto) (*23*) at 2, 4, 6, 8, or 10 ng per reaction mixture. Complexes were formed on 33-mer probes (Table 1) and resolved on a polyacrylamide gel as described above, except the plug was one-eighth the total volume and the gels were run at 350 V for 4.17 h.

The binding of GR monomer was done on a small probe (33-mer) (Table 1) and a large probe (50-mer) comprised of γ-fibrinogen sequence from position −189 to −140 with a G to A point mutation at position −155 to remove a GRE half-site (*16*). Probes were present in equimolar ratios (0.2 ng of small to 0.32 ng of large). Binding reaction mixtures were prepared as previously described (*8*). The GR, T7X556, was present at a range of 25−300 ng per reaction mixture. Since nuclear extract enhances binding of GR to DNA, these reactions required a greater amount of GR in comparison to the XGRAF−GR binding reactions described above. Gels were prepared and run as described for XGRAF binding.

Binding reactions for GR homodimer used the small probes (33-mer) described in Table 1. Binding conditions were the same as for GR monomer except that the range of GR was 5−40 ng per reaction mixture. Reactions were run on a 5% polyacrylamide gel at 250 V for 2.25 h.

Quantitative Analysis of Gel Mobility Shift Assays. The labeled DNA was detected on a Bio-Rad FX phosphorimager and quantitated with Quantity One software (Bio-Rad). The type of quantitation used was dependent upon specific reaction conditions for each type of experiment.

Quantitation of XGRAF and GR binding (Figures 4 and 6) was conducted as follows. Within a single lane, the amount of small or large probe bound to protein was divided by the amount of identical free probe. The ratio of these values for the small to large probes was then calculated (*22*). The final value for each small mutant probe was expressed as a percentage of binding to the small wild-type probe with an identical amount of protein present.

To compare formation of the XGRAF−GR complex on different DNAs (Figure 5), ratios of XGRAF−GR−DNA to total radioactivity were calculated for each lane. The ratios for each mutant probe were then expressed as a percentage of the ratio of complex formed on the wild-type probe under identical reaction conditions.

Binding of the GR dimer to mutant probes (Figure 8) was compared to binding to the GRE probe under identical conditions as follows: dimer bound to mutant probe (% of wild type) = [(dimer bound to mutant probe/free mutant probe)/(dimer bound to GRE probe/free GRE probe)] × 100.

RESULTS

In our previous work, we identified a glucocorticoid responsive region from position −187 to −157 relative to the transcription start site in the *Xenopus* γ-fibrinogen subunit gene (*16*, *17*). This region contains a GRE half-site from position −168 to −163 (see Figure 1), but this half-site is insufficient for conferring full hormone responsiveness to the promoter. An additional sequence within the −177 to −169 segment binds XGRAF and is required for full induction of gene expression. This sequence was examined by mutating each nucleotide individually to the other three possible nucleotides (*15*). The results of this experiment localized the core binding site for XGRAF from position

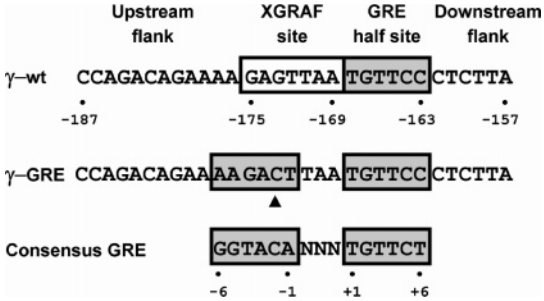

FIGURE 1: *Xenopus* γ-fibrinogen DNA from −187 to −157 relative to the transcription start site at position +1. The XGRAF binding site (white box), the GRE half-site (gray box), and both flanking sequences in the γ-wt sequence are displayed. The γ-GRE mutant and a consensus GRE (*32*) are shown in relation to the γ-wt sequence. The GRE mutation involves conversion of the base at position −173 from G to C (indicated by the arrowhead) which causes the sequence to function as a full GRE.

−175 to −169 (see Figure 1). XGRAF and GR bind the DNA cooperatively at their respective sites and function as a heterodimeric unit (*8*).

The segment of DNA from position −187 to −157, which contains the XGRAF site and the single adjacent half-GRE, remains functional when placed in the heterologous context of the *Xenopus* Bβ-fibrinogen gene promoter and upstream sequence, which has no homology with the γ-fibrinogen gene regulatory region (*17*). Thus, the DNA segment from position −187 to −157 does not require any other sequences within the γ-fibrinogen gene upstream region to function as a glucocorticoid response unit. The possibility remained, however, that sequences within the −187 to −157 segment, upstream or downstream of the core XGRAF (positions −175 to −169) and GR (positions −168 to −163) binding sites, play a role in the activity of this heterodimeric unit.

Since the XGRAF−GR heterodimer is a novel transcriptional regulatory complex, we sought a more complete understanding of the role of the flanking DNA sequences in glucocorticoid responsiveness. For these investigations, we used constructs in which the −187 to −157 fragment of the γ-fibrinogen gene DNA was placed upstream of the −141 to +40 region of the Bβ-fibrinogen gene regulatory region so that the single full GRE from position −162 to −148 that is solely responsible for the hormone responsiveness of the Bβ-gene is removed (*19*). The γ-fibrinogen segment from −187 to −157 was positioned so that the XGRAF−GR core binding sites, plus the two bases at positions −177 and −176, were in the exact location, relative to the transcription start site, normally occupied by the Bβ full GRE.

Effects of the Flanking Sequences on XGRAF−GR Function. To examine the role of the flanking sequences in XGRAF−GR function, the γ-flanks were mutated to match the sequences that flank the GRE of the Bβ-fibrinogen gene. The mutated upstream flank, mutU*, lies near the XGRAF binding site, and the mutated downstream flank, mutD, borders the GR binding site. Simultaneously changing both flanks in mutU*D (Table 1) resulted in an approximately 50% decrease in glucocorticoid responsiveness but did not completely eliminate hormone induction (Figure 2A). To verify that both XGRAF and GR played a role in the remaining hormone responsiveness of the mutU*D construct, the individual XGRAF and GR binding sites were mutated (Table 1). Mutation of the XGRAF binding site in mutU*XD

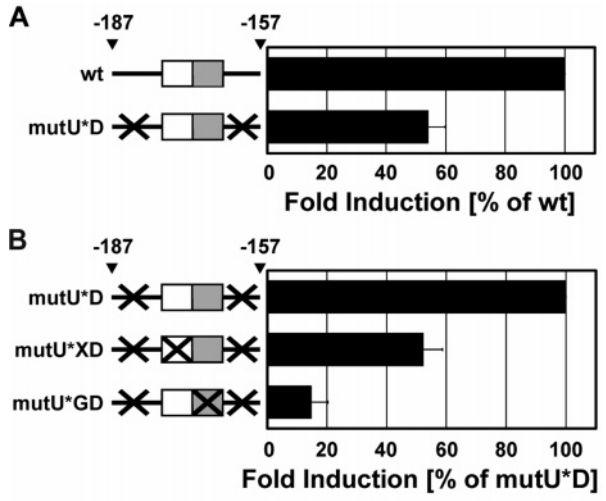

FIGURE 2: Reduced function of the XGRAF−GR core binding sequence following replacement of the flanking sequences. The diagrammatic schemes of the constructs containing the *Xenopus* γ-fibrinogen gene upstream DNA show the presence of the XGRAF binding site (white box), the GRE half-site (gray box), and the mutated sequence (×). See Table 1 for wild-type and mutant sequences. Constructs were transfected into *Xenopus* primary hepatocytes, and glucocorticoid responsiveness was determined as described in Materials and Methods. The fold hormonal induction of each construct is given as a percentage of the wt (A) or mutU*D (B) control. (A) The data are expressed as the average of nine separate experiments ± SEM. The fold induction of the wt construct was 2.3. (B) The data are expressed as the average of 10 separate experiments ± SEM. The fold induction of the mutU*D construct was 1.6. All experimental constructs were significantly different from their controls ($p < 0.05$) as determined by the Wilcoxon signed-rank test (*26*).

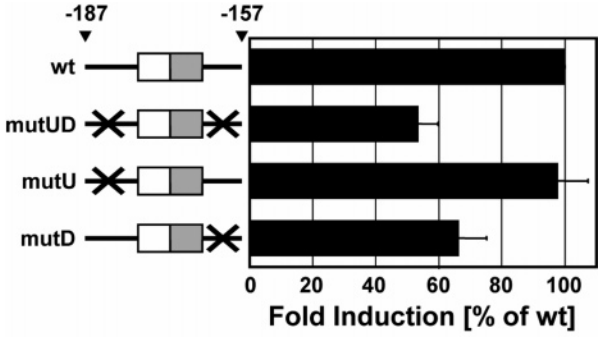

FIGURE 3: Importance of the downstream flanking sequence for XGRAF−GR function. The diagrammatic schemes of the constructs containing the *Xenopus* γ-fibrinogen gene upstream DNA show the presence of the XGRAF binding site (white box), the GRE half-site (gray box), and the mutated sequence (×). See Table 1 for wild-type and mutant sequences. Constructs were transfected into *Xenopus* primary hepatocytes, and glucocorticoid responsiveness was determined as described in Materials and Methods. The fold hormonal induction of each construct is given as a percentage of the wt control. The data are expressed as the average of eight separate experiments ± SEM. The fold induction of the wt construct was 1.8. The mutUD and mutD constructs were significantly different from the control ($p < 0.05$) as determined by the Wilcoxon signed-rank test (*26*).

resulted in a partial decrease in glucocorticoid responsiveness, while mutation of the GRE half-site in mutU*GD nearly eliminated hormone induction (Figure 2B). These results are essentially identical to previous observations made with the native γ-gene flanking sequences intact (*17*). Therefore, while the XGRAF−GR core binding site still retains some responsiveness without the native flanks, the wild-type sequences are necessary for the full function of the XGRAF−GR heterodimer.

Since some of the nucleotides in the upstream flanking Bβ-sequence are identical to the γ-sequence, the upstream flank was further mutated from the Bβ-sequence so that no bases matched the native γ-fibrinogen sequence (mutU, Table 1). These new mutations in mutUD yielded essentially the same reduced glucocorticoid responsiveness as observed in mutU*D (compare Figures 2A and 3). Therefore, we used mutU instead of mutU* in the remaining experiments.

To assess the individual roles of each flanking sequence, we mutated either the upstream or downstream sequence (Table 1). Mutation of the upstream flank in mutU resulted in no appreciable change in hormone induction (Figure 3). In contrast, mutation of the downstream sequence in mutD substantially diminished the glucocorticoid response to a level similar to that observed when both flanks were mutated in mutUD (Figure 3). The reduced hormone responsiveness of mutD and mutUD is comparable to that obtained in previous experiments when binding of XGRAF to its specific site on the DNA was abolished (*8*, *15*−*17*). Therefore, we sought to determine whether the decrease in glucocorticoid induction observed with the flanking sequence mutants was due to elimination of XGRAF binding.

Role of the Flanking Sequences in XGRAF Binding. To correlate function with protein binding, we performed quantitative gel mobility shift assays. Our previous work using gel shift assays has established that XGRAF behaves as a single distinct band and binds with high specificity to a core DNA sequence (*8*, *15*−*17*). We are able to study XGRAF binding without complications from binding of endogenous GR to its adjacent site, because GR is undetectable in our nuclear extract (*16*). To examine the effects of flanking sequence on XGRAF binding, nuclear extract was combined with either a wild-type probe or a mutant probe (SP) with one or both flanking sequences altered. All four probes bound XGRAF in sufficient amounts for quantitation (XGRAF:SP in Figure 4A). Instead of competition studies, we elected to use a large wild-type DNA probe (LP) with an XGRAF binding site as an internal control (XGRAF:LP in Figure 4A). The larger control probe allowed simultaneous detection of the experimental probe within the same sample. This approach has the advantage of increased accuracy, which allows small differences in DNA binding affinity to be detected (*22*). Following quantitation of all four bands, the ratio of XGRAF−SP complex to free SP was divided by the ratio of XGRAF−LP complex to free LP to normalize protein binding within each sample. In Figure 4B, the normalized XGRAF binding for each mutant probe is expressed as a percentage of the normalized XGRAF binding to the small wild-type probe under identical conditions. Mutation of both upstream and downstream flanks in mutUD resulted in a 50% decrease in XGRAF binding (Figure 4B). When only the downstream flank was altered in mutD, there was no decrease in the level of XGRAF binding compared to that of the wild type. In contrast, mutation of the upstream flank alone reduced the level of XGRAF binding to that seen on the double mutant (mutU, Figure 4B). However, it was mutation of the downstream flanking sequence that resulted in a weaker hormone induction (Figure 3). Since the upstream

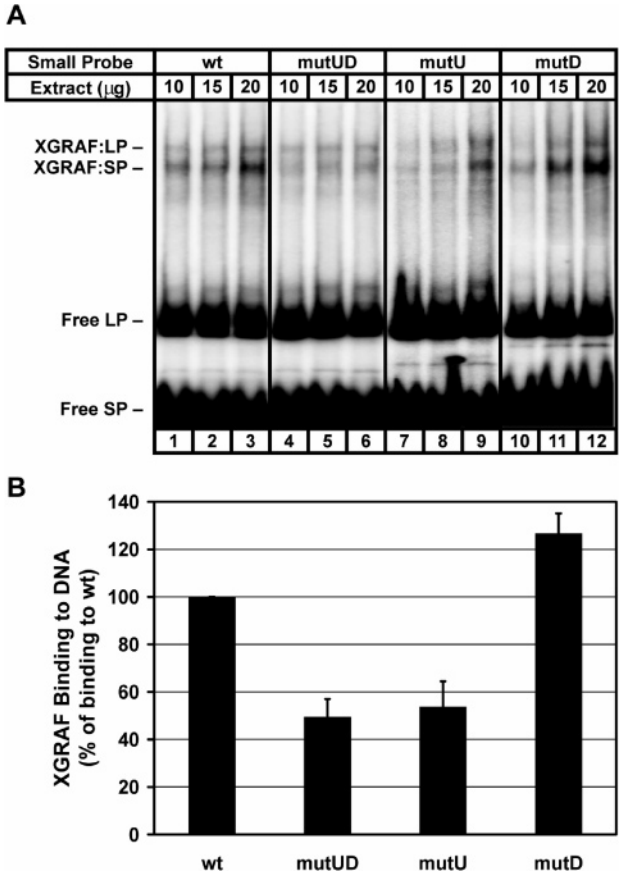

FIGURE 4: Decreased binding of XGRAF to γ-fibrinogen DNA following mutation of the upstream flanking sequence. (A) The gel mobility shift assay was carried out as described in Materials and Methods with a large control DNA probe (LP) present in each lane. The small experimental probes (SP) are described in Table 1. The bands are denoted to the left as XGRAF complexed with either the large or small probe (XGRAF:LP or XGRAF:SP) or as the free individual probes. (B) Data for each experimental probe were analyzed from nine independent samples run on three separate gels as described in Materials and Methods and are expressed as a percentage of XGRAF binding to the small wt control ± SEM. Binding of XGRAF to the mutUD and mutU probes was significantly different from the control ($p < 0.05$) as determined by the Wilcoxon signed-rank test (*26*).

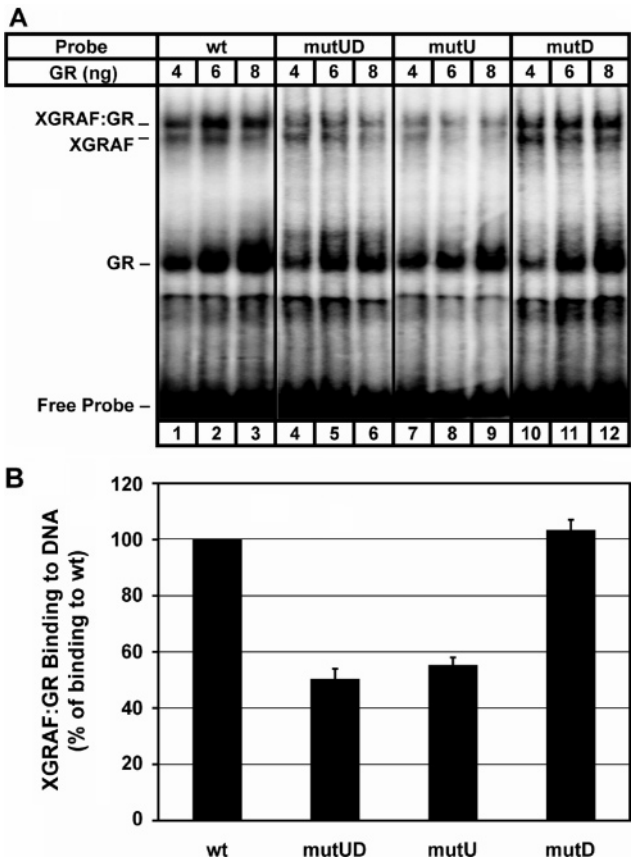

FIGURE 5: Reduced formation of the XGRAF−GR−DNA complex following mutation of the upstream flanking sequence. (A) The gel mobility shift assay was carried out as described in Materials and Methods with the experimental probes described in Table 1. The bands are denoted to the left as XGRAF:GR, XGRAF, or GR complexed with probe or as free probe. (B) Data for each experimental probe were analyzed from 10 independent samples run on three separate gels as described in Materials and Methods and are expressed as a percentage of binding of the XGRAF−GR complex to the wt control ± SEM. Binding of the XGRAF−GR complex to the mutUD and mutU probes was significantly different from the control ($p < 0.05$) as determined by the Wilcoxon signed-rank test (*26*).

flank did not alter function, it is unlikely that decreased XGRAF binding is responsible for the reduced glucocorticoid responsiveness.

Effect of Flanking Sequence Mutations on Formation of XGRAF−GR Heterodimers. An alternative mechanism that can inhibit hormonal activation of transcription is the elimination of the interaction between XGRAF and GR by the insertion of a single nucleotide between their respective binding sites (*8*). Consequently, we examined whether the reduced function brought about by the flanking sequence mutations was due to loss of XGRAF−GR heterodimer formation. A fixed amount of nuclear extract (XGRAF) was combined with varying amounts of GR and either wild-type or mutant probes (Table 1 and Figure 5A). The ratio of XGRAF−GR−DNA to total DNA for each mutant was then compared to the same ratio for the wild-type probe under identical conditions and expressed as a percentage of the wild-type control. Mutation of both flanks in mutUD or the upstream flank alone in mutU resulted in an approximately 50% reduction in complex formation on the DNA (Figure 5B). However, mutation of only the downstream flank in mutD had no effect on the ability of XGRAF and GR to form the heterodimer (Figure 5B). Though formation of the XGRAF−GR−DNA complex was not eliminated by any of the flanking sequence mutations, the smaller amount of complex on the mutU and mutUD mutants parallels the reduced amount of XGRAF−DNA complex available for formation of the XGRAF−GR−DNA complex. However, these reductions in the level of binding do not correlate with the effects of the mutD and mutUD flanking sequence mutations on the functional activity of this regulatory region.

Dependency of GR Monomer Binding on Flanking Sequence Composition. Since neither XGRAF binding nor XGRAF−GR−DNA complex formation accounts for the decreased function associated with mutated flanking sequences, we examined the effects of flanking sequence composition on binding of GR to its half-site. Employing the method used to assess XGRAF binding, we examined binding of the GR monomer to wild-type and mutant probes (Figure 6A). Mutation of both flanking sequences in mutUD resulted in a decrease in the level of GR monomer binding to the single GRE half-site (Figure 6B). Mutation of the upstream sequence alone failed to reduce monomer GR

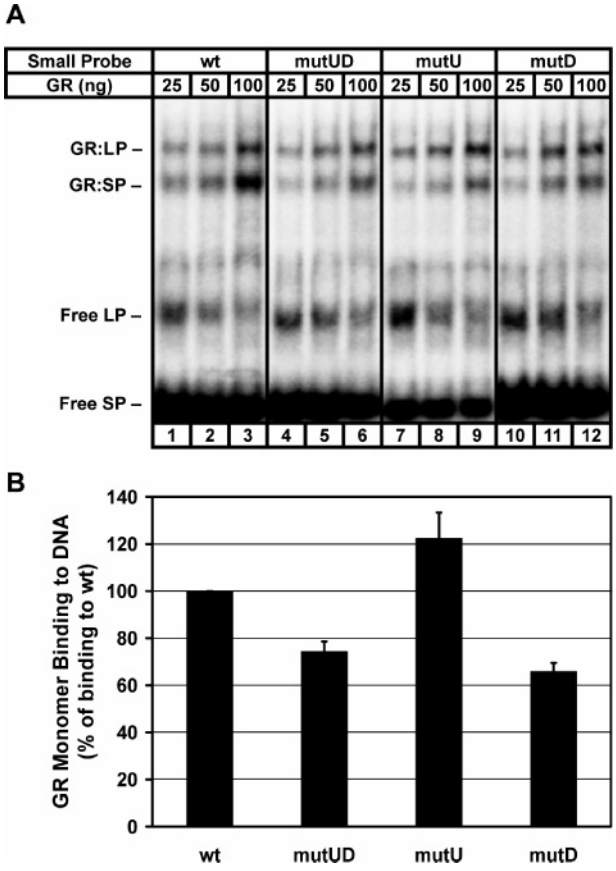

FIGURE 6: Decreased binding of GR monomer to γ-fibrinogen DNA following mutation of the downstream flanking sequence. (A) The gel mobility shift assay was carried out as described in Materials and Methods with a large control DNA probe (LP) present in each lane. The small experimental probes (SP) are described in Table 1. The bands are denoted to the left as GR complexed with either the large or small probe (GR:LP or GR:SP) or as the free individual probes. (B) Data for each experimental probe were analyzed from 15 independent samples run on three separate gels as described in Materials and Methods and are expressed as a percentage of binding of the GR monomer to the small wt control ± SEM. Binding of the GR monomer to the three experimental probes was significantly different from the control ($p < 0.05$) as determined by the Wilcoxon signed-rank test (*26*).

binding (mutU, Figure 6B). In contrast, mutating the downstream sequence resulted in a nearly 40% decrease in the amount of GR bound to the DNA (mutD, Figure 6B). These results link the decreased hormone responsiveness to a decrease in the level of GR binding that can be attributed to the composition of the downstream flanking sequence.

An Essential Flanking Sequence for Optimal GR−GR Binding and Function. It is possible that the downstream sequence is only important for GR binding and function in the context of the XGRAF−GR heterodimer because the binding site relies on a single half-GRE. To ascertain if the downstream flank is important for GR binding in the context of a full GRE, we mutated the base at position −173 to a C (Figure 1, and see the GRE construct in Table 1). We had shown previously that this minimal change resulted in a loss of XGRAF binding accompanied by strong hormone induction suggestive of dimeric GR activity (*15*). In the present context, with the wild-type flanking sequences, this GRE construct responded strongly to glucocorticoid. Mutation of both flanking sequences in GREmutUD (Table 1) resulted in a 60% decrease in hormone induction compared to the

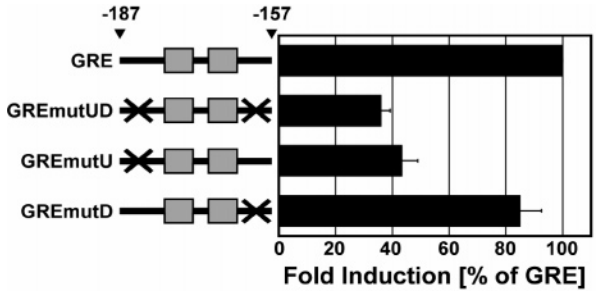

FIGURE 7: Importance of the upstream flanking sequence for GR homodimer function. The diagrammatic schemes of the constructs containing the *Xenopus* γ-fibrinogen gene upstream DNA modified to contain a full GRE show the presence of the GRE half-sites (gray boxes) and the mutated sequence (×). See Table 1 for wild-type and mutant sequences. Constructs were transfected into *Xenopus* primary hepatocytes, and glucocorticoid responsiveness was determined as described in Materials and Methods. The fold hormonal induction of each construct is given as a percentage of the GRE control. The data are expressed as the average of six separate experiments ± SEM. The fold induction of the GRE construct was 14.3. The GREmutUD and GREmutU constructs were significantly different from the control ($p < 0.05$) as determined by the Wilcoxon signed-rank test (*26*).

control (Figure 7). Alteration of the upstream sequence in GREmutU was almost as deleterious as mutation of both flanking sequences in GREmutUD (Figure 7). However, with only the downstream flank mutated, GREmutD retained 85% of its activity in comparison to the GRE construct (Figure 7). These results were interesting because the opposite flank had impacted GR function and binding in the XGRAF−GR heterodimer.

To confirm our prediction that GR binds as a homodimer to the GRE construct and to assess the effect of flanking sequence on GR homodimer binding, we performed quantitative gel mobility shift assays using the GRE probe (Table 1). In Figure 8A, GR bound primarily as a dimer to the GRE and GRE mutant probes. To compare the GR dimer binding on mutant probes to the GR dimer binding on the GRE probe, the ratio of GR dimer to free probe was quantified (Figure 8B). The ratio for each mutant probe was then normalized to the ratio from the GRE probe under identical conditions. Mutation of both flanking sequences resulted in a 50% reduction in GR dimer formation in comparison to the control (GREmutUD, Figure 8B). Binding of GR homodimer to the upstream mutant, GREmutU, was similarly reduced, while no decrease in the level of binding was observed on the downstream mutant, GREmutD (Figure 8B). These binding data correlate with the functional data for the GR homodimer which indicated that the upstream flank is important for hormone induction in this context.

DISCUSSION

The upstream regulatory region of the *Xenopus* γ-fibrinogen gene contains a binding site for an XGRAF−GR heterodimer that is essential for the full glucocorticoid induction of gene expression (*8*, *15*−*17*). A single base pair mutation in this site converts it to a functional GRE able to bind a GR−GR homodimer (*15*). This property enabled us to examine the effects of sequences directly adjacent to the dimer binding site on the binding and function of both XGRAF−GR and GR−GR dimers. Our results indicate that

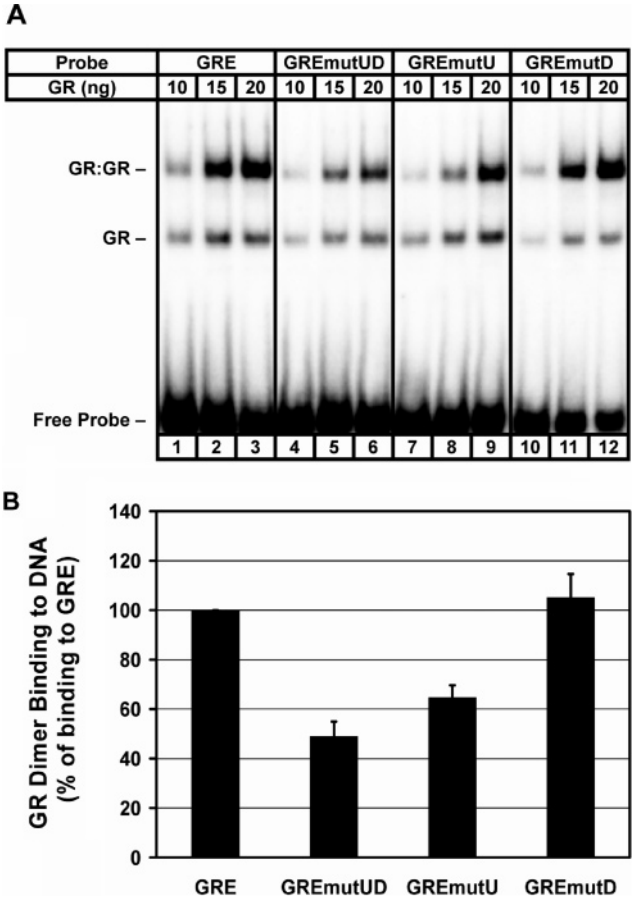

FIGURE 8: Reduced binding of GR homodimer to γ-GRE DNA following mutation of the upstream flanking sequence. (A) The gel mobility shift assay was carried out as described in Materials and Methods with the small experimental probes described in Table 1. The bands are denoted to the left as GR:GR (GR homodimer), GR (GR monomer), and free probe. (B) Data for each experimental probe were analyzed from 15 independent samples run on three separate gels as described in Materials and Methods and are expressed as a percentage of binding of the GR dimer to the GRE control ± SEM. Binding of the GR dimer to the GREmutUD and GREmutU probes was significantly different from the control ($p < 0.05$) as determined by the Wilcoxon signed-rank test (*26*).

flanking sequence composition affects the binding and function of the two dimers in distinctly different ways.

In the native sequence, mutation of the upstream flank adjacent to the XGRAF binding site reduces XGRAF binding and XGRAF−GR heterodimer formation with little effect on transcription. However, we have previously shown that elimination of heterodimer binding was deleterious to transcriptional activation by glucocorticoids (*8*). Apparently, sufficient heterodimer is able to form *in vivo* to maintain normal function, even with the reduced level of XGRAF binding caused by mutation of the upstream flanking sequence. A similar compensatory mechanism does not exist to correct the effects of the downstream mutation on GR binding and function. Reduced GR occupancy resulting from a lower affinity for its binding site can account for the diminished function, even when complex formation is relatively unchanged (Figure 5).

In contrast to the case of the XGRAF−GR heterodimer, GR−GR homodimer formation and function are impacted only by mutations to the upstream flanking sequence. Since the GRE half-site adjacent to the upstream flank is a poor match to the consensus sequence (see Figure 1), the upstream flanking sequence could enhance binding of the homodimer to compensate for the large number of mismatches in this GRE. Curiously, the native downstream flank that facilitates binding of the GR monomer to the wild-type γ-fibrinogen sequence does not play a role in enhancing the binding of GR in the homodimer, even though the downstream half-GRE is thought to be occupied first in homodimer formation (*27*). The different requirements for flanking sequence composition may reflect different conformations adopted by GR in hetero- and homodimers under equilibrium binding conditions.

These differences between the heterodimer and the homodimer demonstrate an important connection between binding partner and flanking sequence specificity. Such complexity may be especially important for the steroid receptor family of which GR is a part, since androgen, progesterone, and mineralocorticoid receptors recognize similar or identical core sequences yet regulate very different patterns of gene expression.

This investigation shows that flanking sequences and dimerization partners influence the binding and function of transcription factors, adding to the difficulty of identifying authentic regulatory elements. Despite such difficulties, the advent of genomics and bioinformatics has made it desirable to develop methods that predict specific elements in vast stretches of DNA. Using established consensus sequences as templates, a number of computer programs for searching for potential transcription factor binding sites have been devised (*28*−*31*). However, to minimize false identification of irrelevant sites and to maximize the detection of actual regulatory elements, the design of these programs must overcome a number of challenges, because of the complexity and unpredictability of the interactions between transcription factors and DNA (*28*−*31*).

In the example given here, mutation of a single base creates a functional full GRE (Figure 1), although this sequence is only a moderate match to the consensus GRE (seven of 12 bases, with three of four critical bases) (*27*, *32*, *33*). This mutant functions well under the influence of a particular flanking sequence, but the binding site might remain undetected in a computer-assisted search due to the marginal overall match to the consensus sequence, even with a search weighted toward known critical bases. It is interesting to note that, previously, we mutated this site to form a 10 out of 12 match to the consensus GRE with three of the four critical bases (GGTAAAnnnTGTTCC) (*16*). However, the resulting sequence was nonfunctional in this particular context, though it would likely be identified as a good candidate in a computer search.

The difference in the requirements for flanking sequences between the XGRAF−GR heterodimer and the GR−GR homodimer at nearly identical sites demonstrates the importance of context in regard to both the binding and function of GR. Transcription factor binding sites that cannot be readily identified by their traditional binding sequence may be functional due to additional complex features such as the presence of a specific flanking sequence. This complexity emphasizes the need to use experimental approaches in close conjunction with computer-based methods to maximize the identification and confirmation of authentic transcription factor binding sites.

NOTE ADDED AFTER ASAP PUBLICATION

The original posting of May 5, 2006 contained incorrect versions of Figures 5 and 8 as well as some minor typographical errors. The correct version is shown in the posting of May 16, 2006.

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