Analysis of the DNA-binding Site for Xenopus Glucocorticoid Receptor Accessory Factor

CRITICAL NUCLEOTIDES FOR BINDING SPECIFICITY IN VITRO AND FOR AMPLIFICATION OF STEROID-INDUCED FIBRINOGEN GENE TRANSCRIPTION*

(Received for publication, October 31, 1997)

Min Li, Xiongwen Ye, Robert N. Woodward‡, Cindy Zhu, LaNita A. Nichols, and Lene J. Holland§

From the Department of Physiology, University of Missouri School of Medicine, Columbia, Missouri 65212

Steroid hormones, which include glucocorticoids and mineralocorticoids from the adrenal cortex and estrogens, progestins, and androgens from the gonads, regulate a vast array of physiological processes that are essential for development, differentiation, growth, metabolism, homeostasis, behavior, and reproduction in vertebrate organisms. In the classical model of steroid hormone action (1), the steroid ligands bind to specific intracellular protein receptors in target cells. The hormone-receptor complexes interact with particular short nucleotide sequences in the chromosomal DNA and modulate transcription of nearby genes. This model, however, cannot account fully for the complex tissue-specific and gene-specific actions of hormones. Transcriptional induction by steroids is influenced by many factors, such as local chromatin structure (2), stages of the cell cycle (3), cellular morphology or differentiation state (4, 5), specific hormone ligand (6), and physiological state (7). Differential hormone responsiveness depends in part on the availability of other transcriptional regulatory proteins including coactivators and corepressors, which do not themselves bind to DNA (8), and accessory factors, which are DNA-binding proteins (9). For glucocorticoid-regulated genes, several accessory factors have been identified (2, 10–17), but the mechanisms by which these proteins potentiate hormonal activation of transcription are not known.

To understand the role of accessory DNA-binding proteins in determining responsiveness to a steroid hormone signal, we are investigating glucocorticoid induction of fibrinogen gene expression in the liver. Fibrinogen is the precursor of fibrin, the major structural protein of a blood clot, and its synthesis is regulated by adrenal steroids both in basal homeostasis and following physiological stresses such as infections, inflammation, surgery, burns, etc. (18, 19). Using primary liver cells from the frog Xenopus laevis as a model system (20), we have demonstrated that glucocorticoids stimulate transcription of the three separate genes coding for the fibrinogen subunits, termed α, β, and γ (21).

Identification of the specific DNA sequences that mediate steroid regulation of the Xenopus fibrinogen genes was accomplished by linking the 5′-flanking DNA of these genes to the firefly luciferase reporter gene and transfecting the DNA into primary Xenopus hepatocytes. Hormonal activation of the β gene occurs through a single element (22) with a close match to the consensus glucocorticoid response element (GRE), GGTACAnnTGTCT (23). Full stimulation of the γ gene, on the other hand, requires three closely spaced weak binding sites for the glucocorticoid receptor (GR) between nucleotides −168 and −135 relative to the start site of transcription (24). These sites have homology only to the downstream portion of the consensus GRE and therefore are referred to as half-GREs. In addition, steroid responsiveness is affected by bases within the sequence AAGAGTAA at positions −177 to −169, immediately adjacent to the 5′-most half-GRE (24). This tract is unrelated to the conventional GRE and does not match other known transcription factor-binding sites. A protein present in Xenopus liver nuclei binds to this DNA sequence in vitro. Experiments with DNA containing blocks of mutations within and around bases −177 to −169 showed that the DNA-protein interaction correlates with hormonal activation of transcription. Thus, we
named the nuclear protein Xenopus glucocorticoid receptor accessory factor (XGRAF). Compared with known GR accessory factors, the location of the XGRAF-binding site is unusual since it replaces the sequence that would normally constitute the upstream half of a GRE.

In this work, we describe the detailed mutational analysis of the 9-bp region to which XGRAF binds. This investigation located the 5′- and 3′-boundaries of the recognition sequence and revealed which nucleotides are most critical for DNA binding in vitro and for glucocorticoid-stimulated transcription of transfected DNA in Xenopus primary hepatocytes.

**Experimental Procedures**

Introduction of Point Mutations into Reporter Gene Constructs—All possible single-point mutations in the potential XGRAF-binding site in the γ gene upstream region were obtained by the following general strategy. 1) The γ gene sequence from positions −187 to +41 was synthesized by the polymerase chain reaction (PCR) using a downstream primer with wild-type sequence and an upstream primer consisting of a mixed population of oligonucleotides with mutations in the XGRAF-binding region from positions −177 to −169. 2) The PCR products were inserted into a luciferase reporter vector, and the DNA was cloned by transformation into bacteria. 3) The nucleotide sequence of the γ DNA in individual clones was determined to ascertain which nucleotide(s) of the XGRAF region had been mutated.

The upstream primer, 5′-GGGTACCCAGACAGAAAGAGTTAAAGTTCCCTCTTATGTTC-3′, was synthesized (Genemed Biotechnologies, Inc.) in a single reaction, with the reservoir for each nucleotide in the potential binding site (underlined) deliberately contaminated to a concentration of 2.5% with each of the three non-designated bases. PCR products from this primer yielded 15 of the 27 possible single-point mutants. A second population of mixed primers was synthesized (Genosys Biotechnologies), with contamination at the desired positions to optimal levels based on the formula described by Derbyshire et al. (25), yielding several additional mutants. The few remaining mutants were synthesized in PCRs with specific individual primers.

The PCR amplification was carried out with the PLLγ−187 construct (24) as the template DNA (which contains γ gene DNA from positions −187 to +41), the upstream primers described above, a downstream primer within the vector, and Pfu polymerase (Stratagene) following the protocol from the manufacturer. The PCR products were digested upstream with KpnI and adjacent to position +41 downstream with HindIII, purified through 2% low-melting-temperature agarose gels (26), and cloned into KpnI- and HindIII-digested pLuc-Link 2.0 (27). Transformation into Escherichia coli DH5a was as described (24). The nucleotide sequences of the γ DNA and of the junctions with vector DNA were confirmed for each mutant. Plasmid DNA was purified over anion-exchange resin and, for transfection, over a cesium chloride gradient (24).

Gel Shift Assays—Both the probe and the competitors for the gel shift assays contained the γ sequence extending from positions −187 to −115 and were generated by PCR using Pfu polymerase. In addition, the molecules included 19 bases of vector sequence upstream of position −187 and a MfeI restriction enzyme site downstream of position −115. Wild-type PLLγ−187 was used as the template for making the probe, and the point mutation constructs described above were the templates for the competitors. The PCR products were purified through 2% low-melting-temperature agarose gels (26), and the probe was 5′-end-labeled (28).

Nuclear extracts were made from X. laevis primary hepatocytes after 4 days in culture exactly as described (24), except that the concentration of HEPES-KOH in buffer C− was 20 mM. The gel shift assays were carried out as described (24) in a final volume of 15 μl with 0.5 ng of radioactive probe (7000–51,000 cpm) and either no specific competitor or a 0.5–500-fold molar excess of specific competitor. Native 5% polyacrylamide gels (24) were run at 350 V for 6–7 h at 4 °C, dried at 80 °C for 2 h, exposed to XAR film (Eastman Kodak Co.) at −80 °C with one Lightning Plus intensifying screen (DuPont), and exposed to a PhosphorImager screen (Molecular Dynamics, Inc.). The data from the phosphorimaging scan were analyzed with ImageQuant 3.3 software (Molecular Dynamics, Inc.).

Data Analysis for Gel Shift Assays—The ability of XGRAF to bind γ DNA with mutations in the putative XGRAF-binding region was determined by competition gel shift assays, which contained a constant amount of radioactively labeled wild-type γ DNA and either no competitor or various concentrations of DNA with a single-point mutation. An example of the gel shift assay with mutant A−171 as the competitor is shown in Fig. 1A. Radioactivity in the shifted DNA/XGRAF complex in each lane was quantitated from the phosphorimaging scan. The total amount of XGRAF in the complex with radioactive DNA in the absence of any competitor (Fig. 1A, lane 2) was defined as 1.0, and the amount of XGRAF in the complex with radioactive DNA in the presence of a 100-fold molar excess of wild-type competitor was defined as zero (Fig. 1A).
The quantity of XGRAF remaining in the complex with radioactive DNA in the presence of mutated competitor (Fig. 1A, lanes 3–17) was expressed as a fraction of 1.0. The difference between the total and the fraction still bound to the radioactive probe equaled the fraction of XGRAF bound to the competitor DNA.

The equilibrium binding equation describes the interaction between XGRAF and DNA (Equation 1),

$$K_d = [DNA/XGRAF]/[DNA]_{total}$$

(Eq. 1)

where [XGRAF] indicates free XGRAF protein concentration, [DNA] represents free competitor DNA concentration, and [DNA/XGRAF] represents concentration of XGRAF complexed with competitor DNA. Since competitor DNA is in excess over XGRAF, the concentration of total competitor DNA, [DNA]$_{total}$, can be substituted for the concentration of free competitor DNA. Also, [XGRAF] can be expressed as [XGRAF], minus [DNA/XGRAF], and the binding equation can be rearranged to the Scatchard equation (Ref. 29) (Equation 2),

$$f/[DNA]_t = (1/K_d) \times f + 1/K_d$$

(Eq. 2)

where $f$ is the ratio of bound XGRAF to total XGRAF, [DNA/XGRAF]/[DNA], is expressed in units of fold excess of unlabeled competitor DNA over total competitor DNA, $C_{op}$ is defined in this way in three independent experiments. Values for [DNA/XGRAF] below 0.1 or above 0.9 were not included in the Scatchard plots. Essentially the same results were obtained when $C_{op}$ was calculated as the negative reciprocal of the slope.

**Methylation Interference Footprinting—**DNA probes containing $\gamma$ gene upstream sequence from positions $\sim$232 to $\sim$6, $\gamma$-P-labeled on the sense strand, and from positions $\sim$232 to $\sim$115, $\gamma$-P-labeled on the antisense strand, were produced by PCR with 0.1 mm primers and Taq enzyme (24). The PCR products were purified through a native 6% polyacrylamide gel (26) followed by organic extraction. The end-labeled DNA fragments ($\sim$1 $\times$ $10^5$ cpmpmol) were partially methylated at the guanine moieties by incubation for 2–3 min with dimethyl sulfate without carrier (30); in some cases underwent organic extraction; were precipitated twice with ethanol; and were dissolved in 10 mm Tris-HCl and 0.1 mm EDTA (pH 8.0). For preparative binding, the reaction volumes described above for the gel shift assay were scaled up 15–30-fold, with $\sim$300,000 cpmp DNA probe, and electrophoresis was carried out at 250 V for 2.5–3.5 h at 4 °C. Wet gels were exposed to XAR film at 250 V for 2.5–3.5 h at 4 °C. Wet gels were exposed to XAR film at 250 V for 2.5–3.5 h at 4 °C. Wet gels were exposed to XAR film at 250 V for 2.5–3.5 h at 4 °C.

**RESULTS**

Identification of specific guanosines involved in XGRAF binding—Previously, we mapped the DNA elements between 177 and 135 bp upstream of the transcription start site that are important for glucocorticoid regulation of the Xenopus $\gamma$-fibrinogen subunit gene (24). Three sites designated half-GRE1 (positions $\sim$168 to $\sim$169), half-GRE2 (positions $\sim$156 to $\sim$155), and half-GRE3 (positions $\sim$140 to $\sim$135) in Fig. 2C have a close match to the downstream half of the consensus GRE, bind to the DNA-binding domain of GR in vitro, and contribute to steroid-induced transcription in primary hepatocytes. Half-

![Fig. 2. Identification of guanosines in the $\gamma$-fibrinogen subunit gene upstream regulatory region that contact XGRAF. A, sense strand methylation interference. The assay used $\gamma$ DNA from positions $\sim$232 to $\sim$6 synthesized by PCR with an end-labeled sense strand primer. Lane G $\rightarrow$ A, DNA sequencing ladder; lane B, protein-bound DNA; lane F, free DNA. Numbers indicate genomic sequence positions. Arrowheads mark the bands with reduced intensity in the protein-bound DNA fraction compared with free DNA (lane B versus lane F). B, antisense strand methylation interference. The conditions were the same as described in A, except that the probe was $\gamma$ DNA from positions $\sim$232 to $\sim$115, $\gamma$-P-labeled on the antisense strand. C, the nucleotide sequence of the $\gamma$ upstream region from positions $\sim$187 to $\sim$130. Arrowheads at positions $\sim$184, $\sim$175, and $\sim$173 indicate those guanosines at which methylation inhibited binding of XGRAF. The squares above the sequence indicate 10-bp intervals. All the elements that contribute to glucocorticoid responsiveness of the $\gamma$ gene are indicated below the sequence: the XGRAF-binding region between positions $\sim$177 and $\sim$169 and the three half-GREs (positions $\sim$168 to $\sim$163, $\sim$156 to $\sim$151, and $\sim$140 to $\sim$135).

Identification of guanosines in the $\gamma$-fibrinogen subunit gene upstream regulatory region that contact XGRAF. A, sense strand methylation interference. The assay used $\gamma$ DNA from positions $\sim$232 to $\sim$6 synthesized by PCR with an end-labeled sense strand primer. Lane G $\rightarrow$ A, DNA sequencing ladder; lane B, protein-bound DNA; lane F, free DNA. Numbers indicate genomic sequence positions. Arrowheads mark the bands with reduced intensity in the protein-bound DNA fraction compared with free DNA (lane B versus lane F). B, antisense strand methylation interference. The conditions were the same as described in A, except that the probe was $\gamma$ DNA from positions $\sim$232 to $\sim$115, $\gamma$-P-labeled on the antisense strand. C, the nucleotide sequence of the $\gamma$ upstream region from positions $\sim$187 to $\sim$130. Arrowheads at positions $\sim$184, $\sim$175, and $\sim$173 indicate those guanosines at which methylation inhibited binding of XGRAF. The squares above the sequence indicate 10-bp intervals. All the elements that contribute to glucocorticoid responsiveness of the $\gamma$ gene are indicated below the sequence: the XGRAF-binding region between positions $\sim$177 and $\sim$169 and the three half-GREs (positions $\sim$168 to $\sim$163, $\sim$156 to $\sim$151, and $\sim$140 to $\sim$135).
Critical Nucleotides for XGRAF Binding and Function

GRE1 is the most critical of the three GREs for hormonal induction. In addition, the tract from positions −177 to −169 is necessary for full hormone responsiveness even though it does not match the consensus GRE. The nuclear protein XGRAF binds to this region of the DNA (Fig. 2C).

To identify bases that have direct contacts with XGRAF, we used the methylation interference assay, which disrupts protein binding by methylation of critical guanines within a recognition site. A radioactively labeled DNA fragment including nucleotides −177 to −169 of the γ gene upstream region was partially methylated in vitro. The DNA was incubated with *Xenopus* liver nuclear extract, and both protein-bound DNA and free DNA were isolated by preparative native gel electrophoresis. The DNA was cleaved at all methylated guanines, and the two populations of DNA were compared for relative abundance of fragments ending at particular positions (Fig. 2). When the sense strand was radioactively labeled (sequence shown in Fig. 2C), the two DNA fragments ending at positions −175 and −173 were significantly reduced in intensity in the protein-bound DNA fraction (Fig. 2A, lane B) as compared with the free DNA fraction (Fig. 2A, lane F), indicating that methylation of these guanines interfered with binding of a protein in the nuclear extract. Thus, bases G−175 and G−173 are important contact points for XGRAF. The intensity of the fragment ending at G−164 was also reduced. This nucleotide is not considered to be part of the core XGRAF-binding site since the intervening bases from positions −178 to −181 are not required for XGRAF binding or function (24). When the antisense strand was radioactively labeled, no differences in intensities of bands were observed (Fig. 2B, compare lanes F and B). Although no guanines are present on the antisense strand within the putative XGRAF-binding site between positions −177 and −169, this experiment confirmed that the binding site does not extend upstream to position −182 or downstream to position −164, where the nearest guanines are located.

**Effect of Point Mutations on XGRAF Binding Ability**—The relative contribution of each nucleotide to the specific interaction between XGRAF and the γ DNA was assessed by saturation mutagenesis of the site from positions −177 to −169, generating all 27 single-point mutants in the 9-bp sequence. The effect of the mutations on DNA binding in vitro was analyzed by the gel shift assay, using the mutated DNA sequences as competitors for binding of XGRAF to radioactively labeled wild-type DNA, as described under “Experimental Procedures.” Binding ability is expressed as C₅₀, the -fold excess of competitor required to displace half of XGRAF from the wild-type probe. For each mutant, the C₅₀ value was calculated in three independent experiments, and the results are presented as the mean ± S.E. of the three determinations (Fig. 3 and Table I). The wild-type DNA had a C₅₀ value of 1.7-fold excess.

At positions −177, −176, and −174, all of the mutants bound strongly to XGRAF, with low C₅₀ values of 1.7−3.8-fold excess. Therefore, these positions are not critical determinants for binding specificity since any nucleotide can be substituted without significantly affecting the interaction with XGRAF. Similarly, changing G−175 to either C or T was not deleterious to binding. However, when this position was mutated to A, the binding ability was reduced, with a C₅₀ of 36-fold excess. It is interesting that the introduction of A at position −175 creates a stretch of six adenosines, which may interfere with binding due to structural alterations rather than the specific nucleotide substitution (33).

In contrast, nearly all changes in the bases from positions −173 to −169 substantially impaired XGRAF binding ability (Fig. 3 and Table I), with C₅₀ values from 20-fold excess for mutant C−171 to 323-fold excess for mutant C−173. The only exception was mutant C−169, which retained relatively strong XGRAF binding (C₅₀ = 6.3-fold excess).

**Effect of Point Mutations on Glucocorticoid Responsiveness**—We also examined the effects of the point mutations in the XGRAF-binding site on glucocorticoid induction of transcription. The mutated γ gene upstream region was inserted into a luciferase reporter vector, and the constructs were transfected into primary *Xenopus* hepatocytes. Transfected cells were divided for plus or minus glucocorticoid treatment for 2 days, and lysates were analyzed for luciferase activity. The total -fold increase in luciferase levels in hormone-treated cells reflects the role of not only the XGRAF site, but also the three half-GREs in the upstream regulatory region of the γ gene (Fig. 2C). As described under “Experimental Procedures,” the effect of the mutations only on the XGRAF contribution to the hormonal stimulation was assessed by comparing each single-point mutant with wild-type DNA, representing 100% induction, and with a triple mutant in the XGRAF-binding site, which eliminated XGRAF binding and therefore represented 0% activity of XGRAF in the induction. The triple mutant, which changed −175GAG−173 to −175ACT−173, has been shown previously to reduce glucocorticoid responsiveness through ef-

![Fig. 3. Relative ability of single-point mutants of the γ DNA from positions −177 to −169 to bind XGRAF. Using the gel shift assay and Scatchard analysis described under “Experimental Procedures” and exemplified in Fig. 1, the ability of each mutant to bind to XGRAF was quantitated. The wild-type (WT) nucleotides at positions −177 to −169 and their corresponding single-point mutations (mut) are indicated along the y axis. C₅₀ on the x axis represents the -fold excess of mutant DNA required as competitor to displace half of XGRAF from the radioactively labeled wild-type DNA probe. The data are shown as the mean ± S.E. of values determined in three independent experiments. For each mutant, three independently produced preparations of competitor DNA and at least two different batches of nuclear extract were used (except for mutant C−174, for which the data were derived from two experiments with two different preparations of competitor and one nuclear extract).](http://www.jbc.org/toc)
The other two exceptions are mutants G177A and C174A, which improved the glucocorticoid-induced transcription in vivo 2-fold. Hence, these positions are not essential for conferring XGRAF function on the γ gene. Among single-point mutations that did not correspond with strong binding and function, the only four exceptions, which will be discussed below, high activity in the gel shift assay correlated with strong binding in vivo.

**FIG. 4.** Relative ability of single-point mutants of the γ DNA from positions −177 to −169 to enhance glucocorticoid induction of transcription. Transfection of Xenopus primary hepatocytes with constructs containing mutations in the XGRAF-binding region of the γ DNA and maintenance of the cells with or without hormone treatment are described under “Experimental Procedures.” The wild-type (WT) nucleotides at positions −177 to −169 and their corresponding single-point mutations (mut) are indicated along the y axis. The data are reported as XGRAF activity, which is defined as the portion of transcription of the single-point mutants in the absence of hormone stimulation attributable to XGRAF (rather than GR) for each mutant compared with that of wild-type DNA (see “Experimental Procedures”). The values are the mean ± S.E. of three independent experiments. The value obtained in each experiment was the average of usually triplicate, and in a few cases duplicate, measurements.

**DISCUSSION**

**Correlation between XGRAF Binding to DNA in Vitro and Stimulation of Transcription**—In Table I, the ability of DNA with single-point mutations at positions −177 to −169 to bind to XGRAF in the gel shift assay is classified as strong (+) if half-maximal competition was achieved with <7-fold molar excess of mutated competitor over wild-type probe or as weak (−) if 20-fold or greater excess competitor was needed. Similarly, in Table I, the ability of DNA with each single-point mutation to enhance glucocorticoid-induced transcription in transfected primary hepatocytes is labeled as high (+) if at least 60% of wild-type XGRAF activity was retained or low (−) if 46% or less of wild-type XGRAF activity was observed. With only four exceptions, which will be discussed below, high activity in the transfection assay correlated with strong binding ability in the gel shift assay, whereas low functional activity corresponded with weak binding of XGRAF to the mutated DNA.

**TABLE I**

| Wild Type | Mut | C50 (fold excess) | Quality of Binding | XGRAF Activity | Quality of Induction | Basal Transcription |
|----------|-----|-------------------|-------------------|---------------|---------------------|---------------------|
|          |     |                   |                   |               |                     |                     |
| −177A    | C   | 2.8 ± 0.9         | +                 | 109 ± 8       | 182 ± 23            | 72 ± 4              |
|          | G   | 5.0 ± 1.6         | -                 | 89 ± 29       | 142 ± 25            | 65 ± 5              |
|          | T   | 2.7 ± 0.7         | +                 | 135 ± 31      | 97 ± 38             | 72 ± 34             |
| −176A    | C   | 3.8 ± 2.5         | +                 | 100 ± 20      | 90 ± 16             | 72 ± 16             |
|          | G   | 1.7 ± 0.6         | +                 | 108 ± 36      | 142 ± 25            | 72 ± 16             |
|          | T   | 2.8 ± 1.5         | +                 | 91 ± 7        | 142 ± 25            | 72 ± 16             |
| −175G    | A   | 36 ± 2            | +                 | 33 ± 14       | -                   | 65 ± 15             |
|          | C   | 1.7 ± 0.1         | +                 | 107 ± 31      | +                   | 74 ± 4              |
|          | T   | 2.4 ± 0.9         | +                 | 85 ± 15       | +                   | 104 ± 1             |
| −174A    | C   | 3.1 ± 0.2         | +                 | 120 ± 22      | +                   | 74 ± 8              |
|          | G   | 2.1 ± 0.8         | +                 | 79 ± 39       | +                   | 94 ± 16             |
|          | T   | 2.3 ± 0.4         | +                 | 121 ± 33      | +                   | 89 ± 10             |
| −173G    | A   | 212 ± 33          | -                 | 32 ± 7        | -                   | 52 ± 7              |
|          | C   | 323 ± 70          | +                 | 453 ± 60      | +                   | 62 ± 7              |
|          | T   | 177 ± 15          | +                 | 24 ± 10       | +                   | 53 ± 5              |
| −172T    | A   | 321 ± 36          | +                 | 15 ± 14       | +                   | 100 ± 13            |
|          | C   | 176 ± 41          | +                 | 24 ± 11       | +                   | 76 ± 9              |
|          | G   | 28 ± 6            | -                 | 62 ± 22       | +                   | 87 ± 26             |
| −171T    | A   | 134 ± 31          | +                 | 40 ± 24       | +                   | 61 ± 15             |
|          | C   | 203 ± 68          | +                 | 11 ± 4        | +                   | 87 ± 41             |
|          | G   | 94 ± 22           | +                 | 46 ± 4        | +                   | 63 ± 27             |
| −170A    | C   | 180 ± 29          | -                 | 27 ± 10       | -                   | 68 ± 22             |
|          | G   | 240 ± 88          | +                 | 40 ± 2        | +                   | 48 ± 7              |
|          | T   | 283 ± 31          | +                 | 14 ± 3        | +                   | 69 ± 14             |
| −169A    | C   | 63 ± 23           | +                 | 25 ± 4        | +                   | 63 ± 23             |
|          | G   | 86 ± 26           | +                 | 11 ± 3        | +                   | 73 ± 23             |
|          | T   | 28 ± 9            | +                 | 25 ± 9        | +                   | 68 ± 14             |

* C50 = fold excess of mutant required to displace half of XGRAF from wild-type DNA.
* C50 values <7-fold excess were defined as strong (+) binding and ≥20-fold excess as weak (−) binding.
* XGRAF activity, hormonal stimulation attributable to XGRAF for mutated DNA as a percentage of that for wild-type DNA.
* XGRAF activity ≥60% was defined as strong (+) and ≤46% as weak (−). Gray shading indicates mutants with a discrepancy between binding and function.

Effects on the XGRAF-binding site rather than the GRE (24). Retention of at least 60% of XGRAF activity was considered normal function, whereas activity below 47% was defined as impaired.

As shown in Fig. 4 and Table I, full XGRAF activity ranging from 79 to 135% of the value obtained with wild-type DNA was achieved for all single-nucleotide substitutions at positions −177, −176, and −174. Hence, these positions are not essential for conferring XGRAF function on the γ gene. At position −175, XGRAF activity was reduced to 33% when the site was mutated to A, but 85 and 107% of wild-type function were attained with the T and C substitutions, respectively. Thus, the only functionally deleterious mutation from positions −177 to −174 is the G to A transition at position −175.

Conversely, almost all mutations at positions −173 to −169 significantly decreased XGRAF activity to between 11 and 48% of function with wild-type DNA (Fig. 4 and Table I). The most dramatic departure from this general pattern is the G to C transversion at position −173, which improved the glucocorticoid induction of XGRAF activity to 45% of normal XGRAF activity. The other two exceptions are mutants C−172 and C−171, which allowed 60 and 71% of wild-type activity, respectively.

**Effect of Point Mutations on Basal Transcription**—The level of transcription of the single-point mutants in the absence of hormone treatment ranged from 48 to 142% of that of wild-type DNA (Table I). Each data point for basal expression was obtained from an independent transfection event, whereas changes due to hormone treatment were assessed on a single cell population that was divided after the transfection.
Cas as a function of the ability of the DNA to bind XGRAF. The portion of the glucocorticoid response attributable to XGRAF, expressed as XGRAF activity with mutant DNA as a percentage of that with wild-type DNA, is plotted as a function of the ability of the DNA to bind XGRAF in vitro, expressed as $C_{50}$. See “Experimental Procedures” and “Discussion” for details.

The most striking discrepancy between physical association with XGRAF and ability to amplify glucocorticoid responsiveness was seen with mutant C$^{173}$, for which the hormonal induction was much higher than for any other construct, while ability to bind XGRAF was the weakest (Table I). This mutant was not included in Fig. 5 because its functional activity was much greater than that of the other mutants. Our method of computation attributed the stimulation of transcription to XGRAF, but we believe that the effect in this case was actually due to GR. The C$^{173}$ mutation generated the following sequence: $\text{C}^{173}\text{AAGACTnmmTGTTC}^{169}$, with two matches to the upstream half of the consensus GRE (GGTACAnnnTGTCT) in addition to the five out of six matches to the downstream half. We have shown previously that the C at position $\text{C}^{173}$ is essential for GR to interact with this site as a dimer and for hormonal stimulation greater than 10-fold (24). Therefore, the strong glucocorticoid response seen with the single-point mutation to C$^{173}$ could be explained by strong GR binding that eliminated the role of XGRAF in the induction. A comparable effect was not expected with any of the other nucleotides because even when all the bases except $\text{C}^{173}$ were changed to match the consensus GRE, no increase in GR dimer binding or hormonal induction was observed (24).

Two other mutants, G$^{172}$ and C$^{171}$, were also classified in Table I as having weak binding while retaining the capacity to stimulate transcription. The levels of XGRAF transcriptional activity were, however, moderate at 60 and 71%, respectively, the lowest values that were still considered positive. The binding abilities ($C_{50}$ values of 24- and 20-fold excess), although defined as weak, were intermediate between the strongest and weakest. In Fig. 5, the data for these mutants are represented by the two points in the center of the plot, which lie close to the line and therefore show good correlation between binding and function.

Mutant C$^{169}$ had a striking disjunction between strength of binding to DNA and ability to amplify glucocorticoid action. XGRAF bound quite well to the C$^{169}$ mutant since only a 6.3-fold molar excess of this DNA was required to displace half of XGRAF from the wild-type probe in the gel shift assay (Table I). Nonetheless, hormonal stimulation was very poor, with only 25% of wild-type XGRAF activity. The distinction between the C$^{169}$ mutant and all other constructs is evident from the anomalous position of the C$^{169}$ data in the lower left portion of Fig. 5. These results cannot be explained by changes in the interaction of GR with the DNA since the mutation lies within the 3-bp region between the two half-sites of the GRE, which is not critical for GR binding and function (23). Although the C$^{169}$ mutant binds fairly tightly to XGRAF in vitro, we hypothesize that it is incapable of conferring a functionally important conformational change on the protein that would occur upon binding to the natural recognition sequence.

Another important question is whether XGRAF had general effects on transcription independent of its amplification of GR action. The level of basal transcription for each of the single-point mutants is shown in Table I. Basal transcription was inherently more variable than fold hormonal induction in the transfection assay because each data point was derived from an independently transfected sample. When transcriptional activity in the absence of hormone treatment was plotted versus binding ability (Fig. 6), only a slight correlation was observed. Therefore, we conclude that XGRAF may have a small effect on general transcription of the γ subunit gene, but that the major function of XGRAF is to enhance glucocorticoid induction in response to GR. This specificity is in contrast to many other glucocorticoid receptor accessory factors (such as nuclear factor-1; activator protein-1; cAMP response element-binding protein; hepatocyte nuclear factor-1, -3, and -4; and chicken ovalbumin upstream promoter transcription factor), which also stimulate basal transcription (2, 10–17).

Consensus Sequence for the XGRAF Recognition Site—Based on the physical and functional data presented here, a consensus sequence can be derived for the XGRAF-binding site that reflects the most favorable nucleotide at each position. Bases $\text{C}^{177}$ and $\text{C}^{176}$ are no longer considered part of the recognition sequence since no substitutions at these positions affected XGRAF binding or activity. For nucleotides $\text{C}^{175}$ to $\text{C}^{169}$, the consensus sequence is BNGTTAA ($B = \text{C, G, or T}; N = \text{A, C, G, or T}$). Even with this more well defined site, we found no striking matches to recognition sequences in the transcription factor site data base TRANSFAC 3.2 (34) using the TESS
Models for Interaction of XGRAF and GR with Contiguous or Overlapping Binding Sites—Previously, we presented four possible models for the interaction of XGRAF and GR with DNA at closely juxtaposed sites (24). Model 1 depicted simultaneous binding of XGRAF to its site at nucleotides 175 to 169 and GR binding as a dimer at nucleotides 177 to 163, which would constitute a full-length GRE. To bind these sites concurrently, XGRAF must contact the DNA in the minor groove since GR is known to occupy the major groove (35). However, the methylation interference experiment (Fig. 2) established that XGRAF also binds in the major groove since modification of the N-7 positions of guanines, which are accessible only in the major groove, interfered with binding. Therefore, Model 1 is not a likely mechanism for interaction of GR and XGRAF with their respective sites.

Model 2 proposed an interaction between XGRAF and a monomer of GR. Model 3 depicted a trimeric complex consisting of one molecule of XGRAF and a dimer of GR, with GR contacting only the downstream half of the GRE. Both of these scenarios are possible but must take into account that the XGRAF- and GR-binding sites are directly contiguous. In Model 4, binding of XGRAF and GR was sequential rather than simultaneous, which would obviate problems of steric hindrance for two protein molecules binding to abutted recognition sites. Experiments are in progress to distinguish between these mechanisms.

Classically, the presence of a receptor defined a tissue as a target for a steroid hormone, and the presence of a high affinity receptor-binding site on the DNA was a prerequisite for a responsive gene. It is becoming increasingly clear that steroid hormone action is dramatically influenced by many other aspects of the local cellular environment and the structure of the gene regulatory region. Accessory DNA-binding proteins such as XGRAF can play as important a role as the receptor in determining the extent of hormonal induction. The fact that diverse cellular responses rely on unique combinations of accessory DNA-binding proteins, coactivators, corepressors, and other factors makes it possible for multiple control mechanisms to regulate genes differentially in the same cell in response to a single hormonal stimulus.

Acknowledgments—We gratefully acknowledge insightful discussions with Drs. Mark Milanick and Mark Hannink and helpful comments on the manuscript from Brian Morin.

REFERENCES

1. Tsai, M.-J., and O’Malley, B. W. (1994) Annu. Rev. Biochem. 63, 451–486
2. Archer, T. K., Lefebvre, P., Wolford, R. G., and Hager, G. L. (1992) Science 255, 1573–1576
3. Hsu, S. C., Qi, M., and DeFranco, D. B. (1992) EMBO J. 11, 3457–3468
4. Pockwinse, S. M., Stein, J. L., Lian, J. B., and Stein, G. S. (1995) Exp. Cell Res. 216, 244–260
5. Schmidhauser, C., Bissell, M. J., Myers, C. A., and Casperson, G. F. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9118–9122
6. Katzenellenbogen, J. A., O’Malley, B. W., and Katzenellenbogen, B. S. (1996) Mol. Endocrinol. 10, 119–131
7. DeBjrk, R. H., Petrides, J., Deuster, P., Gold, P. W., and Sternberg, E. M. (1996) J. Clin. Endocrinol. Metab. 81, 228–235
8. Horwitz, K. B., Jackson, T. A., Bain, D. L., Richer, J. K., Takimoto, G. S., and Tung, L. (1996) Mol. Endocrinol. 10, 1167–1177
9. Lucas, P. C., and Graner, D. K. (1992) Annu. Rev. Biochem. 61, 1113–1117
10. Diamond, M. I., Miner, J. N., Yoshinaga, S. K., and Yamamoto, K. R. (1990) Science 249, 1266–1272
11. Rigoua, G., Roux, J., Pictet, R., and Grange, T. (1991) Cell 67, 977–986
12. Imai, E., Miner, J. N., Mitchell, J. A., Yamamoto, K. R., and Graner, D. K. (1993) J. Biol. Chem. 268, 5353–5356
13. Hall, R. K., Sladek, F. M., and Graner, D. K. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 412–416
14. Roux, J., Pictet, R., and Grange, T. (1995) DNA Cell Biol. 14, 385–396
15. Wang, J.-C., Stromstedt, P.-E., O’Brien, R. M., and Graner, D. K. (1996) Mol. Endocrinol. 10, 794–800
16. Suh, D.-S., Zhou, Y., Oei, G. T., and Rechler, M. M. (1996) Mol. Endocrinol. 10, 1227–1237
17. Scott, D. K., Mitchell, J. A., and Graner, D. K. (1996) J. Biol. Chem. 271, 31909–31914
18. Kushner, I. (1988) Methods Enzymol. 163, 373–383
19. Munck, A., Guyre, P. M., and Holbrook, N. J. (1984) Endocr. Rev. 5, 25–44
20. Bhattacharya, A., and Holland, L. J. (1991) Mol. Endocrinol. 5, 587–597
21. Roberts, R. L., and Holland, L. J. (1993) Endocrinology 132, 2563–2570
22. Roberts, R. L., Nichols, L. A., and Holland, L. J. (1993) Biochemistry 32, 11627–11637
23. Beato, M. (1989) Cell 56, 335–344
24. Woodward, R. N., Li, M., and Holland, L. J. (1997) Mol. Endocrinol. 11, 563–576
25. Derbyshire, K. M., Salvo, J. J., and Grindlay, N. D. F. (1986) Gene (Amst.) 46, 145–152
26. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., pp. 6.30–6.31, 6.46–6.47, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. d’Emden, M. C., Okimura, Y., and Maurer, R. A. (1992) Mol. Endocrinol. 6, 581–588
28. Holland, L. J., Wall, A. A., and Bhattacharya, A. (1991) Biochemistry 30, 1965–1972
29. Meisterernst, M., Gander, I., Rogge, L., and Winnacker, E. L. (1988) Nucleic Acids Res. 16, 4419–4435
30. Garabedian, M. J., LaBaer, J., Liu, W.-H., and Thomas, J. R. (1993) in Gene Transcription: A Practical Approach (Hames, B. D., and Higgins, S. J., eds) pp. 243–263, IRL Press/Oxford University Press, Oxford
31. MacGregor, G. R., and Caskey, C. T. (1989) Nucleic Acids Res. 17, 2365
32. Goodsell, D. S., Kopka, M. L., Cascio, D., and Dickerson, R. E. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2930–2934
33. Wingender, E., Kel, A. E., Keil, O. V., Karas, H., Heinemeyer, T., Dietze, P., Knuppel, R., Romaschenko, A. G., and Kelso, W. N. (1997) Nucleic Acids Res. 25, 265–268
34. Schug, J., and Overton, G. C. (1997) TESS: Transcription Element Search Software on the WWW, Technical Report CBIL-TR-1997-1001, Version 0.0, Computational Biology and Informatics Laboratory, School of Medicine, University of Pennsylvania, Philadelphia.
Analysis of the DNA-binding Site for *Xenopus* Glucocorticoid Receptor Accessory Factor: CRITICAL NUCLEOTIDES FOR BINDING SPECIFICITY IN VITRO AND FOR AMPLIFICATION OF STEROID-INDUCED FIBRINOGEN GENE TRANSCRIPTION

Min Li, Xiongwen Ye, Robert N. Woodward, Cindy Zhu, LaNita A. Nichols and Lené J. Holland

*J. Biol. Chem.* 1998, 273:9790-9796.
doi: 10.1074/jbc.273.16.9790

Access the most updated version of this article at http://www.jbc.org/content/273/16/9790

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 33 references, 7 of which can be accessed free at http://www.jbc.org/content/273/16/9790.full.html#ref-list-1