DNA binding analysis of glucocorticoid receptor specificity mutants

Iris Alroy and Leonard P. Freedman*
Cell Biology & Genetics Program, Sloan-Kettering Institute and Cornell University Graduate School of Medical Sciences, 1275 York Ave, New York, NY 10021, USA

Received December 13, 1991; Revised and Accepted January 28, 1992

ABSTRACT
The glucocorticoid receptor (GR) DNA binding domain consists of several conserved amino acids and folds into two zinc finger-like structures. Previous transactivation experiments indicated that three amino acids residing in this region, Gly, Ser and Val, appear to be critical for target-site discrimination. Based on the solved crystal structure, these residues are at the beginning of an amphipathic \( \alpha \)-helix that interacts with the DNA's major groove; of these, only valine, however, contacts DNA. In order to examine their functional role directly, we have substituted these residues for the corresponding amino acids from the estrogen receptor (ER), overexpressed and purified the mutant proteins, and assayed their binding specificity and affinity by gel mobility shifts using glucocorticoid or estrogen response elements (GRE or ERE, respectively) as DNA probes. We find that all three residues are indeed required to fully switch GR's specificity to an ERE. The contacting valine in GR is of primary importance. The corresponding residue in ER, alanine, is less important for specificity, while glutamic acid, four amino acids towards the N-terminus, is most critical for ER discrimination. Finally, we show that the GR DNA binding domain carrying all three ER-specific mutations has a significantly higher affinity for an ERE than the ER DNA binding domain itself. We interpret these results in the context of both the data presented here and the crystal structure of the GR DNA binding domain complexed to a GRE.

INTRODUCTION
The glucocorticoid receptor (GR) is a member of a superfamily of ligand-inducible transcription factors. GR shares many of its functional and structural features with other members of this family, which include receptors for steroid and thyroid hormones, vitamin \( D_3 \), the retinoids, peroxisomal activators, and several proteins whose ligands have not yet been identified (for reviews, see refs. 1–5). In addition, several cDNAs for steroid-like receptor proteins have recently been isolated from Drosophila (for review, see ref. 6). As direct signal transducers, the hormone-receptor complex localizes to the nucleus, where it binds to specific DNA response elements leading to an enhancement or inhibition of the transcription of linked genes. In general, nuclear receptors can be dissected into domains of discrete functions corresponding to ligand binding, DNA binding and transcriptional activation.

While subtle but important differences in DNA binding affinity may be influenced by combinatorial effects of full-length receptors with each other and/or other nuclear factors (7–10), the specificity of nuclear hormone receptors for target sequences in regulated genes has been shown to be encoded entirely by the DNA binding domain (11). The amino acid sequence of this region displays considerable homology between members of the nuclear receptor superfamily. Several residues are absolutely conserved, including two sets of four cysteines that were shown, in the case of GR, to each coordinate a \( Zn^{2+} \) ion (12) in an arrangement reminiscent of the 'zinc finger' coordination scheme originally proposed for transcription factor IIIA of Xenopus (13–15). Mutagenesis of the glucocorticoid and estrogen receptors (ER) have indicated that specific residues within the zinc finger region are critical for DNA binding specificity (16–18), DNA-dependent dimerization (18, 19), and positive control of transcription (20). These results have recently been expanded upon by three-dimensional structural analysis (21–23). In particular, the crystal structure of the GR DNA binding domain complexed to a glucocorticoid response element (GRE) (23) shows that only three residues make specific base contacts, and are localized exclusively to an \( \alpha \)-helix that begins at the carboxyl terminal end of the first finger (Gly 458) and extends to Glu 469. Two of the three residues, Lys 461 and Arg 466, are absolutely conserved in the nuclear receptor superfamily, while the third, Val 462, is not, suggesting that it alone might be responsible for target site discrimination. On the other hand, mutagenesis experiments of this region indicate that in addition to Val 462, two neighboring amino acids, Gly 458 and Ser 459, are important in conferring DNA binding specificity. For example, changing these amino acids to the corresponding residues of ER converts the specificity of the mutant GR so that it now transactivates more strongly from an estrogen response element (ERE) than a GRE (17, 18). The reciprocal swaps in the context of ER result in similar specificity changes (16).
Since the original mutagenesis experiments that affected GR and ER specificity were assayed for in vivo effects on activation of reporter constructs following transfection, detailed questions regarding binding affinities and specificities of the various mutants could not be addressed. Given that the mutagenesis results indicate a role for residues that do not necessarily make contacts in the complex crystal structure, a complete understanding of this region of the DNA binding domain will require additional structural and functional analyses of both the estrogen receptor complex and GR specificity mutants. As a first step towards that goal, we have generated three such mutants in the context of the GR DNA binding domain, overexpressed and purified these mutant proteins to homogeneity, and directly studied their DNA binding to palindromic GREs and EREs as well as to a chimaeric element, using gel mobility shift assays.

**METHODS**

**In vitro mutagenesis**

Oligo-directed mutagenesis was carried out by subcloning the GR DNA binding domain as an Xba I fragment from the overexpression plasmid pT7 GR440-525 (23, 24) into the ampicillin repair phagemid vector pSELECT-1 (Promega). Single-stranded template was generated by superinfection with helper phage. Mutations were then introduced into the second strand by double-primer annealing of a mutagenic oligo and an ampicillin repair oligo designed to convert a mutated β-lactamase gene to wild-type. Mutant construction was done in three stages. First, GR Val 462 was mutated to alanine using a 'GSA' oligo (5' GAA AGC TTA GGC TTC ACA TGT CAC CCC 3'). The GR-GSA DNA was then used as template for a second round of mutagenesis in which Gly 458 and Ser 459 were simultaneously mutated to glutamate and glycine, respectively, using an 'EGA' oligo (5' GCT CTT TTA AAG AAA GCT TTG CCC 3'). The GR-EGA DNA was then used to generate a third mutant, GR-EGV by converting the alanine back to Val-462, with an 'EGV' oligo (5' GCT CTT TTA AAG AAT ACT TTG CAG CCT CCA C 3'). Following mutagenesis, clones were selected for ampicillin resistance, and screened for the appropriate mutations by dideoxy DNA sequencing. Confirmed mutated DNA was subcloned back into the original pT7 overexpression vector, and pT7 GR440-525 mutants reconfirmed by sequencing following subcloning.

**Overexpression and Purification of the Wild-type and Mutant**

**GR 440-525 Proteins**

BL21(DE3)/pLysS, a strain carrying a stable integrant of T7 gene 1 (T7 RNA polymerase) under the control of the lac UV5 promoter (25), was transformed with either pT7GR 440-525 (23, 24), pT7GR440-525-GSA, pT7GR440-525-EGG or pT7GR440-525-EGA, and then induced at mid-logarithmic growth (O.D.600=0.6) by addition of IPTG to a final concentration of 0.5mM. After 3 hours of induction, cells were collected by centrifugation, resuspended in three volumes of lysis buffer (50mM Tris-HCl, pH 7.5, 1mM EDTA, 10% glycerol, 500 mM NaCl, 4mM CaCl2, 40 mM MgCl2, 5mM DTT, 0.5 mM PMSF, lysozyme to 0.13 mg/ml and DNase I to 20µg/ml) and incubated on ice for 10 minutes. Na deoxycholate was added to 0.05%, and the solution was then mixed for 15 minutes at 4°C. The lysate was centrifuged at 12,000×g for 30 minutes in an SS-34 rotor, and the pellet discarded. The supernatant was precipitated with ammonium sulfate to 50%, stirred for 10 minutes at 4°C, and centrifuged at 27,000×g. The pellet was resuspended in 0.05% NP40, 10% glycerol, 5 mM DTT, 50 mM NaCl) and dialyzed against TGED50. The dialysate was applied to a Bio-Rex 70 (Biorad) column equilibrated with TGEDZ50 buffer (10% glycerol, 50µM ZnSO4), and eluted with a 50–600 mM NaCl gradient. SDS PAGE indicated that a peak fraction eluting at approximately 300 mM NaCl contained virtually homogeneous GR440-525 derivatives.

**In Vitro DNA Binding**

DNA binding was assayed by gel mobility shift electrophoresis. Complementary strands of synthetic oligonucleotides were resuspended in 5 mM sodium cacodylate/50mM NaCl, and annealed by heating for 2 minutes to 65°C, followed by slow cooling to room temperature. 50 ng duplex oligo was end-labeled with 20 µCi γ-32P-ATP (3000 Ci/mmol) using T4 polynucleotide kinase. Purified GR 440–525 and mutant protein derivatives were incubated with 0.5 ng (10,000 cpm) of the labeled, double-stranded oligonucleotide at 33°C for 30 minutes, together with 0.5 ng poly (dl:dC), and a binding buffer to give a final concentration of 20 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.05% NP40, 10% glycerol, 50 mM KCl and 1 mM DTT. Protein-DNA complexes were then resolved by electrophoresis on 10% nondenaturing acrylamide gels (75:1 acrylamide:bis) run at 20 V/cm at 4°C. Gels were dried, and subjected to autoradiography for 3 hrs. Individual bands were recorded on a phosphor screen for 2 hours and scanned using a PhosphorImager 400-E (Molecular Dynamics) set to detect 32P radioactive emissions; the signals were digitized and quantified with Image Quant Software (Molecular Dynamics) and corrected for background.

For gel shifts comparing the binding of the ER DNA binding domain and GR-EGA, a 51 bp restriction fragment from pATCl (kindly provided by Dr. Ann Nardulli, Univ. Illinois), containing a consensus ERE flanked by polylinkers, was used. pATCl was digested with SphI and BamHI, fractionated on a 4% NuSieve agarose gel, and a 51 bp ERE-bearing fragment...
was cut out and purified using a Spin-X column (Costar). The purified fragment was filled in with Klenow enzyme in the presence of dATP, dCTP, dGTP and α-32P-TPP (3000 Ci / mmol). ER-DNA binding domain (provided by Dr. J. Schwabe, MRC, Cambridge) and GR-EGA were incubated with 2 ng (20,000 cpm) of the labeled restriction fragment at room temperature for 30 min, together with 0.5 ng poly (dl:dc), and a binding buffer to give a final concentration of 15 mM Tris-HCl, pH 7.9, 0.2 mM EDTA, 10% glycerol, 80 mM KCl and 0.4 mM DTT, as specified for ER-DBD. Protein-DNA complexes were then resolved by electrophoresis on 5% nondenaturing acrylamide gels (75:1 acrylamide:bis) run at 26 V/cm at 4°C. Bands were detected and quantified as described above.

RESULTS

The DNA binding domain of the estrogen and glucocorticoid receptors (ER and GR, respectively) is a highly conserved region which determines the specificity of target gene recognition (Fig. 1). In addition, functional consensus GREs and EREs differ by only two base pairs in each half site (26); T(5)T(6) of a GRE becomes A(5)C(6) in an ERE (Fig. 2). Thus it is here that binding discrimination must take place within the response element. Combining the results of transient CAT transactivating assays from three laboratories (16—18), it is apparent that three amino acids, located at the C-terminal side of the first finger (Fig. 1, residues in bold), play a key role in distinguishing between a GRE and ERE. A substitution of two of these amino acids in the context of GR to the corresponding ER residues (Gly-Ser to Glu-Gly) switches the receptor specificity from a GRE to an ERE (Fig. 2). Thus it is here that binding discrimination must take place within the response element. Combining the results of transient CAT transactivating assays from three laboratories (16—18), it is apparent that three amino acids, located at the C-terminal side of the first finger (Fig. 1, residues in bold), play a key role in distinguishing between a GRE and ERE. A substitution of two of these amino acids in the context of GR to the corresponding ER residues (Gly-Ser to Glu-Gly), switches the receptor specificity from a GRE to an ERE (Fig. 2). Thus it is here that binding discrimination must take place within the response element.

Figure 2. DNA sequences of consensus glucocorticoid and estrogen response elements (GRE and ERE), and an intermediate GRE/ERE element. Functional GREs or EREs consist of two hexameric half-sites oriented as inverted repeats (enclosed by arrows) spaced by three base pairs. Boldfaced bases in each half-site indicate the only differences between the GRE and ERE sequences used in this study. A chimeric element, EGRE, contains one ERE half-site and one GRE half-site, with a spacing of three nucleotides.

Mutant Binding to a GRE

The wild-type GR DNA binding domain typically binds with high affinity and cooperatively as a dimer to the two half-sites that comprise a functional GRE (a sharp jump to the second shifted species even at low protein concentrations) (Fig. 4). The recently solved crystal structure of this same GR derivative clearly shows that the protein, monomeric in solution (21, 27), dimerizes upon DNA binding by forming reciprocal salt bridges, hydrophobic interactions, and hydrogen bonds between residues in each monomer that reside exclusively within the second finger. When we introduced one amino acid change towards ER in the amino terminal α-helix of the GR DNA binding domain (GR-GSA), it lowered the protein’s apparent DNA binding affinity for a GRE ($K_d$ dimer$= 2.45 \times 10^{-7}$ M versus $7.4 \times 10^{-8}$ M for GR), but the

Figure 3. GR 440—525, GR-GSA, GR-EGV and GR-EGA protein derivatives following E. coli overexpression and purification. The DNA binding domains of the wild-type GR (GR-GSV; lane 2) and mutant derivatives (GR-GSA, lane 3; GR-EGV, lane 4; and GR-EGA, lane 5) were expressed in E. coli using the T7 system and purified to virtual homogeneity as described in the Methods section. Proteins were resolved on a 15% polyacrylamide-SDS gel and visualized by Coomassie blue staining. Equal amounts of protein were loaded in each lane, as determined by Bradford assay. Molecular weight markers are indicated in lanes 1 and 6.
mutant protein still bound cooperatively (Fig. 4). Two different amino acid changes towards ER (GR-EGV) reduced the binding affinity for the GRE even further (at least three-fold), but the protein still preferred to bind as a dimer (Fig. 4). Three amino acid changes towards ER (GR-EGA) abolished binding to the GRE completely (Fig. 4). The crystal structure of the GR DNA binding domain complexed to a shorter version of the same GRE used in these gel shift analyses showed a direct van der Waals contact between the methyl group of Val 462 and the 5-methyl group of thymine at position 5 in the GRE (see Fig. 2), but no direct contact between DNA and either Gly 458 or Ser 459. The gel shift assays shown here support the importance of Val 462 for GRE recognition, since the substitution of valine for alanine in GR-GSA clearly lowers GR's affinity for the GRE, and at the same time the presence of valine in GR-EGV confers some affinity to the GRE relative to what is seen with GR EGA. Nevertheless, GR-GSA can still bind preferentially to a GRE. GR-GSA, despite its lacking the valine, has twice the binding affinity of GR-GSA for the GRE relative to that seen with GR EGA.
affinity for the GRE than GR-EGV, possibly because the ER-specific Glu might give rise to a disruptive interaction with the carbonyl groups of the T(5)T(6) sequence in the GRE. Indeed, Truss et al. (28) recently examined ER association with GRE/ERE oligonucleotides (see below) and concluded that a T at position +5 or +6 may sterically hinder ER binding.

Mutant Binding to an ERE

The same set of mutants were analyzed for their binding properties to a palindromic ERE oligonucleotide duplex, which was designed to be identical to the GRE we used except that two central bases within each half site, T(5)T(6), was changed to A(5)C(6) (Fig. 2); these changes have been shown to be sufficient to convert a GRE to a functional ERE (26). GR-EGA, which contains all three ER changes within the context of the GR DNA binding domain, binds cooperatively and with high affinity to the ERE oligo (K_d^dimer = 1.7 x 10^{-7} M) (Fig. 5), but, as shown above, not at all to the GRE (Fig. 4). Thus a complete specificity change requires the presence of all three ER residues (glutamic acid, glycine, and alanine). From molecular modelling, it appears that replacing Gly with Glu at position 462 in GR permits Glu’s direct interaction with the amino group of C(6) in the ERE (W.X. Xu and B.F. Luisi, personal communication; see Discussion and Fig. 8b). When Val 462 is retained (GR-EGV), the mutant protein’s affinity for the ERE is decreased as compared to GR-EGA (K_d^dimer = 2.95 x 10^{-7} M). We suspect that this decrease in binding might be due to steric interference by Val with the proposed interaction of Glu 458 with C(6) of the ERE. Finally, in contrast to the complete specificity shift of GR-EGV, GR-EGV has a low but detectable affinity for the GRE (Fig. 4), which is most likely due solely to the contact made by the valine side-chain to T(5) in the GRE.

It appears that the amino acid at position 462 plays a different role in GR and ER, since the valine in GR-EGV confers some affinity for a GRE (Fig. 4), whereas the presence of alanine in GR-GSA does not contribute to ERE affinity (Fig. 5). Thus alanine’s role in directing specificity to an ERE, both in a mutant protein or wild-type ER, might not be to directly enhance protein binding, but rather to permit glutamic acid to contact C(6). On the other hand, the importance of a direct role for glutamic acid (and glycine) in ER recognition of an ERE is demonstrated in the results presented here in that GR-EGV clearly prefers to bind an ERE over a GRE (Fig. 5 versus Fig. 4, respectively). It is interesting to note that the wild type GR protein has a low but detectable affinity for the ERE (K_d^dimer = 8 x 10^{-7} M). We speculate that the Val 462 side chain may interact with the A(5) of the ERE. Consistent with this, GR-GSA, which lacks the valine, has no detectable affinity for the ERE (Fig. 5).

Mutant binding to a GRE/ERE hybrid

We designed a third oligonucleotide binding site in order to analyze our mutant proteins that was comprised of one GRE half site and one ERE half site, in the typical inverted repeat orientation and separated by three base pairs (Fig. 2). We have called this oligonucleotide ‘EGRE’. It was recently shown that full-length estrogen and progesterone receptors (the latter having the same specificity as GR), bind to an EGRE with similar affinities, and both can transactivate a responsive reporter gene to the same extent (28). Our rationale for using such an oligonucleotide, which we call an ‘EGRE’, was that since each of the mutant derivatives has a clear preference for one binding site over the other, we would expect monomer binding predominating over cooperative dimerization, and in that way separate the free energy contribution of dimer binding from the intrinsic affinity each mutant has for a given ERE or GRE half-site. Previous, unpublished experiments indicate that the contribution of dimerization to the overall binding affinity of the GR DNA binding domain is a gain of approximately two orders of magnitude over monomer binding (L.P.F., unpublished).

Thus, the wild type GR DNA binding domain binds noncooperatively to the EGRE, with a K_d^dimer = 3.4 x 10^{-7} M (Fig. 6). GR-GSA, which has no affinity for an ERE and low affinity for a GRE (Figs. 5 and 4, respectively), also binds very poorly to the EGRE oligo (Fig. 6). Because GR and GR-GSA

---

**Figure 6.** In vitro binding of purified GR 440–525, GR-GSA GR-EGV and GR-EGA to an EGRE. Gel mobility shift assays of GR and the mutant proteins to an intermediate element, EGRE, containing one ERE half site and one GRE half site, with the sequence (top strand) 5' GATCGACCGAGG TCAAGATGT TCTG-TCGAG 3'. Annotations are indicated as in Figure 4.
While both proteins dimerize strongly to a 51 bp ERE fragment species with a higher affinity for an ERE than ER itself. GR DNA binding domain, we have generated a protein binding derivative (29) overproduced and purified independently of the conditions for the ER derivative. Identical results were generated experiments presented in this study; data not shown), GR-EGA cooperatively to the 31 bp ERE oligo used in the other binding per se that for GR-EGA, binding to the recognition element the rest of the structural parameters effecting the function of the GR DNA binding domain will not dimerize sequences; the ER DNA binding domain will not dimerize with one GRE half-site (data not shown), suggesting that it has a higher intrinsic affinity (i.e., minus any contribution by dimerization) for an ERE than GR does for a GRE. This implies that for GR-EGA, binding to the recognition element per se contributes more to the overall affinity than is the case for GR. Even more surprising, GR-EGA has an even higher apparent affinity for an ERE than the ER DNA binding domain itself (22) (kindly provided by J. Schwabe, MRC-Cambridge) (Fig. 7). While both proteins dimerize strongly to a 51 bp ERE fragment (carrying the ERE consensus sequence surrounded by polylinker sequences; the ER DNA binding domain will not dimerize cooperatively to the 31 bp ERE oligo used in the other binding experiments presented in this study; data not shown), GR-EGA binds with a significantly higher affinity than the ER DNA binding domain itself (22, 23) essentially confirms this model, the functional roles of these sub-domains have been recently dimerization (18, 19) and positive control of transcription (20). Structures of both the GR and ER DNA binding domains reveal that in the first a-helix, which, as predicted, restricted to the first a-helix; it is in the first a-helix that the specificity-switch mutations are found. By modeling, this amino terminal a-helix can fit into the major groove of one of two half-sites that comprise a GRE or ERE (21, 22). Thus amino acid side-chains in the first a-helix could make base-specifying contacts, conferring target-site recognition to this small part of the domain. The recently solved crystal structure of the GR DNA binding domain complexed to a GRE (23) essentially confirms this model, but also raises questions regarding the role of particular amino acids shown by mutagenesis to be important for discrimination. For example, the crystal structure indicates that only three residues make direct or water-mediated contacts with bases within the GRE; they are, as predicted, restricted to the first a-helix. Two of the three, Arg 461 and Lys 466, however, are conserved throughout the nuclear receptor superfamily and therefore could not be mediating hormone response element specificity. That leaves Val 462, which in the crystal structure makes a van der Waals contact via one of its 7-methyl side chain groups with the 5-methyl group of thymine at position 5 in the GRE (Fig. 8a), leaves Val 462, which in the crystal structure makes a van der Waals contact via one of its 7-methyl side chain groups with the 5-methyl group of thymine at position 5 in the GRE (Fig. 8a), to confer specificity of DNA binding to the zinc finger region. Nevertheless, two additional residues within the a-helix, which, like the valine, are not conserved among members of the superfamily, appear from previous work and this study to be critical in determining whether a receptor will bind a GRE or

<table>
<thead>
<tr>
<th>Receptor Derivative</th>
<th>GRE (TGTTCT)</th>
<th>ERE (TGACCT)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR</td>
<td>+ +++</td>
<td>+</td>
<td>this study</td>
</tr>
<tr>
<td>GR</td>
<td>(+++ +++)</td>
<td>(-)</td>
<td>17, 18</td>
</tr>
<tr>
<td>ER-EGV</td>
<td>++</td>
<td>(+ + + + + +)</td>
<td>18</td>
</tr>
<tr>
<td>GR-ESA</td>
<td>(+)</td>
<td>(+ + +)</td>
<td>30</td>
</tr>
<tr>
<td>GR-EGV</td>
<td>+</td>
<td>+ + + + + +</td>
<td>18, 17</td>
</tr>
<tr>
<td>ER-EGV</td>
<td>(++)</td>
<td>(+ + +)</td>
<td>16</td>
</tr>
<tr>
<td>GR-EGA</td>
<td>-</td>
<td>+ + + + + +</td>
<td>this study</td>
</tr>
<tr>
<td>ER</td>
<td>(--)</td>
<td>(+ + + + + +)</td>
<td>16</td>
</tr>
</tbody>
</table>

+ or + denotes the relative binding or lack of binding by the indicated receptor or mutant receptor DNA binding domain to a GRE or ERE oligonucleotide; (+) or (+) denotes the ability of the indicated full-length receptor or mutant receptor transiently expressed in a cotransfection experiment to activate transcription of the CAT gene off a GRE or ERE-dependent reporter plasmid.

**DISCUSSION**

The glucocorticoid receptor DNA binding domain folds into a rather compact structure anchored by two zinc ions that are each tetrahedrally coordinated by four cysteine sulfurs (12). Mutagenic analysis of this domain indicated that residues within the first zinc finger has a profound effect on discrimination of DNA binding (11, 16-18, and summarized in Table 1), while portions of the second finger might be important for DNA-dependent dimerization (18, 19) and positive control of transcription (20). The functional roles of these sub-domains have been recently borne out by structural analyses (21-23). 2-D NMR-solved structures of both the GR and ER DNA binding domains reveal that the end of each finger gives rise to an amphipathic a-helix; it is in the first a-helix that the specificity-switch mutations are found. By modeling, this amino terminal a-helix can fit into the major groove of one of two half-sites that comprise a GRE or ERE (21, 22). Thus amino acid side-chains in the first a-helix could make base-specifying contacts, conferring target-site recognition to this small part of the domain. The recently solved crystal structure of the GR DNA binding domain complexed to a GRE (23) essentially confirms this model, but also raises questions regarding the role of particular amino acids shown by mutagenesis to be important for discrimination. For example, the crystal structure indicates that only three residues make direct or water-mediated contacts with bases within the GRE; they are, as predicted, restricted to the first a-helix. Two of the three, Arg 461 and Lys 466, however, are conserved throughout the nuclear receptor superfamily and therefore could not be mediating hormone response element specificity. That leaves Val 462, which in the crystal structure makes a van der Waals contact via one of its 7-methyl side chain groups with the 5-methyl group of thymine at position 5 in the GRE (Fig. 8a), to confer specificity of DNA binding to the zinc finger region. Nevertheless, two additional residues within the a-helix, which, like the valine, are not conserved among members of the superfamily, appear from previous work and this study to be critical in determining whether a receptor will bind a GRE or
an ERE: Gly 458 and Ser 459. Swapping these amino acids for the corresponding amino acids present in ER, Glu and Gly, is sufficient to change binding specificity to an ERE, even though the specifying GR valine is still present (GR-EGV; Fig. 5). On the other hand, making a single substitution for the valine with the corresponding residue from ER, alanine, does not convert the protein (GR-GSA) to an ERE-binding species. The conclusion we draw from these mutants is that while residues within the first α-helix of both the GR and ER DNA binding domains are clearly critical for discrimination, their positions are not equivalent. That is to say, while valine at position 462 within GR is necessary for GRE recognition, the corresponding alanine in ER is not the determining residue for ERE recognition. Rather, alanine may be contributing to target site discrimination not by increasing affinity to specific bases, but by providing effective repulsion to a non-target site (i.e., a GRE; compare wild type and GR-GSA binding to a GRE in Fig. 4). An alternative role for Ala might be to sterically permit another residue, such as glutamic acid, to make a direct side-chain contact with DNA. Indeed, based on the results presented here, it is probable that the ER-specific Glu, five amino acids more N-terminal than the alanine (Fig. 1), is what specifies protein binding to an ERE. This could be done via a direct side-chain contact of Glu to the amino group of C(6) in the major groove (Fig. 8b), one of two bases within an ERE half-site that distinguishes it from a GRE (Fig. 2). Supporting this idea is work by Zilliacus et al. (30), who showed that a GR-ESA mutant protein has a higher affinity for a GRE-like oligo (C substituting for T at position 6; TGTCCT) than to a GRE; changing C(6) of an ERE to a T (i.e. more GRE-like; TGTAC-T) abolishes binding of GR-ESA to this hybrid oligo. The fact that GR-GSA still recognizes a GRE suggests that the side-chain methyl group of alanine might, like Val 462, make a van der Waals contact to T(5) (Fig. 8c). Alternatively, it might provide a hydrophobic pocket that would favor burial of the T(5) methyl group. This interaction would be weaker than valine’s van der Waals, and hence account for the observed lower affinity of GR-GSA for a GRE (Fig. 4).

Two factors determine GR’s overall DNA binding properties: dimerization and the protein’s intrinsic affinity for a GRE half-site. The GR DNA binding domain’s affinity for a GRE half-site is lower, per se, than that of GR-EGA for an ERE half-site (data not shown), while it appears that the contribution of dimerization to overall affinity is the same for both proteins (compare Figs. 2 and 3). Given this, our observation that GR-EGA has a substantially higher ERE binding affinity than does the ER DNA binding domain itself (Fig. 6) might be accounted for by the combination of ER’s higher ERE affinity (due to the EG- A residues), and the GR DNA binding domain’s dimerization capability, that are both resident in this mutant protein. This would in turn imply that ER’s dimerization interface contributes less, while its intrinsic affinity for an ERE sequence contributes more, to overall binding affinity than these same parameters in GR.

The results presented here highlight the importance, but not preeminence, of one amino acid each in the GR and ER DNA binding domains for determining DNA recognition. Both residues, valine in GR and glutamic acid in ER, lie in the recognition (amino terminal) α-helix, but are at different positions within that helix. Two other amino acids shown here and in previous work to contribute to specificity, glycine and serine, do not appear from the GR crystal structure to contact the DNA, so we presume that their effect on specificity is indirect. For example, Ser 459 interacts with the conserved Arg 489, which in turn contacts phosphates within the GRE DNA (23). This interaction may not be critical for specificity, but may affect affinity. The presence of glycine either at the second (for GR)
or third (for ER) position from the beginning of the helix (in both cases between two of four Zn$^{2+}$ coordinating cysteines [Fig. 1]) might be important in allowing the α-helix to fit optimally into the major groove, owing to glycine's small size, or as an initiator of the α-helix due to its relative flexibility (31). A glycine is present at one of these two positions in every member of the nuclear receptor superfamily. This and other models await further refinement of existing structures and the structures of mutant proteins such as those presented in this work.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Ben Luisi (MRC Virology Unit-Glasgow) for invaluable help with computer-generated graphics; he and Weixin Xu (Yale University-HHMI) for helpful comments and insights, Dr. John Schwabe (MRC-Cambridge) for purified ER DNA binding domain protein, and Dr. Ann Nardulli (University of Illinois) for plasmid pATCl and purified ER DNA binding domain protein.

REFERENCES