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# Functional dissection of the hormone and DNA binding activities of the glucocorticoid receptor

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**We have identified two separate regions of the 795 amino acid rat glucocorticoid receptor that interact with hormonal ligands and DNA respectively. The functional regions were defined by direct assays of segments of the receptor coding sequence translated *in vitro*. Hormone affinity measurements suggested that residues near the receptor C-terminus are the primary determinants of ligand binding, whereas sequence-specific DNA binding activity resides between amino acids 440 and 546. DNA binding efficiency was stimulated only modestly by prior hormone binding. The receptor regions identified in these *in vitro* studies correspond to those that mediate ligand-dependent transcriptional enhancement *in vivo*.**

*Key words:* DNA-binding region/enhancer activating protein/ligand-binding region/steroid receptor

## Introduction

Glucocorticoids are steroid hormones that govern a wide range of processes affecting development and physiological homeostasis; their actions are mediated by the glucocorticoid receptor, an intracellular protein produced at low levels in nearly all mammalian cell types. The receptor monitors circulating glucocorticoid levels by binding with high affinity to cognate ligands. In turn, this interaction triggers a structural change in the receptor, termed 'transformation', resulting in its stable nuclear association, and in the selective increase or decrease in the efficiency of transcription initiation at particular promoters (see Yamamoto, 1985, for review). The selectivity of receptor action reflects in part its capacity to associate with specific DNA sequences close to regulated promoters. These binding sites, first detected *in vitro* on mouse mammary tumor virus (MTV) DNA with highly purified rat glucocorticoid receptor (Payvar *et al.*, 1981; Scheidereit *et al.*, 1983), operate *in vivo* as glucocorticoid response elements (GREs) (Chandler *et al.*, 1983), mediating the strong stimulation of MTV transcription by the receptor (Fasel *et al.*, 1982; Buetti and Diggelmann, 1983; Ucker and Yamamoto, 1984; Firzlaff and Diggelmann, 1984); in fact, the MTV GREs are receptor-dependent transcriptional enhancers that can confer hormonal regulation upon linked heterologous promoters (Chandler *et al.*, 1983; Zaret and Yamamoto, 1984; Ponta *et al.*, 1985).

A full understanding of receptor transformation, and of the mechanisms by which the receptor transduces the hormonal signal into specific modulations in gene activity will require detailed characterizaton of the receptor itself. Conventional biochemical and genetic studies (e.g. Scheidereit *et al.*, 1983; Wrange *et al.*, 1984; Yamamoto *et al.*, 1976), while illuminating, have been compromised by limiting material and by the uncertainties of somatic cell genetics. Recently, glucocorticoid receptor cDNAs from rat (Miesfeld *et al.*, 1984, 1986), human (Hollenberg *et al.*, 1985) and mouse (Danielsen *et al.*, 1986) have been cloned and sequenced, revealing strong evolutionary conservation, and facilitating direct analyses of this regulatable enhancer activating factor. Regions of the protein that encompass the binding sites for the hormone (Gigurere *et al.*, 1986; Danielsen *et al.*, 1986; Godowski *et al.*, 1987) and for specific DNA sequences (Godowski *et al.*, 1987) have been broadly delineated. Here we define them more precisely, and describe a quantitative analysis of the hormone and DNA binding domains of the 795 amino acid rat glucocorticoid receptor.

## Results

### *Experimental strategy*

Our general approach was to construct recombinant plasmids carrying specific portions of the receptor coding region that can be transcribed *in vitro* with SP6 RNA polymerase, producing transcripts for *in vitro* translation in reticulocyte lysates. The resultant receptor fragments were then tested for hormone and DNA binding capacity. In general, receptor derivatives were immunoenriched with monoclonal antibodies either before or after the binding reactions (see below). Each translation reaction (or a portion thereof) was carried out in the presence of [$^{35}$S]-methionine; immunoprecipitation and gel electrophoresis of these products (for example, see Figure 2B) provided assessments of reaction efficiencies, the size and stability of each derivative, and their relative specific activities for homone or DNA binding.

Receptor derivatives lacking the N-terminus were fused in-frame with sequences encoding the first three or the first 97 amino acids of herpes simplex virus thymidine kinase (see Materials and methods). Receptor mutants were named according to the following conventions: those beginning with N extend from the N-terminus through the amino acid number given (thus, N795 is the intact receptor); those ending with C begin at the given amino acid number and extend through the C-terminus; those beginning with X extend from amino acid 407 through the amino acid number given; those denoted by two hyphenated numbers include the region encompassed by the amino acid numbers given; those beginning with Δ lack the region encompassed by the amino acid numbers given; those ending with EBU carry amino acids 407–423 fused downstream of amino acid 793 (see Figure 1C).

### *Epitope mapping*

Epitopes for two monoclonal antibodies, BUGR1 (Eisen *et al.*, 1985) and 250 (Okret *et al.*, 1984), were first mapped provisionally by measuring the reactivity of receptor derivatives produced in *Escherichia coli* as β-galactosidase fusion proteins. Thus, fusions to the X795 or 406C segments were strongly detected by monoclonal antibody BUGR1, whereas fragment 418C was nonreactive (Figure 1A and B). Subsequent immunoprecipitations of *in vitro* translation products labeled with [$^{35}$S]methio-

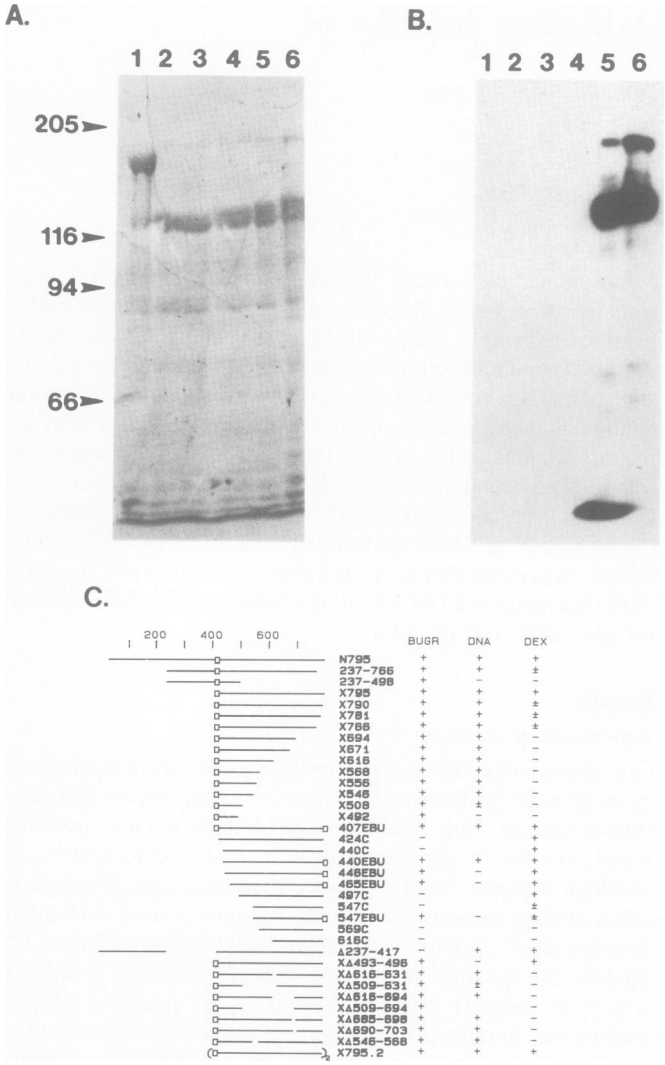


**Fig. 1.** Immunoreactivity of receptor fragemnts. (**A** and **B**) Receptor fragments were produced in *E. coli* as β-galactosidase fusion proteins (see Materials and methods). **Lane 1**, 446−738; **lane 2**, 427C; **lane 3**, 424C; **lane 4**, 418C; **lane 5**, X795; **lane 6**, 406C. Migration of mol. wt markers (kd) is shown on **panel A**, representing (in descending order) myosin, β-galactosidase, phosphorylase b, bovine serum albumin. Transformed cell lysates were electrophoresed in SDS polyacrylamide gels, blotted, and reacted with monoclonal antibody BUGR1 and $^{125}$I-labeled goat anti-mouse serum (**panel B**); the filter was then stained with amido black to visualize the transferred proteins (**panel A**). Bands above the 116 kd β-galactosidase marker represent fusion proteins. Note that, except for the 446−738 fusion, most of the receptor moiety was rapidly degraded in four protease-defective *E. coli* strains tested, leaving only short receptor 'tails' attached to β-galactosidase (unpublished observations); this instability has not been investigated further. (**C**) Receptor deletion mutants expressed in reticulocyte lysates (see text for nomenclature conventions), and their immunoreactivities with monoclonal antibody BUGR1. '+' indicates a positive signal on immunoblots and/or >80% immunoprecipitation of the [$^{35}$S]methionine labeled translation products; '−' denotes the absence of reactivity by either assay. Immunoprecipitation results are shown in various forms in Figures 2−4. DNA and DEX columns summarize most of the findings shown in Figures 3 and 2, respectively. Numbers at the top indicate amino acid positions. The indicated receptor segments are contained in each mutant. The open box represents amino acids 407−423, which encompass the BUGR1 epitope. X795.2 is a tandem dimer of fragment X795.

nine or [$^3$H]dexamethasone (see Figures 2−4) confirmed that the BUGR1 epitope resides between amino acids 407−423; in similar experiments (not shown), we concluded that the epitope for monoclonal antibody 250 is encompassed by amino acids 119−273.

Miesfeld *et al.* (1985) showed previously that the DNA and hormone binding regions are both in the C-terminal half of the receptor, suggesting that BUGR1 may be particularly useful for our experiments; moreover, Eisen *et al.* (1985) established that BUGR1 interferes with neither hormone nor DNA binding by the receptor. Remarkably, as summarized in Figure 1C and shown below (see Figures 2 and 3), the BUGR1 epitope is accessible for immunoprecipitation of the nondenatured forms of every receptor derivative tested that carries the 407−423aa region, whether at its normal position within the intact receptor, at the N-terminus of derivatives lacking amino acids 1−406, or transferred to the receptor C-terminus at amino acid 793. Thus, the BUGR1 epitope allows characterization of many different receptor derivatives with a single immune reagent.

*The hormone binding region*

Two ligands, dexamethasone and dexamethasone mesylate, were used to define and characterize the hormone binding domain of the receptor. [$^3$H]Dexamethasone mesylate is an electrophilic affinity labeling derivative of dexamethasone that associates covalently with cysteine residues available at sites of protein binding (Eisen *et al.*, 1981; S.Simons, unpublished data). Proteins labeled by [$^3$H]dexamethasone mesylate can be fractionated by SDS polyacrylamide gel electrophoresis and visualized by fluorography. As expected, the binding specificity of dexamethasone mesylate is low compared with that of dexamethasone (Figure 2A, lane T). To identify the *bona fide* receptor derivative, a portion of each labeling reaction (0.2 μM [$^3$H]dexamethasone mesylate) also contained excess (10 μM) unlabeled dexamethasone, which competes only at the saturable, high affinity sites of the receptor.

Figures 2A and B (reactions 1−3) shows that the C-terminal half of the receptor (X795) is indistinguishable in hormone binding capacity from the intact receptor, N795, whereas no specifically labeled proteins are detected in a translation reaction programmed with nonreceptor RNA. Specific, competable labeling is maintained with mutants lacking as many as 14 C-terminal amino acids (reactions 4 and 5), but truncation of 29 amino acids yields a derivative, X766 (reaction 6), that is labeled even in the presence of excess dexamethasone (see Discussion). No binding was detected with derivatives bearing more extensive C-terminal deletions, although the proteins themselves were produced competently (Figure 2A and B). N-terminal deletion derivatives extending beyond amino acid 407 were analyzed by transferring the BUGR1 epitope to the receptor C-terminus; this epitope transfer affected neither immunoprecipitation efficiency nor hormone binding capacity (cf. reactions 3, 18 and 19). Specific binding activity is retained by the 440EBU and 465EBU derivatives (reactions 19 and 20), whereas 547EBU binds with reduced efficiency (reaction 21); under the conditions used, 547EBU has a 15- to 20-fold lower binding capacity (normalized to input concentrations) than the derivatives extending further toward the N-terminus. A series of mutants with internal deletions in the region between amino acids 422 and 704 yielded results consistent with these findings (reactions 7, 8 and 10−17). Thus, [$^3$H]dexamethasone mesylate identifies a region of the receptor between amino acid ~550 and ~781 that is important for hormone binding.

In an approach independent of the affinity labeling and immune reagents, receptor derivatives were synthesized in the presence of various concentrations of [$^3$H]dexamethasone; hormone binding was measured in a filter assay (Miesfeld *et al.*, 1986), yielding a family of binding curves (Figure 2C) that represents a quantitative view of the effects of the different receptor

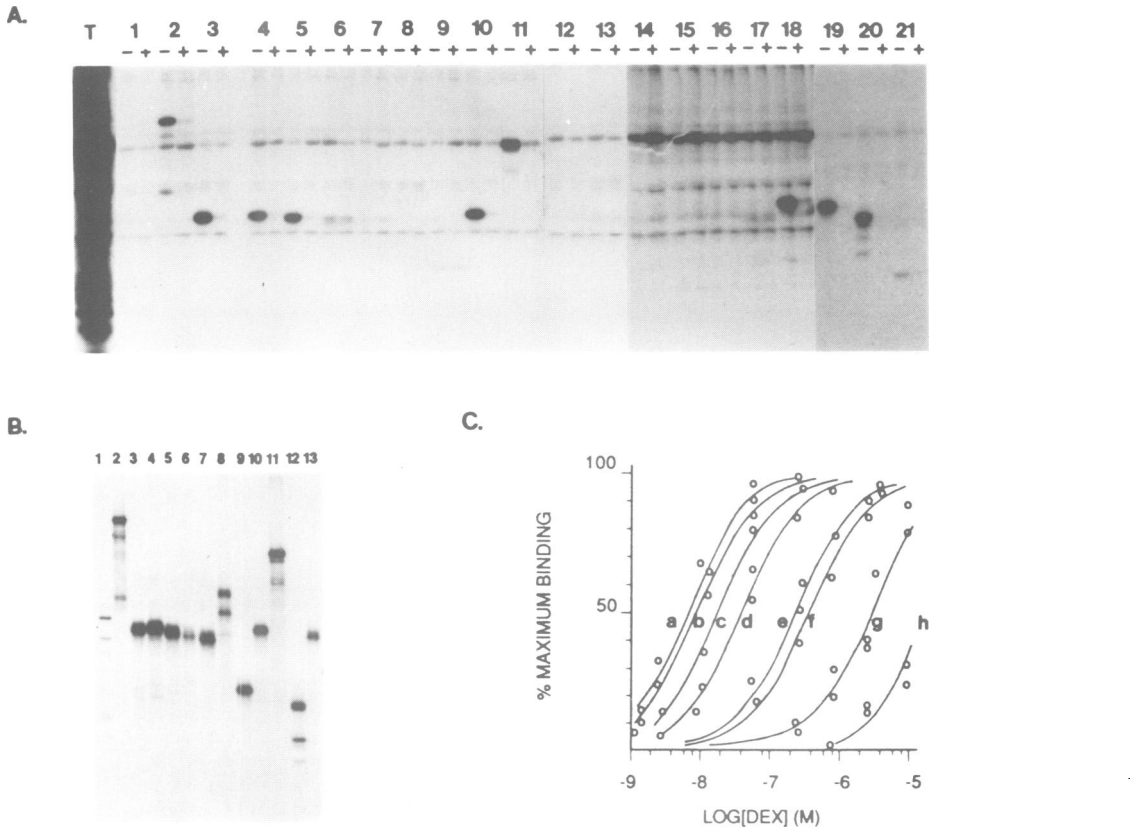


**Fig. 2.** Hormone binding by receptor derivatives synthesized *in vitro*. (**A**) Unlabeled *in vitro* translation reaction products were incubated with 0.2 $\mu$M [$^3$H]-dexamethasone mesylate in the presence (+) or absence (−) of 10 $\mu$M unlabeled dexamethasone. Receptor derivatives were immunoprecipitated, electrophoresed and fluorographed. Reactions: T, total reaction without immunoprecipitation; 1, reticulocyte lysate programmed with tk RNA; 2, N795; 3, X795; 4, X790; 5, X781; 6, X766; 7, XΔ685-698; 8, XΔ690-703; 9, X694; 10, XΔ493-496; 11, Δ237-417; 12, XΔ423-637; 13, XΔ616-631; 14, 237-671; 15, XΔ509-631; 16, XΔ616-694; 17, XΔ545-568; 18, 407EBU; 19, 440EBU; 20, 465EBU; 21, 547EBU. (**B**) A portion of each translation reaction was labeled with [$^{35}$S]methionine to assess translation and immunoprecipitation efficiencies; as examples, reactions 1−13 are shown. (**C**) Translation reactions were carried out in the presence of various concentrations of [$^3$H]dexamethasone, and receptor binding was assayed as described in Materials and methods. For each derivative, maximum binding level was interpolated from Scatchard plots. Curves: a, N795; b, X795; c, 440C; d, 465C and 497C; e, X790; f, X781; g, 547C; h, X766.

lesions. Thus, the apparent equilibrium dissociation constant of receptor derivative X795 is virtually identical to that of the intact receptor, ~7 nM, and C-terminal truncations result in dramatic reductions in affinity: X790 and X781 have an apparent $k_d$ ~0.2 $\mu$M, whereas binding to X766 is barely detectable, with an estimated $k_d$ >10 $\mu$M. In contrast, N-terminal deletions have more gradual effects: 440C, 465C and 497C bind with affinities within 2- to 5-fold of maximal, and 547C displays a $k_d$ ~2 $\mu$M. Taken together, the results obtained with the two procedures confirm and extend earlier suggestions that glucocorticoid binding activity resides in the C-terminal ~30% of the receptor (Giguere *et al.*, 1986; Godowski *et al.*, 1987).

### *The DNA binding region*

To assess DNA binding by the receptor fragments, [$^{35}$S]-methionine-labeled translation products were immunoenriched for receptor by precipitation with Staph A-antibody complexes, and then incubated with $^{32}$P-end-labeled *Xho*II digestion products of plasmid pTK1A2, which include six fragments between 2.1 kb and 90 bp, one of which (340 bp) contains an MTV GRE. Each reaction was divided into three subreactions containing various amounts of unlabeled pBR322 or calf thymus competitor DNA. Bound DNA was eluted and displayed on agarose gels; receptor levels were measured separately by scintillation counting or by autoradiography of SDS polyacrylamide gels (data not shown), confirming that all derivatives tested were immunoprecipitated efficiently, and allowing estimates of specific activities.

The results (Figure 3, reactions 2, 3 and 9) reveal that the intact receptor and derivative X795 each bind to the GRE-containing DNA fragment with apparent efficiencies and selectivities that are indistinguishable from purified rat liver receptor; as expected, binding in the presence of competitor DNA is not observed in the absence of receptor-derived translation products (reaction 1). In general, derivatives bearing progressive deletions from the C-terminus to amino acid 546 bind with selectivities and efficiencies that approach full length receptor (reactions 4−7 and 10), whereas the apparent binding efficiency of X508 is several-fold lower (reaction 11), and X492 displays no binding (reaction 8). Interestingly, the specific activities of DNA binding by receptor derivatives with C-terminal deletions between amino acids 766 and 616 appeared slightly lower than those with deletions extending further beyond the hormone binding domain; this is consistent with *in vivo* results (Godowski *et al.*, 1987) that suggest the role of ligand binding in receptor transformation (see Discussion). Using the 'epitope transfer' derivatives described above, progressive deletions downstream from amino acid 407 were also tested: 440EBU is nearly fully active (reac-

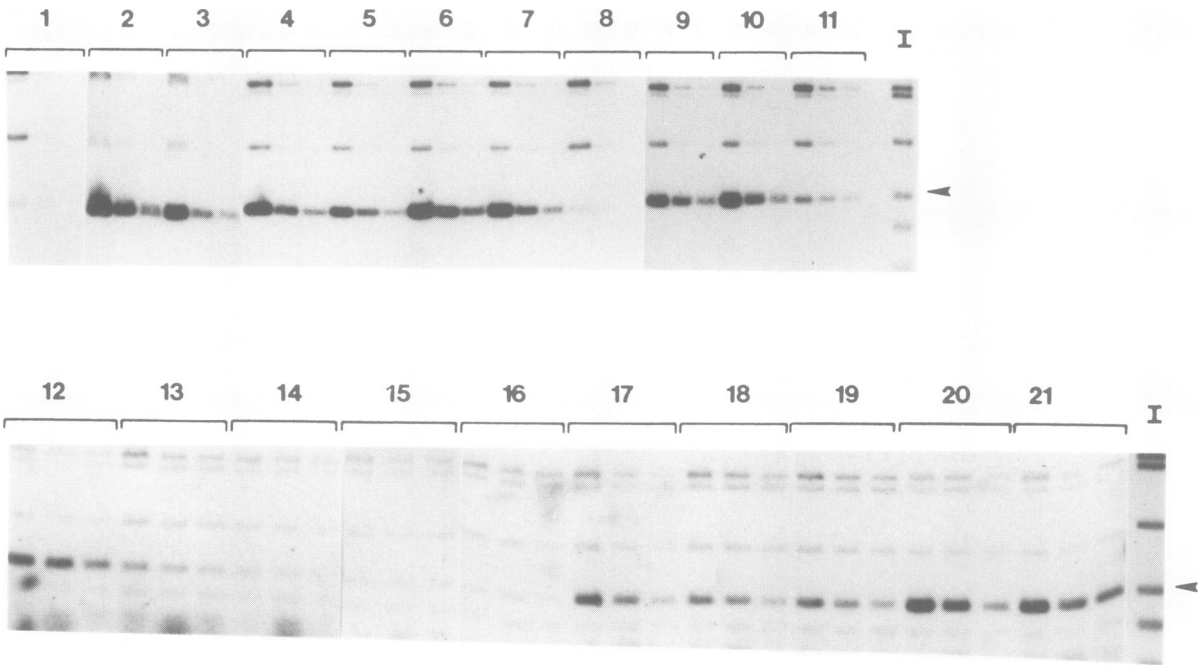


**Fig. 3.** DNA binding by receptor derivatives synthesized *in vitro*. Receptor derivatives were enriched by immunoprecipitation (BUGR1, reactions 1–20; 250, reaction 21); complexes were incubated with $^{32}$P-end-labeled DNA restriction fragments together with different amounts of nonspecific competitor DNA. Bracketed subreactions, left to right, indicate 0, 10 and 30 ng, respectively, of pBR322 DNA (**upper panel**), or 20, 100 and 500 ng of sheared calf thymus DNA (**lower panel**). Receptor levels were approximately equimolar in these reactions. Shown are autoradiograms of the bound and eluted DNA fragments after agarose gel electrophoresis. The arrow denotes the position of the 340-bp GRE-containing fragment. Reactions: 1, reticulocyte lysate programmed with tk RNA; 2, N795; 3, X795; 4, X694; 5, X671; 6, X568, 7; X556; 8, X492; 9, purified rat liver glucocorticoid receptor (Wrange *et al.*, 1984); 10, X546; 11, X508; 12, 440EBU; 13, 446EBU; 14, 465EBU; 15, 547EBU; 16, XΔ493-496; 17, XΔ616-631; 18, XΔ546-568; 19, XΔ616-694; 20, XΔ685-698; 21, Δ237-417. I, input labeled DNA fragments, *Xho*II cleavage products of pTK1A2.

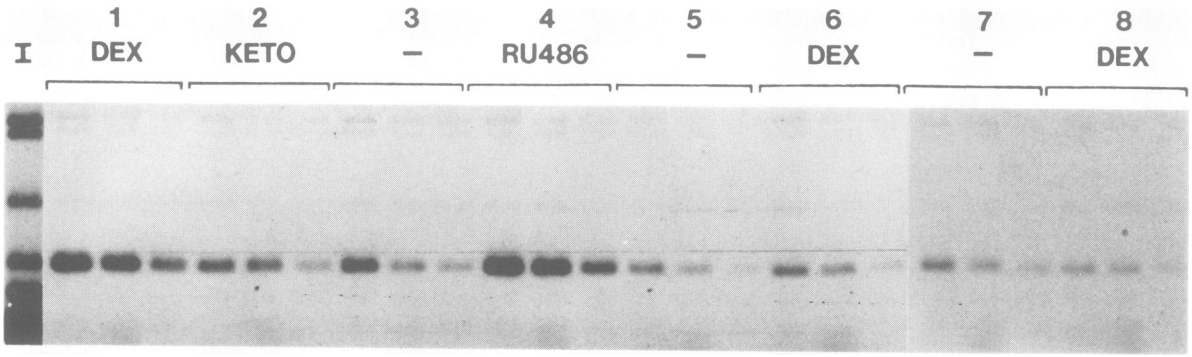


**Fig. 4.** Effects of bound ligands on DNA binding by receptor derivatives translated *in vitro*. *In vitro* translation reactions were as follows: 1, X795.2 synthesized in the presence of 10 nM dexamethasone; 2, X795.2, 10 nM dexamethasone, 5 μM ketoconazole; 3, X795.2, no ligands; 4, X795.2, 10 nM RU486; 5, X790, no ligands, 6, X790, 1 μM dexamethasone; 7, X766, no ligands; 8, X766, 1 μM dexamethasone. DNA binding reactions were carried out as described in Figure 3 with calf thymus DNA as unlabeled competitor in the bracketed subreactions.

tion 12), whereas 446EBU and 465EBU fail to bind (reactions 13 and 14). A series of internal deletion/substitution mutations yielded results consistent with the deletion mutants; most strikingly, DNA binding activity is abolished in XΔ493-496, which substitutes four wild-type amino acids (Pro-Ala-Cys-Arg) with five others (Arg-Ala-Ser-Ser-Pro; reaction 16), whereas mutants that retain the 440–508 segment display specific binding capacity.

We conclude that the DNA binding domain of the receptor appears nearly fully active even as small fragments, suggesting that it autonomously assumes a relatively stable conformation. Sequences important for maximal binding under our *in vitro* conditions reside between amino acids 440 and 546, and selective binding at reduced efficiency is observed upon further truncation to amino acid 508.

### *Effects of ligand binding on specific DNA binding*

It is well established that hormone binding increases substantially the affinity of receptor for nonspecific DNA sequences (Yamamoto *et al.*, 1974), but direct tests of hormone effects on specific DNA binding have not been reported. We therefore carried out preliminary experiments to determine whether GRE binding by receptor derivatives produced *in vitro* is affected detectably by three ligands that interact specifically with the hormone binding site of the receptor: (i) dexamethasone, a potent glucocorticoid; (ii) ketoconazole, a nonsteroidal glucocorticoid

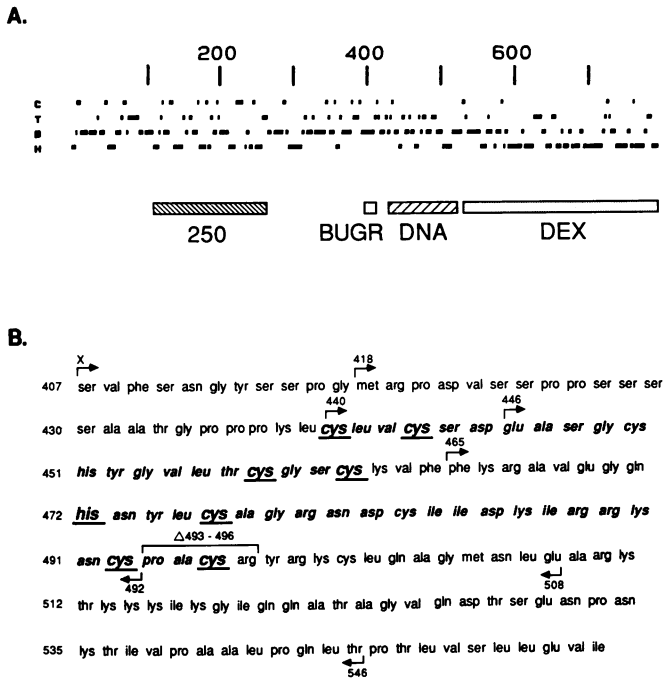


**Fig. 5.** Primary and secondary structural features of the DNA and hormone binding regions. (**A**) Closed boxes denote computer-predicted secondary structures (PREDICT; Finer-Moore and Stroud, 1984): C, random coil; T, turn; B, $\beta$-sheet; H, $\alpha$-helix. Diagrammed below are regions of the receptor competent for interaction with monoclonal antibodies 250 and BUGR1, and for specific DNA binding and specific ligand binding; these regions include sequences that may contribute indirectly as well as directly to the corresponding interactions. (**B**) Primary sequence of the DNA binding region of the receptor. Two potential zinc finger motifs are shown in boldface; underlined Cys and His residues would form coordination complexes with zinc ions according to this view (see text). The end points of several receptor deletion derivatives are shown; $\delta$493−496 introduces five amino acids, Arg-Ala-Ser-Ser-Pro, at the position of the four amino acids deleted.

antagonist (Loose *et al.*, 1983); and (iii) RU486, a glucocorticoid antagonist that also displays weak agonist activity (Raynaud and Ojasoo, 1983).

Only modest effects were observed. In fact, for unknown reasons, the best results were obtained with the X795 derivative (or with an X795 dimer, X795.2, as shown in Figure 4). The dexamethasone−receptor complex binds to the GRE-containing DNA fragment 3- to 4-fold more efficiently than does the unliganded receptor (Figure 4, reactions 1 and 3), whereas dexamethasone together with a 500-fold excess of ketoconazole yields levels of specific binding at or below that seen with receptor alone (reaction 2). In contrast, RU486 seems to stimulate specific binding as much or more than does dexamethasone (reaction 4), perhaps suggesting that the anti-glucocorticoid activities of ketoconazole and RU486 may be accomplished by different mechanisms.

Consistent with our hormone binding studies (Figure 2C), dexamethasone increased the capacity of receptor fragment X790 to bind to specific DNA sequences only if the ligand concentration was increased by 100-fold, to 1 $\mu$M (Figure 4, reactions 5 and 6). Even at this concentration, the hormone had no effect on specific DNA binding by receptor fragment X766, again as predicted from the hormone affinity determinations, and from the failure of this derivative to activate GRE-mediated enhancement *in vivo* (Godowski *et al.*, 1987). These results indicate that the hormonal effects observed, while perhaps suboptimal (see Discussion), clearly involve interactions with the *bona fide* hormone binding site of the receptor.

## Discussion

The ligand binding regions of the glucocorticoid (Giguere *et al.*, 1986; Danielsen *et al.*, 1986) and the estrogen (Kumar *et al.*, 1986) receptors have been inferred indirectly from the positions of lesions that yield a nonbinding phenotype in transiently transfected cells. Such loss-of-function mutants potentially include those with general defects in protein folding or conformational stability, RNA or protein turnover, or intracellular localization. Therefore, we employed assays that require retention of binding capacity by receptor fragments produced *in vitro*, under conditions that permit measurements of relative specific activities. In addition, we utilized two ligands − [$^3$H]-dexamethasone mesylate, which enabled visualization of bound complexes on SDS gels and facilitated ligand binding by mutant sites, and [$^3$H]dexamethasone, which allowed quantitation of apparent dissociation constants. In general, our results confirm and extend the earlier studies, and the two hormonal derivatives yielded complementary information. For example, excess dexamethasone failed to compete [$^3$H]dexamethasone mesylate labeling of X766; this implies that the C-terminal deletion in X766 might increase the rate of dexamethasone dissociation, consistent with the finding that X766 binds dexamethasone with three to four orders of magnitude lower affinity than does X795.

The conclusions derived from deletion mapping are supported fully by the recent N-terminal sequence determination ($Gly_{518}$) of a ~30-kd tryptic fragment of the receptor that retains prebound steroid (J.Carlstedt-Duke, P.-E.Stromstedt, O.Wrange, T.Bergman, J.-A.Gustafsson and H.Jornvall, unpublished results), and by the elucidation of the site ($Cys_{656}$) of covalent labeling by dexamethasone mesylate (S.Simons, J.Pumphrey, S.Rudikoff and H.Eisen, unpublished). We speculate from our binding measurements that the amino acids that actually contact the ligand may be close to the receptor C-terminus. That is, ligand affinity falls steeply with C-terminal truncation − 30-fold upon deletion of five amino acids (791−795), ~$10^4$-fold with a 29aa deletion (767−795). Indeed, binding to these derivatives is observed at 4°C, but is unstable under our normal room temperature binding conditions. In contrast, more modest changes accompany N-terminal truncations − 6-fold with a 90aa deletion (407−496) and 300-fold with a 140aa deletion (407−546) − implying that these sequences may act indirectly, perhaps contributing conformational stability to the hormone binding site.

The DNA binding domain was similarly localized with positive functional assays. Extending preliminary studies in our laboratory (Rusconi *et al.*, 1987; Godowski *et al.*, 1987), we found that the 407−546 fragment differed little from the intact receptor in its efficiency and selectivity of binding to a GRE-containing DNA fragment. In fact, all receptor derivatives that retained wild-type segment 440−508 displayed DNA binding activity, whereas all mutants with alterations in the 440−508 segment lacked activity (Figure 3). Thus, these findings are consistent with speculation by Weinberger *et al.* (1985) that the 440−495 region may be structurally analogous to the 'zinc finger' motif proposed by Miller *et al.* (1985) to correspond to the nucleic acid binding region of transcription factor TFIIIA. On the other hand, the primary sequence and chemical properties of the two putative fingers in the glucocorticoid receptor (Figure 5B) differ substan-

tially both from each other and from those proposed for TFIIIA and other putative 'finger proteins' (Berg, 1986); in particular, the first putative receptor finger is highly hydrophobic, whereas the TFIIIA fingers are quite hydrophilic.

In any case, even small polypeptide fragments that encompass the DNA binding region display binding activities approaching that of the intact protein, conceivably reflecting structural stability imparted by zinc coordination complexes analogous to those envisioned by Miller *et al.* (1985). Although other interpretations are not excluded, sequences oustide of the putative fingers, between amino acids 508 and 546, may also contribute substantially to DNA binding activity. Included within this segment is a highly basic (six of eight residues) peptide between 510 and 517 which resides in a region (503−515) of predicted (Finer-Moore and Stroud, 1984) strong α-helical character (Figure 5A). Indeed, the 440−525 region also carries *in vivo* activities for nuclear localization (D.Picard, personal communication) and GRE-mediated transcriptional enhancement (Miesfeld *et al.*, 1987). In fact, the receptor sequences involved in DNA binding and enhancer activation have not yet been genetically distinguished (Miesfeld *et al.*, 1987), despite a recent claim by Giguere *et al.* (1986) to the contrary.

Godowski *et al.* (1987) showed that receptor derivatives bearing C-terminal deletions extending to amino acid ~560 display constitutive rather than hormone-regulated enhancer activation activity *in vivo*; these results are fully consistent with our *in vitro* results, and suggest that receptor transformation represents the unmasking (derepression) of regions of the receptor that mediate its transcriptional effects. Interestingly, a transition in predicted secondary structure (from predominantly β-sheet to α-helical character) also occurs at ~aa560 (Figure 5A). Together with the accessibility of protease cleavage sites close to this region (Carlstedt-Duke *et al.*, 1982), these findings imply that this portion of the receptor may serve as a hinge during receptor transformation.

The transformation phenomenon increases receptor binding to nonspecific DNA sequences *in vitro*, and correlates *in vivo* with stable nuclear localization and enhancer activation. Circumstantial evidence (Zaret and Yamamoto, 1984; Becker *et al.*, 1986) indicates that transformation might also increase dramatically the affinity of the receptor for GRE sequences. If so, the very modest hormonal stimulations of specific DNA binding observed here suggest that our experimental conditions are far from optimal, perhaps reflecting the relatively low efficiencies of our assays, partial ligand-independent transformation during *in vitro* synthesis, or the absence of post-translational modifications important for the transformation phenomenon. While we presently favor this class of explanations, our results are also consistent with the possibilities that receptor transformation does not affect GRE binding (Willmann and Beato, 1986), or that it involves the association or dissociation of nonreceptor factors from the receptor complex (Dahmer *et al.*, 1985; Mendel *et al.*, 1986), or that the primary effect of transformation is to derepress receptor determinants for nuclear localization or enhancer activation, rather than solely to unmask the specific DNA binding region. In this context, it is interesting that the two anti-glucocorticoid ligands tested behaved differently in our assays. Conceivably, the binding of dexamethasone and other active glucocorticoids induces or stabilizes a receptor conformation that derepresses multiple activities of the types discussed above, whereas each anti-glucocorticoid may derepress only a ligand-specific subset of the activities.

## Materials and methods

*Plasmid constructions*

Specific DNA fragments of the cloned rat glucocorticoid receptor coding region (Miesfeld *et al.*, 1986) were obtained by complete restriction digestion at convenient sites, by partial digestion with *MnlI* and *AluI*, by exonuclease BAL31 resection, or by a combination of these procedures. The DNA fragments were first inserted into polylinker containing vectors (pSP64, pSP65; Melton *et al.*, 1984) in order to determine their exact boundaries by DNA sequencing and to provide convenient terminal restriction sites for transfer into expression vectors (see below). Internal deletions were generated by single-step ligation of appropriate in-frame N-terminal and C-terminal fragments into an expression vector.

Two series of *in vitro* expression vectors were constructed. The pTK3 series (pTK3.010, pTK3.NO2, pTK3.120, pTK3.2N1) contained a 104-bp DNA fragment with 5′ untranslated sequences and the first three codons of herpes simplex virus thymidine kinase (tk) inserted into pSP64 immediately downstream of the SP6 promoter and upstream of a polylinker (*Bam*HI, *Sma*I, *Sac*I and *Eco*RI) fused in each of the three translational reading frames. The pTK97 series was similar, except it contained a 390-bp fragment that includes the first 97 codons of tk. Most experiments described here utilized pTK3 derivatives, but no systematic differences have been observed with the pTK97 derivatives tested to date. Translation of C-terminal deletion derivatives extended to termination codons either within pSP64 sequences, resulting in addition to 6−34 nonreceptor amino acids, or within an inserted synthetic oligonucleotide, adding 5−7 nonreceptor amino acids. The prokaryotic expression vectors used in Figure 1 were pUR290, 291, 292 (Rüther and Müller-Hill, 1983), which yield in-frame fusions of inserted receptor sequences to the C-terminus of β-galactosidase;

*In vitro transcription and translation*

Transcription reactions (30 μl) contained 0.3 pmol *Pvu*II-linearized template DNA, 1 mM each ribonucleotide triphosphate, 1 U/ml SP6 RNA polymerase in the buffer recommended by the supplier (Boehringer). After 40 min at 37°C, nucleic acids were extracted with 1:1 phenol−chloroform and precipitated from ethanol. The pellet (~10 pmol RNA and 0.3 pmol DNA) was dissolved in 30 μl TE (10 mM Tris−HCl pH 7.4, 1 mM $Na_2$ EDTA).

Translation reactions (50 μl) contained 5−10 μl DNA/RNA solution, 30 μl reticulocyte lysate (Promega), and 10−15 μl 'substrate mix'. Substrate mixes for various types of reactions yielded final concentrations as follows: 20 μM each of 19 amino acids (met depleted); 1 μCi/ml [$^{35}$S]methionine (NEN, >800 Ci/mM; not included in hormone binding analyses); 10 mM β-mercaptoethanol (not included in [$^3$H]dexamethasone-mesylate binding assays); 10 μM unlabeled methionine (for isotope dilution in DNA binding assays; the endogenous methionine pool was estimated from dilution curves to be ~6 μM); hormones were added as indicated (see also below). Reactions were incubated 20 min at 32°C. Typical yields were 10−40 fmol/μl; residual DNA templates had no detectable effects on translation efficiency.

*DNA binding assay*

*Buffers.* TEGN50, TEGN150 and TEGN250 (20 mM Tris−HCl pH 7.6, 1 mM $Na_2$EDTA, 20% glycerol, 10 mM β-mercaptoethanol, NaCl at mM concentration indicated by the buffer number); TBS (10 mM Tris−HCl pH 7.5, 150 mM NaCl, 1 mM $Na_2$ EDTA); other additions are as indicated.

*DNA.* pTK1A2 DNA (Jones, 1986) was restricted with *Xho*II to generate a 340-bp fragment containing the MTV LTR GRE, plus control fragments of 2.1, 1.6, 0.77, 0.2, 0.09, 0.017 and 0.008 kb. The restricted DNA was phosphatased and subsequently kinased in the presence of [γ-$^{32}$P]ATP (ICN) according to standard procedures (Maniatis *et al.*, 1982).

*Immunobilized antibodies.* Fifty microliters of ascites fluid containing monoclonal antibody BUGR1 (Eisen *et al.*, 1985) or 250 (Okret *et al.*, 1984), was added to 2 ml inactivated *Staphylococcus aureus* A cells (10% v/v suspension in PBS, 2.5% BSA; Pansorbin, Calbiochem). After 2 h of gentle mixing at 4°C, the mixture was centrifuged; the pellet was rinsed once with TBS, 2.5% BSA, and resuspended in 2 ml of the same buffer. StaphA-adsorbed antibody is stable for several weeks at 4°C.

*Reaction.* Translation products (0.2−0.5 pmol) from a 50-μl reaction were immunoprecipitated by addition of 45 μl StaphA-antibody suspension followed by incubation (20 min, room temperature) in TEGN250, 2.5% BSA (total volume 400 μl), and centrifugation. The complexes were rinsed once with TEGN250 and resuspended in 155 μl TEGN150, 2.5% BSA; for each DNA binding reaction, 50 μl of this suspension was added to 50 μl end-labeled DNA (0.4−0.8 ng/μl and either 0, 10 or 30 ng pBR322 (for bracketed left, middle and right subreactions, respectively, in Figure 3, upper panel) or 20, 100 or 500 ng sonicated calf thymus DNA (for left, middle and right subreactions, respectively in Figure 3, lower panel, and in Figure 4) in TEGN150, 4 mM $MgCl_2$; hormones were added as indicated. After 2−3 h at 4°C with gentle mixing, unbound DNA was removed

by centrifugation plus one rinse of the pellet with TEGN150, 300 μg/ml insulin. Bound DNA was eluted from the complexes by resuspending 10 min at room temperature in TEGN50, 17 mM $MgCl_2$, followed by centrifugation. The supernatant was transferred to a fresh tube, supplemented with 10 μg tRNA, and nucleic acids were ethanol precipitated and redissolved in 20–40 μl TE. Aliquots were mixed 5:1 with loading buffer containing 100 μg/ml RNase A, electrophoresed in a 1.5% agarose gel, and dried onto DE81 paper (Whatman) for autoradiography. Selectivity of binding was evaluated either by direct counting of excised bands or by densitometric scanning of autoradiograms. Residual bound DNA was eluted from the immobilized receptor with 500 mM NaCl, and the level of antibody-bound [$^{35}$S]methionine-labeled receptor was determined either by direct scintillation counting (Safety-Solve, RPI), or by SDS polyacrylamide gel electrophoresis and autoradiography, thereby allowing estimates of relative DNA binding efficiency.

*Hormone binding assays*

To measure dexamethasone binding, [$^{3}$H]dexamethasone (NEN, >40 Ci/mmol) was added at the indicated concentration, in the presence or absence of 200-fold excess unlabeled dexamethasone, to *in vitro* translation mixes (15–20 μl) containing 20 μM unlabeled methionine in place of [$^{35}$S]methionine. After translation, reactions were diluted to 120 μl with ice-cold TEGN40, 0.1% BSA (supplemented with labeled and unlabeled dexamethasone as above) and incubated 30 min, 0°C. Subsequent steps (charcoal treatment, GF/C filter loading and rinsing) were as previously described (Miesfeld *et al.*, 1986), except in the case of the C-terminal deletion mutants, for which the following modifications were adopted: charcoal treatment and centrifugation were carried out for 45–60 s, filter loading was completed in 1 min at 4°C, and rinsing, 3 × 1 min. Specifically bound hormone was calculated as the difference in bound radioactivity between the uncompeted and competed reactions; results were normalized to receptor levels estimated from filter-bound control radioactivity in a parallel translation reaction containing [$^{35}$S]methionine.

Affinity labeling with dexamethasone mesylate (Eisen *et al.*, 1981) was performed by diluting *in vitro* translation products (15–20 μl, unlabeled) into 100 μl of TEGN40 (pH 9.0) containing 0.2 μM [$^{3}$H]dexamethasone mesylate (NEN), with or without 10 μM unlabeled dexamethasone. After 2–4 h at 0–4°C, the reactions were brought to 40 mM β-mercaptoethanol, and the receptor derivatives immunoprecipitated essentially as described for the DNA binding assays (~1 μl StaphA-antibody per μl translation mix), except the samples were rinsed, progressively, with 500 μl TBS, 1% BSA; 500 μl TBS, 0.3% insulin; 500 μl TE. The washed pellets were resuspended in loading buffer and electrophoresed in SDS polyacrylamide gels. After fixation with 5:1:5 methanol:acetic acid:water, gels were soaked 30 min in $H_2O$ and 30 min in 1 M sodium salicylate, then dried and autoradiographed at −70°C, 1–10 days.

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