Influence of a steroid receptor DNA-binding domain on transcriptional regulatory functions

Jeffrey A. Lefstin,¹ Jay R. Thomas,¹ and Keith R. Yamamoto

Departments of Pharmacology, and Biochemistry and Biophysics, Program in Biological Sciences, Biochemistry and Molecular Biology Program, University of California at San Francisco, San Francisco, California 94143-0450 USA

We have isolated two independent mutations in the DNA-binding domain of the rat glucocorticoid receptor, P493R and S459A, that implicate DNA binding in the control of attached transcriptional activation domains, either that of the receptor itself or of VP16. The mutants are capable of activating transcription normally, but unlike wild-type receptors, they interfere with particular transcriptional activators in yeast and mammalian cells, and inhibit growth when overexpressed in yeast. The mutant residues reside at positions within the three-dimensional structure of the receptor that could, in principle, transduce structural changes from the DNA-binding surface of the receptor to other functional domains. These findings, together with the salt dependence of specific and nonspecific DNA binding by these receptors, suggest that specific DNA acts as an allosteric effector that directs the functional interaction of the receptor with targets of transcriptional activation and that the P493R and S459A mutants mimic the allosteric effect of specific DNA, allowing the receptor to interact with regulatory targets even in the absence of specific DNA binding.

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The interaction of DNA and site-specific DNA-binding proteins was classically considered as a "rigid body" association determined by the interaction of two preexisting and stable interfaces. However, it is now apparent that many DNA-protein interactions are accompanied by structural changes. DNA structure may be altered by interaction with proteins, and site-specific DNA-binding proteins may undergo conformational changes upon binding to DNA (Spolar and Record 1994). The dynamic nature of protein-DNA interactions raises the possibility that the final structure of the protein might depend upon the particular sequence to which it binds, that is, specific DNA sequences might act as allosteric effectors to determine the catalytic or regulatory activities of the protein. Restriction endonucleases, for example, are DNA-binding proteins whose catalytic functions are inactive on nonspecific DNA and active at specific sites. Allosteric effects of DNA sequence on endonuclease activity have been proposed for *EcoRI* (Heitman 1992) and demonstrated for *NaeI*, *NarI*, *BspMI*, *HpaII*, and *SacII* (Oller et al. 1991).

Might the activities of transcriptional regulatory proteins similarly be governed or influenced by the DNA sequences to which they bind? The magnitude of activation or repression by a given factor commonly varies

with "DNA context." In general, it has not been determined whether such context effects reflect the contributions of other transcriptional regulators bound nearby, or instead indicate a direct effect of DNA sequence on the disposition of a regulator. However, the correlation between site-specific conformations and site-specific activities of the transcription factors PRTF/MCM1 (Tan and Richmond 1990) and the p50 subunit of NF- κ B (Fujita et al. 1992; Hay and Nicholson 1993) supports the idea that DNA-induced conformational changes can modulate the function of transcriptional regulators. In the case of the tumor suppressor gene p53, long-range conformational changes occur upon DNA binding, and certain oncogenic mutants of p53 assume the DNA-bound conformation in solution, suggesting that point mutations can mimic both the structural and the functional effects of specific DNA binding on p53 (Halazonetis et al. 1993).

In contrast with these ideas, transcriptional regulatory proteins have traditionally been conceptualized as simple fusions of two distinct and wholly independent functions: a DNA-binding domain, whose sole function is to tether the protein in the vicinity of a promoter, and modulatory domains, which activate or repress transcription. This view derives from the empirical ease of constructing functional chimeric regulators by fusing an activation domain from one regulator to the DNA-binding domain of another (Brent and Ptashne 1985; Keegan et al.

¹These authors contributed equally to this work.

1986). According to this paradigm, DNA-binding domains are passive structures that merely deliver modulatory domains to particular promoters.

Although site-specific delivery of modulatory domains certainly serves an essential role in transcriptional regulation, DNA-binding domains typically harbor multiple activities. For example, the 795 amino acid rat glucocorticoid receptor (GR), a member of the intracellular receptor superfamily of regulators (Tsai and O'Malley 1994), binds to glucocorticoid response element (GRE) DNA sequences through a strongly conserved ~70 amino acid zinc-binding motif (Giguere et al. 1986; Godowski et al. 1987; Freedman et al. 1988). This same zinc-binding region also mediates nuclear localization (Picard and Yamamoto 1987), DNA-induced dimerization (Tsai et al. 1988; Luisi et al. 1991), transcriptional activation (Hollenberg et al. 1987; Miesfeld et al. 1987; Hollenberg and Evans 1988; Freedman et al. 1989), and interactions with other transcriptional regulators such as AP-1 and cAMP response element binding (CREB) protein (Miner and Yamamoto 1991) and the SWI complex (Yoshinaga et al. 1992). The zinc-binding region has been mutagenized extensively (Hollenberg and Evans 1988; Severne et al. 1988; Schena et al. 1989; Thomas 1993), and the structures of several steroid receptor zinc-binding regions have been determined (Härd et al. 1990; Luisi et al. 1991; Baumann et al. 1993; Schwabe et al. 1993b). Such analyses revealed residues involved in nuclear localization, dimerization, and DNA binding and implied that dimerization activity and local folding of the zinc-binding region may be induced upon specific binding to GRE sequences. In addition, positive control mutants, which compromise transcriptional activation but not DNA binding, have been isolated in the zinc-binding region, supporting a role for this domain in transcriptional regulation (Godowski et al. 1989; Schena et al. 1989; Zandi et al. 1993).

Does DNA binding modulate the structure and function of the zinc-binding region? Might specific GRE sequences, acting through the zinc-binding region, modify the transcriptional regulatory properties of steroid receptors? We have studied a positive control mutant termed LS7 (Godowski et al. 1989) that binds DNA *in vitro* and represses transcription in transfected tissue culture cells like wild-type receptor, but fails to enhance transcription. One strategy was to exploit the fact that steroid hormone receptors are functional when expressed in the yeast *Saccharomyces cerevisiae* (Metzger et al. 1988; Schena and Yamamoto 1988; Mak et al. 1989; Wright et al. 1990). Unexpectedly, we found that expression of the LS7 mutant in yeast, unlike wild-type or any other mutant tested previously, inhibited cell growth in the presence of hormone; subsequently, we found that a second mutation in the zinc-binding region, S459A, displayed phenotypes indistinguishable from LS7. Interestingly, the residues affected in the two mutants reside near a region that structural studies have implied may change conformation upon binding to specific DNA (see below). Therefore, we set out to examine the relationships among the positive control phenotype observed in trans-

fect animal cells, the hormone-dependent growth defect seen in yeast, and the potential role of specific DNA binding in those activities.

Results

Mutants in the zinc-binding region that inhibit the growth of yeast

Two independent mutations in the rat GR zinc-binding region, proline 493 to arginine (P493R) and serine 459 to alanine (S459A) (Fig. 1A), cause a hormone-dependent growth defect in yeast when expressed from 2 μ -based vectors (Fig. 1B). P493R is one of two alterations in LS7, a mutant that displays a positive control phenotype in transfected tissue culture cells (Godowski et al. 1989); comparison of LS7, P493R, and the second change, A494S, revealed that P493R is entirely responsible for the LS7 phenotypes (data not shown). S459A was constructed after the mutation S459R was recovered as a loss-of-function allele in a random mutagenesis of the zinc-binding region (Thomas 1993). By all criteria tested (see below), the P493R and S459A mutants are phenotypically indistinguishable. Under these same conditions, the wild-type receptor inhibits yeast growth slightly, but the effects of the mutants are substantially more dramatic. Like transcriptional activation, the growth defect is hormone-dependent in the context of the full-length mutant receptors, but truncation of the carboxy-terminal signaling domain produced receptor derivatives that inhibited yeast growth constitutively upon transformation with the expression plasmid. The growth defect has been seen in all yeast strains tested and does not require the presence of a reporter plasmid; the phenotype is exacerbated at 37°C and almost abolished at 18°C (data not shown). Immunoblotting of the receptor protein (see Fig. 5A, below) revealed no detectable changes in the levels or integrity of the mutant receptors.

The mutant receptors interfere with particular yeast upstream activating sequences

Because the GR is a potent transcriptional activator when expressed in yeast, we considered the possibility that the growth defect might reflect interference by the mutant receptors with transcription of normal yeast genes (Wright et al. 1991). Such an effect might arise from "squelching," in which the mutant receptors would interact with one or more target factors also required for the action of one or more yeast regulators; these interactions would presumably preclude interaction of the target factors with the yeast regulators, thereby limiting transcription from yeast promoters under their control.

To test for transcriptional interference, we examined a promoter activated by the regulatory protein Gal4. We measured mRNA from the galactose-inducible *GAL1* locus (Johnston 1987) following 20 min of hormone treatment, with the last 10 min in the presence of galactose

Figure 1. (A) Schematic diagram of the rat GR zinc-binding region. The P493R and S459A mutations are indicated; italicized residues are mutated in Tables 1 and 2. (B) The P493R and S459A mutants cause hormone-dependent growth defects in yeast. Yeast strain YPH500 was transformed with 2 μ plasmids expressing full-length glucocorticoid receptor cDNA from the GPD promoter. Transformants were streaked on selective plates containing no hormone (–) or 1 μ M deacetylcortivazol (DAC) (+) and grown for 3 days at 30°C.

B

(Fig. 2a). Under these conditions, the P493R and S459A mutant receptors interfered with the production of *GAL1* mRNA. Induction of *GAL1* mRNA does not require prior synthesis of new gene products (Perlman and Hopper 1979), indicating that the observed interference reflects a direct inhibition of transcriptional induction. Similar results were obtained with reporter constructs bearing either the *GAL1* upstream activating sequence (UAS_G) (Guarente et al. 1982) or a single Gal4-binding site upstream of the yeast *CYC1* TATA box fused to the β -galactosidase gene (Fig. 2b,c). As with the growth defect, we observed modest transcriptional interference by the wild-type receptor, implying that the P493R and S459A phenotypes might merely exaggerate an existing characteristic of the wild-type receptor rather than representing a true gain of function. We conclude from these results that inhibition of galactose-inducible transcription by the mutant receptors operates by direct interference with Gal4 function; it is independent of chromosomal location, choice of TATA box, or synergy between complex promoter elements.

In contrast, we found virtually no inhibition of induction of the copper-activated *CUP1* promoter (Thiele and Hamer 1986): Neither the wild-type nor the P493R or S459A mutant receptors affected the copper response (Fig. 2d). This demonstrates that the transcriptional interference phenomenon is selective, affecting only a subset of yeast activators.

The mutant phenotype requires a transcriptional activation domain

By expressing different receptor segments, we found that the growth inhibitory activity required the amino-terminal half of the protein (data not shown), but not the carboxy-terminal signaling domain (see Fig. 4, below); the

amino-terminal segment of the receptor includes a transcriptional enhancement function denoted *enh2* (Godowski et al. 1988) or *tau-1* (Hollenberg and Evans 1988). To investigate the relationship of the transcriptional interference and growth inhibition phenotypes with the *enh2* enhancement region, we examined a series of deletions within the amino-terminal segment in the context of the P493R mutation. The results (Fig. 3) showed that the severity of transcriptional interference and growth inhibition displayed by the various deletions in the P493R receptor paralleled roughly the *enh2* activity of the same deletions in the wild-type receptor. These findings indicate that the mutations in the receptor zinc-binding region promote a squelching phenomenon in which one or more transcriptional activation surfaces embedded in *enh2* might associate inappropriately with their target factors.

To assess whether the effects of the mutant zinc-binding regions are *enh2* specific, we replaced the *enh2* region of the P493R receptor with the acidic activation domain of herpes virus protein VP16 (Triezenberg et al. 1988). This chimeric mutant receptor (Fig. 3) and its S459A counterpart (data not shown) displayed moderate transcriptional interference and growth inhibitory activities, whereas the same receptor–VP16 chimera in the context of the wild-type zinc-binding region caused neither growth inhibition (Fig. 4) nor squelching of UAS_G (data not shown). The wild-type receptor–VP16 chimera, however, strongly activated transcription from a GRE-linked reporter gene in the presence of hormone (Fig. 3). Thus, the phenotypic effects of the P493R and S459A mutations of the zinc-binding region of the receptor are retained when the region is linked to a heterologous activating region.

We were surprised by the failure of the wild-type receptor–VP16 chimera to inhibit growth or squelch tran-

Figure 2. Transcriptional interference by wild-type and mutant receptors. (a) RNA of the chromosomal *GAL1* gene. Yeast strain CY341, expressing GRs or an empty expression vector (pG-1) from 2 μ plasmids, was grown in nonrepressing (2% glycerol–2% ethanol) medium and treated with 1 μ M DAC (0.1% of a 1 mM stock solution in ethanol) or 0.1% ethanol alone. Following 10 min of hormone treatment, galactose was added at 2% final concentration to induce the *GAL1* promoter. RNA was isolated after 10 min of galactose induction. Primer extension detects the *GAL1* transcript. (b) Activity of the *GAL1* UAS_G. Cultures of CY341 containing 2 μ receptor expression vectors and the reporter plasmid pLGSD5 (UAS_G–CYC1–lacZ) were treated with galactose and hormone or ethanol as in a. β -Galactosidase enzymatic activity was determined after 6 hr of induction. Data are averaged from three transformants; error bars represent the standard error of the mean. (c) Activity of a single GAL4 site. Cultures of CY341 were grown, treated, and assayed as in b, but the reporter plasmid (pSV14) contained a single 17-mer GAL4-binding site instead of the complete UAS_G and yeasts were grown in glucose medium before shifting to a galactose medium. (d) Activity of the *CUP1* promoter. Yeast strain YPH499 expressing the indicated glucocorticoid receptor from 2 μ plasmids was grown in glucose and treated with 1 mM copper sulfate (final) and hormone or ethanol; β -galactosidase activity from a *CUP1*–lacZ reporter plasmid (pLDA-241) was determined after 6 hr.

scription, as fusions of VP16 to the DNA-binding domains of Gal4 (Berger et al. 1992) or the estrogen receptor (Gilbert et al. 1993) are highly toxic in yeast. One interpretation is that the wild-type zinc-binding region of the GR (but not the P493R and S459A mutants) somehow inactivates transcriptional activation domains when the receptor is not bound to a GRE, and that the Gal4 and estrogen receptor DNA-binding regions lack this capacity. Alternatively, perhaps the Gal4 and the estrogen receptor segments of those fusions contribute to VP16 toxicity in some way that wild-type glucocorticoid receptor does not.

The mutant receptors can activate normally but self-squelch when overexpressed

The results described above were obtained with the receptor expressed in yeast from high copy, 2 μ -based vectors (10–40 copies/cell). In contrast, when the mutant receptors were expressed from low copy, CEN/ARS vectors (one to two copies per cell), they caused neither the growth defect nor squelching of UAS_G (data not shown). We expressed the wild-type and mutant receptors at low or high copy (Fig. 5A) and compared their ability to activate transcription of a GRE-linked reporter gene (Fig. 5B). Under low copy conditions, the mutants activated transcription as well as the wild-type receptor; thus, the mutants are indistinguishable from wild-type when bound to GREs. Under high copy conditions, however, the mutants displayed two- to threefold less activity than wild type. We suggest that these results reflect self-squelching: At high levels of expression, mutant receptors bound at nonspecific sites may titrate targets away from the mutant receptors bound at GREs. Their wild-type behavior at low concentration implies that the mutant receptors occupy GREs in vivo with affinities and specificities similar to the wild-type receptor and indicates that transcriptional interference does not reflect site-specific repression by the mutant receptors.

The self-squelching mechanism also suggested a simple unifying explanation for the positive control phenotype of P493R in transfected animal cells (Godowski et al. 1989). We tested this notion by transiently transfecting mouse F9 embryonic carcinoma cells with P493R expression vector at various levels of DNA. As expected, we found that at a high level (1 μ g) of receptor plasmid, the mutant receptors were severely compromised relative to wild type for transcriptional enhancement. However, at low levels of receptor plasmid (10 or 100 ng), the mutant receptors activated transcription as efficiently as the wild type (Fig. 5C). We conclude that squelching is the most parsimonious explanation for all of the phenotypes discovered for the P493R and S459A mutant receptors, including the positive control phenotype described in animal cells, and the transcription and growth inhibition phenotypes in yeast. The similarity of the mutant phenotypes in yeast and mammalian cells also indicates that squelching is intrinsic to the mutant receptors and not an idiosyncrasy of receptor expression in yeast. Finally, these findings underscore the pitfalls of interpreting transient transfection experiments, in which transfected cDNAs may produce protein products in amounts that greatly exceed their normal levels.

It is notable that in the absence of hormone, the P493R and S459A mutants, but not the wild-type receptor, induced a low level of gene activity (Fig. 5B), as well as modest but reproducible squelching in the absence of hormone (Fig. 2b). This constitutive activity suggests that the mutant aporeceptors may spontaneously assume an active conformation similar to that of the hormone-bound receptor, relieving the inactivation of the receptor normally imposed by the unliganded signaling domain.

Figure 3. Correlation of the growth inhibition and transcriptional interference phenotypes with a transcriptional activation domain. Deletion derivatives of the GR were expressed in YPH499 from 2 μ plasmids and assayed for GRE-linked transcriptional activation (WT) or transcriptional interference with the *GAL1* UAS_G (P493R) in the presence of 1 μ M DAC. Receptor-driven transcriptional activation was measured using a reporter plasmid (pAS-26X) containing three repeated GREs from the tyrosine aminotransferase promoter driving a *CYC1-lacZ* fusion, and is expressed as percent of full-length receptor activity after 6 hr of hormone treatment. UAS_G activity was determined as in Fig. 2b, except that cells were grown in glucose prior to galactose induction, and is expressed as the fraction of the β -galactosidase activity observed in cells containing the empty expression vector pG-1 instead of a receptor expression plasmid. The results for the 70–300 deletion showed considerable variability in the experiments shown, but this deletion behaved similarly to the 154–406 deletion in several other experiments. Data are averaged from three transformants and the standard error of the mean is reported. The growth defect was qualitatively evaluated from growth on plates containing 1 μ M DAC.

Role of DNA binding and dimerization

The X-ray crystallographic structure of the GR zinc-binding region complexed with a GRE (Luisi et al. 1991) identified interactions of particular amino acids with specific bases, with the DNA phosphodiester backbone, and with a second receptor monomer to form a dimer. We mutated residues implicated in these functions (see Fig. 7, below, white residues) and tested them in the context of the P493R mutation for the growth inhibition and squelching phenotypes (Table 1). We also monitored the relative effects of these mutations on nonspecific DNA binding by the T7X556 receptor derivative, which encompasses the zinc-binding region (amino acids 407–556) (Freedman et al. 1988; Table 2). This was accomplished by DNA–cellulose chromatography using bulk salmon sperm DNA substrate, as nonspecific DNA affinity correlates with the NaCl concentration required to elute the receptor from DNA–cellulose (Yamamoto and Alberts 1974).

We began by mutating residues of the recognition helix that protrudes into the major groove of the GRE. Strikingly, mutating a specific base contact, the extremely conserved residue K461, to alanine had no effect on the squelching phenotypes of the P493R receptor (Table 1), although the K461A mutant in the context of the wild-type zinc-binding region displays <1% of wild-type transcriptional activation at a GRE-linked promoter (Thomas 1993). However, mutation of K461 to glutamate, rather than alanine, abolished the growth inhibitory phenotype (Table 1). Interestingly, the K461A mutant eluted from DNA–cellulose indistinguishably from wild-type receptor, whereas nonspecific DNA affinity was reduced significantly by the K461E mutation (Table 2); elution of the K461E receptor at ~70 mM NaCl lower concentration than that required for wild type and

K461A corresponds to an ~10-fold reduction in nonspecific DNA affinity (Yamamoto et al. 1976). Replacement of the positively charged K461 by the bulky, negatively charged glutamate residue may preclude DNA binding.

As with K461, mutation of the base contact residue V462 to glutamate abolished growth inhibition by the P493R receptor and impaired nonspecific DNA binding. Finally, we mutated residue R466, which appears to

Figure 4. Control of VP16 toxicity by the receptor zinc-binding region. Strain YPH499 was transformed with 2 μ plasmids expressing receptor derivatives or GAL4–VP16 under control of the GPD promoter and incubated on selective plates for 4 days at 30°C. VP16 chimeras are as in Figure 3, but the receptors (N-VP16-556 and N-VP16-556P493R) lack the signaling domain (amino acids 557–795); such truncations are constitutively active. Microscopic examination showed an equivalent number of microcolonies for N-VP16-556P493R and GAL4–VP16.

Figure 5. Self-squelching by the mutant receptors in yeast and mammalian cells. (A) Protein levels. The indicated receptors were expressed from 2μ or CEN/ARS expression vectors in yeast strain YPH500 and detected by immunoblotting 50 μg of cell extract. (wt) Wild-type; (P) P493R; (S) S459A. Migration positions of molecular weight markers are shown. (B) Transcriptional activation in yeast. GRE-linked transcriptional activation in the strains from A was measured from a 3×GRE-CYC1-lacZ reporter (pΔS-26X) in the presence (solid bar) or absence (stippled bar) of 1 μM DAC and expressed as a percentage of β-galactosidase activity obtained from the wild-type receptor expressed on a 2μ plasmid. Data are the average of two transformants, and error bars represent standard error of the mean. (C) Transcriptional activation in mammalian cells. Transcriptional activation from a 3×GRE-Adh-luciferase reporter was assayed in mouse F9 cells transiently transfected with the indicated amount of receptor expression vector. Dexamethasone was included in all experiments at a concentration of 0.1 μM. Luciferase activity was normalized to a β-galactosidase expression plasmid as a control for transfection efficiency. Data are the average of two transfections and error bars represent standard error of the mean.

make both a specific base contact and a nonspecific phosphate contact. The R466A mutant, which presumably eliminates those contacts, abolished both growth inhibition and squelching associated with P493R, and significantly reduced the nonspecific DNA affinity of the zinc-binding region. Thus, three independent second-site mutants that significantly diminished nonspecific DNA binding also abrogated the P493R mutant phenotype (Tables 1 and 2).

We next tested mutations that affect the dimer interface, R488A and the double mutant R479A/N491A; these lesions abolish cooperative binding of receptor monomers in vitro and transcriptional activation in vivo (Thomas 1993; J.R. Thomas, J.N. Miner, W. Liu, M.d.M.

Vivanco, and K.R. Yamamoto, in prep.). We found that the dimer interface mutations had no effect on the squelching of UAS_G when tested in the context of the P493R mutant receptor; dose-response studies confirmed this equivalence over a wide range of hormone concentrations (data not shown). However, the dimerization mutants substantially reduced the magnitude of growth inhibition by P493R. Thus, receptor dimerization may be important for growth inhibition but not for squelching of UAS_G. This suggests that although both squelching of UAS_G and growth inhibition may reflect titration of transcription factors by the P493R receptor, the two phenotypes may involve distinct targets (see Discussion).

Table 1. Requirements for receptor DNA binding and dimerization

Mutant	Contact ^a	Growth defect	UAS _G activity
Experiment 1			
wild type		–	0.87 ± 0.10
P493R		+++	0.26 ± 0.01
P493R + K461A	specific base	+++	0.23 ± 0.03
P493R + K461E		–	N.D.
P493R + V462E	specific base	–	N.D.
P493R + R466A	specific base and non-specific PO ₄	–	1.14 ± 0.07
Experiment 2			
P493R		+++	0.18 ± 0.01
P493R + R479A/N491A	dimerization	+	0.10 ± 0.02
P493R + R488A	dimerization	+	0.16 ± 0.02

Double mutants of P493R and other residues in the zinc-binding region were tested for the growth defect and squelching of UAS_G as in Fig. 3. Squelching was slightly stronger in the experiment testing the dimerization mutants; hence, the experiment is listed separately. Values represent the average determined from two transformants, and the standard error of the mean for each experiment is reported. (N.D.) Not determined. ^aThe role of the second mutation only, based on Luisi et al. (1991), and W. Xu and P. Sigler (pers. comm.).

Taken together, these results indicate that specific DNA binding is not required for squelching of UAS_G or growth inhibition by P493R and suggest that the mutant phenotypes do not arise from binding of the receptor to particular sites in the yeast genome. In contrast, mutations that disrupt nonspecific DNA binding abolish the P493R phenotypes, perhaps indicating that receptor

Table 2. DNA–cellulose chromatography of zinc-binding region mutants

T7X556 derivative	Elution from DNA–cellulose (mM NaCl)
wild type	220–235
P493R	295
S459A	295–310
K461A	235
K461E	160
V462E	160
R466A	160
R479A/N491A	235
R479A/N491A/P493R	295

Crude protein extracts were prepared from *E. coli* expressing wild-type or mutant T7X556 derivatives of the GR, which encompass the zinc-binding region. Approximately 200 ng of T7X556 in 125 µg of total extract protein was loaded onto a 1-ml DNA–cellulose column at 4°C and eluted with a 100–400 mM NaCl gradient. One-milliliter fractions representing a 15 mM portion of the gradient were analyzed by dot blotting and immunodetection with the receptor-specific monoclonal antibody BuGR2; the peak elution of T7X556 was identified by densitometry.

must be nonspecifically bound to exert its effects; conceivably, of course, these mutations might also affect other potential activities of the zinc-binding region not tested here. In any case, the correlation between the mutant phenotypes and nonspecific DNA binding might reflect either interactions of the receptor with other DNA-bound proteins, or a requirement for nonspecific DNA contacts to stabilize receptor structure.

Specific and nonspecific binding energetics

Our mutational analysis suggested that an important difference between the P493R and S459A mutants and the wild-type receptor might be found in their behavior on nonspecific DNA. This inference was reinforced by the elevated NaCl concentrations required for their elution from DNA–cellulose (Table 2), indicating additional interactions on nonspecific DNA sufficient to produce roughly a 10-fold increase in affinity.

To better understand the differences in binding, we sought to dissect the DNA interactions into phosphate contacts with the DNA backbone and interactions with the DNA bases. We examined the salt dependence of wild-type and P493R-specific and nonspecific binding. Salt dependence of protein–DNA affinity reflects the stoichiometric participation of salt cations in protein–DNA interactions [the polyelectrolyte effect (Record et al. 1976)]. Proteins binding to the phosphate backbone of DNA displace bound cations (0.88 monovalent cations per phosphate). The dependence of the logarithm of the equilibrium binding constant K_A on the logarithm of the salt concentration directly reflects the stoichiometry of cation displacement in the binding reaction: Hence, the slope of this plot is equal to the number of displaced cations, or the number of phosphate contacts multiplied by 0.88.

To determine the salt dependence of receptor binding, we turned to fluorescence polarization, a solution-based technique in which the molecular volume of a fluorescent probe, excited by a polarized source, is measured by the effect of its tumbling rate on the polarization of emitted fluorescence. With a fluorescently labeled DNA probe, the technique yields quantitative information about the affinity and stoichiometry of protein binding (Fernando and Royer 1992). We directly measured specific binding to a fluorescein-labeled GRE; to determine the nonspecific affinity, we titrated a fixed concentration of receptor and GRE with increasing amounts of nonspecific competitor, calf thymus DNA (Linn and Riggs 1972). A plot of the logarithm of the apparent association constants, which subsume any cooperativity, versus the logarithm of the KCl concentration is shown in Figure 6. This analysis ignores receptor dimerization, but a two-site cooperative computer model, explicitly considering dimerization and utilizing the entire binding curve, yielded similar results (C. Royer, pers. comm.). The slopes of wild-type and P493R binding to specific and nonspecific DNA were all similar, indicating that a similar number of phosphate contacts are made in each case. [The number of phosphate contacts predicted from the

slope of the wild-type receptor binding to a GRE is 5.0 ± 0.2 per monomer, in agreement with the five phosphates contacted by the specifically bound monomer in the GR-GRE complex crystallographic structure [Luisi et al. 1991].

Because each binding reaction examined in Figure 6 involved the same number of phosphate contacts, the differences in affinity (i.e., the vertical displacement of each plot) probably result from nonelectrostatic forces. Thus, the 1700-fold preference of wild-type receptor for GREs over nonspecific binding sites would appear to derive from such forces as hydrophobic effects, hydrogen bonds, and van der Waals contacts. Interestingly, the interaction of P493R with nonspecific DNA, compared to nonspecific binding by the wild-type receptor, also seemed to involve increased nonelectrostatic forces. In this respect, the P493R mutant could be viewed as binding to nonspecific DNA in a manner reminiscent of the wild-type receptor bound to specific DNA.

Evolutionary patterns within the intracellular receptor superfamily

We examined the conservation of positions 459 and 493 in the intracellular receptor gene superfamily, which is represented in all metazoan phyla studied to date [Amoro et al. 1992; Laudet et al. 1992]; interestingly, no superfamily members have been found in yeast. Comparison of the equivalent positions of S459 and P493 in the conserved zinc-binding regions of all other members of the superfamily (Table 3) revealed two remarkable patterns.

First, the position 459 and 493 equivalent residues are well-conserved: 53 of the 72 members of the superfamily have a glycine at 459 and a glutamine at 493. Besides the

Table 3. Comparison of position 459 and 493 equivalents in the intracellular receptor superfamily

	Members	459 Equivalent	493 Equivalent
GR family	4	S	P
FTZ-F1 family	4	S	P
MB67	1	G	P
HNF4, B280.8, <i>tll</i>	3	G	R
<i>knirps</i> , <i>knrl</i> , <i>egon</i>	3	G	K
ERR1, ERR2	2	A	Q
ZK418.1, <i>odr-7</i>	2	A	R
All other superfamily members	53	G	Q

Sequence data are from the complete nonredundant peptide sequence data base of the National Center for Biotechnology Information as of July 12, 1994; the *odr-7* sequence was a personal communication from P. Sengupta, H. Colbert, and C. Bargmann. Alternative splice products, oncogenic mutants, and obvious homologs across species were not scored as separate members. Italics indicate substitutions that lead to transcriptional interference by overexpressed GR.

GR pattern of a serine at 459 and proline at 493, the only other variations are alanine at 459 (ERR1, ERR2) and arginine [*tailless* (*tll*), HNF-4, *Caenorhabditis elegans* open reading frame (ORF) B280.8] or lysine [*knirps*, *knirps-related* (*knrl*), *embryonic gonad* (*egon*)] at 493. The *C. elegans* ORF ZK418.1 and *odr-7* gene possess both alanine at 459 and arginine at 493. Several other *C. elegans* unpublished superfamily members show identical patterns (A. Sluder, pers. comm.). Alanine and arginine are precisely the residues at 459 and 493, respectively, that yield the squelching phenotype in the GR. A lysine, but

Figure 6. Apparent affinity of wild-type (WT) and P493R receptors for specific and nonspecific DNA as a function of KCl concentration. DNA binding by purified WT and P493R T7X556 receptor derivatives was determined from the increasing fluorescence polarization of a 27-bp duplex oligonucleotide containing an idealized palindromic GRE and bearing a fluorescein at each 5' end (see Materials and methods). The apparent K_d of the receptor for specific binding at various KCl concentrations was measured by titrating receptor protein (1–1800 nM) onto the fluorescent DNA (1 nM) and identifying the concentration of receptor that increased fluorescence polarization to 50% of the level of the specific binding plateau. For nonspecific binding, calf thymus DNA was titrated (1 μ M to 2 mM bp) into an assay containing 1 nM fluorescent GRE and 500 nM purified T7X556; the con-

centration of competitor DNA that reduced polarization to 50% of the specific binding plateau ($D_{1/2}$) was identified. The apparent K_d of receptor for the calf thymus DNA was computed at each KCl concentration by the formula $K_d(\text{nonspecific}) = 2K_d(\text{specific})D_{1/2}/[2(\text{GR}) - (\text{GRE}) - 2K_d(\text{specific})]$ (Lin and Riggs 1972). The necessary assumption that an insignificant fraction of the competitor is bound was satisfied at all competitor concentrations. The standard error in the slope, and in the y -intercept (extrapolated K_A at 1 M KCl), of the least-squares fit to the experimental points is indicated.

not other amino acids, at position 493 of the GR also produced growth inhibition and squelching, albeit less effectively than did P493R; S459R did not inhibit growth (Thomas 1993). We do not have enough biochemical information about these superfamily members to infer how these substitutions might affect function, but the observed patterns suggest that the S459A and P493R mutations produce regulators with specific functional conformations, rather than merely aberrant proteins.

Second, with the exception of the recently discovered human MB67, all superfamily members that have a serine at the 459 equivalent position have a proline at 493, and vice versa. This correlation encompasses the closely related members of the GR family, receptors for mineralocorticoids, progestins and androgens, as well as the more distantly related FTZ-F1 family, which includes the *Drosophila* FTZ-F1 regulator and its mouse homolog ELP. This coconservation of S459 and P493 suggests that they form a functional connection in the operation of the zinc-binding region, perhaps serving in concert as determinants of protein conformation.

Discussion

A model: DNA-mediated allostery regulates transcriptional activation domains

We have isolated two mutants in the zinc-binding region of the GR, S459A and P493R, that interfere with transcriptional activation by certain regulatory proteins when overexpressed in yeast or mammalian cells. We have shown that this effect does not require specific DNA binding by the receptor, that it operates in different promoter contexts, and that the mutant receptors are activators, not repressors, when bound in the vicinity of a promoter. Furthermore, the behavior of the mutant receptors is similar in yeast and mammalian cells and is dependent on the intracellular concentration of the receptor. Therefore, transcriptional interference by the mutant receptors cannot arise from effects on general cell physiology, or from aberrant activation or site-specific repression of a particular locus.

The requirement for an activation domain suggests that transcriptional interference operates by squelching, whereby the receptor sequesters targets required by other transcriptional activators. We conclude that the mutant receptors bind one or more such targets under circumstances in which the wild-type receptor does not. Based in part on the position of the mutants in the structure of the zinc-binding region (see below) and on the assumption that the wild-type receptor must contact its targets when it occupies a GRE, we suggest that specific DNA acts as an allosteric effector to regulate receptor interaction with transcriptional targets. There are two key postulates in this scheme: (1) The receptor undergoes a conformational change when it binds specifically to a GRE; and (2) only GRE-bound receptor contacts its targets.

Our interpretation of the P493R and S459A mutations is that they mimic the effect of specific DNA. Conse-

quently, all of the hormone-receptor complexes in the nucleus assume the active (GRE-bound) conformation and can bind to target proteins. Under conditions of receptor overexpression, only a small fraction of total cellular receptor actually occupies specific sequences, particularly in yeast, which has no known natural GREs. Hence, the P493R and S459A mutations would increase drastically the number of receptors that bind their protein targets, resulting in self-squelching and in squelching of cellular promoters.

It is likely that virtually all receptors not bound to GREs are bound nonspecifically to DNA, as the high intranuclear DNA concentration will drive those low-affinity interactions (Lin and Riggs 1975; Yamamoto and Alberts 1975). In that context, it is notable that second-site mutations incompatible with nonspecific DNA binding abrogated the mutant phenotypes. This implies that the two states of the receptor postulated in our model correspond to the specifically and nonspecifically bound conformations of the receptor. That is, binding to specific GREs, but not to nonspecific DNA, triggers the active conformation; in contrast, the mutant receptors may assume the active conformation even on nonspecific DNA.

An alternate interpretation is that nonspecific DNA binding is sufficient to facilitate contact between the receptor and its targets, and that the mutants simply increase the numbers of receptors nonspecifically bound. Although such a hypothesis is not explicitly excluded by our experiments, we regard it as untenable on three grounds. First, experiments with chimeric receptors (J.A. Lefstin and K.R. Yamamoto, unpubl.) indicate that the GR zinc-binding region uniquely potentiates *enh2* toxicity, and even heterologous DNA-binding domains with greater DNA affinity and more active nuclear localization functions cannot substitute. Second, the R479A/N491A mutation selectively reduces growth inhibition without reducing nonspecific affinity; consequently, the mutants' nonspecific affinity alone cannot account for the growth defect. Third, functional studies (Fig. 5B,C) suggest indistinguishable DNA occupancy *in vivo* by the full-length wild-type and mutant receptors.

DNA binding by the wild-type and mutant receptors

The *in vitro* DNA-binding properties of the wild-type and P493R receptors were consistent with the hypothesis that the nonspecifically bound mutant receptors mimic certain properties of the specifically bound wild-type receptor. At 140 mM salt, we found that sequence-specific binding by the wild-type receptor involved 4.4 kcal/mol of apparently nonelectrostatic contacts, providing ~1700-fold binding selectivity for GREs relative to nonspecific DNA. The P493R and wild-type receptors appeared to make the same number of phosphate contacts; however, P493R interacted with nonspecific DNA with an additional 1.2 kcal/mol of free energy relative to the wild-type receptor. One interpretation is that P493R, unlike wild-type, may be able to make close contacts in the major groove of nonspecific DNA, perhaps mediated

by water molecules, which might be forbidden to the wild-type receptor. Alternatively, the added free energy might reflect a more favorable conformation for P493R that makes nonspecific contacts similar to wild type. Either scheme is consistent with differential binding energetics in which the P493R mutant on nonspecific DNA resembles the specifically bound wild-type receptor. Whether such behavior is truly the product of conformational change awaits structural analysis of the mutants.

Nature of the P493R and S459A mutants

In the structure of the receptor–GRE complex (Fig. 7), P493 occupies a turn between two helical regions of the zinc-binding region and S459 is on the back side of the recognition helix, facing away from DNA. Rather than contacting base pairs in the major groove, S459 connects

by hydrogen bonds the recognition helix to other portions of the zinc-binding region (residues 445, 489, and 496). Mutation of S459 to alanine would disrupt this hydrogen-bonding network, perturbing the interaction of the recognition helix with the rest of the protein. In addition, S459 is one of three residues of the zinc-binding region responsible for discriminating between GRE and estrogen response element (ERE) half sites (Zilliacus et al. 1992). Because S459 does not contact DNA directly, its effects on specificity may be indirectly transmitted through the conformation of the zinc-binding region.

P493 and S459 are linked by a region of the receptor (amino acids 486–491, shown in blue in Fig. 7) that forms a distorted helix in the crystal structure of the glucocorticoid receptor zinc-binding region–GRE complex. Conflicting nuclear magnetic resonance (NMR) studies have argued both that this portion of the receptor may be extended in solution (Härd et al. 1990; van Tilborg et al.

Figure 7. The structure of the GR zinc-binding region bound to a palindromic GRE, as inferred by X-ray crystallography (Luisi et al. 1991). (Yellow) P493 and S459. The distorted helix (486–491) shown in blue may be relatively unstable in the absence of specific DNA and may be stabilized upon DNA binding. (White) Dimerization and DNA contacts mutated in Table 1. The residues in the central dimerization interface are (top to bottom) R479, R488, and N491; the base contacts protruding from the recognition helices are (outer to inner) K461, V462, and R466. R489 (violet).

1995) and that it is helical in solution (Baumann et al. 1993); the occurrence of both helix and loop forms in the crystal structure of the estrogen receptor zinc-binding region–ERE complex suggests that this helix may be metastable, perhaps induced or destabilized by protein–protein or protein–DNA contacts (Schwabe et al. 1993a). We suggest that the P493R and S459A mutations might simulate GRE-mediated allosteric changes in the zinc-binding region by modulating the state of the distorted helix or by altering the conditions under which it interacts with other regions of the receptor protein.

Roles of the zinc-binding region

Although activation domains like *enh2* and VP16 efficiently enhance transcription when fused to heterologous DNA-binding domains like those of LexA and Gal4, we propose that these same activation domains can be functionally modulated by the DNA-binding domain of the GR. This apparent paradox may be rationalized in part by the multiple activities resident in the receptor zinc-binding region, together with the multifaceted complexity of activation domains and of transcriptional initiation machineries; different surfaces of these multiprotein structures may correspond to distinct activities that operate only in certain molecular contexts. By this view, transcriptional activation from a simple test promoter may use only a subset of the functions of the activation domain. However, we also note that perhaps not all *enh2* functions are modulated by the zinc-binding region.

Dimerization by the GR zinc-binding region might potentiate the formation of certain transcriptional activation surfaces. Unlike Gal4(1–147) (Carey et al. 1989), the zinc-binding region of the receptor fails to dimerize in solution (Hård et al. 1990) but dimerizes cooperatively on GREs. Conceivably, the P493R and S459A mutants might mimic a GRE-induced conformational change, leading to dimerization on nonspecific DNA, formation of an activation surface, and contact with a target factor in the absence of a GRE. In support of this model, LexA(1–87)–VP16 chimeras, which lack a strong dimerization domain, are not growth inhibitory in yeast, whereas LexA(1–202)–VP16 chimeras, which include a dimerization domain, inhibit growth (N. Silverman and L. Guarente, pers. comm.). We found that mutation of zinc-binding region dimerization contacts reduced growth inhibition by P493R, implying that the mutant receptors are dimerized when they exert their effects.

However, dimerization alone cannot account for the mutant phenotypes, as the P493R mutant with altered dimer contacts remained fully capable of squelching UAS_G. *Enh2* does not contain a single discrete, activation domain (Fig. 3) but may consist of several distinct transcriptional regulatory functions (Tasset et al. 1990). We speculate that one activation surface produced upon dimerization may contact a target important for yeast viability; in contrast, a separate activation surface, requiring distinct determinants in the zinc-binding region, might contact a target required by Gal4. The zinc-bind-

ing region might itself generate an activation surface that can contact target factors and act synergistically with the second domain. The latter possibility is consistent with the finding that the zinc-binding region alone can activate transcription, albeit weakly, in certain contexts in vivo and in vitro (Hollenberg et al. 1987; Miesfeld et al. 1987; Hollenberg and Evans 1988; Freedman et al. 1989).

Another explanation for the effects of the mutants is that the zinc-binding region might negatively affect transcriptional activation domains unless specifically bound to DNA; such inhibition might operate directly, by masking activation domains, or indirectly, by association with proteins that block interactions of activation domains with their targets. Analogous inhibition of native and heterologous transcriptional activation domains has been described for yeast heat shock factor (HSF); mutants that release activation domains from low-temperature repression are clustered in the DNA-binding domain of HSF (Bonner et al. 1992).

Implications

The precise relationship of *enh2* and the zinc-binding region, and the perturbations induced in the zinc-binding region by P493R and S459A, await more detailed structural studies. Clearly, however, the yeast growth defect induced by the P493R and S459A mutants affords an opportunity to define genetically other proteins that interact with the GR: Yeast mutants that suppress growth inhibition by these mutants might define targets of transcriptional activation or factors involved in receptor signaling.

Our model extends the general notion that the activity of transcriptional regulators is not fixed but, instead, depends on other components, including the DNA sequences that they occupy. Conceivably, such a mechanism might serve to prevent squelching effects in vivo; however, we suspect that squelching effects observed under conditions of receptor overexpression are insignificant at physiological receptor levels.

Our work suggests an alternative rationale, based on the notion that the GR carries a suite of potential functional surfaces that can be exposed when it binds to specific DNA sites. In principle, the choice of functional surfaces displayed by the GR might be determined not merely by specific DNA binding, but by the particular sequence to which the receptor binds: That is, there may be a repertoire of GREs that can specify various unique functional conformations of the GR. A single factor could achieve considerable regulatory versatility if a combination of both protein–protein and protein–DNA contacts specified its contribution to regulation of the transcription complex.

Materials and methods

Yeast strains and media

Yeast strains YPH499 (*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*) and YPH500 (*MATα ura3-52 lys2-*

801 *ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*) have been described (Sikorski and Hieter 1989). Strain CY341 was derived from YPH499 but contains a fully functional *GAL2* allele (gift of C. Peterson, University of Massachusetts, Worcester). Yeast strains were grown at 30°C in minimal medium with amino acids and 2% glucose, except for galactose induction experiments. The appropriate amino acids were omitted to select for retention of plasmids. Yeast strains were transformed by the lithium acetate method (Gietz et al. 1992). For plate assays of the growth defect, the synthetic glucocorticoid deacetylcortivazol (DAC, gift of S. Simons, National Institutes of Health, Bethesda, MD) was added to plates at a final concentration of 1 μM from a stock solution of 10 mM dissolved in ethanol. The growth defect was assayed after 3 days of growth at 30°C.

Yeast plasmids

TRP1 plasmids (2 μ) expressing the 795 amino acid rat GR from the GPD promoter were based on pG-1 (Schena et al. 1991). A construct replacing amino acids 154–406 of the receptor with amino acids 413–490 of VP16 (pG-N-VP16-795) was a gift of P. Godowski (Genentech). A deletion of residues 154–406 (Δ6) was accomplished by digesting this construct at the genomic *SalI* site and a *BamHI* site introduced at residue 407, blunt-ending with Klenow fragment, and religation; this operation inserts the nonreceptor residues RSP between 153 and 407. The Δ1, Δ2, Δ4C1, and Δ5 deletions have been described (Miesfeld et al. 1987). For expression of the receptor on CEN/ARS plasmids (pRS314-GN795), a *SpeI*-*NaeI* fragment of pG-N795 containing the GPD promoter, GR cDNA, and phosphoglycerokinase terminator was inserted into the polylinker of pRS314 (Sikorski and Hieter 1989) cut with *SpeI* and *SmaI*. P493R, S459A, and other zinc-binding region mutants were constructed by oligo-directed mutagenesis. The pG-GAL4-VP16 plasmid (gift of S. Yoshinaga, Amgen) was constructed by inserting a *BglIII*-*BclI* fragment of a GAL4(1–147)-VP16(413–454) fusion (gift of M. Carey, University of California, Los Angeles) into the *BamHI* site of pG-1. Reporter plasmids pΔS-26X (3×GRE-CYC1-*lacZ*) (Garabedian and Yamamoto 1992), pLGSD5 (UAS_G-CYC1-*lacZ*) (Guarente et al. 1982), pSV14 (GAL4 17-mer-CYC1-*lacZ*) (Giniger et al. 1985), and pLDA-241 (*CUP1-lacZ*) (Durrin et al. 1992) have been described.

Immunoblotting

Exponentially growing 10-ml cultures of YPH500 bearing GR expression plasmids were harvested, washed in 1 ml of high salt extraction buffer (400 mM NaCl; 10 mM Tris-HCl at pH 7.5; 1 mM EDTA, 0.1% Triton X-100; 1 mM DTT; 1 μg/ml of aprotinin, pepstatin A, and leupeptin; 1 mM PMSF) and resuspended in 200 μl of high salt extraction buffer. Glass beads were added and the cells shaken for 20 min at 4°C. The extract was collected by puncturing the bottom of the tubes and performing centrifugation into another tube. Extracts were centrifuged at 14,000g for 10 min to remove insoluble material, and the protein concentration (7–8 μg/μl) was determined by the Bio-Rad Protein Microassay. Western blots were probed with the BuGr2 anti-receptor monoclonal antibody (Gametchu and Harrison 1984).

RNA analysis

Cultures (100 ml) of yeast strain CY341 transformed with GR expression plasmids were grown to an OD₆₀₀ of ~0.5 in selective 2% glycerol–2% ethanol medium. Each culture was split into two 50-ml aliquots and treated with 50 μl of 1 mM DAC dissolved in ethanol or 50 μl of ethanol alone. After 10 min at 30°C, 2.5 ml of a 40% galactose solution was added. After 10

min more at 30°C, yeast cells were harvested by centrifugation, frozen in liquid nitrogen, and stored at –80°C. RNA was isolated from the frozen cells by vortexing with glass beads in phenol–chloroform followed by ethanol precipitation. Primer extension analysis of *GAL1* and *U5* RNA was carried out as described (Peterson and Herskowitz 1992). The oligonucleotides GCGCTAGAAATTGAACTCAGG and AAGTTCCAAAAAAT-ATGGCAAGC were used to detect the *GAL1* and *U5* transcripts, respectively. Quantitation of a primer extension of the long-lived *U5* transcript (using a Molecular Dynamics PhosphorImager), which was not affected by the experimental conditions, was used to normalize the amount of RNA extended with the *GAL1* probe (~12 μg).

β-Galactosidase assays

Cultures (2 ml) in the appropriate selective media were grown overnight, after which 0.5 ml was added to 2 ml of fresh media containing either 2.5 μl of 1 mM DAC in ethanol or 2.5 μl of ethanol alone. For assays of Gal4 activity, 1.5 ml of culture grown in glucose-containing medium was harvested, washed with 1 ml of water, and resuspended in 100 μl of water. Then 50 μl was added to 2 ml of 2% galactose medium containing DAC or ethanol. Assays of *CUP1* promoter activity included copper sulfate at 1 mM. Yeast were cultured for 6–8 hr at 30°C and 1.5 ml were then harvested for β-galactosidase assay. A modified permeabilized cell assay (Garabedian and Yamamoto 1992) was used to assay β-galactosidase activity. Enzymatic units were computed by the formula $(1000 \times \Delta OD_{420}) / (OD_{600} \times \text{culture volume assayed (ml)} \times \text{time (min)})$.

Transfections

F9 mouse embryonic carcinoma cells were grown and transiently transfected as described (Miner and Yamamoto 1992). Expression vectors for the GR (p6R-GR) and β-galactosidase transfection controls (p6R-βgal) were based on the plasmid p6R, containing the Rous sarcoma virus enhancer and promoter region and the SV40 transcription termination and polyadenylation sequences. The reporter plasmid pΔTAT3-luciferase contained three copies of the GRE from the tyrosine aminotransferase gene upstream of the *Drosophila Adh* distal promoter (–33 to +53) driving firefly luciferase. Each transfection included 2 μg of reporter plasmid and 0.2 μg of β-galactosidase control plasmid; empty p6R expression vector was added so that each pool of transfected cells received 3.2 μg of DNA.

Luciferase activity was determined by resuspending PBS-washed cells in 120 μl of reporter lysis buffer (Promega) and incubating at room temperature for 15 min. Cellular debris was removed by centrifuging for 4 min at 15,000g at 4°C. Then 10 μl of supernatant was assayed for luciferase activity using 100 μl of luciferase assay (Promega) in a Monolight 2001 luminometer, counting integrated light for 30 sec at room temperature. After background subtraction, luciferase activity was normalized to the β-galactosidase internal transfection control. Activity of the RSV long terminal repeat used to express the GR and β-galactosidase was not affected by experimental conditions.

DNA–cellulose chromatography

Crude protein extracts were prepared from 100-ml cultures of *Escherichia coli* BL21(DE3)/pLysS expressing T7X556 derivatives by deoxycholate lysis, polyethyleneimine precipitation, and ammonium sulfate fractionation as described by Freedman et al. (1988). T7X556 represents a receptor fragment encompassing the zinc-binding region, amino acids 407–556. Extracts (200

μ l) were dialyzed against HGED50 buffer (50 mM HEPES at pH 7.6, 10% glycerol, 0.5 mM EDTA, 2.5 mM DTT, 0.5 mM PMSF, 50 mM NaCl), and aliquots were stored at -80°C . Immunoblotting with the BuGR2 monoclonal antibody confirmed the presence of the T7X556 protein; ~ 40 ng/ μ l of T7X556 was present in extracts containing 25 μ g/ μ l of total protein. No cross-reacting *E. coli* proteins could be detected. Specific GRE binding of wild-type, P493R, and S459A T7X556 extracts was confirmed by gel mobility shift analysis.

DNA-cellulose chromatography was carried out at 4°C on a Pharmacia FPLC system. Five microliters of crude extract diluted in 200 μ l of HGED50 plus 50 μ M zinc sulfate and 10 μ g/ml of BSA carrier was loaded onto a 1-ml DNA-cellulose column (1 mg/ml of salmon sperm DNA) at 0.1 ml/min, and the column was washed with 4 ml of buffer at 0.2 ml/min. A 100–400 mM NaCl gradient extending over 20 ml was then applied to the column at 0.2 ml/min, and 1-ml fractions were collected. Samples (200 μ l) of each fraction were transferred to Immobilon-P membranes using a Milliblot dot blotter. The amount of T7X556 in each fraction was determined by developing the membrane as described for immunoblotting and computerized scanning of the developed membrane. Samples of purified T7X556 proteins eluted at identical positions in the gradient as the T7X556 in crude extracts.

Determination of DNA-binding affinities

Recombinant T7X556 derivatives of wild-type and P493R were expressed in *E. coli* BL21(DE3)/pLysS and purified to apparent homogeneity as described by Freedman et al. (1988). Protein concentration was determined relative to BSA standards by the Bio-Rad Protein Microassay. GRE oligonucleotides were synthesized by the Howard Hughes Medical Institute (University of California at San Francisco) with a 5' fluorescein-ON group and purified by OPC columns (Applied Biosystems). The GRE probe was a 27-bp complementary duplex, having fluorescein groups at both 5' ends, with the sequence (top strand, receptor half-sites in boldface) F-GTTGCC**AGA**ACATGATGTTCTAATC-TG; flanking sequences were chosen to minimize hairpin loop formation and cryptic receptor binding. Oligonucleotides were quantitated by ultraviolet absorption and annealed in 500 mM KCl. For specific affinity determination, 1.5-fold serial dilutions of T7X556 were added to 20 borosilicate glass tubes containing 1 nM fluorescent GRE oligonucleotide, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 100 mM KCl (final volume 1.2 ml) such that the final receptor concentration varied from 1800 to 1 nM. The fluorescence polarization of each sample was determined at 23°C using a PanVera Beacon Fluorescence Polarization System (PanVera Corp, Madison, WI); each sample was read twice and the average polarization used. Aliquots of 1 M KCl were added to each sample (final concentrations, 100–183 mM KCl), and the measurements were repeated, following equilibration (<1 min), at the higher salt concentration; the added salt diluted the samples by $<10\%$ at the highest salt concentration. The apparent K_d for receptor binding was determined graphically from a plot of polarization versus the logarithm of protein concentration. Nonspecific higher order complexes were apparent at elevated receptor concentrations (>100 nM at 100 mM KCl) but did not interfere with identification of the specific binding plateau. Competition experiments were conducted similarly, except that a fixed concentration of receptor (500 nM final) was added to reactions containing 1 nM of fluorescent GRE and twofold dilutions of calf thymus DNA (300–3000 bp). Concentration of competitor DNA was calculated assuming that each base pair initiated a potential nonspecific binding site.

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J A Lefstin, J R Thomas and K R Yamamoto

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