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THE CASE FOR DNA MEDIATED ALLOSTERIC REGULATION OF THE GLUCOCORTICOID RECEPTOR

by

JAY RAJAN THOMAS

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY

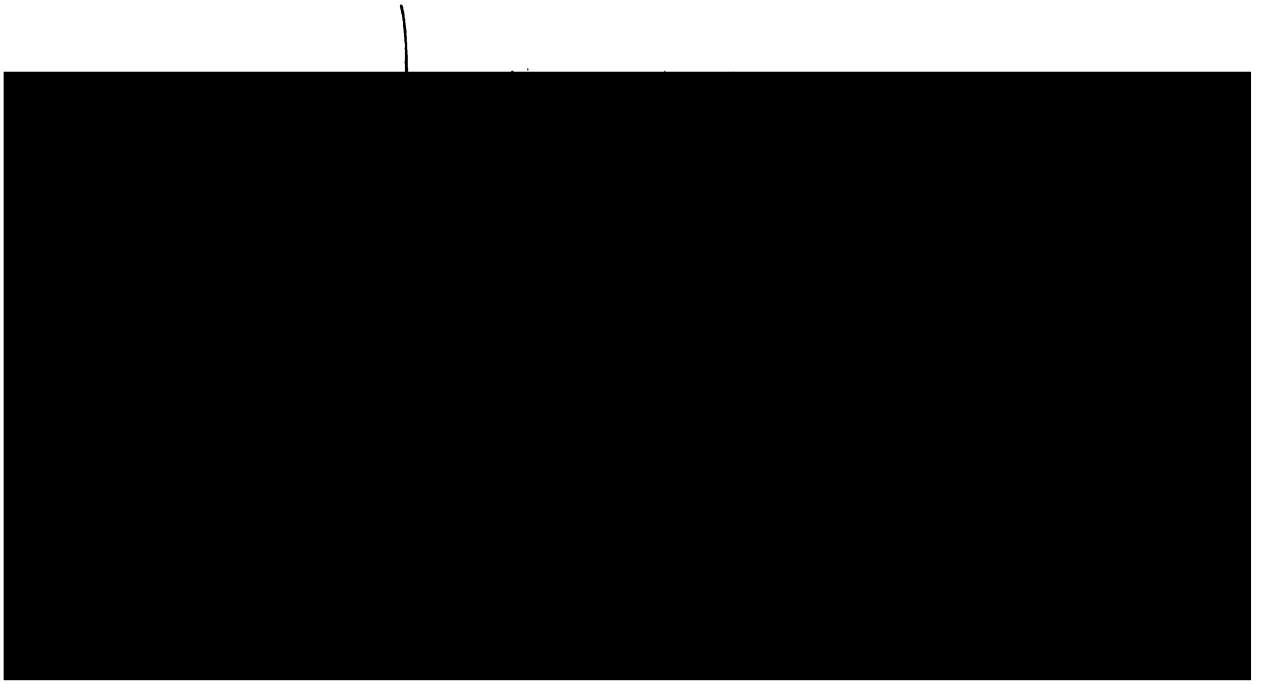
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UNIVERSITY OF CALIFORNIA

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SOLI DEO GLORIA

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I would like to gratefully thank many people for making my graduate career and thesis possible. Celia Schiffer unselfishly provided much time to assist me in manipulating structural information for display and analysis of GR by computer generated graphics. Steve Yoshinaga generously provided purified T7-X556 to use as a standard for Western quantitation and as a control for in vitro DNA binding.

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The Yamamoto lab has been a great environment to work. Soledad DeGuzman exemplifies the quality of people in the lab. She has lavished her love upon all of us with her time, hard work, concern, and lumpia. Jeffrey Lefstin has been instrumental in the advancement of the yeast growth defect mutants project. I hope he has as much fun and reward in carrying on the project as I have had in working with him. I have thoroughly enjoyed my association with all my fellow post-docs and graduate students over the years. They have all been not only excellent scientific colleagues, but people I have been proud to call friends. I would list all their names here if not for space considerations, but my memories of them are firmly etched in my heart and mind.

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Finally, I would like to thank my dear wife, Sandra Holley. Her love and support has filled a void in my life, made me a better person, and given me the courage and strength to pursue what are now our dreams.

THE CASE FOR DNA MEDIATED ALLOSTERIC REGULATION OF THE GLUCOCORTICOID RECEPTOR

BY

JAY RAJAN THOMAS

DR. KEITH R. YAMAMOTO

PH.D. ADVISOR

THESIS ABSTRACT

The glucocorticoid receptor (GR) is a hormone-dependent transcription factor. At a simple glucocorticoid response element (GRE), GR alone enhances transcription. At a composite GRE, GR, together with other proteins, can either enhance or repress transcription. This functional diversity implies regulation. A clue to this regulation comes from structural studies that imply that simple GRE binding promotes dimerization by inducing a conformational change in the GR Zn-finger region—the DNA binding domain.

To test the functional importance of GR dimerization, I mutagenized residues predicted to mediate dimerization. These mutations decreased GR's cooperative binding at a simple GRE without affecting half-site affinity. Functional analysis demonstrated that both simple and composite GRE activation require dimerization. However, composite GRE repression did not require dimerization, implying a GR monomer suffices to form a repressive complex. Because GR is monomeric in solution but can function either as a monomer or a dimer on DNA, its quaternary state may be regulated. DNA sequence and/or protein context at a sequence are the likely regulators.

To test the hypothesis that DNA is an allosteric regulator of GR function, I mutated the Zn-finger region and screened in yeast for altered phenotypes. I

found two point mutants, each of which caused a hormone-dependent yeast growth defect. These mutants had an altered conformation that appears to mimic a conformation that may be induced in wild-type GR at a simple GRE. The functional consequence of this altered conformation was "squelching"—transcriptional inhibition of promoters by sequestering required factors. The phenotype of these Zn-finger region mutants requires the N-terminal enhancement domain, Enh2. This observation together with the fact that isolated Enh2 can cause a similar phenotype implies that the Zn-finger region may regulate the activity of Enh2. Thus, these mutations may induce structural and functional changes that wild-type GR attains only upon simple GRE binding, strongly implying that a simple GRE is an allosteric regulator of GR function.

In general, DNA mediated allosteric regulation of transcription factor activity provides a mechanism for different activities of a factor at different sites, and for avoiding squelching by allowing enhancer function only at specific sites.

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INTRODUCTION

Part I

The Biological Question and Experimental Approach

GR is a hormone-inducible transcription factor that resides in the cytoplasm in the absence of hormone (Picard and Yamamoto, 1987). Upon hormone binding, GR undergoes a temperature dependent "transformation" making it competent to translocate to the nucleus and bind to specific DNA sites, termed glucocorticoid response elements (GREs) (Yamamoto, 1985). From a GRE, GR regulates the transcriptional activity of linked promoters. The type of transcriptional regulation is determined by the class of GRE to which GR is bound, which can be either a simple or a composite GRE.

At a simple GRE, GR is the sole DNA binding factor necessary to activate transcription (Godowski et al., 1988a). A simple GRE has a strong consensus sequence, composed of two inverted half sites separated by three base pairs. In vitro binding analysis has demonstrated that GR binds cooperatively as a homodimer to this site (Perlmann et al., 1990).

At a composite GRE, GR requires other protein factors for activity. For example, at a composite GRE from the proliferin promoter, GR requires the proteins, Jun and Fos, which together comprise the phorbol ester-activated transcription factor, AP1 (Diamond et al., 1990). Despite the fact that this composite GRE has no strong sequence homology to the consensus simple GRE, GR alone can bind to it. Moreover, Jun and Fos can bind to this element. In fact, DNase I footprinting and gel retardation analysis suggest that GR and the AP1 factors can co-occupy the composite element (unpublished observations, M. Diamond and J. Miner). The important observation is that the nature of the complex formed at this site determines its functional attributes. GR, in conjunction with

Jun, directs transcriptional activation. GR, in conjunction with Jun and Fos, at this same composite element, directs transcriptional repression. The stoichiometry of GR, Jun, and Fos in these complexes is unknown. These observations are schematized in Figure 1.

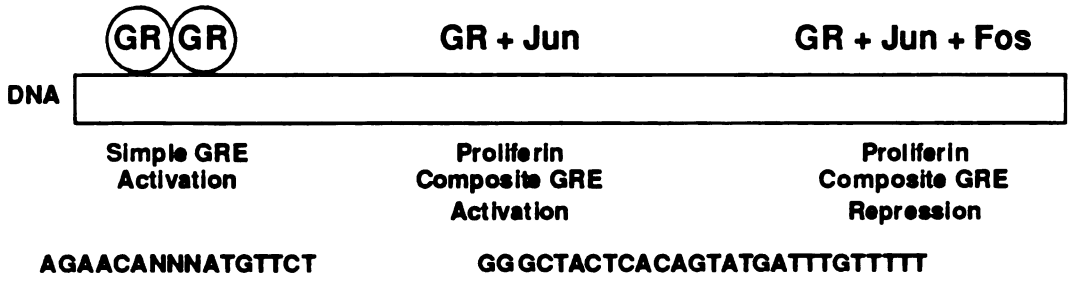


Figure 1

Classes of Glucocorticoid Response Elements

The fact that GR can function so diversely at simple and composite elements implies regulation. The nature of this regulation is the subject of this thesis. In theory, this regulation could be mediated by the DNA site, the protein context at a DNA site, or a combination of both.

Structural studies of the Zn-finger region of GR as a monomer in solution (by 2D-NMR) (Hard et al., 1990) and as a dimer at a simple GRE (by X-ray crystallography) (Luisi et al., 1991) provide a clue to this regulation. A comparison of these two structures led to the hypothesis that a simple GRE may induce a conformational change that promotes dimerization. Therefore, in theory, a DNA site, through inducing a conformational change, could regulate GR function. This phenomenon of DNA mediated allosteric regulation could explain in part how GR is able to function so diversely.

To test this hypothesis of DNA mediated allosteric regulation, I mutagenized the GR Zn-finger region. First, to test the functional role of GR dimeriza-

tion, I engineered mutations at residues predicted from structural analysis to mediate dimerization. I tested these mutants for their ability to bind to both simple GRE half- and full-sites in vitro and to activate and repress transcription in vivo. Second, to find functional support for the hypothesis of DNA mediated allosteric regulation, I mutagenized the Zn-finger region and screened for phenotypes in yeast.

INTRODUCTION

Part II

The Structure of the GR Zn-Finger Region and the Structural Basis For the Hypothesis That DNA May Induce a Conformational Change That Promotes Dimerization

Figure 2a shows the primary sequence of the GR Zn-finger region (amino acids 440-525). Figure 2b shows the structure of a monomer derived from the X-ray study of a dimer of this region at a simple GRE. Figure 2c shows the structure of a monomer of this region in solution derived from 2D-NMR. The region is comprised of two Zn-fingers, each of which uses four cysteines to coordinate a Zn^{2+} ion (shown as a yellow + in Figures 2b and c) with tetrahedral geometry. Each Zn coordination center is followed by an amphipathic α-helix. One of the amphipathic α-helices, composed of residues 457-469, fits into the major groove where it makes sequence specific base contacts at a simple GRE. This DNA recognition helix will hereafter be referred to as helix R and is shown in yellow in Figures 2b and c, looking down the helix. The residues involved in base contacts are denoted with black arrows in Figure 2a. The other amphipathic α-helix, composed of residues 492-503, packs perpendicular to helix R though an extensive hydrophobic core. This perpendicularly packing helix will hereafter be referred to as helix P and is shown in red in Figures 2b and c, as viewed from the side.

Residues 476-491 comprise a region of the second Zn-finger from which all dimer contacts are made. The residues involved in dimer contacts are denoted with a black dot in Figure 2a. This region is shown in cyan in Figures 2b and c. A comparison of this region between the two structures shows a dramatic difference. In Figure 2b, from the X-ray structure of a dimer at a simple GRE, residues

486-491 form a distorted α-helix that does not appear in the solution monomer structure. This distorted helix will hereafter be referred to as helix D (shown in cyan in Figure 2b, looking down the helix). Helix D makes phosphate contacts through residues R489 and K490 (denoted by black rectangles in Figure 2a), makes dimer contacts through residues R488 and N491, and makes intramolecular contacts with other parts of the Zn-finger region through residue R489.

Despite the fact that the 2D-NMR structure was determined at millimolar concentrations of the Zn-finger region, there was no evidence of dimer formation. As a dimer at a simple GRE, the residues involved in dimerization appear to have a different conformation than they have as a monomer in solution. In particular, helix D appears to form. Because helix D makes phosphate contacts, it is possible that DNA binding stabilizes this alternate conformation, thereby facilitating dimer contacts between certain helix D residues.

Based of these structural studies, I propose that a simple GRE may induce a GR conformational change that promotes its dimerization. I have tested the functional importance of dimerization and have screened in yeast for GR mutants that may extend the notion of DNA mediated allostery.

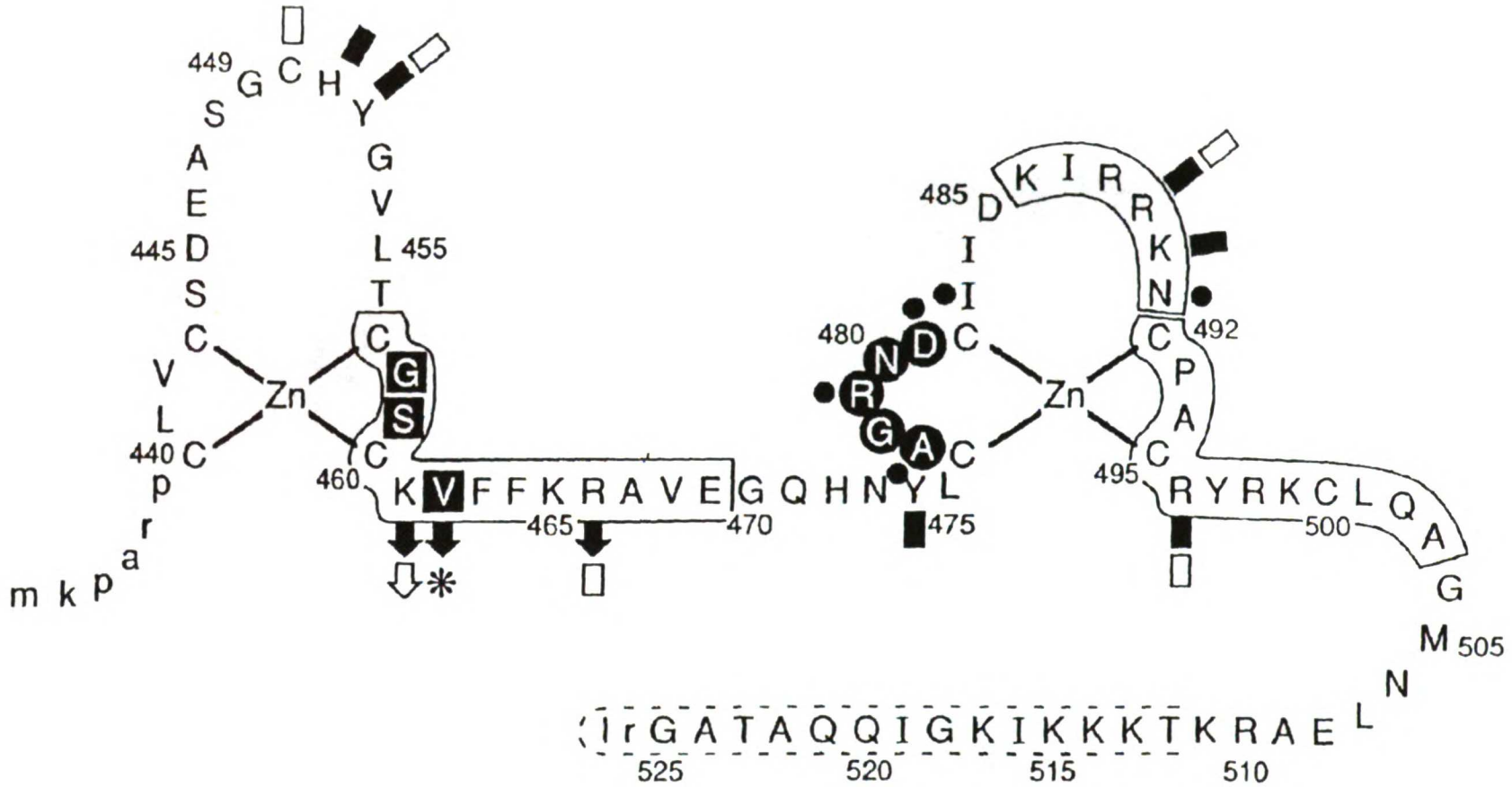


Figure 2a
Primary Sequence of the GR Zn-Finger Region
From Luisis et al. Nature 352:497-505 (1991)

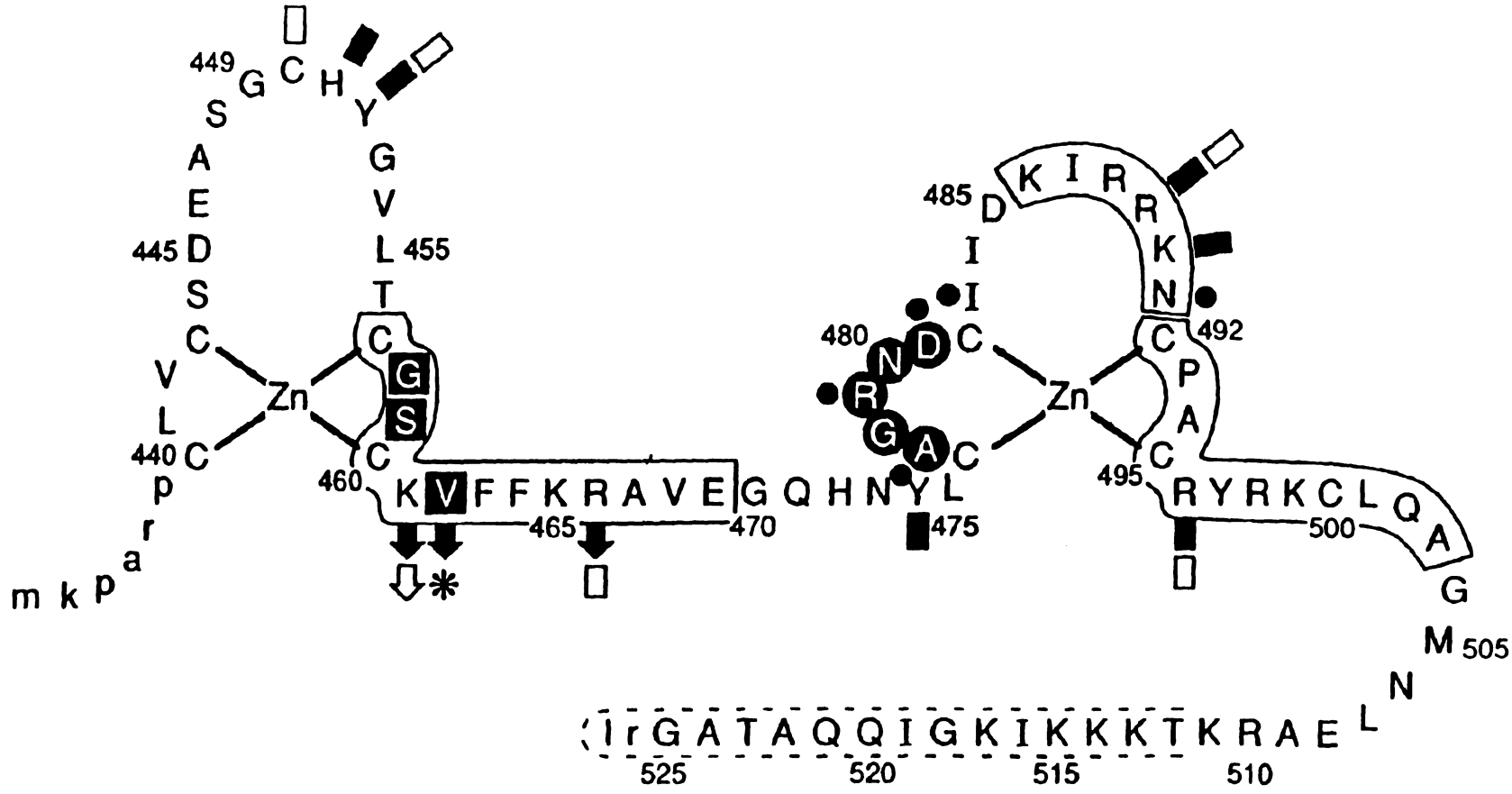


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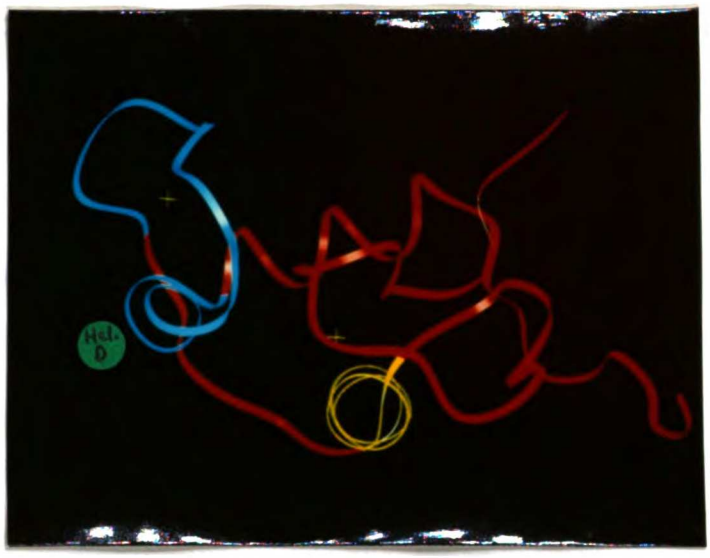


Figure 2b

The Zn-Finger Region Structure Derived from X-ray Crystallography of a Dimer at a Simple GRE

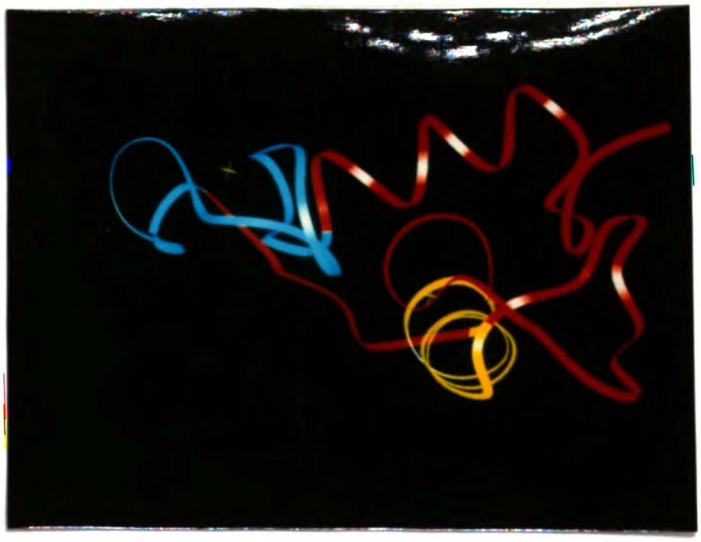


Figure 2c

The Zn-Finger Region Structure Derived from 2D-NMR from a Monomer in Solution

CHAPTER 1

Transcriptional Activation and Repression By The Glucocorticoid Receptor Have Different Requirements For Dimerization

Abstract

X-ray crystallographic analysis of the Zn-finger region of GR has predicted the residues that mediate homodimerization. I have mutated these residues and characterized the mutants for DNA binding in vitro and transcriptional activity in vivo. I found that mutations of the predicted dimer contacts in fact cause a selective defect in cooperative binding to a glucocorticoid response element (GRE) in vitro, without affecting recognition of a GRE half site. Functional analysis in vivo demonstrated that these Zn-finger region mutants caused phenotypes even in native GR; therefore, the Zn-finger dimer interface is likely the predominant mediator of GR dimerization. This in vivo analysis also demonstrated that transcriptional enhancement from both a simple GRE and a proliferin composite glucocorticoid response element (GRE) required dimerization; however, repression from the composite GRE did not. Thus, a monomer of GR is sufficient to form a repressive complex with AP1 at the composite GRE. Because GR is monomeric in solution but can function both as a monomer and a dimer on DNA, its quaternary state may be regulated. I speculate DNA sequence or protein context at a DNA site or a combination of both regulates the quaternary state of GR.

Introduction

GR is a hormone dependent transcription factor, which upon binding hormone, translocates from the cytoplasm to the nucleus where it binds specific DNA sites, termed glucocorticoid response elements (GREs) (Yamamoto, 1985). Dependent upon the type of GRE, GR is able to activate or repress transcription from linked promoters.

At a simple GRE, for which there is a strong consensus sequence, GR is the sole DNA binding factor necessary to activate transcription (Godowski et al., 1988a). An idealized simple GRE is composed of two half-sites that are inverted repeats separated by three base pairs (AGAACANNNTGTTCT). In vitro analysis has demonstrated that GR binds cooperatively to this element as a homodimer (Perlmann et al., 1990).

At a composite GRE from the proliferin gene, GR is able to regulate transcription in conjunction with the AP1 family members Jun and Fos (Diamond et al., 1990). In conjunction with Jun, GR is able to activate transcription, and in conjunction with Jun and Fos, GR is able to repress transcription. DNase I footprinting analysis and gel retardation analysis support the idea that GR and AP1 co-occupy the proliferin element. The stoichiometry of these complexes is unknown.

Using X-ray crystallography, Luisi et al. have solved the structure of the GR Zn-finger region as a homodimer bound to a simple GRE (Luisi et al., 1991). They identify residues from the Zn-finger region that appear to constitute the dimer interface. As predicted from finger swap experiments (Umesono and Evans, 1989), this interface includes residues from the D box, residues 477-481 that comprise a knuckle of the second Zn-finger. In addition, dimer contacts are also made from a substructure, helix D, that is COOH-terminal to the D box. In theory, these predicted dimer contacts provide the energy to drive cooperative

DNA binding and may be responsible for restricting binding to appropriately spaced half-sites.

In this study, I sought to determine whether the dimer contacts predicted from structural analysis actually mediate cooperative binding. In addition, I investigated the functional importance of dimerization for GR activation and repression in vivo. To this end, I have mutated the residues seen to constitute the dimer interface in the crystal structure. I have assessed the ability of the mutants to bind as monomers to a simple GRE half site and to bind cooperatively as dimers to a simple GRE full site in vitro. In parallel, I have monitored their function at simple and composite GREs in vivo.

Results

The X-ray crystal structure implies the following dimer contacts: Within the D box, there exist a symmetrical pair of intersubunit salt bridges between the sidechains of Arg 479 (hereafter referred to as R479) and Asp 481 (D481), and a symmetrical pair of intersubunit backbone hydrogen bonds between the carbonyl group of Ala 477 and the amine group of Ile 483. Within helix D, there exists a symmetrical pair of intersubunit hydrogen bonds between the carbonyl group of Asn 491 (N491) and the reciprocal N491 side chain. The positions of the participating residues in the primary sequence are shown in Figure 2a, and the X-ray structure of dimers at a simple GRE is shown in Figure 3, with the side chains involved in dimerization highlighted.

I chose to mutate to alanine the residues whose sidechains make dimer contacts. This amino acid has a small side chain, minimal potential for chemical interaction, is unlikely to perturb any surrounding secondary structure, and has been successfully used in similar mutagenesis. I used site-directed mutagenesis to create the following single and double mutants: R479 to Ala (denoted R479A), D481A, N491A, R479A/N491A, and D481A/N491A.

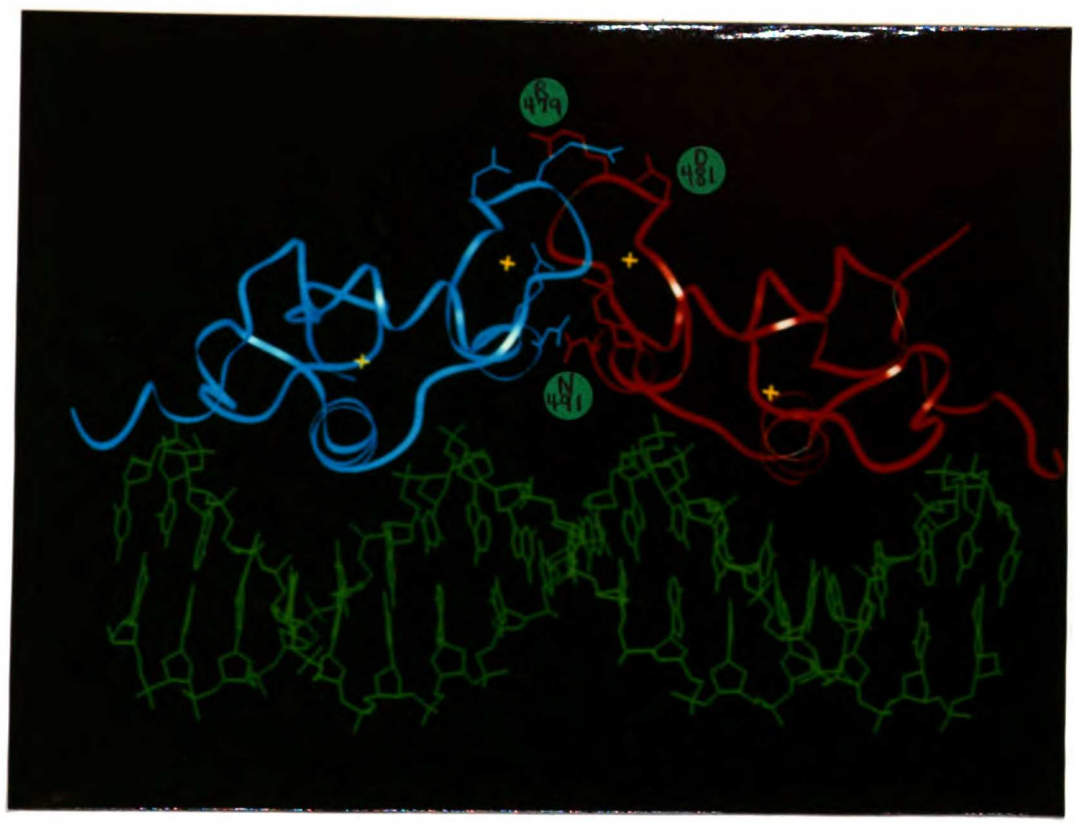


Figure 3

The Dimer Interface of the GR Zn-Finger Region

at a Simple GRE

To assess DNA binding in vitro, I expressed these mutants as T7-X556 derivatives in E. coli. The wild-type form of this derivative, which expresses the Zn-finger domain of GR (amino acids 407-556), is stable, readily purified, and binds cooperatively to an idealized simple GRE (Freedman et al., 1988). All mutants expressed in this form were also stable and present at wild-type levels as assessed by Western blotting (data not shown).

Partially purified E. coli extracts containing wild-type or mutant T7-X556 were used in gel retardation assays with two different GREs. To examine the affinity of a monomer for a half site, the extracts were incubated with radiolabeled GRE-S4, an oligonucleotide that has idealized simple GRE half-sites separated by four base pairs, as opposed to the optimal three base pairs. This oligonucleotide fails to bind GR cooperatively; instead, it binds GR predominantly as a monomer. Figure 4a shows a representative gel shift with this oligonucleotide, along with quantitation from three independent experiments. All mutants bound GRE-S4 as monomers essentially with wild-type affinity.

In order to assess cooperative DNA binding, the extracts were then incubated with radiolabeled GRE-S3, an oligonucleotide containing an idealized simple GRE, with the half-sites separated by three base pairs. This oligonucleotide binds GR cooperatively, giving predominantly a dimer gel shift with wild-type GR. Figure 4b shows a representative gel shift with this oligonucleotide along with quantitation from three independent experiments. Single lesions of the D box residues (R479A or D481A) caused only a moderate loss of cooperative binding, despite the predicted loss of two salt bridges. N491A, predicted to cost two hydrogen bonds, had only 16% of wild-type cooperative binding. Double mutants of either D box residue (R479A or D481A) with N491A caused the most severe loss of cooperative binding (8-11% of wt).

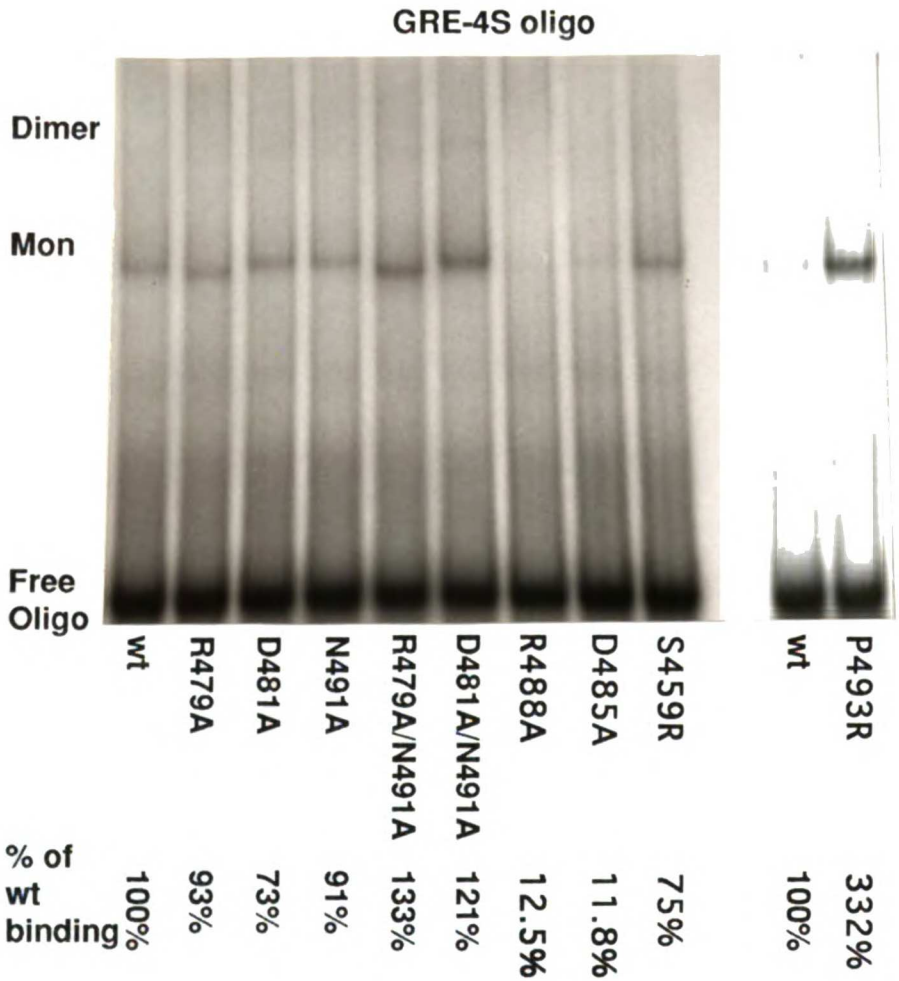


Figure 4a
Half-Site Affinity of Wild-type and Mutant T7-X556 Derivatives

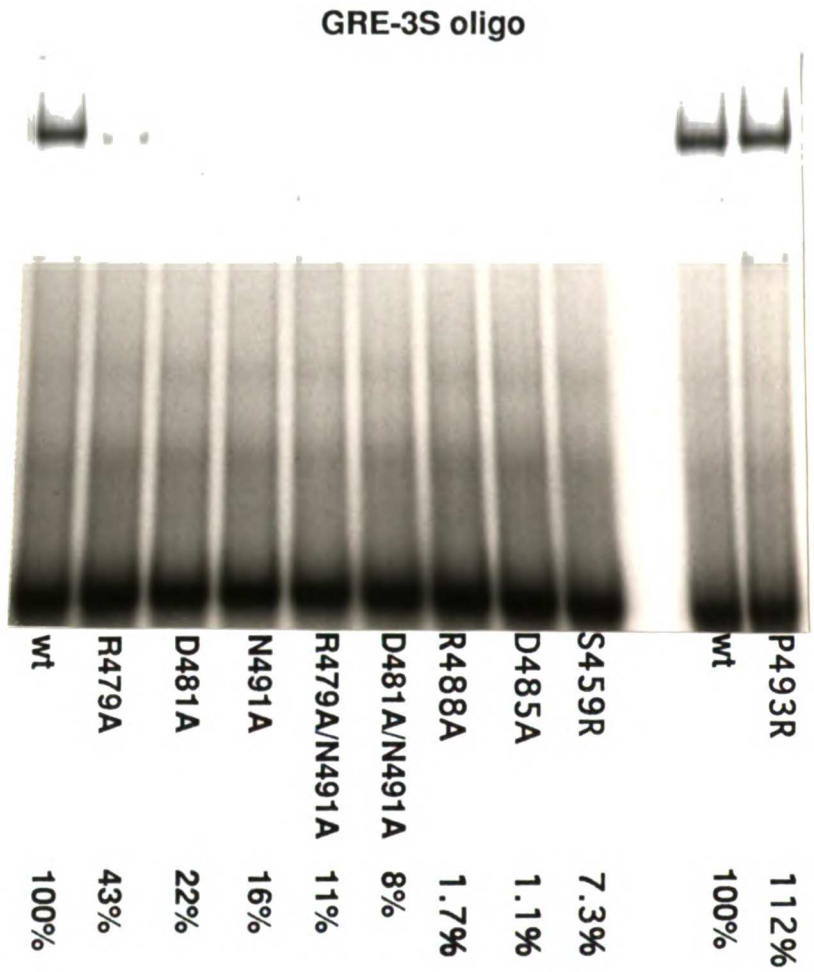


Figure 4b
Cooperative Binding of Wild-Type and Mutant T7-X556 Derivatives

Having characterized the DNA binding activities of the mutants in vitro, I used transfections into tissue culture cells to assess their transcriptional regulatory activity in vivo. To this end, the mutants were expressed as full length GR (amino acids 1-795, termed N795) from the mammalian expression vector, p6R. I monitored transcriptional activation by co-transfecting wild-type or mutant GR expression vectors with an appropriate chloramphenicol acetyl-transferase (CAT) reporter under GRE control. CAT activity is assumed to reflect the transcriptional activity of the wild-type and mutant GR derivatives. To test enhancement from a simple GRE, CV-1 cells were co-transfected with GR and the reporter pΔ(1GS3)DCO. This reporter contains a single idealized simple GRE, identical to the one used for in vitro binding, cloned upstream of a minimal ADH promoter driving the CAT gene. To test enhancement from a composite GRE, F9 cells were co-transfected with GR, Jun, and the reporter plfG3 CAT. This reporter contains three proliferin composite elements cloned upstream of a minimal ADH promoter driving the CAT gene. To test repression from a composite GRE, F9 cells were co-transfected with GR, Jun, Fos, and the reporter plfG3 CAT.

Figure 5a shows the average of the results from three independent transfections for simple and composite activation. The activities of the mutants are shown as a percent of wild-type activity. Figure 5b shows the average of the results from three independent transfections for composite repression.

In general, the mutants demonstrate a similar spectrum of loss of transcriptional activation at both the simple and the proliferin composite GREs. However, despite the fact that this set of mutants is crippled to varying extents for cooperative binding in vitro and activation in vivo, they have completely wild-type activity for repression at the composite GRE.

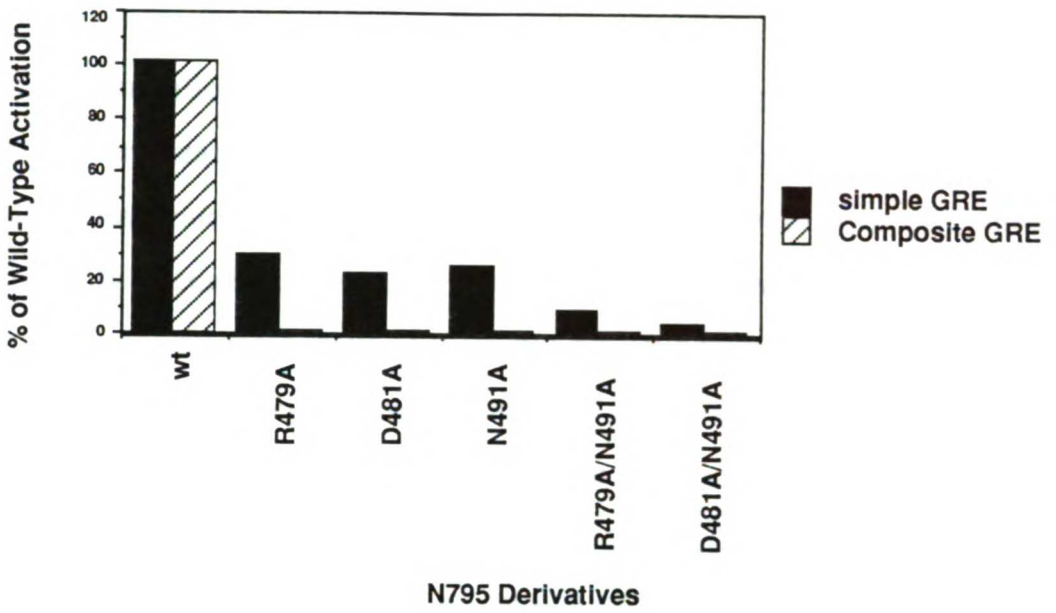


Figure 5a
Dimer Mutant Activation at Simple and Composite GREs

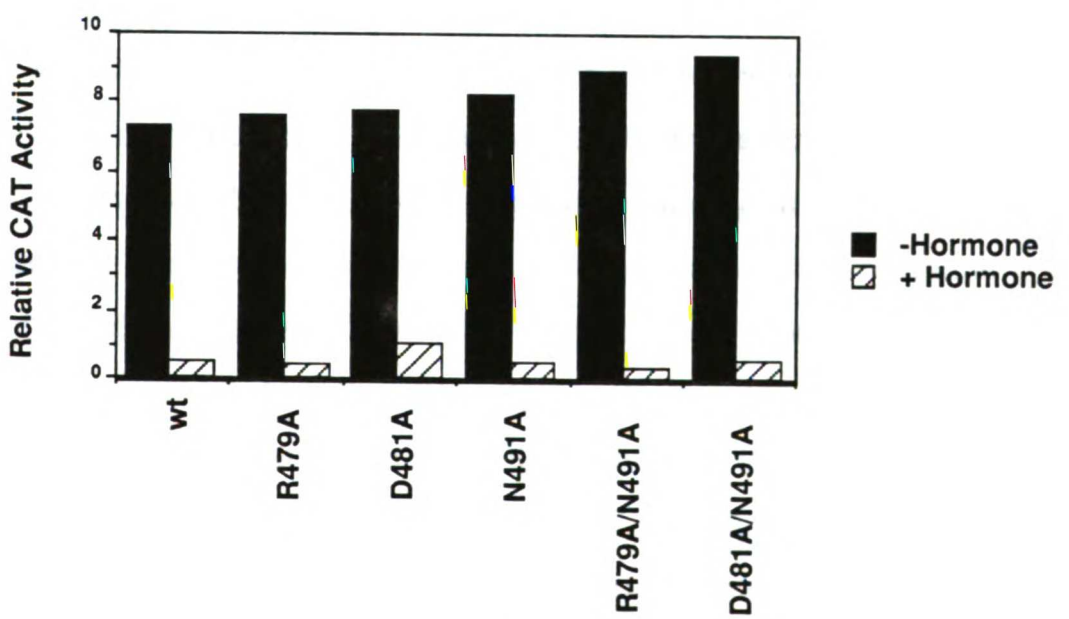


Figure 5b
Dimer Mutant Repression at a Composite GRE

Discussion

The mutations introduced at sites predicted from the crystal structure to mediate dimerization have the predicted phenotype—the mutants were able to bind as monomers to half-sites with wild-type affinity, but were impaired in their ability to confer cooperative binding of a second monomer. Importantly, because all the mutants retained two wild-type functions, affinity for a half-site in vitro and repression at a composite GRE in vivo, their lack of cooperative binding at a full site was not the result of the mutations having global deleterious effects.

In vivo, the effects of the mutations on simple GRE activation by full length GR paralleled their effects on cooperative binding in vitro by T7-X556 derivatives, i.e., wt > R479A ≈ D481A > N491A > R479A/N491A ≈ D481A/N491A. The mutations also severely compromised composite GRE activation. In vitro binding studies demonstrate that the composite GRE is a weaker binding site for GR than a simple GRE (unpublished observations, M. Diamond and Shih-Teng Jeng). Therefore, the loss of cooperativity may have a more severe effect on occupancy at the composite GRE than the simple GRE.

I conclude that the loss of cooperative binding in vitro is sufficient to explain the loss of transcriptional activation in vivo; therefore, activation at both simple and composite GREs requires dimerization. Moreover, the Zn-finger region mutants produce their phenotypic effects in the context of the intact receptor, implying that the Zn-finger dimer interface is likely the predominant dimerization motif in GR.

The estrogen receptor has been reported to have a strong dimerization function in the hormone binding domain. Conceivably, GR might have a similar activity, and the mutants presented here might be defective in some post-dimerization event mediated by the Zn-finger "dimer interface". However, deletion

analysis of GR does not support this notion. Truncation of the GR hormone binding domain produces a constitutive transcriptional activator termed N556 (Godowski et al., 1987). The activation potential of this GR derivative is quantitatively similar to the native full length form, implying that no function essential for transcriptional activation resides in the deleted region.

Despite the fact that the dimerization mutants are deficient for cooperative binding in vitro and transcriptional activation in vivo, they are wild-type for transcriptional repression. The implication is that a monomer of GR is able to form a functional repressive complex with Jun and Fos at the proliferin element. In vitro crosslinking results showing native GR:Jun:Fos stoichiometry to be 1:1:1 support this idea (J.N.M. unpublished observations). Therefore, GR can perform distinct functions bound to DNA as a monomer and a dimer.

The following results argue that GR is predominantly monomeric prior to GRE binding. Perlmann et al. used a gel shift assay to investigate the rate of subunit exchange between potential full length GR dimers and potential dimers of a truncated form of GR in a mixing experiment (Perlmann et al., 1990). They found rapid exchange, which implies that GR is predominantly monomeric or weakly dimeric in solution. This result contrasts with a similar kinetic exchange analysis done with estrogen receptor (ER) (Kumar and Chambon, 1988). This analysis demonstrated that full length and truncated ER dimers exchanged slowly, implying the formation of stable dimers in solution. This difference further supports the idea that GR does not have an ER-like hormone binding domain dimerization motif and that GR may exist as a monomer in solution. Thus, if GR is predominantly monomeric in solution and can function on DNA as both a monomer and a dimer, its quaternary state may be regulated.

Indeed, structural analysis of the Zn-finger region not only supports the idea that GR may exist as a monomer in solution, but also implies that DNA

binding may be a regulatory trigger that induces dimerization. The Zn-finger region of GR, which has been expressed and purified, clearly exists as a monomer (unpublished observations, L. Freedman). Like full length GR, this region is able to bind cooperatively to a simple GRE as a dimer. The structure of a monomer in solution has been determined by 2-D NMR (Hard et al., 1990), and the structure of a dimer on a simple GRE has been determined by X-ray crystallography (Luisi et al., 1991). Interestingly, even at the millimolar concentrations used to determine the NMR structure, no evidence of dimer formation is found. This result is particularly striking given the results presented here that the Zn-finger region contains the predominant dimerization motif of GR. Moreover, the X-ray crystallographic analysis shows that GR dimerizes rather than bind in correct register with its cognate half-sites on a GRE with a spacing of 4 base pairs between half sites. This result implies a strong dimer interface.

Despite this strong dimer interface on DNA, there is no evidence for dimer formation in solution. To explain this discrepancy, Luisi et al. proposed that DNA binding induces dimer formation through a conformational change (Luisi et al., 1991). They identify a substructure, a distorted α-helix (helix D), that is present in the dimer form on DNA in the X-ray crystal, but not seen in the solution monomer by NMR. This substructure not only makes contacts with DNA, but also provides important dimer contacts. Thus, the stabilization of this region's secondary structure by DNA contacts could at the same time promote the formation of a competent dimerization interface.

Therefore, I propose that GR exists as a monomer prior to GRE binding. Upon finding a GRE, regulatory events occur that determine the oligomerization state of GR. At a simple GRE, a GR monomer binds and proceeds on to homodimerization. At a composite GRE, a GR monomer is bound cooperatively by either Jun or Jun/Fos that pre-exist at the site. In a complex with Jun, GR

proceeds on to homodimerization. However, in conjunction with Jun and Fos, GR remains a monomer. This regulation may be based on the sequence of the site and/or the protein context of the site. The experiments described here do not address this question. I have genetically and biochemically characterized other Zn-finger region mutants that support the hypothesis that DNA is an allosteric regulator of GR function (see Chapters 2 and 3). The question of whether additional regulation is conferred by protein context is also being explored (Speculations in Chapter 4).

In summary, residues predicted from structural analysis of the Zn-finger domain to mediate dimerization, in fact do affect cooperative binding in vitro when mutated. This Zn-finger dimer interface is the predominant dimerization motif of native GR. For the GREs tested, transcriptional activation requires dimerization, but repression does not. The implication is that the quaternary state of GR is regulated. I speculate that DNA sites and/or protein context at a site determine the quaternary state of GR.

CHAPTER 2

Genetic and Biochemical Analysis of the Zn-finger Domain of the Glucocorticoid Receptor Supports the Hypothesis that DNA Is an Allosteric Regulator of GR Function

Abstract

Structural analysis has identified a region in the GR Zn-finger domain that differs between a monomer in solution and a dimer on a GRE. This region may become organized upon DNA binding into a substructure, a distorted α-helix (helix D), that has been predicted to be important in mediating DNA induced dimerization. In the GR:GRE complex, this region is highly ordered, making phosphate contacts with DNA, dimer contacts with another subunit, and intramolecular contacts with other regions of the same subunit. In theory, the DNA contacts could stabilize helix D, and once formed, the helix could induce conformational changes in other parts of the molecule.

I have tested the importance of helix D by mutating residues in this substructure and residues that may interact with it. Mutants that had a phenotype in yeast were further characterized for DNA binding in vitro and transcriptional activity in vivo. I describe mutants in helix D, that are not part of the dimer interface, yet are defective for cooperative binding and transcriptional activation, while being wild-type for repression. In addition, I describe a mutant that binds a half site with wild-type affinity, but is defective for both activation and repression. These phenotypes are consistent with but do not alone prove the model that this region is involved in DNA induced dimerization. In addition, I describe two point mutants each of which caused a hormone dependent growth defect in yeast. I speculate that these mutations cause an altered conformation that aberrantly activates GR, bypassing the normal reg-

ulatory step of binding at a GRE. Further analysis of these gain of function mutants may provide a definitive test of the hypothesis that DNA is an allosteric regulator of GR function.

Introduction

As discussed in a previous chapter, it is likely that hormone bound, nuclear GR, prior to GRE binding, exists as a monomer. Luisi et al. hypothesize that DNA induces GR dimerization (Luisi et al., 1991). They identify a region in the Zn-finger domain that appears to differ between a monomer in solution (Hard et al., 1990; personal communication from R. Kaptein) and a dimer bound to a simple GRE. In the X-ray dimer structure, this region forms a distorted α-helix (helix D) that is not seen in the solution monomer. Helix D makes phosphate contacts with DNA, intersubunit dimer contacts, and intrasubunit contacts with other key regions of the molecule. The secondary structure of helix D could be stabilized by these DNA contacts and intramolecular contacts and thus promote the formation of a competent dimer interface. In theory these contacts could not only help stabilize this substructure but also transduce information of specific DNA binding to it or from it.

In addition, there is a position near helix D that seems interesting from prior experimentation. Paul Godowski created a linker scanning mutation, called LS7, that changed P493 to arginine (P493R) and A494 to serine (A494S) (Godowski et al., 1988b). These residues lie near a hinge between helix D and the amphipathic α-helix (helix P) that lies perpendicular to the recognition α-helix (helix R). In mammalian cells, Godowski found that this double mutant fails to activate transcription, but is competent for repression at the prolactin negative GRE. In addition, he found that it has wild-type affinity for a simple GRE in vitro.

Thus, helix D warrants further study for several reasons. First, it may undergo a conformational change upon binding DNA. Second, the crystal structure identifies intramolecular interactions with helix D that may help stabilize it and/or transduce information to it or from it. Finally, a mutant (LS7) already exists nearby at an interesting hinge position.

I have sought to test the importance of this region by mutating the residues that constitute helix D and the residues that interact with it. I used a phenotypic screen in yeast to select mutants for further study, that includes characterization of DNA binding in vitro and transcriptional activity in vivo.

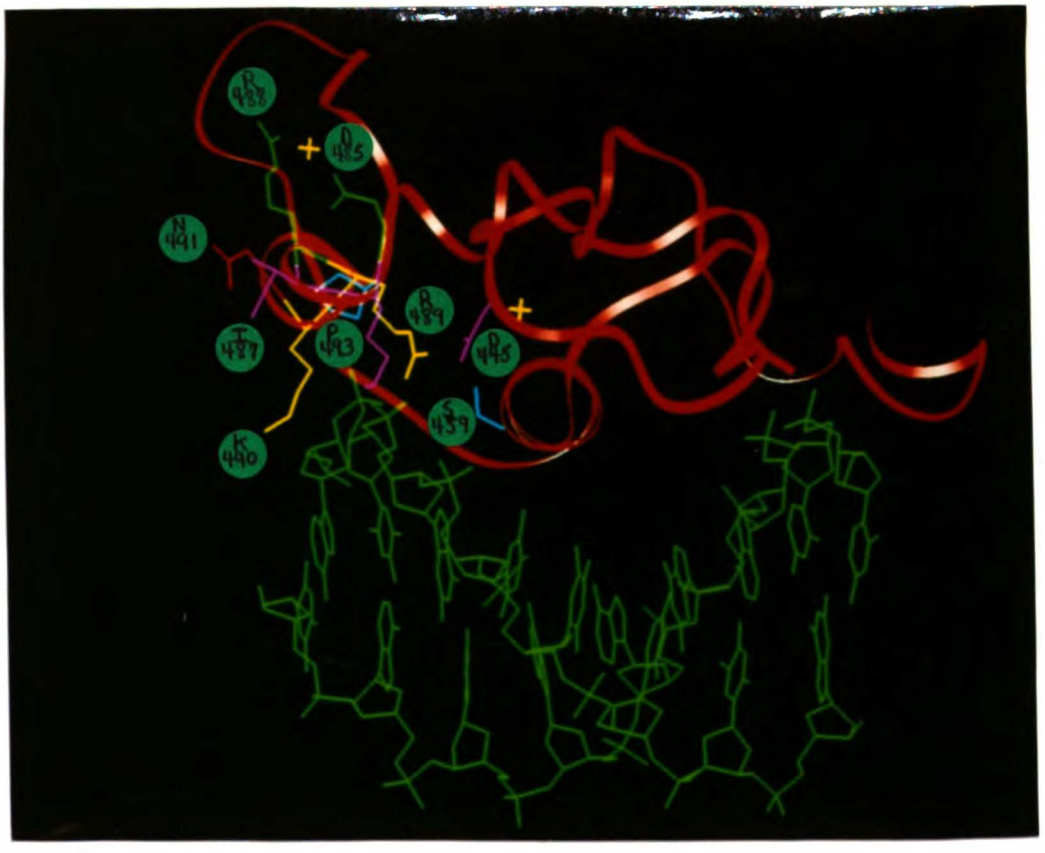


Figure 6

Helix D and Its Interactions

Results

Figure 6 shows helix D, in the second Zn-finger, which encompasses amino acids 486-491. These residues are predicted to make the following contacts. R489 and K490 (shown in yellow) make phosphate contacts with the DNA. R488 and N491 make dimer contacts: R488 (shown in green) makes a hydrogen bond with the backbone carbonyl group of C476 of the other subunit, and N491 (shown in red) makes a pair of hydrogen bonds with the reciprocal N491 residue of the other subunit. In addition to its dimer contact, R488 (shown in green) also makes an intramolecular salt bridge with D485 (shown in green), a residue immediately adjacent to helix D. In addition to its phosphate contact, R489 also makes intramolecular hydrogen bonds with S459 (shown in cyan), a residue on the back side of helix R, and D445 (shown in magenta), another residue from the first Zn-finger. P493 (shown in cyan) lies near a hinge between helix D and helix P.

I used site-directed mutagenesis to change targeted residues to alanine for the reasons stated in the previous chapter. The exceptions are as follows. In order to make a non-conservative change, I mutated I487 to arginine in addition to alanine. In an attempt to identify a single amino acid alteration responsible for Godowski's LS7 phenotype, I mutated P493 to arginine. In addition to making S459A, I also obtained a mutation of S459 to arginine through random chemical mutagenesis. Therefore, the mutated residues from helix D are: K486A, I487A, I487R, R488A, R489A, K490A, and N491A. The mutated residues that interact with helix D are D445A, S459A, S459R, and D485A. Finally, I made P493R.

To screen these mutants, I initially characterized their transcriptional activation potential in yeast. To this end, I expressed wt or mutant full length GR from the yeast expression vector, pG-1. Transcriptional activation potential was inferred from the levels of β-galactosidase expressed from the integrated re-

porter, pI-G26.1, which has three simple GREs from the tyrosine amino-transferase gene (TAT) controlling a minimal CYC1 promoter driving the Lac-Z gene. Figure 7 shows the average of the results of 3 independent experiments. β-galactosidase levels before and after hormone induction are expressed as a percent of wild-type hormone activated levels.

D445A, K486A, I487A, I487R, and K490A have essentially wild-type activity, and they were not analyzed further. N491A has 22% of wild-type activity. S459R, D485A, R488A, and R489A show no activation potential. P493R and S459A gave a common phenotype. S459A had 28% and P493R had 35% of wild-type hormone induced activation and both demonstrated a significant increase (~ten-fold) in transcription in the absence of hormone. Interestingly, they also both caused a severe hormone dependent growth defect in yeast (data shown in next chapter). Further characterization of these growth defect mutants (S459A and P493R) will be deferred to the next chapter.

To characterize further the mutants that had a phenotype in yeast, I used a gel retardation assay, as in a previous chapter, to assess the affinity of the mutants for a half site and a full site. Immunoblotting confirmed that all the mutant X556 derivatives used were stable and present at comparable levels (data not shown). R489A has not yet been analyzed for DNA binding. The data for the remaining mutants is presented in Figure 4a and b, along with quantitation. To summarize, S459R had wild type affinity for a half-site, but was deficient for cooperative binding at a full site. D485A and R488A had a common phenotype in being severely reduced for both half and full site affinity. N491A has been previously discussed in its context as a dimer contact.

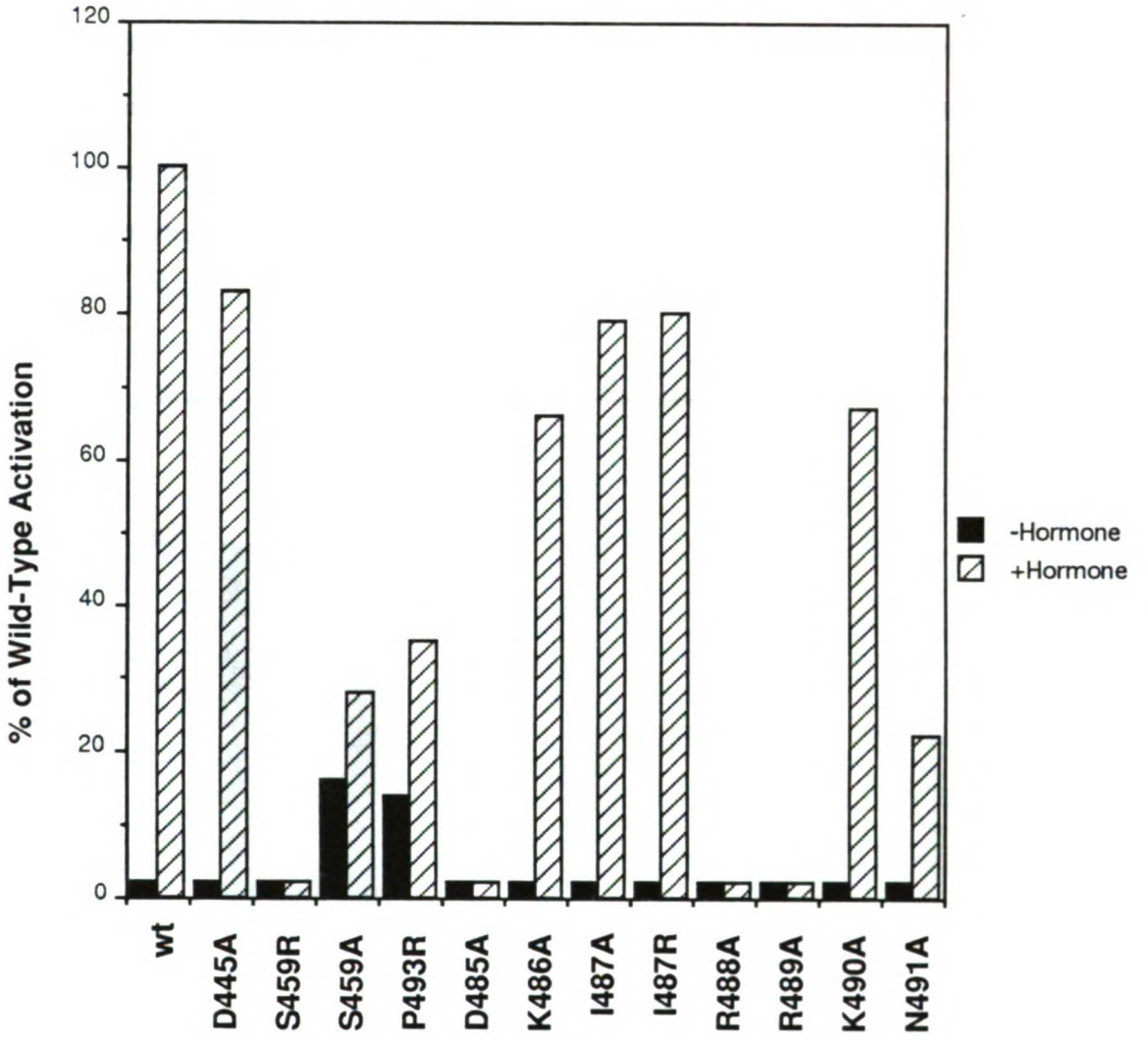


Figure 7

Yeast Activation Potential of Mutants in Helix D and Interacting Residues

As described in a previous chapter, I used mammalian cell transfection to assess the transcriptional activation and repression potential of the mutants. All mutants with phenotypes in yeast, except R489A, underwent this analysis. Figure 8 shows the results for activation at a simple GRE in CV-1 cells. In general, the mammalian activation results at a simple GRE reflected the activation results from yeast at a simple GRE. As in yeast, S459R, D485A, and R488A were significantly down for activation.

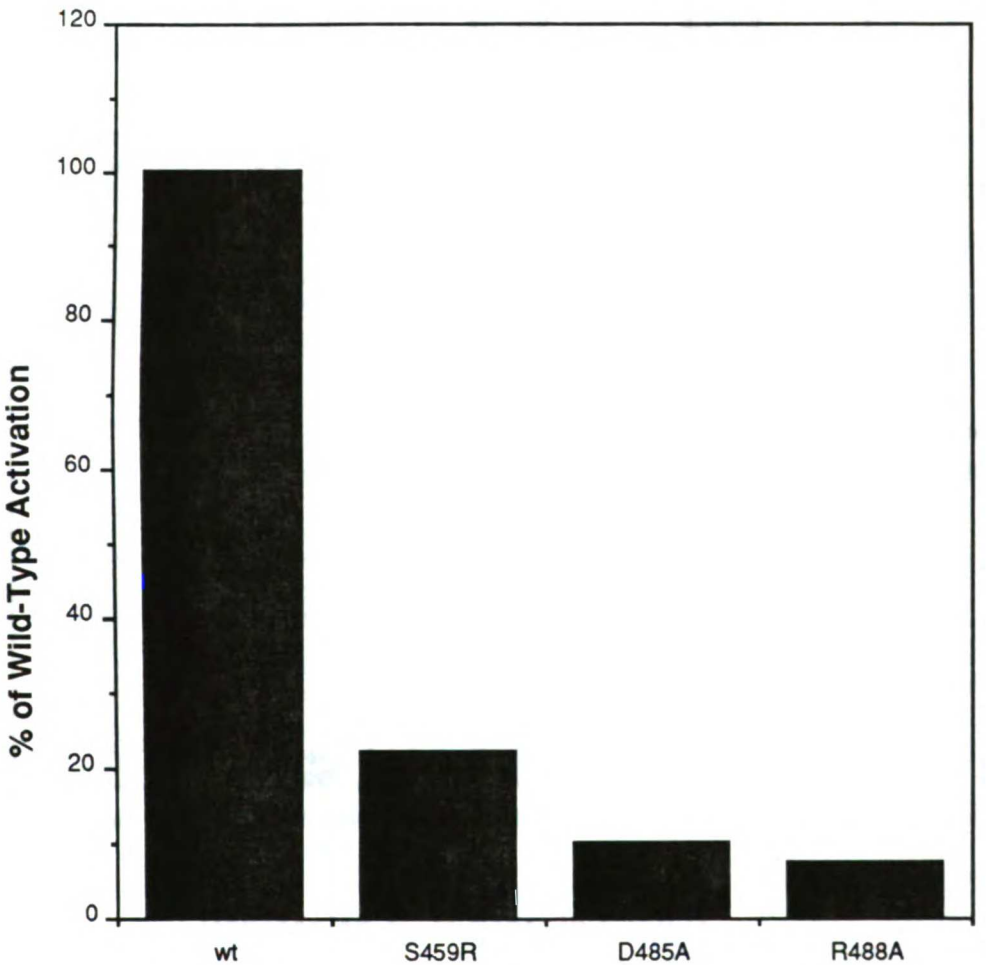


Figure 8
Activation by Mutants in Helix D and Interacting Residues in Mammalian Cells

Figure 9 shows the repression activity of the mutants at the proliferin composite GRE in F9 cells. D485A and R488A were completely wild-type for repression. S459R was only able to repress the Jun/Fos basal level of transcription ~two-fold. Compared to the wild-type repression of ~15-fold, S459R retains only 13% of wild-type repression.

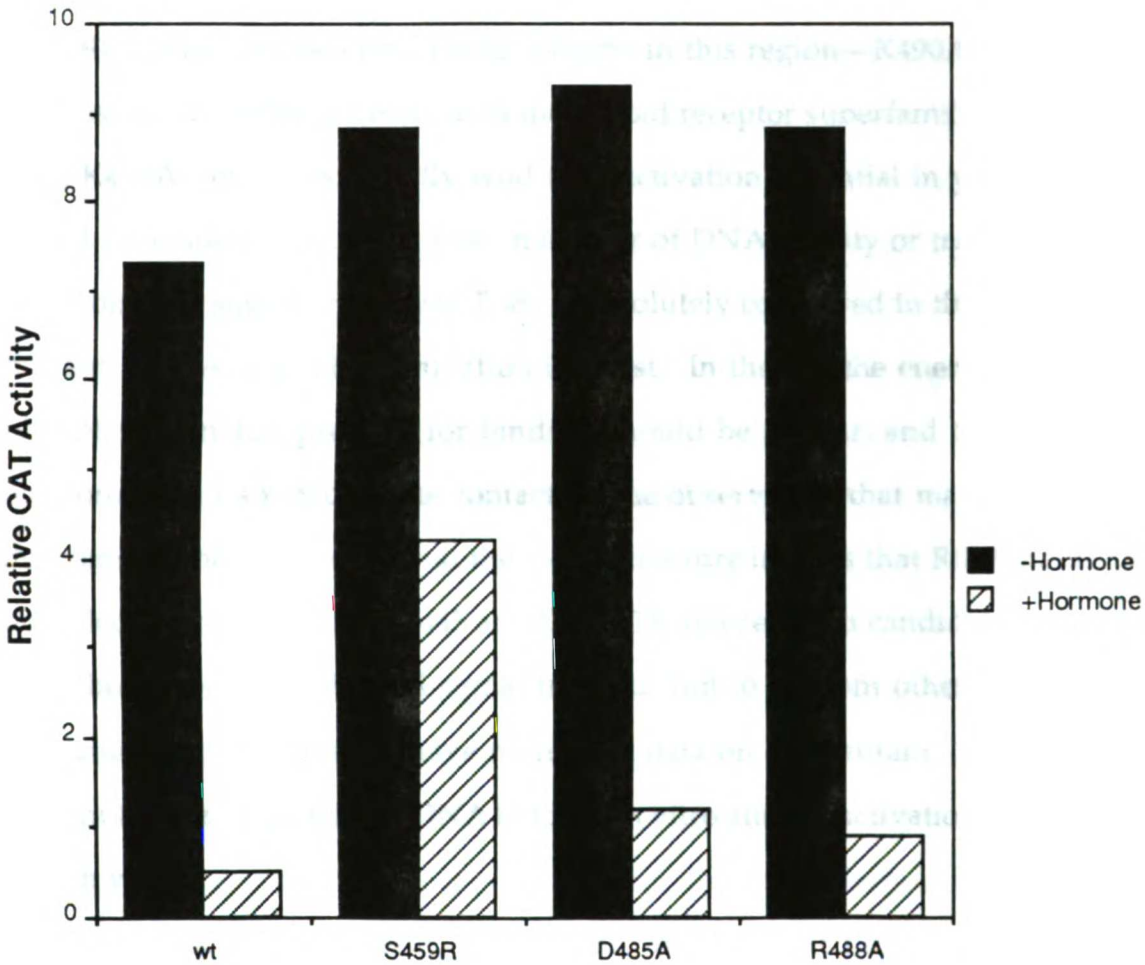


Figure 9
Repression by Mutants in Helix D and Interacting Residues

Discussion

According to the model that DNA induces GR dimerization through conformational change, the sensing of DNA by GR must trigger some event that promotes dimerization. The seven phosphate contacts and three specific base contacts that GR makes with a specific site are prime candidates for this sensing role. I have mutated most of these DNA contacts, and the results are summarized in Appendix II. For the purpose of this chapter, I focus on helix D residues 486-491. There are two phosphate contacts in this region—K490A and R489A. K490 is not absolutely conserved in the steroid receptor superfamily and its mutant, K490A, retains essentially wild-type activation potential in yeast. Therefore, I conclude that it is not a key mediator of DNA affinity or transducer of a DNA binding signal. However, R489 is absolutely conserved in this family and mutant, R489A, abolishes activation in yeast. In theory, the energy these two phosphate contacts provide for binding should be similar, and they are both only one out of six phosphate contacts. One observation that may explain the difference in phenotypes is that the X-ray structure implies that R489 makes intramolecular contacts with D445 and S459. Therefore, it is a candidate for mediating both DNA affinity and signal transduction to or from other parts of the molecule. Unfortunately, I have no further data on this mutant. It will be important to characterize it for DNA binding in vitro and for activation and repression in vivo.

Mark Schena, however, has another mutation at this position, R489K (Schena et al., 1989). He finds this conservative change mutant to be cold sensitive and deficient for activation in yeast. It also binds poorly to a simple GRE in vitro. Interestingly, this mutant remains wild-type for repression at a prolactin negative GRE. Given my previous analysis of the dimerization requirement for activation but not repression, I suggest that this mutation may

disrupt the function of helix D causing a loss in the ability to dimerize. This defect would abolish activation but would not affect repression. Thus, this phenotype is consistent with the idea that R489 may be both a sensor of DNA binding and a transducer of information required for dimerization.

N491 and R488 are residues that reside in helix D and appear to make dimer contacts. The mutant N491A has been previously described. In summary, this mutant is predicted to cost two hydrogen bonds. It has wild-type affinity for a half-site, but is partially deficient for cooperative binding at a full site. In vivo, it is partially deficient for activation, but is wild-type for repression. Despite also being a dimer contact, R488A had a different phenotype. This mutant is predicted to cause the loss of only one dimer interface hydrogen bond, yet it was deficient for binding at both a half-site and a full site. However, it is important to note that R488 also appears to makes a salt-bridge with D485, a residue immediately adjacent to helix D. Interestingly, D485A gave the same phenotype as R488A. It, too, was deficient for binding at both half and full sites in vitro. Additionally, in vivo, they were both deficient for activation but wild-type for repression. Because they both retained one wild-type function, they cannot have global structural defects. I suggest that the salt-bridge between D485 and R488 stabilizes the formation of helix D upon DNA binding, and abolition of either partner of this pair, selectively destabilizes this region so that it cannot make appropriate phosphate or dimer contacts. Therefore, these mutants have reduced affinity for a simple GRE half-site, in addition to being unable to form dimers. This phenotype was similar to that of R489K—the mutant phosphate contact from helix D. Apparently any disruption of this region, either by removing a critical phosphate contact or preventing it from being formed, is sufficient to cause this phenotype.

The X-ray structure also predicts helix D interactions with D445 and S459. D445 is a residue that is highly conserved in the steroid receptor superfamily (43 out of 46 sequences) (Amero et al., 1992).. The only change is a conservative one to Glu (3 of 46). Its mutant form, D445A, causes a drastic change from a charged amino acid to a small hydrophobic one. Nevertheless, D445A had wild-type activity for activation in yeast, and was not further tested in this study. However, given its absolute conservation and its interesting contact with helix D, it will be important to test this mutant for other functions. I speculate that a DNA mediated signal may be transduced through D445 to the first Zn-finger affecting some function other than activation, such as repression.

The other residue that is predicted to interact with helix D is S459. One mutant form, S459R, retained wild-type affinity for a half-site, but was significantly down (7% of wt) for cooperative binding at a full site. In vivo, both activation and repression were also significantly down. Again, since this mutant retained one wild-type function (half-site affinity), it cannot have global deleterious effects. It is this wild-type affinity for the half-site that made this mutant particularly interesting. Because the S459R monomer bound a half-site with wild-type affinity, its ability to recognize specific DNA was unimpaired. Yet despite the fact that this mutation does not lie at the dimer interface, it cannot cooperatively bind another monomer. This failure to cooperatively bind DNA explains its in vivo defect for activation. However, mutations at the dimer interface which have wild-type affinity for a half site but are deficient for cooperative binding, retain wild-type activity for repression. S459R does not. Therefore, S459R may cause another defect in addition to blocking dimerization.

S459 lies within helix R, which makes sequence specific base contacts. but it resides on the backside of this helix away from the DNA. From this position, it appears to interact with R489, a helix D residue that also makes a phosphate

contact. This contact may allow communication between helix R and helix D. I suggest that the S459R mutation blocks this communication. The predicted consequence of this blockage is that helix D fails to form properly so that homodimerization fails to occur and activation is disrupted. Interestingly, repression is also impaired. The implication is that some communication and presumably conformational change is required for both activation and repression. Whether this conformational change is the same for activation and repression is not addressed by this mutant.

Of course, a competing interpretation is that the S459R mutation simply disrupted the structure of the monomer in an uninteresting way so it could not activate or repress. But because S459 is part of the recognition helix (helix R) and half-site affinity is wild-type, this disruption of structure would have to occur in a way in which the structure in which the residue itself resides is unaffected. I believe this scenario to be unlikely. Moreover, another mutation affecting the same residue, S459A, supports the idea that S459 may be a critical node of intramolecular communication. When expressed in yeast, S459A produced a hormone dependent growth defect. Thus, this mutant had a gain of function phenotype. Remarkably, a second hormone dependent yeast growth defect mutant was also found—P493R.

These two growth defect mutants (S459A and P493R) display identical phenotypes for every criterion tested to date (see next chapter). P493 lies near a hinge between helix D and helix P. This position in the crystal structure is quite removed from the position of S459. There is no discernible physical interaction; presumably, the only link is functional. To explain how these two disparate mutants caused an identical phenotype, I hypothesize that these mutations produce a common altered conformation. To explain the growth defect, I hypothesize that this altered conformation is similar to the normal activated form GR attains

at a specific GRE. The mutations bypass the need for the normal regulatory event of binding at a GRE and cause aberrant, unregulated activation. The consequence of this aberrant activation is that GR binds its normal protein target(s) promiscuously, sequestering it (them) from vital yeast promoters, causing a growth defect. The hormone dependence of the phenotype is likely due to the subcellular location of GR. In the absence of hormone, GR is predominantly cytoplasmic; however, in the presence of hormone, GR translocates to the nucleus, where the putative target protein exists. It is also possible that hormone binding may relieve an inhibitory influence that the hormone binding domain exerts on the activity of the rest of the molecule (Picard et al., 1988). I present data in the next chapter to support this model. If this altered conformation hypothesis proves true, then, in addition to dimerization, transcriptional activation could also be regulated by DNA binding.

In summary, the case for DNA allosteric regulation of GR is as follows. First, it is likely GR exists as a monomer prior to GRE binding. A comparison between the NMR structure of a solution monomer and the X-ray structure of a dimer on a GRE, reveals a region, helix D, that is predicted to undergo a conformational change upon DNA binding that could promote dimer formation. In this study, I have described loss of function mutations in this region (S459R, D485A, and R489A), which are not part of the dimer interface, but that abolish dimerization in vitro and activation in vivo, while retaining at least one wild-type function. In addition, the phenotype of S459R also implies DNA may induce some conformational change that is required for repression. Although these mutants are consistent with the model that helix D and its contacts mediate a DNA induced conformational change, they cannot exclusively prove it. However, given the implied structural differences between a monomer in solution and a dimer on DNA, I believe the model presented here to be most likely.

Moreover, the isolation of two gain of function mutants from this region that caused growth defects in yeast supports the circumstantial case for DNA regulation of GR function. In the next chapter, I present an analysis of the growth defect mutants that further strengthens the idea that DNA is an allosteric regulator of GR function.

Chapter 3

Analysis of Two Point Mutants in the GR Zn-Finger Domain That Cause a Hormone-Dependent Growth Defect in Yeast— The Case for DNA Mediated Allosteric Regulation of the Glucocorticoid Receptor

Abstract

I identified two point mutants (P493R and S459A) in the Zn-finger region of GR each of which caused a hormone dependent growth defect in yeast. Temperature dependence of the phenotype, column chromatography, and circular dichroism imply that a GR fragment containing the P493R mutation (T7-X556/P493R) has a different conformation than the corresponding wild-type fragment. Because T7-X556/P493R retains the ability to bind a simple GRE, these structural differences are not the result of simple denaturation. Moreover, because T7-X556/P493R has a three-fold increased affinity for a simple GRE half-site, its conformation may be similar to that induced in wild-type upon simple GRE binding. In vivo in yeast, both mutants caused transcriptional "squelching". Deletion analysis demonstrated that the hormone binding domain was not required for the phenotype, but the N-terminal region was required. Further analysis of the P493R mutation in conjunction with other mutations indicated that specific recognition of a GRE was not required for the phenotype, but that non-specific DNA binding may be important.

Based on these observations, I propose that each mutant has an altered conformation that mimics the activated form that wild-type GR normally attains only at a simple GRE. The consequence of this aberrant activation is that GR promiscuously binds its putative protein target(s). This sequestration of target protein(s) deprives essential yeast promoters of needed factor(s), thus causing

the growth defect. The implication is that the functional competence of wild-type GR is normally regulated by specific recognition of a simple GRE and that these mutations bypass this regulation. Since the N-terminal region is required for the growth defect, I speculate that information of simple GRE binding is transduced through the Zn-finger domain to the N-terminal region, which contains activation domain Enh2. Thus, DNA mediated allosteric regulation of Enh2 function may explain in part how GR can function differently at different sites. In addition, it may explain how the function of a potent transactivation domain is restricted to those molecules of GR that specifically bind at a GRE, thereby avoiding the deleterious consequences of squelching.

Introduction

Transcriptional activators contain enhancement domains which are often modular in nature. These enhancement domains can confer activation potential on heterologous DNA binding domains themselves incapable of activation (Brent and Ptashne, 1981; Godowski et al., 1988a). Enhancement domains are thought to function by making protein-protein contacts with target proteins, whose concentrations may be rate-limiting for transcriptional initiation. When a transcriptional activator binds its cognate site near a regulated promoter, it is thought to tether its target protein near the promoter. This tethering should effectively increase the local concentration of the target and increase initiation by overcoming the rate-limiting step.

Gill and Ptashne describe a phenomenon, termed squelching, where overexpression of an activator paradoxically leads to transcriptional inhibition of promoters that contain no site of action for the activator (Gill and Ptashne, 1988). They proposed that overexpression forces inappropriate interactions between

the activator and its target protein, thus sequestering the target away from promoters that depend on it for function.

The fact that a transcription factor can squelch a promoter at which it has no site of action implies that there are multiple activators that use the same target protein to mediate enhancement. In theory, if there are enough of these multiple activators, all constitutively binding a common target protein, squelching should occur even under normal cellular conditions. The question is how does a cell avoid squelching?

Regulation of the function of an enhancement domain is a potential solution. Ideally, the function of an enhancement domain, which may be the binding of a target protein, should be limited to its site of action, its cognate binding site linked to a promoter. In theory, DNA mediated allosteric regulation of enhancement activity would fulfill these criteria. In fact, there is growing evidence that some transcription factors undergo a conformational change upon DNA binding. These transcription factors include GCN4 (O'Neil et al., 1991; Weiss et al., 1990), C/EBP (O'Neil et al., 1991; Shuman et al., 1990), TFIID (Lieberman et al., 1991), and PRTF (Tan and Richmond, 1990).. Presumably the DNA binding domains of these proteins are capable of sensing correct site DNA binding and initiating a conformational change.

The glucocorticoid receptor is an ideal transcription factor with which to pursue this hypothesis of DNA mediated allosteric regulation. It is a hormone-inducible transcription factor that is capable of squelching (Tasset et al., 1990). In fact, the Gustafsson lab has demonstrated that an isolated GR enhancement domain expressed at high levels in yeast can not only cause squelching, but cause a growth defect that presumably is the result of squelching (Wright et al., 1991). It is possible that the intrinsic activity of this enhancement domain may be regulated in the context of the native receptor. Moreover, the GR Zn-finger

region, which mediates DNA binding and potentially may trigger a conformational change, has been purified and structurally characterized. The structure of this region as a monomer in solution and as a dimer on simple GRE has been solved. In fact, a comparison of these structures implies there is a conformational change upon DNA binding. Furthermore, GR can enhance transcription in yeast, providing a powerful system to characterize GR function genetically.

GR is also a prime candidate to assess the hypothesis of DNA mediated allosteric regulation because of the functional diversity it displays in vivo. At a simple GRE, GR can activate transcription, and from a composite GRE, it can both activate and repress transcription. In theory, these functional differences may be determined by the DNA site.

In the previous chapter, I presented the results of an analysis where the phenotypes of GR Zn-finger region point mutants were screened in yeast. This analysis identified two point mutants, each of which caused a hormone-dependent growth defect in yeast. In this chapter, I characterize these mutants in more detail to examine further the hypothesis of DNA mediated allosteric regulation of GR function. Specifically, I pursue structural characterization to determine if an altered conformation is induced by the mutations. Next, I assess transcriptional inhibition of heterologous promoters that contain no site of GR action to determine if squelching is occurring. Other characteristics such as transcriptional activity in mammalian cells and in vitro binding were monitored. Finally, in an attempt to dissect mechanism, genetic characterization of the growth defect mutants in combination with other point and deletion mutants was performed.

Results

Yeast containing either wild-type or mutant full length GR (N795) expressed from the high copy 2μ vector pG-1 were grown to similar cell densities. Ten-fold serial dilutions were prepared and spotted on plates with or without hormone. The plates were incubated at 30°C for three days. Figure 10 displays the result for N795/P493R; however, N795/S459A behaved identically. Both mutants were severely retarded for growth relative to wild-type in a hormone dependent way. Preliminary Western blot data demonstrated that the mutant receptors were expressed at wild-type levels (data not shown).

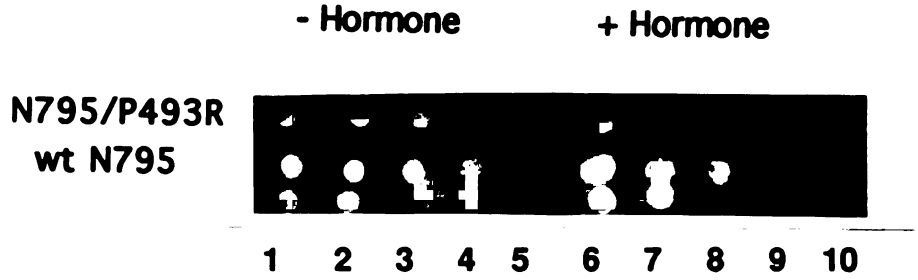


Figure 10
The Hormone Dependent Growth Defect Phenotype

To quantify the growth rate difference, I measured absorbance of suspension cultures at a wavelength of 600nm as a function of time to obtain growth curves. Under identical growth conditions wild-type and mutants had the same doubling time in the absence of hormone. However, in the presence of hormone, wild type had a doubling time of 3.4 hours while N795/P493R had an increased doubling time of 6.4 hours.

The effect of temperature on the growth defect for colony formation was analyzed. It was found that incubation at 37°C exacerbated the growth defect; however, incubation at 18°C almost abolished the growth defect (data not shown, J. Lefstin).

Transcriptional activation potential of GR in yeast was monitored by measuring the amount of β-galactosidase produced from the integrated reporter pI-G26.1. This reporter contains the Lac-Z gene transcribed by the CYC1 promoter under the control of three TAT simple GREs. In Figure 11a, 100% activity is defined as the activity of wild-type GR, expressed from a high copy 2μ vector, in the presence of hormone. Wild-type GR in the absence of hormone displayed <1% activity, whereas both mutants displayed a ten-fold increase in basal activity. However, in the presence of hormone, both mutants were impaired for activation, displaying 28-35% of wild-type activity.

In Figure 11b, 100% activity is defined as the activity wild-type GR, expressed from a low copy CEN-ARS vector, in the presence of hormone. Under these conditions, wild-type and mutants all had a low basal activity of <1%, and full induction upon hormone addition. Moreover, low-copy expression of the mutants in the presence of hormone did not cause the growth defect observed with high-copy expression. However, even with low-copy expression, growth of both mutants at 37°C was defective (data not shown, J. Lefstin).

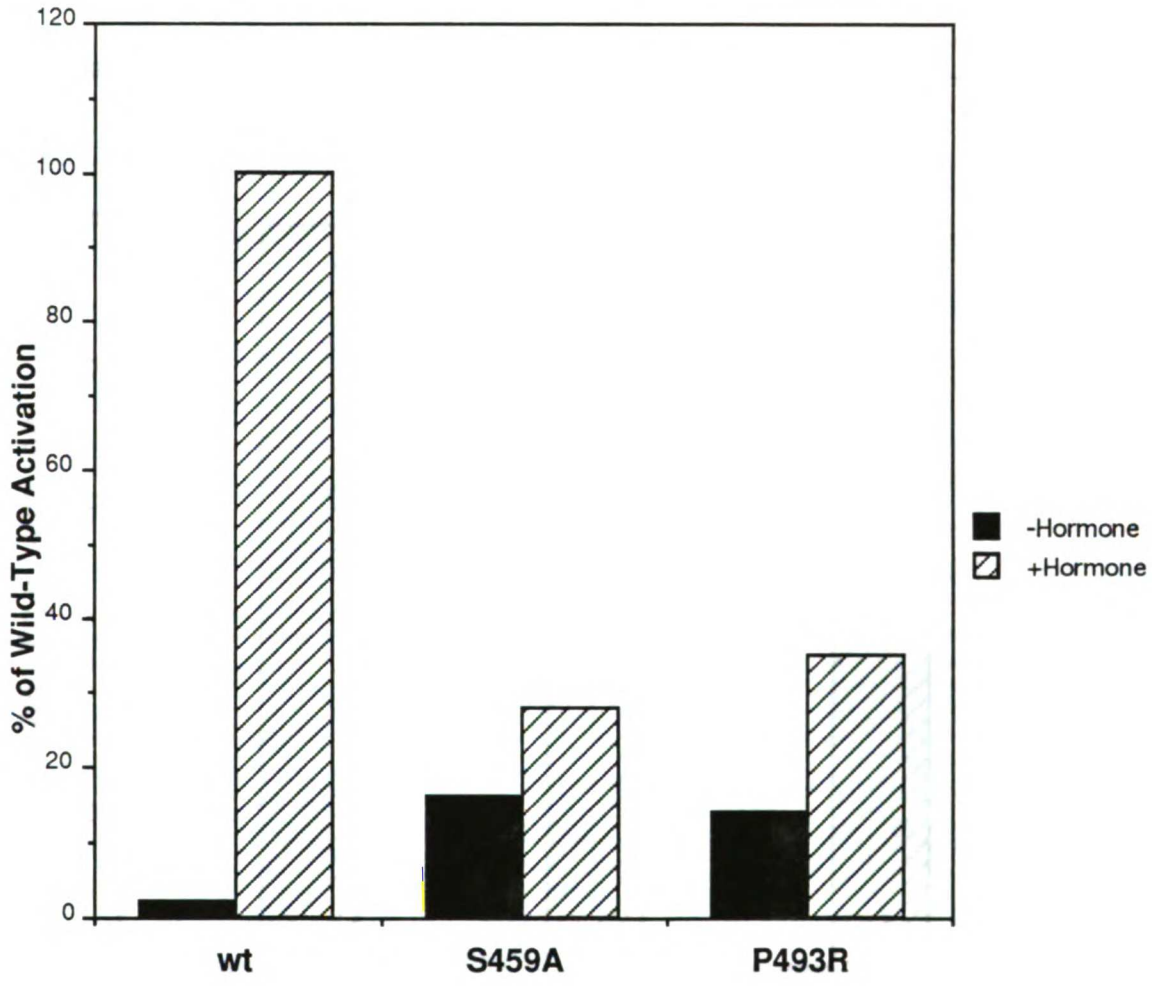


Figure 11a
Transcriptional Activity at a Simple GRE
Under High Copy Expression Conditions

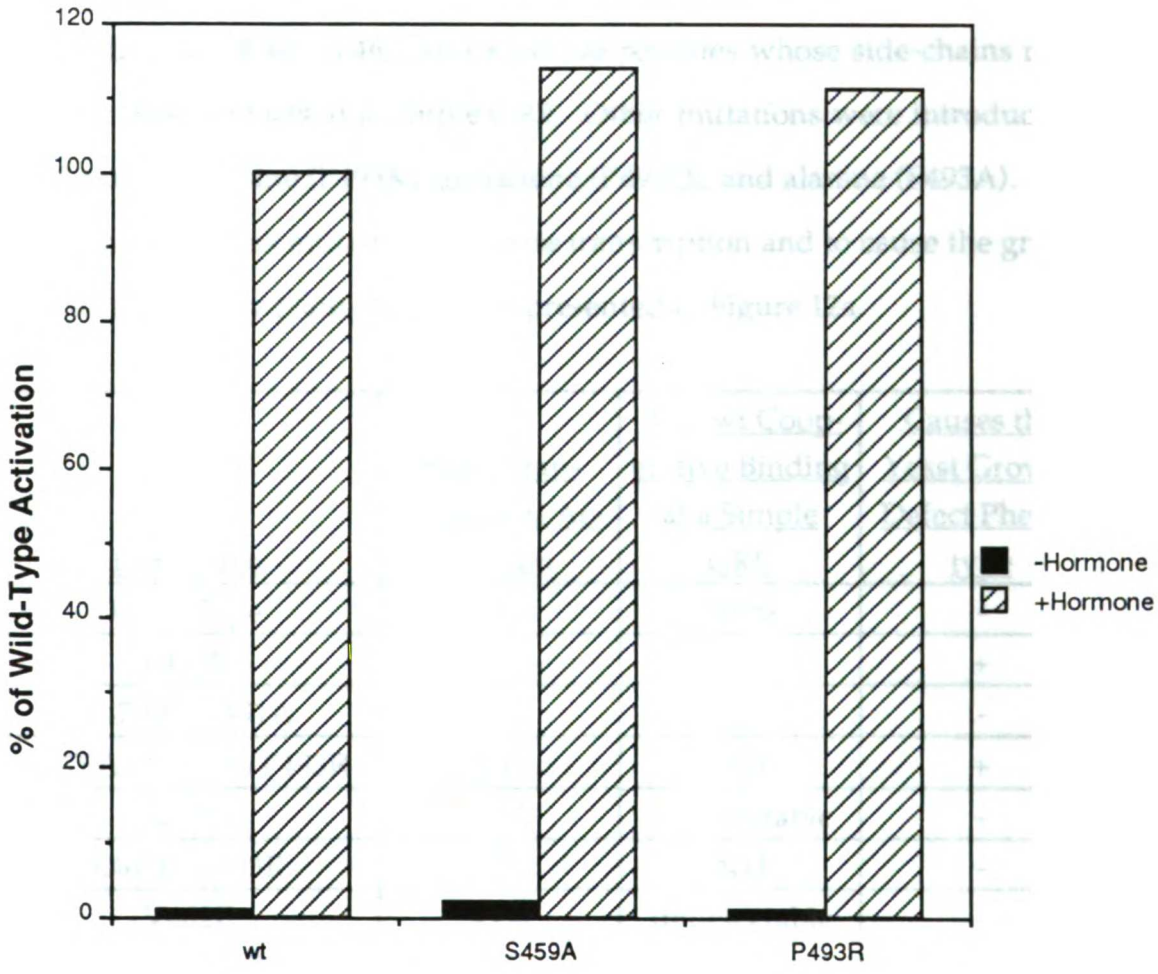


Figure 11b
Transcriptional Activity at a Simple GRE
Under Low Copy Expression Conditions

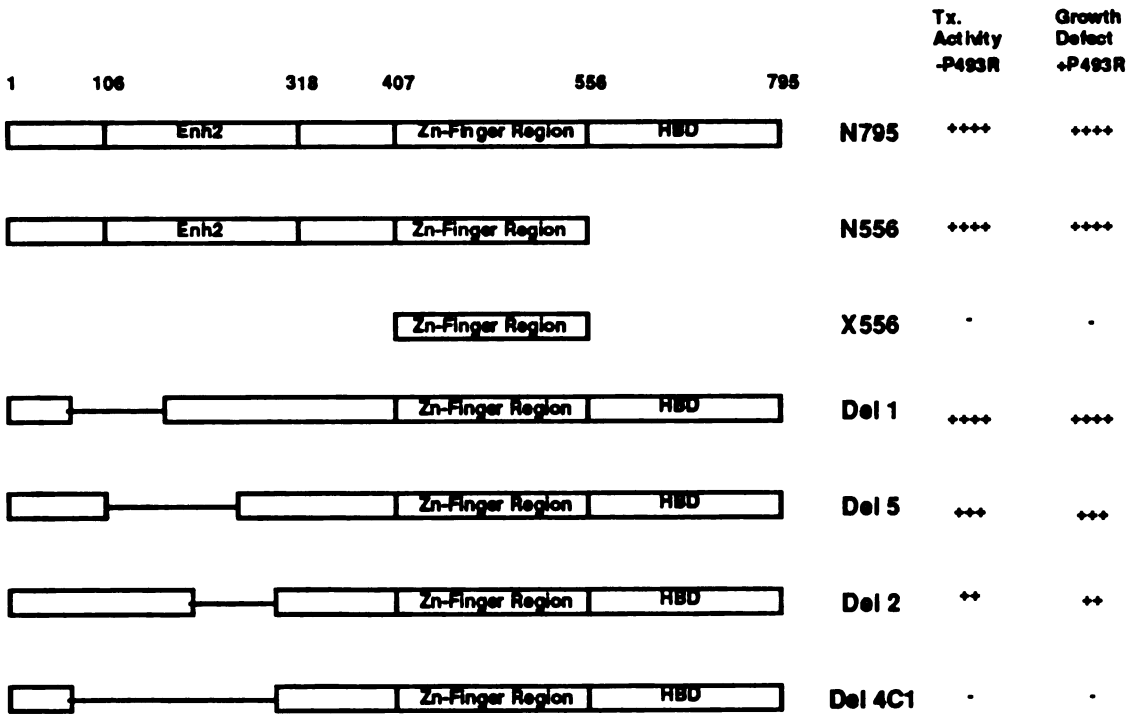
The potential of N795/P493R to cause the growth defect was further tested by combining it with other Zn-finger region mutations or by expressing it in various truncated GR forms. The following multiple mutants were made: P493R/R479A/N491A, P493R/K461E, P493R/V462E, P493R/K461A, and P493R/R466A. R479A and N491A are dimer interface contacts that together cause a severe reduction in cooperative binding in vitro and transcriptional activation in vivo. K461, V462, and R466 are residues whose side-chains make important base contacts at a simple GRE. Other mutations were introduced at the P493 position: lysine (P493K), glutamine (P493Q), and alanine (P493A). The ability of these various mutants to activate transcription and to cause the growth defect, as assayed by plate growth, are presented in Figure 12a.

Derivative	Yeast Transcriptional Activation	% of wt Cooperative Binding at a Simple GRE	Causes the Yeast Growth Defect Phenotype
Wild-Type	100%	100%	-
P493R	35%	112%	+
R479A/N491A	20%	11%	-
R479A/N491A/P493R	ND	ND	+
K461E	<1%	undetectable	-
K461E/P493R	ND	ND	-
V462E	<1%	undetectable	-
V462E/P493R	ND	ND	-
K461A	<1%	ND	-
K461A/P493R	8%	ND	+
P493A	ND	ND	-
P493Q	ND	ND	-
P493K	ND	ND	+

Mutations To Dissect the Mechanism of the Growth Defect

Figure 12a

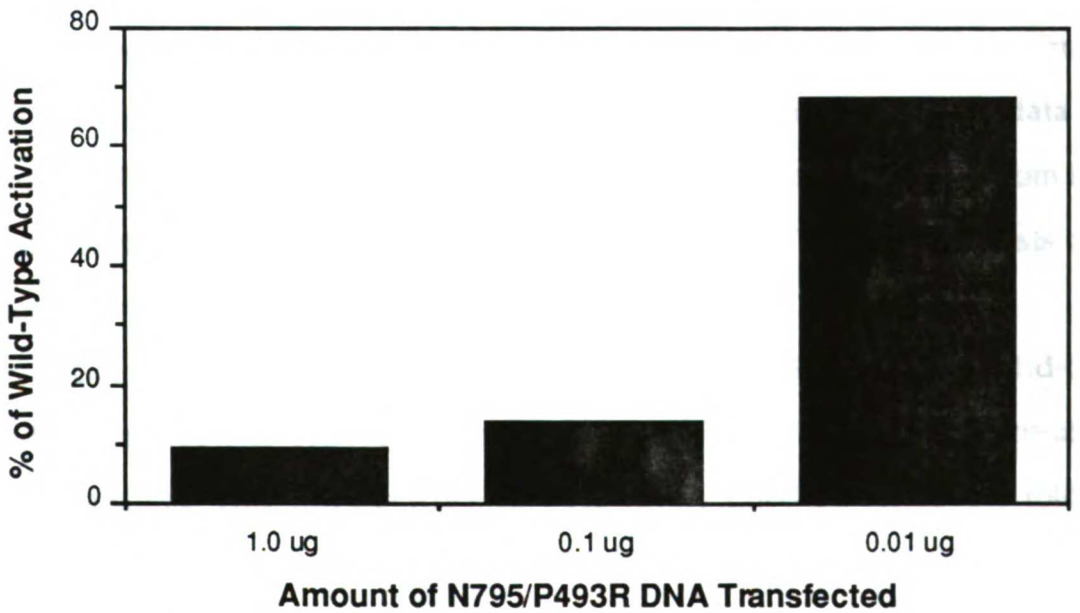
The P493R mutant was also expressed in two deleted forms of GR—N556 and X556. N556 is a constitutive transcriptional activator that lacks the COOH-terminal hormone binding domain. X556 expresses the Zn-finger region, encompassing residues 407-556. Several internal N-terminal region deletions were also combined with the P493R mutation (unpublished observations, J. Lefstin). These include del 1 (deletion of AAs 70-130), del 2 (deletion of AAs 150-300), del 4C1 (deletion of AAs 70-300), and del 5 (deletion of AAs 107-237). The ability of these derivatives to activate transcription without the P493R mutation and their ability to cause the growth defect with the P493R mutation are shown in Figure 12b.



The Effect of Deletions on the Growth Defect

Figure 12b

Transcriptional activation in mammalian cells was monitored by measuring levels of chloramphenicol acetyltransferase (CAT) produced from co-transfections of full length GR with the reporter GMCS in CV-1 cells. This reporter contains the CAT gene under the control of the mouse mammary tumor virus promoter and GREs. Basal levels of transcription in the absence of hormonal induction were identical between wild-type and mutant. Figure 13 displays the percent of wild-type hormone-induced activation produced by the mutants when relatively high levels (1 μg) of expression vector were co-transfected. Both mutants retained only 5-10% of wild-type activity. However, when the amount of transfected expression vector was reduced (0.1 μg and 0.01 μg), the mutant N795/P493R displayed a progressive increase in activity. At 0.1 μg, the mutant displayed 13% of wild-type activity; at 0.01 μg, the mutant displayed 68% of wild-type activity. Paradoxically, activation potential of the mutant relative to wild-type increased as the its levels of expression decreased.



Mammalian Cell Transcriptional Activation by N795/P493R

Figure 13

For structural studies and to assess in vitro binding characteristics of wild-type and mutant Zn-finger regions, the T7-X556 forms of the proteins were expressed in and purified from E. coli. Wild-type T7-X556 is purified by binding to and eluting from BioRex 70 and Cibacron Blue. Strikingly, T7-X556/P493R would not bind to BioRex 70 and would not elute from Cibacron Blue (unpublished observations, B. Maler).

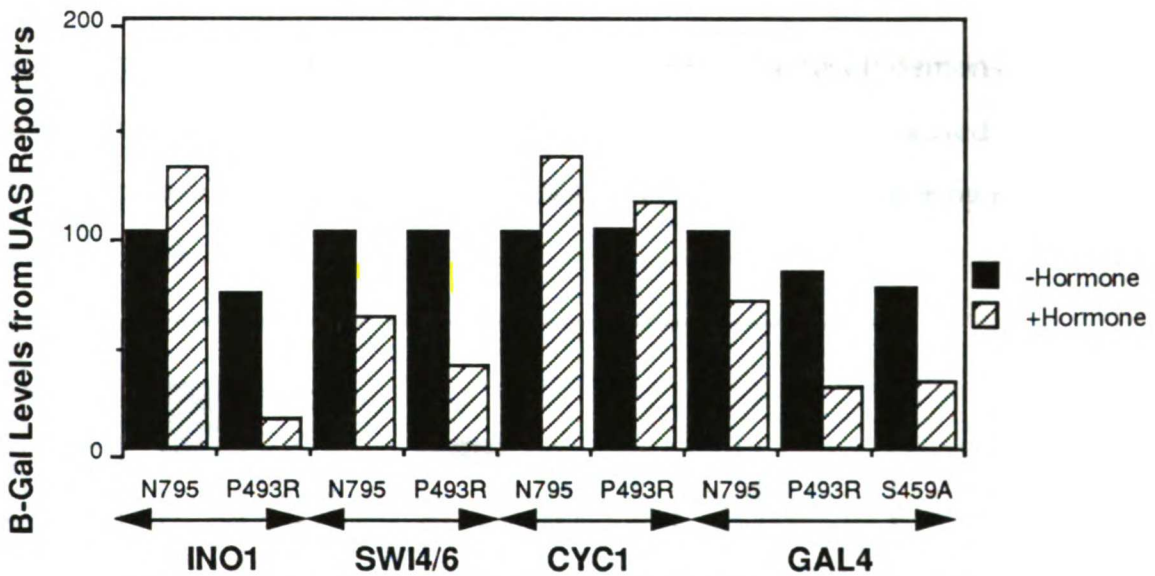
Thus, the P493R point mutation drastically alters the biochemical properties of the T7-X556 GR derivative. A new purification scheme for T7-X556/P493R that utilized yellow agarose and DNA-cellulose proved successful. The purified proteins were then subjected to preliminary circular dichroism analysis (unpublished observations, J. Lefstin and J. Soellen). Figure 14 plots molar ellipticity versus wavelength of light. The peptide bond absorbs most strongly in the region 200-230 nm. Absorbance in this region reflects the α-helical content of proteins. This analysis demonstrated that there was a structural difference between the purified wild-type T7-X556 and T7-X556/P493R as monomers in solution. These proteins were further compared by sucrose gradient sedimentation. There was no apparent difference in the profiles (data not shown). Wild-type T7-X556 is known to be a monomer by calculation from its S value and its radius of gyration. Therefore, T7-X556/P493R also exists as a monomer in solution.

Gel retardation analysis was used to assess the GRE affinity of wild-type and mutant species. See Figures 4a and b. T7-X556/P493R had wild-type affinity for a simple GRE full site (GRE-3S). However, it had a three to four fold increase in affinity for a simple GRE half-site (GRE-4S). Because this analysis was done in the presence of a vast excess poly dI-dC, binding by the mutant was sequence specific.

Molar Ellipticity
0.00e+0
-1.00e+6
-2.00e+6
200
210
220
230
240
250
260
Wavelength, nm
P493R
wt
Circular Dichroism of Wild-Type vs. P493R T7-X556 Derivatives

Figure 14

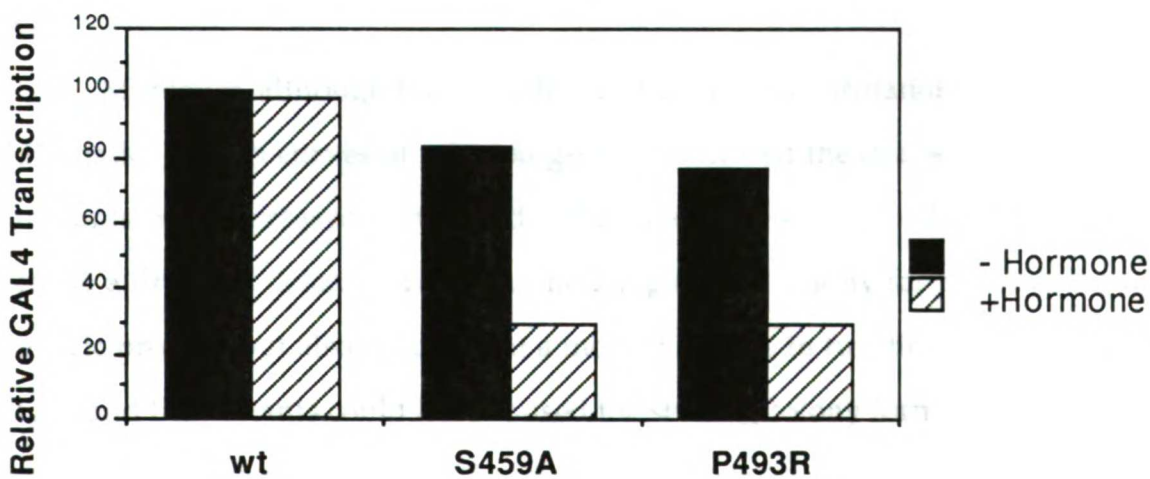
The effect of the growth defect mutants on other yeast activator proteins was assessed. To monitor this activity, a panel of four yeast reporter vectors was used. Each vector had a different upstream activating sequence (UAS) driving the CYC1 promoter/LACZ gene transcription unit. None of the vectors contained GREs. Activity of each UAS was gauged by the amount of β-galactosidase produced. The results are presented in Figure 15. Neither wild-type nor mutant GR had a significant effect on the CYC1 UAS. However, mutant GR expression inhibited the SWI 4/6 cell-cycle box, the INO1 UAS, and the GAL4 UAS. Moreover, wild-type GR had an effect on the SWI 4/6 and GAL4 UASs.



The Effect of the Growth Defect Mutants on Yeast UASs

Figure 15

To assess whether these effects reflect transcriptional inhibition, and to examine their time course, primer extension analysis was done on RNA expressed from the chromosomal GAL1 promoter under control of the GAL4 UAS at early times after galactose induction (unpublished observations, J. Lefstin). Specifically, yeast containing wild-type or mutant GR, expressed from high copy vectors, were grown under noninducing conditions for GAL1 expression. To these cells, hormone was added to activate GR. Ten minutes later, galactose was added to induce expression from the GAL1 promoter. Another ten minutes after this galactose induction, RNA was isolated from the cells for primer extension analysis. The pol II transcript U5 was monitored as an internal control. Its levels were unaffected by all experimental conditions. Figure 16 presents the effect of wild-type and mutant GR on galactose induction of this transcription unit normalized to U5 transcription. The result demonstrated that even at very early times after induction, the mutant caused the same transcriptional inhibitory effect on RNA levels as observed later by monitoring protein levels.



Transcriptional Inhibition of the GAL4 UAS by Primer Extension

Figure 16

Discussion

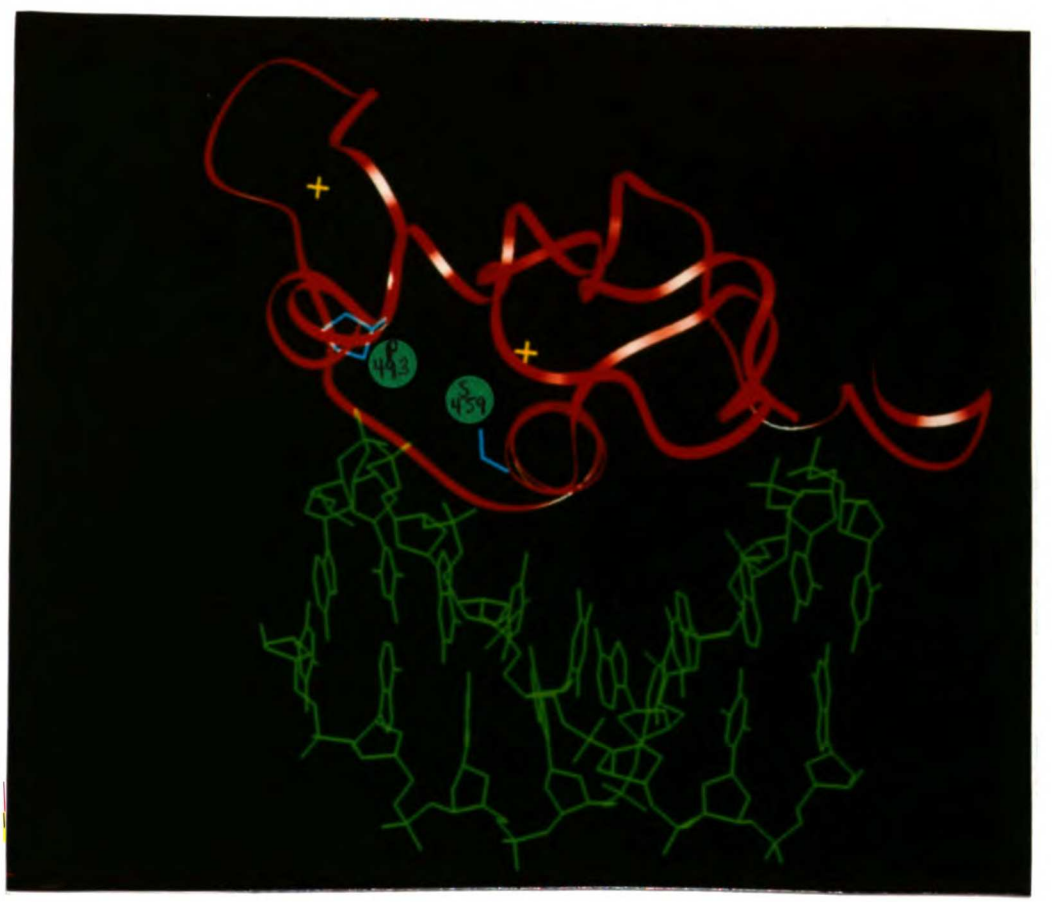
The Two Point Mutants May Cause the Growth Defect Through a Common Mechanism That Is Related to Wild-Type Function

The two Zn-finger region point mutants, P493R and S459A, cause the striking gain of function phenotype of a hormone dependent growth defect in yeast. Several observations imply that this novel mutant phenotype may be related to a wild-type GR function. Wild-type GR causes a very slight growth retardation in certain yeast strains in the presence of hormone (for example BJ2168 or YPH, unpublished observation). Compare lanes 4 and 8 of wild-type colony formation in the absence and presence of hormone in Figure 10. In another yeast strain (W303-1B), Schena notes that the constitutive form of GR, N556, causes a growth defect (Ph.D. dissertation, Mark Schena, 1990). It appears the expression levels of GR in this strain are much higher. Therefore, the effect of the point mutants may have been to exaggerate a characteristic already intrinsic to wild-type GR.

Moreover, although the growth defect for colony formation on plates was dramatic, growth curves of solution growth indicated the doubling time of the mutants only increased ~ two-fold. This change would have been enough to cause a dramatic colony formation phenotype, since colony formation is a geometric process, but also argued that a modest change in function between wild-type and the mutants could have caused the striking colony formation defect.

Despite the fact that P493 and S459 are located in different parts of the three-dimensional Zn-finger structure (shown in Figure 17), and that there is no apparent physical interaction between them, they appear to be functionally related. In this study, the mutants P493R and S459A behaved identically for every

criterion tested. These criteria include growth, transcriptional activation in the absence of hormone in yeast, hormone induced transcriptional activation potential in yeast and mammalian cells, and squelching. Therefore, it is likely that they caused their growth defects by a common mechanism.



Positions of S459 and P493 in the X-ray Crystal Structure

Figure 17

Evolutionary Analysis Suggests S459 and P493 May Have Been Co-selected

Sequence comparisons across the Zn-finger region of the nuclear receptor family indicates that P493 and S459 have been evolutionarily co-selected. In this family, the predominant amino acid at position 459 is Gly (36 out of 46), and the predominant amino acid at position 493 is Gln (34 out of 46). In every case where position 493 is Pro, position 459 is Ser (7 occurrences). There is only one case where Ser occurs at position 459 without Pro at 493. This co-variation is observed in GR and its immediate family relatives (progesterone receptor, mineralocorticoid receptor, and androgen receptor), and in the distantly related Drosophila gene ftz-f1 and its murine homologue ELP. The implication is that these two residues have been co-selected for a particular common function. Either mutant, P493R or S459A, would disrupt this common function.

Interestingly, the amino acids substituted in the mutants, Arg at 493 and Ala at 459, appear in several functional proteins in the superfamily. Of the 46 family members sequenced, the following amino acids occur: at position 459, 36 Gly, 8 Ser (the GR amino acid), and 2 Ala (the growth defect change in GR) and at position 493, 34 Gln, 7 Pro (the GR amino acid), 3 Lys (shown in this study to also cause a growth defect phenotype in GR), and 2 Arg (the growth defect change in GR). Throughout evolution, the only amino acids found at these positions other than the predominant ones are the residues found in GR and residues that when introduced into GR cause a growth defect in yeast. Thus, functional proteins exist that contain the mutant amino acids discovered in this analysis. Therefore, chemically, they are not intrinsically disallowed from participation in a functional Zn-finger structure. Furthermore, the functional attributes of these amino acids may have already been used in evolution. I con-

clude that these GR mutations cause the growth defect through a subtle functional difference that may be an extension of wild-type function.

The Model

The data presented here support the hypothesis that the mutants, P493R and S459A, cause a common altered conformation that leads to inappropriate activation of GR. This conformation may be one that is normally attained by GR, but only upon binding to a simple GRE. The consequence of this normal activation at a GRE may be that GR becomes competent to bind its target protein(s). These point mutants may cause a bypass of this GRE regulation. In this case, every GR molecule in the cell may be inappropriately activated, independently of simple GRE binding. It has been estimated that when expressed from high copy vectors, there are ~10^4 GR molecules per yeast cell. If every one of these molecules became promiscuously active and had the potential to bind its normal protein target(s), the target protein(s) could become sequestered away from other yeast promoters where it (they) is (are) required. Therefore, squelching would occur and the growth defect would be the consequence.

Evidence for Conformational Change

Several observations suggest that a conformational change is induced by the point mutants. First, both mutants produce a partial stimulation of transcription even in the absence of hormone in yeast. The wild-type apo-GR, in the absence of hormone, normally resides in the cytoplasm where it is complexed with Hsp 90. Hormone binding is thought to trigger a temperature dependent conformational change that causes release of Hsp 90 and translocation to the nucleus. Thus, the partial activation by the mutant aporeceptors implies that they may carry structural changes that result in partial Hsp 90 release and nu-

clear translocation. Note, however, that basal activity was not increased in mammalian cells, perhaps reflecting a difference between their respective Hsp 90s. This notion can be tested in yeast because human Hsp 90 can be substituted for the yeast homologue and the effect on basal transcription monitored.

Second, the fact that incubation at 37°C exacerbated the growth defect, while incubation at 18°C lessened it, implies there may be an energy barrier for the growth defect to occur. Conformational change could be the energy requiring event responsible for this observation.

Finally, there is direct physical evidence that the mutants are structurally distinct from wild-type. Column chromatography demonstrated that T7-X556/P493R behaves differently than wild-type. The failure of T7-X556/P493R to bind to BioRex 70 was particularly interesting because the mutation causes a net increase in positive charge. This change would have been expected to increase binding to the cation exchange resin rather than decrease binding. In addition, preliminary circular dichroism measurements implied differences in α-helical content. Because T7-X556/P493R retains a wild-type function, cooperative binding at a simple GRE, these physical differences are not a result of simple denaturation. Moreover, because wild-type and mutant give identical sedimentation profiles on sucrose gradients, the mutant exists as a monomer in solution like wild-type. This result rules out the possibility that the mutations cause aberrant dimerization in solution as the basis of the purification and CD differences. Thus, although wild-type and mutant differ in structure as monomers in solution, the mutant in solution has not attained the conformation required for dimerization that occurs at a simple GRE. There is evidence to support the idea that full conformational activation of the mutant requires association with DNA.

T7-X556/S459A has not yet been structurally characterized. Since P493R and S459A have behaved similarly for all other criteria, I predict that structural analysis will confirm that they have a common structure. Despite the lack of this information, it is clear that at least for the P493R mutant the first part of the model was correct—there was an altered conformation.

Evidence for Squelching

The second part of the model predicts the functional consequence of this structural alteration to be squelching. Squelching is defined as transcriptional inhibition due to an activator binding a target protein in such large amounts that the target protein is sequestered away from other promoters that are dependent on it for activity. Indeed, there was evidence to support this idea. In yeast, native receptor containing the P493R mutation (N795/P493R), expressed from a high copy 2μ vector, caused hormone dependent inhibition of the function of the SWI 4/6, INO1, and GAL4 UASs, as judged by β-galactosidase expression levels driven from these promoters. In addition, for the GAL4 UAS, primer extension analysis of RNA demonstrated that this inhibition was transcriptional and occurred at a very early time after galactose induction. Since galactose induction requires no new gene expression, this inhibition was likely direct. Because there are no binding sites for GR on these reporter vectors, the inhibition is likely mediated by protein-protein interactions. It is possible, but unlikely, that GR interacts directly with each of the activator proteins that operate at these UASs. Rather, the mechanism of inhibition seems more likely to have occurred through some common target protein. This latter scenario seems more likely because different activators with different binding specificities may have exploited the same evolutionarily successful enhancement motif that

mediates an interaction with a common target. Therefore, the phenotype of the P493R growth defect mutant fits the definition of squelching.

In addition, because this transcriptional inhibition occurred very early, while the growth rate was still unaffected, it is unlikely the state of slow growth caused the transcriptional inhibition. It is more likely the transcriptional inhibition caused the slow growth.

Interestingly, the CYC1 UAS appears to be unaffected by either wild-type or mutant GR. This result demonstrates that there is no general, non-specific shutdown of all RNA polymerase II transcription. The implication is that the CYC1 UAS may bind a different target protein(s) than GR and the other activators tested. This conclusion is further supported by the fact that the copper inducible transcription factor, CUP1, is also not inhibited by wild-type or mutant GR (unpublished observation, J. Lefstin). Thus, there may be different classes of activator proteins that bind different target proteins and use different mechanisms to activate transcription.

It is also interesting to note that although the mutants had a larger quantitative effect than wild-type, wild-type GR had a qualitatively similar hormone dependent inhibitory effect on some of the UASs. This observation is consistent with the idea that the point mutations may have been shifting an equilibrium toward a conformation that wild-type GR can also normally attain. This partial squelching by wild-type may explain why some yeast strains show a slight slowing of growth in the presence of hormone and why one strain (W303-1B) which may express very high levels of wild-type GR may be severely growth retarded by the N556 derivative. This argument is based on the idea that all the conformations of a protein are in equilibrium. When the total amount of protein is increased, the absolute concentration of each conformation in equilibrium is

increased. At a certain threshold concentration, sufficient amounts of even wild-type GR may attain the conformation which causes the growth defect.

This line of reasoning is related to a further prediction of squelching. This prediction is that squelching should be dependent on the amount of mutant GR expressed. Thus, when mutant GR was expressed from a high copy 2μ vector (40-100 copies/cell), the growth defect and squelching were observed. However, when mutant GR was expressed from a low copy CEN-ARS vector (1-2 copies/cell), there was no growth defect at 30°C, and squelching was not observed (unpublished observations, J. Lefstin). This result mechanistically links the growth defect with squelching. It is intriguing that even under low-copy expression conditions at 37°C, the mutants displayed the squelching effect and the growth defect phenotype (unpublished observation, J. Lefstin). This result may indicate that the increased temperature favors formation of the altered conformation in the mutant molecules and may imply there is a sensitive threshold level of "activated" GR below which the phenotype does not occur.

Further support for the prediction of dose-dependent squelching comes from an analysis of the ability of the mutants to activate transcription at a simple GRE in the presence of hormone. In yeast, at high copy, the mutants retained only 28-35% of wild-type activation potential. However, at low copy, they attained wild-type levels of activity. These results suggest self-squelching. That is, at high-copy the subset of mutant GR molecules that successfully bind a GRE may be deprived of a target protein that is sequestered elsewhere in the cell by other mutant GR molecules. At low-copy, a subset of GR molecules still successfully bind a GRE but because the sequestration elsewhere in the cell is decreased, sufficient target protein is available to mediate full activation. The low-copy result also demonstrates that the mutants have no intrinsic defect in their ability to occupy a simple GRE in vivo and have no intrinsic defect in their

activation apparatus. Rather, unregulated function of a normal transcriptional apparatus may have caused the apparent defect in the ability of the mutants to activate under high copy conditions.

This dependence of phenotype on the amount of mutant GR also explains the mammalian cell activation results. Under conditions when relatively high levels of DNA expressing mutant GR were transfected (1 μg), transcriptional activation was only 5-10% of wild-type, as compared to 35% of wild-type in yeast from high-copy vectors. I attribute this difference to the likely greater level of protein produced per cell under transfection conditions of mammalian cells (an assumption based on protein levels from similar mammalian transfections). Higher levels of mutant protein led to a higher degree of self-squelching. As in yeast, when relatively lower levels of GR were transfected, the mutant N795/P493R actually displayed a higher percentage of wild-type function. At the lowest level of DNA transfected, the mutant displayed 68% of wild-type activation potential. Studies to assess the ability of the growth defect mutants to squelch other activator proteins in mammalian cells are now in progress.

Deletion Analysis Implies Intramolecular Communication and Regulation

Deletion analysis was performed to identify the domains of GR that are required for the growth phenotype. Truncation of the C-terminal hormone binding domain had no effect: expressed from high copy vectors, the constitutively active forms of the mutants, N556/P493R and N556/S459A, caused a hormone-independent growth defect. This result is reminiscent of the observation that wild-type N556 could prevent the growth of the W303-1B strain, where GR may be expressed at very high levels.

To assess the requirement for the N-terminal region, the derivative X556/P493R was made. This derivative encompasses the Zn-finger region (residues 407-556). Despite the fact that X556/P493R retains 44% of wild-type N556 activity at a simple GRE in yeast (data not shown), it did not cause the growth defect. The fact that N556 caused the growth defect but X556 did not implies that either the N-terminal region or a combination of the N-terminal region and the Zn-finger region may be required for the phenotype. Furthermore, because a Zn-finger region mutant required the N-terminal region for its effect, the inference was that there may be communication between these two domains.

The N-terminal region includes a strong transcriptional activation domain termed Enh2. To test whether this function is the required activity, several internal deletions of this N-terminal region were combined with the P493R mutation. A variety of growth rates were observed. A direct correlation was found between activation potential and the ability to cause the growth defect, i.e., the deletions that retained the most activation potential caused the slowest growth. Formally, squelching activity needs to be correlated with these N-terminal deletions. However, I predict that they will be directly related. This supposition is reasonable given the expectation that an activation domain should bind a target protein. It is this interaction that could mediate squelching.

Wright et al. provided further support for the idea that the activation activity of the N-terminal region is involved in the growth defect (Wright et al., 1991). They expressed the human GR N-terminal activation region (amino acids 77-262) in yeast. The equivalent rat GR residues are amino acids 96-281. This region confers transcriptional activation potential on heterologous DNA binding domains themselves incapable of activation. They observed that this isolated domain expressed from very high copy vectors (500 vector copies/cell) in yeast caused squelching and a growth defect. The similarity in phenotype to that of

S459A and P493R strongly suggests that these Zn-finger region mutations cause their phenotype through the N-terminal activation region. Moreover, because the isolated N-terminal region can cause the phenotype, it must be innately structurally and functionally competent. Furthermore, because this region presumably has no capacity to bind DNA, it must cause the phenotype in solution.

Under high copy expression conditions in yeast, the fact that wild-type GR does not cause the growth defect, but the mutants S459A and P493R do, implies that the innate activity of N-terminal region may be regulated by the Zn-finger domain. I propose that the Zn-finger region normally inhibits the intrinsic activation potential of the N-terminal region until simple GRE binding triggers a conformational change that releases this inhibition. The growth defect mutants would bypass this control. If this were true, DNA would be acting as a true allosteric mediator of GR function, having long range effects away from its own site of interaction.

The Growth Defect Mutants Do Not Require Specific DNA Binding, But Appear To Require Nonspecific DNA Binding

Formally, the growth defect mutants could have caused their phenotype by their action in solution, on specific DNA, or on nonspecific DNA. To assess the role of DNA binding, the P493R mutant was combined with point mutations at residues that are predicted by the X-ray structure to make specific base contacts (K461, V462, R466). The mutants K461E/P493R and V462E/P493R failed to elicit the growth defect. Glutamic acid is a large, negatively charged amino acid that may have caused steric and charge repulsion from DNA. Indeed, K461E and V462E, as point mutants, failed to bind a simple GRE in vitro as T7-X556 derivatives. Although this binding result was a negative one, the stability and levels of these point mutants were circumstantial evidence that the derivatives

were not simply denatured. Furthermore, because these residues point out into solution from an α-helix, in theory, they should have been readily accommodated in the structure without perturbing it. Thus, the result with the double mutants (K461E/P493R and V462E/P493R) implies that DNA affinity is required to cause the defect. Therefore, a mutant in solution is insufficient to cause it. This conclusion needs testing by in vitro binding analysis of the combination mutants.

The mutation of the residues that make specific base contacts to glutamic acid was a severe one. The size and charge of Glu could have abolished both nonspecific and specific DNA binding. In order to investigate the requirement for these two modes of DNA binding, I engineered more subtle mutations at the DNA recognition helix. I made the mutants K461A and R466A. These mutations should selectively remove favorable interactions with DNA bases without perturbing the helix in which they reside or creating any unfavorable interactions.

First, the double mutant K461A/P493R was made and tested. It was still able to cause the growth defect, although it was severely reduced for activation at a simple GRE in yeast (8% of wild-type). The implication is that the growth defect does not require specific recognition of and activity at a simple GRE. In vitro binding analysis of this mutant has not yet been done.

The double mutant R466A/P493R was also made and tested. This mutant combination abolished both the growth defect and activation at a simple GRE in yeast. The interpretation of this mutant is complicated by the fact that R466 may play a dual role, being involved in both nonspecific and specific DNA binding. Inspection of the X-ray crystal structure of a dimer of the Zn-finger region of GR on an oligonucleotide with two GRE half sites separated by four base pairs promotes the idea of this dual role. In this structure, one monomer binds in correct

register with its half site, and R466 makes a specific contact with a guanine. The other monomer makes appropriate dimer contacts, and therefore is out of register with its half site. This subunit makes predominantly nonspecific DNA contacts. In this subunit, R466 makes a phosphate contact with the DNA backbone. It is widely believed that specific DNA binding proteins must have a non-specific binding mode in order to find their sites efficiently. It is hypothesized that specific DNA binding proteins scan two-dimensionally along the major groove in a nonspecific binding mode, predominantly making phosphate contacts, while attempting to make specific contacts. Once a specific site is found, the protein is thought to flip into a specific DNA binding mode making high affinity contacts with bases. Since R466 may be involved in both of these binding modes, the phenotype of the double mutant, R466A/P493R, may merely indicate that some DNA affinity is required for the growth defect.

Thus, the K461A/P493R mutant argued that specific recognition of a GRE was not required for the growth defect. In addition, there were other multiple mutants that further supported the idea that the growth defect phenotype is not dependent on recognition of and function at a specific GRE. P493R was also combined with the double mutant R479A/N491A. This double mutant of the Zn-finger region dimer interface has been previously shown to severely reduce cooperative binding in vitro and activation in vivo, while retaining wild-type repression in vivo. The combination mutant R479A/N491A/P493R as an N556 derivative retained the ability to cause the growth defect. Its activation potential in yeast and its in vitro DNA binding properties have not yet been determined. This multiple mutant was stable, present at wild-type levels in yeast, and clearly not denatured because it retained the ability to cause the growth defect. Because the dimer interface mutations actually abolished the residues involved in making dimer contacts, it was highly unlikely the combination with P493R somehow

restored dimerization. Therefore, the implication is that a monomer containing the P493R mutation is sufficient to cause the growth defect. However, in vitro binding studies need to be done to verify the predicted loss of cooperative binding. But assuming this result is obtained, this mutant would have been unlikely to occupy a GRE in vivo because of its predicted loss of cooperative binding. Thus, this dimerization defective growth mutant supported the idea that specific recognition of a GRE is not required for the growth defect. Together with the K461A/P493R mutant that poorly activated at a GRE but still caused the growth defect, current evidence suggests that function at a GRE is not required.

One more line of experimentation separates the ability of GR to activate at a simple GRE from the growth defect. Steve Yoshinaga has found that activation at a simple GRE in yeast is completely dependent on SWI 1, 2, and 3 (manuscript submitted). When the P493R mutant was expressed in a strain deleted for SWI 1, 2, and 3, the growth defect still occurred (data not shown). Therefore, transcriptional activation at any SWI dependent GRE was not required for the growth defect. Moreover, SWI 1, 2, and 3 were not the targets of the mutant squelching.

The above results bear on the formal possibility that the P493R and S459A mutations may have caused the growth defect in yeast by binding to some site in the yeast genome. This genomic site could be a fortuitous GRE or the mutations could be causing a new DNA specificity. Once bound to this site, GR could either activate or repress some gene that in turn led to the growth defect. The results with the base contact (K461A/P493R) and dimerization (R479A/N491A/P493R) mutants, together with the SWI deletion strain result, argued strongly against the idea that the mutants bind at a fortuitous GRE and activate or repress some gene. Furthermore, the fact that at a very early time after hormonal induction, transcriptional inhibition occurred at the GAL1

promoter implies that no intermediate genomic gene expression or repression was required; therefore, the effect is direct. Because squelching also occurred on episomes on which there are no known GREs, steric repression of transcription due to GR binding affecting local complexes is unlikely.

Altered DNA binding seems unlikely because T7-X556/P493R still retains wild-type affinity for a simple GRE. Moreover, if the mutants had an altered DNA specificity that allowed them to cause the growth defect by binding to a genomic site, it would be highly unlikely that the panel of different UAS episomes would have this same site. Finally, since the mutants also had the same self-squelching phenotype in yeast and mammalian cells, the yeast phenotype was not a simple yeast artifact. Again, the transcriptional inhibition of the mammalian reporter vectors was unlikely to have been caused by a site in common with the yeast genome and the yeast episomes.

Thus, I conclude that neither a specific GRE nor some new specific site is required for the growth defect. The hypothesis that the transcriptional inhibition is caused by sequestration of a common target protein (squelching) remains the best hypothesis. However, the phenotypes of the other DNA binding mutants (K461E/P493R, V462E/P493R, K461E/V462E/P493R, and R466A/P493R) all argue that some DNA affinity was required for the growth defect. Based on negative results, this inference is weak and will require further experimentation.

In theory, there could be two reasons for this apparent requirement for DNA. First, the point mutants could induce the activated GR conformation in solution, but the interaction with the target protein may only occur on DNA. The fact that the isolated N-terminal enhancement region can cause a growth defect in yeast apparently without the need for DNA binding makes this hypothesis less likely. Moreover, although T7-X556/P493R displays a conformational

difference with wild-type in solution, it does not dimerize in solution, implying that it may not be fully conformationally activated. The second hypothesis is then that the association with nonspecific DNA may promote the full conformational change required for the growth defect.

Speculation on the Requirement for Nonspecific DNA Binding

To understand the apparent need for nonspecific DNA binding by the mutant to cause the phenotype, I will consider first how wild-type GR might find a site. A wild-type monomer is predicted to bind DNA first in a nonspecific binding mode, I propose the change from a monomer in solution to a nonspecifically bound form is accompanied by a primary conformational change. Previously, I have presented the idea that helix D may form upon DNA binding. Because helix D makes only phosphate contacts, it or other substructures could form even under nonspecific DNA binding conditions. This nonspecifically bound form would then scan the major groove looking for a specific site. Upon binding a specific GRE, I propose the specific contacts trigger a secondary conformational change that leads to functional competency. The key point is that both primary and secondary conformational changes must occur for function. I believe the growth defect mutants bypass the need for the specific GRE site trigger. However, they would still be dependent on the conformational change induced by nonspecific binding to lead to functional competency. Mutations that abolish nonspecific binding would then prevent the growth defect by P493R and S459A.

The Conformation of the Growth Defect Mutants May Mimic the Conformation Wild-type Attains at a Simple GRE

If the model that a specific GRE site triggers a conformational change were true, there would be an energy requirement. Presumably the energy from the

interaction of side chains with bases and backbone phosphates would drive this putative conformational change. In theory, the DNA site could be viewed as an enzyme and GR as a substrate for this enzyme. Studies of catalysis argue that an enzyme can stabilize the transition state of its substrate thus catalyzing a reaction. Transition state analogs therefore have higher affinities for the active site of their enzymes than the natural substrates. The P493R and S459A mutants could be viewed as transition state analogs. Indeed, T7-X556/P493R had a three fold increased affinity for a simple GRE half site. The implication is that the conformation that the GRE normally induces had already been attained due to the mutation. Because the mutant was in the high affinity conformation already, no energy was expended in inducing the conformation and the result was better binding. Thus, there is experimental support for the idea that a GRE induces a conformational change and that the growth defect mutants bypass the need for this regulation by having already caused the appropriate conformational change.

However, it must be noted that T7-X556/P493R did not demonstrate a proportional binding increase at a full simple GRE in vitro. The in vitro assay may have been unable to reflect increased binding. Further in vitro experimentation is required. However, there is some evidence that the P493R mutant may direct increased affinity for a simple GRE in vivo. The GR derivative X556a ("a" refers to non-receptor amino acids encoded by the vector) is a weak activator in yeast, providing only ~12.5% of the activation potential of N556a. The GR derivative X556a/P493R displays ~44% of the activation potential of N556a. Because X556a/P493R lacks the N-terminal region and does not cause the growth defect, there is probably no self-squelching occurring. Thus, the increased activation potential of X556a/P493R may reflect increased occupancy of a simple

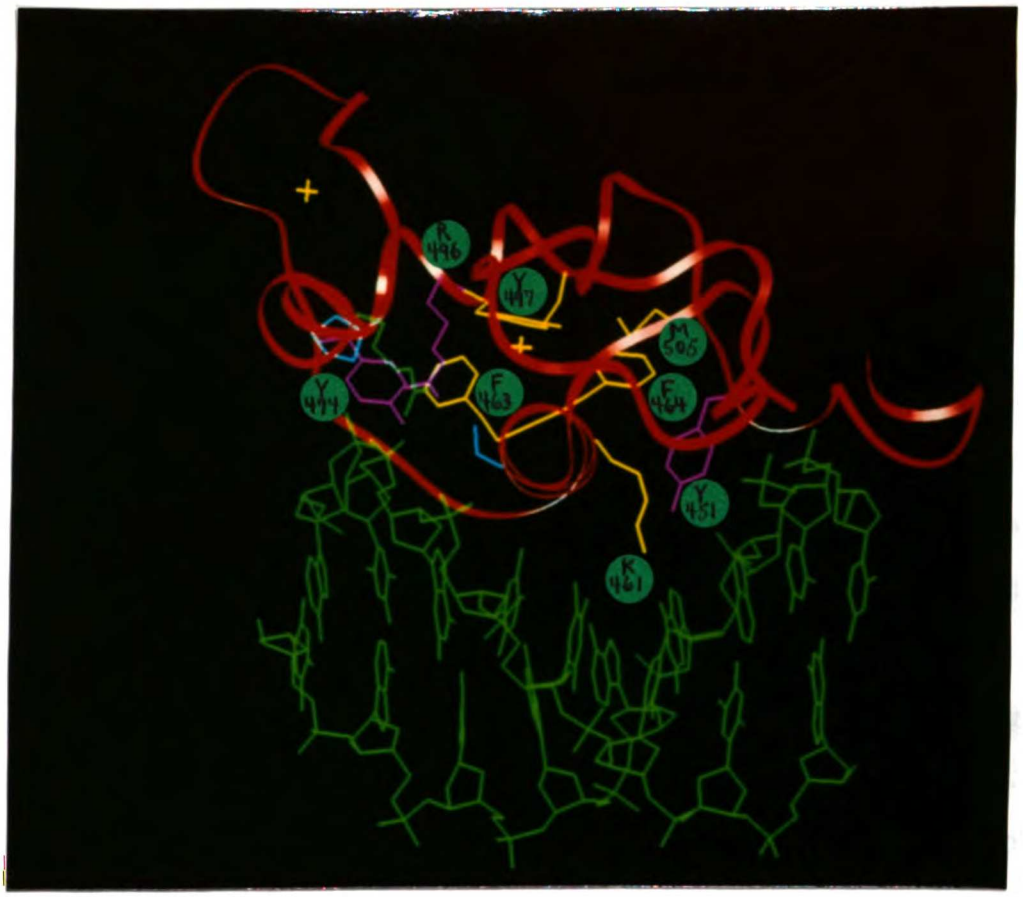
GRE in vivo. This ~three fold increase in activation in vivo is similar to the ~three fold increase in binding to a simple GRE half-site in vitro.

The fact that the double mutant K461A/P493R still causes the growth defect implies that K461 may be part of the GR sensing apparatus that detects GRE binding and triggers a conformational change. There is evidence to support this idea. K461 normally makes hydrogen bonds with a guanine of the GRE. Its mutant form K461A had no detectable activity at a simple GRE in yeast. However, the double mutant K461A/P493R had 8% of wild-type activation and was still capable of causing the growth defect. Since self-squelching was probably occurring, the appropriate comparison for the activation potential of the K461A/P493R mutant is P493R. P493R had 35% of wild-type activation potential under similar self-squelching conditions. Thus, in the context of the P493R mutation, the mutant K461A was able to recover a significant fraction (~20%) of its transcriptional activation potential. The implication is that the K461-GRE interaction is important for inducing the conformational change at a simple GRE. When the conformational change is induced by the P493R mutation, the need for the K461-GRE interaction is decreased. Because the double mutant did not recover full activity, the energy of the K461-GRE interaction probably plays a significant role in GRE affinity as well as GRE induced conformational change.

The importance of K461 as a potential trigger is highlighted by the fact that it is absolutely conserved in the steroid/thyroid receptor superfamily except for one member (45 out of 46). This exception proves to be extremely interesting. In the tll (Drosophila tailless) protein, the position equivalent to K461 is Ala, the mutation that abolishes activation in GR. Interestingly, the residue at the position equivalent to P493 is Arg, the mutation that causes a growth defect in GR and appears to partially compensate for the deleterious effect of K461A in

GR. Thus, nature has created its own functional version of my K461A/P493R GR mutant.

Conformational changes such as this one proposed for GR may explain a difference observed between bacterial repressors and eukaryotic transcription factors. Bacterial repressors have high specific to nonspecific binding ratios (~100,000:1), while eukaryotic transcription factors have 100-fold lower ratios in general. GR has a ratio of ~2000:1 (Ph.D. dissertation, Josh LaBaer). I speculate this difference may be caused by the energy requirements for conformational change that eukaryotic transcription factors like GR may have. Bacterial repressors need high affinity to maximize occupancy. Eukaryotic transcription factors may sacrifice affinity for a site in order to gain regulatory control through conformational change. Because of this trade-off, eukaryotic promoters may require multiple activators in order to increase fractional occupancy through cooperative binding or to increase fractional occupancy by having multiple independent but redundant activator sites that overall maintain a certain level of occupancy.



Residues Potentially Involved in Mediating Conformational Change

Figure 18

How Interaction With DNA Could Cause Conformational Changes

I speculate that structural studies will reveal that the result of GRE binding is to trigger a conformational change that causes an alteration in the relative orientation of helix R and helix P. Moreover, the mutants, S459A and P493R, may be found to cause a similar conformational change independently of GRE binding. There is a precedent for this type of conformational change. Point mutations in the bacterial activator CRP have been found that make it constitutively active in the absence of its normal activator, cAMP (Garges and Adhya, 1985).. These point mutations have been found to lie near a hinge region between two α-helices that interact through residues near the hinge. One helix takes part in the domain responsible for binding cAMP and the other helix takes part in the domain that binds DNA. The hypothesis has been that the point mutants disrupt the interaction between these critical helices altering their relative orientations in a way that mimics the result of cAMP binding.

In the GR Zn-finger region, helix R packs against helix P through hydrophobic contacts in the highly conserved core of the domain (Figure 18). This hydrophobic core also contains residues that both take part in the hydrophobic core and make phosphate contacts with DNA (shown in magenta: Y451, Y474, and R496). I speculate nonspecific DNA binding may alter the hydrophobic core of the domain through some of these residues, impacting on the nature of the interaction between helices R and P. In addition, nonspecific DNA binding may also be sufficient to promote the formation of helix D which makes phosphate contacts and dimer contacts. These changes may constitute a primary conformational change that may put the interaction of the two amphipathic α-helices under stress. However, full conformational activation does not occur until a second conformational change is triggered at a specific GRE.

At a specific GRE, phosphate contacts may also be part of the trigger. In theory, it is possible some phosphate contacts cannot be made until a conformational change is initiated at a GRE. After this initial change, phosphate contacts that are also part of the hydrophobic core could take part in a further concerted change in conformation. Contacts with bases, such as the interaction of K461 with a guanine, may be part of this initial trigger. This interaction could affect the structure of helix R, in which K461 resides. In theory, it is possible that some of the energy of the K461-GRE interaction is used to cause the K461 residue to move in space, i.e. attaining the transition state from the enzyme-substrate analogy. This structural perturbation is predicted to locally affect the disposition of residues that comprise helix R. These residues include F463, F464, and S459. F463 and F464 are absolutely conserved hydrophobic residues that take part in the hydrophobic core and make direct contacts with absolutely conserved residues in and just downstream of helix P. F463 appears to be in position to interact with R496, another absolutely conserved residue in the other helix. There could be either a favorable Van der Waal interaction between the hydrocarbon side chain of R496 and the aromatic ring of F463 or an unfavorable interaction between the positive charge of R496 and the aromatic ring of F463. F464 appears to make a favorable Van der Waal contact with M505, again an absolutely conserved residue. Finally, both F463 and F464 appear to interact with Y497, again a residue which resides in helix P. Thus, there appears to be an extensive hydrophobic interaction between these two helices which may be affected by specific GRE binding.

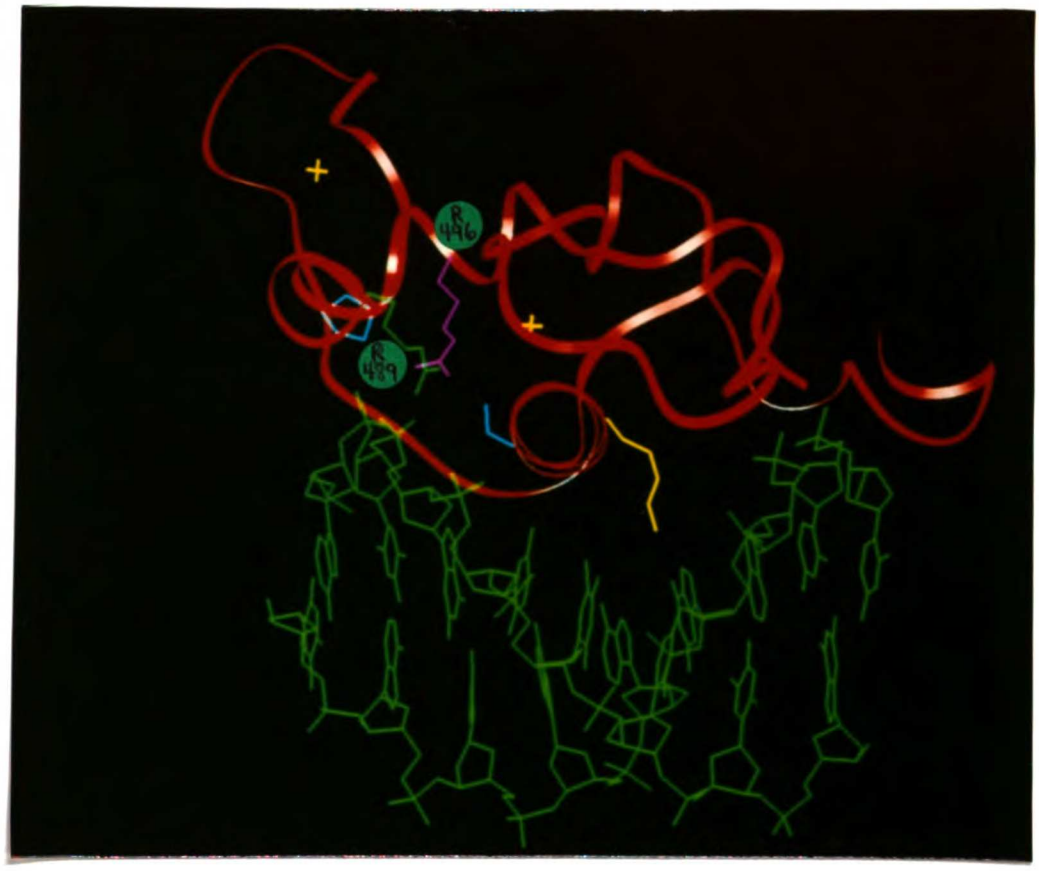


Figure 19

Potential Interactions of S459

How S459A and P493R May Cause Structural Changes

S459, which resides in helix R, appears to make intramolecular contacts. I have argued in a previous chapter that S459 is a critical node of intramolecular communication. When mutated to Arg (S459R), it compromised activation and repression while retaining wild-type affinity for a half site. When mutated to Ala (S459A), it caused a growth defect. Because Ala is small and has minimal potential for interaction, I believe S459A caused its phenotype by the removal of a normal interaction of which the Ser was capable, rather than the formation of a new aberrant interaction or steric disruption of the local structure.

Inspection of the X-ray crystal structure of the Zn-finger region gives some clues about the nature of the potential interactions of S459 (Figure 19). As a dimer on a GRE, S459 appears to make a hydrogen bond with R489. This residue is located in helix D that putatively forms upon DNA binding. R489 also makes a phosphate contact. Interestingly, this same phosphate is also contacted by R496, an absolutely conserved residue from helix P. Therefore, R496 is very close in space to S459, just out of hydrogen bond range. I speculate that S459 interacts with R496 prior to specific DNA binding. Upon specific DNA binding, S459 is induced to move to a position that favors interaction with R489 that has recently taken a position nearby upon formation of helix D and is stabilized in this position by a phosphate contact. R496 makes a new interaction with the same phosphate from the DNA backbone. R496 may be stabilized in this new position through favorable interactions between its hydrocarbon side chain and the aromatic ring of F463. It may have been destabilized in its original position by an unfavorable interaction between its positively charged amine group and the same aromatic ring of F463.

The result of these concerted changes is the disruption of an interaction between helix R (contains S459) and helix P (contains R496). Together with

other concerted changes caused by residues that take part in both phosphate contacts and hydrophobic core contacts (Y452 and Y474), hydrophobic residues from helix R (F463 and F464), and hydrophobic residues from helix P (Y497, C500, M505), the relative orientation of the two amphipathic α-helices may be altered. The importance of R496 is underscored by the fact that it is absolutely conserved in every superfamily member sequenced to date.

The P493R mutation may cause the growth defect through a similar change in conformation. P493 takes part in helix P, and lies near a hinge between helix P and helix D, that putatively forms upon DNA binding. Thus, P493 takes part in the same helix in which R496 resides. From its position near this hinge, P493 is also predicted to take part in the hydrophobic core. Its hydrocarbon side chain is positioned very closely to Y474, another member of the hydrophobic core that also makes a phosphate contact. In theory, this interaction could transduce information of DNA binding. The importance of this potential interaction is highlighted by an analysis of their evolutionary relationship in the superfamily. Y474 is highly conserved, occurring in 39 of 46 family members. In every case, position 493 is a Pro, position 474 is Tyr. There are five occurrences where position 493 is either Arg or Lys (the amino acids that caused a growth defect in GR). In four out of these five occurrences, position 474 is no longer a Tyr. The changes include Ser, Ala, and Cys.

I have mutated P493 to Gln, Ala, and Lys. The change to Gln (P493Q), which is the predominant amino acid at this position in the superfamily, did not cause the growth defect. The change to Ala (P493A) also did not cause the growth defect. Therefore, the simple absence of the Pro residue is not sufficient to cause the phenotype. However, the change to Lys (P493K) did cause a growth defect, although it was less severe than the P493R defect. I concluded that the mutant positive charge may be what is essential to cause the growth de-

fect. Because P493 is predicted normally to take part in the hydrophobic core, the introduction of a positive charge would be quite disruptive, potentially causing a rearrangement of the hydrophobic core. R496 is nearby in helix P. Thus, P493R and P493K may disrupt the same putative R496-S459 interaction that the S459A mutation does and also affect the arrangement of residues in the hydrophobic core, leading to the same conformational change and the same consequence—a growth defect.

Of course, these speculations are probably wrong in detail; however, the mutant P493R (and presumably S459A), through its altered conformation, squelching, and dependence on the N-terminal region for its phenotype, has strongly implied that a simple GRE is an allosteric regulator of GR function.

A model of how wild-type GR may function is shown in Figure 20a. First, it shows GR as a monomer in solution. Upon binding to DNA nonspecifically, a primary conformational change is induced. Upon binding at a simple GRE, a secondary conformational change is induced that promotes dimerization, releases an inhibition of the Zn-finger region on the N-terminal enhancement region, Enh2, and allows binding of a target protein. This conformational activation triggered by a simple GRE would be true allosteric regulation.

A model of how the growth defect mutants may have caused their yeast phenotype is shown in Figure 20b. It shows monomeric GR bound nonspecifically to DNA to already have undergone full conformational activation. Solution monomers appear to have an altered conformation from in vitro analysis, but appear unable to cause the phenotype in vivo (not illustrated). A large fraction of the estimated 10,000 molecules of GR/cell may be in the nonspecifically bound mode. I show GR as a monomer in this mode because the presumptive dimerization defective mutant, R479A/N491A/P493R, can cause the growth defect. Thus, although the point mutant P493R may exist as a dimer

on nonspecific DNA, dimerization appears not to be a requirement for the phenotype. For wild-type GR, dimerization may be required for maximal occupancy at a simple GRE, not activation per se. The consequence of this aberrant activation is squelching of other yeast promoters, including those linked to simple GREs, that require the sequestered target protein. GR, however, is shown as competent to occupy a simple GRE. Indeed, under low copy expression conditions, N795/P493R or S459A were wild-type for activation at a simple GRE, and in vitro, T7-X556/P493R was able to bind at wild-type or higher affinity to half- and full site simple GREs.

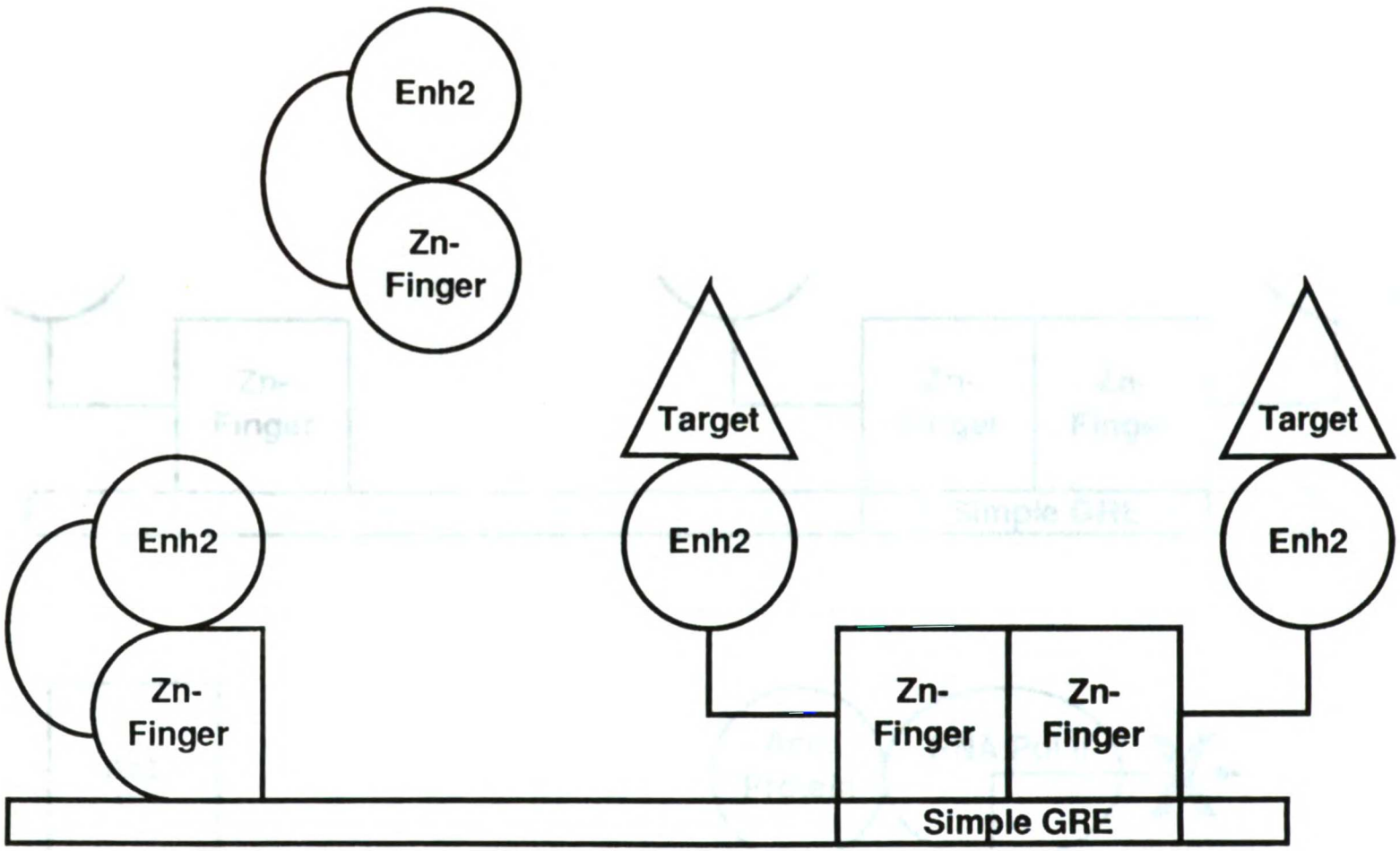
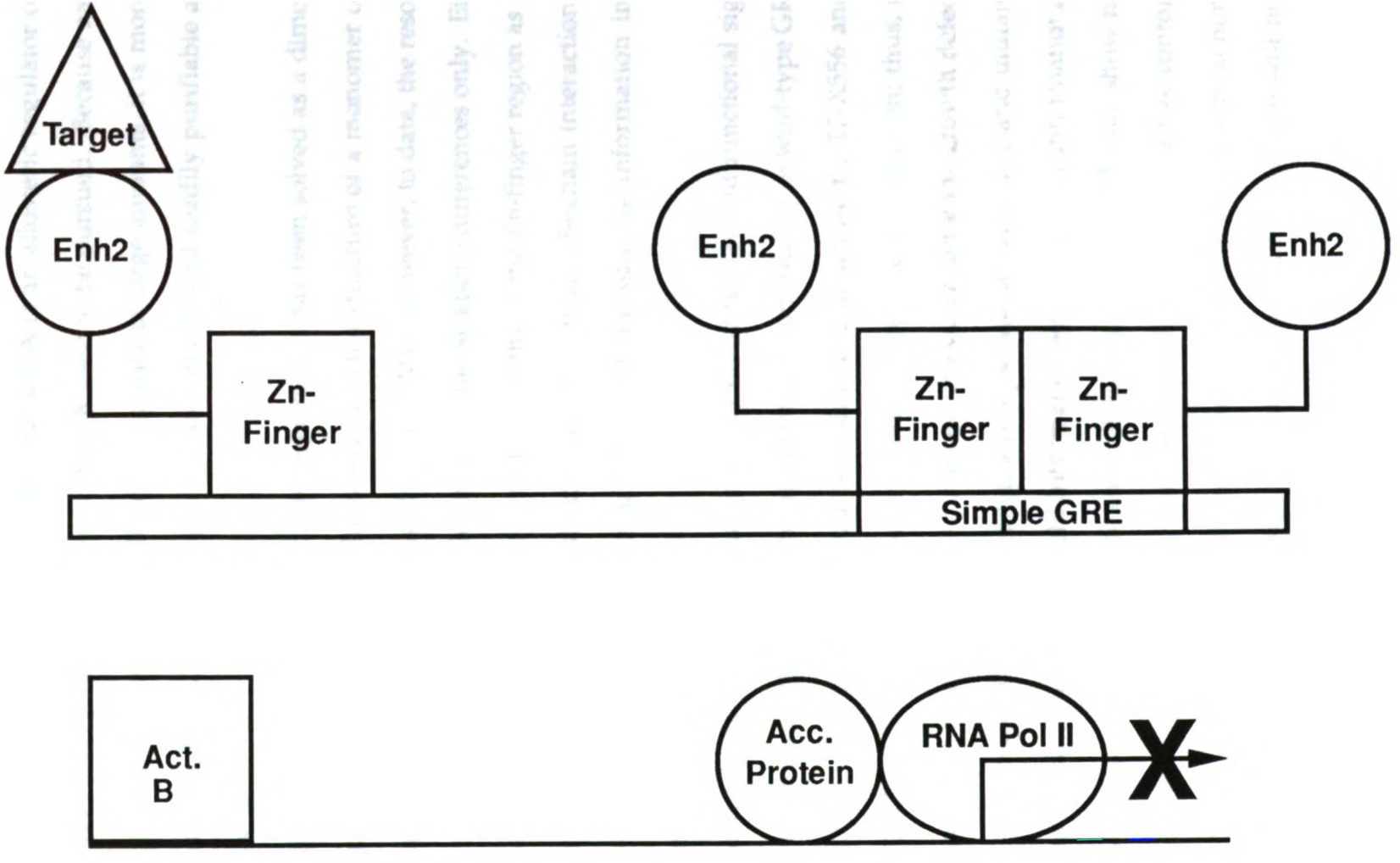


Figure 20a
A Model of Simple GRE Mediated Allosteric Regulation of GR



How the Growth Defect Mutants May Cause Their Phenotype
Figure 20b

Future Directions

To definitively test the hypothesis that DNA is an allosteric regulator of GR function, direct physical studies of the GR need to be pursued. Because native GR is a large protein that is difficult to purify in large amounts, it is more feasible to work with GR derivatives that are smaller and readily purifiable at high levels.

The structure of the isolated Zn-finger region has been solved as a dimer on a simple GRE by X-ray crystallography, and the structure of a monomer of this region in solution has been solved by 2D-NMR. However, to date, the resolution of the NMR structure is sufficient to define backbone differences only. Either higher resolution NMR or X-ray crystallography of the Zn-finger region as a monomer in solution will be required to define critical side-chain interactions that may be responsible for sensing DNA and transducing information intramolecularly.

Structural analysis of the growth defect mutants may add functional significance to interactions predicted to be important from studies of wild-type GR. Preliminary circular dichroism studies indicated that wild-type T7-X556 and mutant T7-X556/P493R differed in structure as monomers in solution; thus, it will be interesting to analyze their structures. However, since the growth defect may require nonspecific DNA binding, a comparison of wild-type and mutant monomers in solution may not yield information about the full conformational change. A comparison of wild-type and mutant on a simple GRE may show no structural difference. Both would be expected to have the same active conformation. Therefore, a more informative analysis would be a comparison on nonspecific DNA. Under this condition, the prediction is that wild-type will not have the fully active conformation, while the mutant will.

To test the hypothesis that the Zn-finger region regulates N-terminal enhancement activity, the GR derivative EX556 may prove useful. This derivative contains Enh2 (amino acids 106-318) connected by a synthetic linker to the Zn-finger region (amino acids 407-556). EX556 can activate transcription in vivo and in vitro, and it is readily purified at high levels from E. coli. If EX556, containing the S459A or P493R point mutations, causes the yeast growth defect, structural analysis should be pursued. An examination of a monomer of EX556 in solution may reveal the contacts between the N-terminal region and the Zn-finger region, and examination of a complex on a simple GRE may confirm that the N-terminal region is released upon DNA binding, proving that DNA is a true allosteric regulator of GR.

In addition to providing a molecular handle on the question of DNA mediated allostery, the growth defect mutants provide a yeast phenotype that might be used to elucidate other GR functions. For example, high copy expression of the gene that codes for the GR target protein might supply enough of the protein to overcome the sequestration by the mutant GR, restoring transcriptional activation and normal growth. Because there was a sensitive threshold amount below which the mutant GR did not cause the phenotype, this suppression should be possible. Low copy expression of the mutant GR at 30°C did not cause the phenotype. However, the same low copy expression of the mutant at 37°C did cause the phenotype. This result argued the amount of GR expressed from low copy vectors is very near the threshold, so overexpression of the target should have a very good chance of overcoming the sequestration and restoring normal growth.

In addition, the yeast growth defect might also prove useful for analysis of other aspects of GR function. Because the growth defect is hormone dependent for N795/P493R or S459A, yeast mutations that block the growth defect may

CHAPTER 4

Speculations About Composite GRE Repression

My analysis strongly supports the hypothesis that a simple GRE acts as an allosteric regulator of GR to promote dimerization and transcriptional activity. At a proliferin composite GRE, a GR dimer, in conjunction with Jun, activates transcription. At the same element, a GR monomer, in conjunction with Jun and Fos, represses transcription. What is the basis for this behavioral difference at the composite element? In theory, there are two explanations for this phenomenon.

First, the composite GRE, which has no strong sequence homology to a simple GRE, could have different allosteric consequences for GR. The site may not promote dimerization, instead binding GR as a monomer in an intrinsic "repressive" conformation. Occupancy as a monomer may be aided by protein-protein interactions with Jun or Jun/Fos. Together with Jun and Fos, the complex would retain its inherent repressive nature. However, with Jun, the state of GR induced by the site could be overcome, leading to homodimerization and transcriptional activation.

Second, the composite site, despite no strong sequence homology to a simple GRE, could have allosteric effects similar to a simple GRE on GR. According to this view, the composite sequence to which GR binds is capable of inducing dimerization and transcriptional activation. Because the site is poorly homologous to a simple GRE, occupancy of the initial GR monomer may be aided by protein-protein interactions with Jun or Jun/Fos. In a complex with Jun, GR would proceed to dimerize and the complex would activate transcription. However, in complex with Jun and Fos, GR would remain monomeric, and the complex would be intrinsically "repressive".

The D485A and R488A mutants imply that the means by which GR occupies a site may differ at composite and simple GREs. These mutants bind poorly at a simple GRE half-site, fail to activate transcription at a simple GRE in vivo, but are wild-type for repression at the composite GRE. D485 is adjacent to and R488 resides in helix D and may stabilize its formation through a salt-bridge they form with each other. Helix D makes phosphate contacts that may aid affinity for a simple GRE and has contacts that form part of the dimer interface. It is likely these mutants prevent formation of helix D, thereby decreasing affinity for a half-site and preventing dimerization. Because they are still wild-type for repression, the means by which GR occupies a composite site must be different. There are two hypotheses of how this occupancy may differ. First, the GR-DNA contacts at a composite GRE may be similar to those at a simple GRE, but the affinity lost from helix D may be compensated for by cooperative binding with Jun or Jun/Fos. Second, GR may make different DNA contacts at a composite element that no longer require helix D interactions. I have engineered point mutations at most of the residues GR uses to make phosphate and base contacts. These mutants may be useful in distinguishing binding modes at simple and composite GREs.

Whatever the binding mode, the S459R mutation supports the idea that repression at a composite GRE requires a conformational change. I have argued that S459 is a critical node of intramolecular communication for GR. It resides on the backside of helix R where it makes contacts with R489 of helix D. When mutated to alanine (S459A), a hormone dependent yeast growth defect phenotype is caused. When mutated to arginine (S459R), the mutant retains wild-type affinity for a GRE half-site, but is compromised for cooperative binding in vitro and activation and repression in vivo. Thus, S459R may block signal transduction of DNA binding information and block conformational changes required

for activation and repression. Whether these conformational changes are the same for activation and repression cannot be determined from this mutant.

A clue to the conformation GR may attain at a composite GRE may be gleamed from Godowski's mutation LS7. LS7 is a double mutant (P493R/ A494S) in the Zn-finger region. Because of its interesting phenotype in mammalian cells, I tested the point mutant P493R and discovered its yeast growth defect phenotype. Testing LS7 in yeast demonstrated that it too was capable of causing a hormone-dependent growth defect. Therefore, the genetic and biochemical information accumulated for P493R likely holds for LS7. LS7 has been tested for repression at the proliferin composite GRE, and it was found to be wild-type (unpublished observation, Jeff Miner and Marc Diamond). Although P493R has not yet been tested for repression, the similarity between LS7 and P493R for the growth defect in yeast argues P493R would likely repress at a composite GRE as well. Experiments are in progress to determine if this assumption is true.

Assuming P493R is in fact capable of repression at a composite GRE, what does it imply about the mechanism of repression and the conformation of GR at a composite GRE? The nature of the P493R mutation leads to the idea that the conformational change that the molecule has undergone is dominant to the sequence to which it may be binding. The substitution of Arg at position 493 may introduce a positive charge into the hydrophobic core, leading to a structural rearrangement that causes aberrant activation. P493R may then be insensitive to the sequence to which it is bound, locked in its aberrantly active state. There is evidence to support the idea that even on nonspecific DNA GR may be in the "activated" form. If this is the case, then GR may potentially still be in the "activated" form in a complex with Jun/Fos under repressing conditions.

Thus, if the assumption that P493R is locked in a DNA site insensitive "activated" conformation is correct, the fact that LS7 represses at wild-type levels implies that a composite GRE may induce the same conformational change as a simple GRE. The further implication is that the protein context of the site determines GR function. In the presence of Jun, dimerization and activation would proceed normally. However, in the presence of Jun/Fos, GR would remain monomeric and activation would be somehow prevented, presumably by GR and Jun/Fos nullifying each other's intrinsic activation potential.

There are several observations compatible with this model. DNaseI footprint analysis of GR at a composite GRE show a large area of protection more consistent with a dimer than a monomer (unpublished observations, Marc Diamond). In addition, preliminary gel shift analysis indicates that GR dimerizes on the composite element, albeit much less efficiently than at a simple GRE (unpublished observations, Shih-Teng Jeng) Therefore, the default event at a composite GRE, in the absence of other factors, may be dimerization. Composite GRE point mutants, that abolish the binding sites for Jun/Fos without perturbing the GR binding site, may elucidate the functional GR default mode at a composite element in vivo. If protein context is the determinative factor, Jun/Fos binding sites mutants may convert a composite GRE into a simple GRE. In addition, in vitro studies with wild-type and dimerization mutants on the composite element in the presence and absence of Jun or Jun/Fos may help elucidate affinity, stoichiometry, and conformation of the different complexes.

Further support for the model that protein context may determine function at a composite GRE comes from David Pearce (a post-doc in the Yamamoto lab) and Bill Matsui (a medical student in the Yamamoto lab). They have paired a simple GRE with a consensus AP1 site at different center to center distances (termed paired elements, PE). Matsui found that at a 26 base pair center to cen-

ter distance between the simple GRE and consensus AP1 site (PE_{26}), GR activated transcription both in the presence of Jun alone and in the presence of Jun/Fos. Pearce found that at a 16 base pair distance between the simple GRE and consensus AP1 site (PE_{16}), the behavior mimics that of a composite GRE (unpublished observation). In the presence of Jun, GR activates transcription; however, in the presence of Jun/Fos, GR represses from this paired element. The implication is that the protein context of the site may determine GR action even when GR is binding at a simple GRE, which is capable of inducing the conformation for transcriptional activation. The spacing would be important for protein-protein interactions to occur properly.

Pearce has tested my dimerization defective mutants on PE_{16}. He finds that the dimerization mutants fail to activate transcription from this paired element with Jun, but are wild-type for repression with Jun/Fos. This result implies the mechanism of the proliferin composite element is identical to that of PE_{16}.

Finally, Pearce has demonstrated that the N-terminal region of GR is essential for repression with Jun and Fos at the composite GRE (manuscript in preparation). It may be that this region interacts directly or indirectly with Jun and Jun/Fos. My model of simple GRE allosteric regulation of GR predicts that the N-terminal region is under the inhibitory control of the Zn-finger region. Upon simple GRE binding, this inhibition may be released allowing the N-terminal region Enh2 to bind its protein target. Because interaction with Jun/Fos may occur in this N-terminal region, this interaction may also be inhibited by the Zn-finger region, and this inhibition may be similarly released upon GRE binding. Therefore, activation and repression may require the same conformational change. Because the N-terminal region may interact both with a protein target for activation and Jun/Fos, the two interactions could be mutually

exclusive. In this way, interaction with Jun/Fos could prevent activation even though GR may be in the "activated" conformation. In some way, the interaction with GR would also have to inhibit the transcriptional activation potential of Jun/Fos.

Thus, a combination of DNA site mediated allostery and protein context at the site seem to determine function at a composite GRE.

CONCLUSION

In summary, I have demonstrated that GR dimerization is required for both simple and composite GRE activation. However, a monomer of GR is able to repress transcription from a composite GRE. Given experimental evidence that GR exists as a monomer in solution, together with the knowledge that GR can function as both a monomer and a dimer on DNA, the implication is that the dimer state of GR is regulated. Structural studies implied that a simple GRE could induce a conformational change in GR to promote dimerization. My mutagenesis of the GR Zn-finger region has produced mutants whose characterization gives strong functional support to the hypothesis that a simple GRE is an allosteric regulator of GR function. These mutants provide motivation to pursue structural studies to definitively test this hypothesis. Moreover, the yeast growth defect provides a phenotype that can be exploited to learn more about GR function. In particular, the isolation of putative protein targets for GR transcriptional activation may be possible.

In general, the phenomenon of DNA site mediated allosteric regulation gives the cell a powerful regulatory tool. The cell can restrict the function of a protein until it has successfully reached an appropriate site of action. Moreover, in theory, a cell could elicit different functions from a protein dependent upon what site the protein bound. An extension of this idea is that the protein context at a DNA site may have regulatory significance as well.

My mutants imply a combination of DNA allostery and protein context at a site are important for regulating GR function. Further genetic, structural, and biochemical analysis may elucidate the mechanistic basis of the functional diversity GR displays at simple and composite GREs.

MATERIALS AND METHODS

Strains and Growth Conditions

The following bacterial strains were used. The E. coli strain DH5α (F^-, φ80dlacZΔM15, Δ(lacZYA-argF)U169, recA1, endA1, hsdR17, (r_k^-,m_k^+), supE44, λ-, thi-1, gyrA, relA1) was used for cloning and for plasmid preps. The strain 71-18 [Δ(lac-proAB), thi, supE, (F', proAB, lac I^q, lacZΔM15)] was used to propagate M13 bacteriophage for isolation of double-stranded and single stranded DNA. The E. coli strain BL21 (Freedman et al., 1988) was used for regulated T7 polymerase expression of the GR derivative X556 and for purification of the derivative. All strains were grown in LB media.

The following yeast strains were used: BJ2168 (a, pep4-3, prc1-407, prb-1122, ura3-52, trp1, leu2) (Jones, 1977), and BJ-G26.1, a derivative of BJ2168 with the vector pI-G26.1 (Schena et al., 1989), which has three GREs driving a CYC1/lacZ fusion.

Site-Directed Mutagenesis and Creation of Mutant GR Derivatives

An oligonucleotide-directed in vitro mutagenesis system (Amersham # RPN 1523) (Sayers et al., 1988) was used to create site-directed mutants. Briefly, single-stranded M13 DNA, containing the region coding for rat GR amino acids 414-556, was hybridized to an oligonucleotide that coded for a specific amino acid change. This oligonucleotide was extended by Klenow polymerase in the presence of dCTPαS and then ligated with T4 DNA ligase. The wild-type strand was selectively nicked with Nci I and chewed back with Exo III, creating a gap opposite to the mutation. The gap was then filled in and ligated with DNA polymerase I and T4 DNA ligase respectively, fixing the mutation in the original

strand. This dsDNA was then transformed into the bacterial strain 71-18 to provide a source of RF M13 phage. A XhoI/SstI restriction fragment containing the mutation was isolated from the RF M13 DNA and cloned in by exchange to a yeast GR expression vector pG-D (Schena et al., 1989), which produces GR derivative N556, or into the bacterial expression vector T7-X556 (Freedman et al., 1988), which produces the GR derivative X556. To create mutant full length GR, a BamHI/Mae III fragment encoding the 5' half of GR from pG-D was combined with a Mae III/BamHI fragment encoding the 3' half of GR from the GR mammalian expresion vector p6R-GR and cloned into a BamHI site of the yeast expression vector pG-1 (Schena et al., 1991). The resultant vector is named pG-N795. To create a full length GR mammalian expression vector, the BamHI fragment encoding GR from pG-N795 was shuttled in by exchange to the mammalian expression vector p6R-GR.

Random Chemical Mutagenesis

To randomly mutagenize the Zn-finger region and the activation region Enh2, the following cloning manipulations were used to generate ssM13 substrates for mutagenesis.

To generate a substrate for mutagenizing the Zn-finger region, an EcoRI/XhoI fragment from pG-D, coding for rat GR amino acids 414-556, was cloned into the corresponding sites in BS+. A KpnI/BamHI fragment from this vector was then cloned into the corresponding sites of M13mp18, yielding the construct named M13-DB/E1-T. Isolation of ssM13 DNA from this bacteriophage provides the top strand of GR.

To generate a substrate for mutagenizing Enh2, a XhoI site was engineered at amino acid 414 and a Bcl I site was engineered at amino acid 311 of pG-EX556a by site-directed mutagenesis, yielding a vector named pG-BXEX556a. Next, an NcoI linker (CCCATGGG) was placed at the SmaI site of BS+, yielding BS/Nco. An NcoI/XhoI fragment of pG-BXEX556a, encoding rat GR amino acids 106-414, was then cloned into the corresponding sites of BS/Nco. A KpnI/SstI fragment from this vector was then cloned into the corresponding sites of M13mp18, yielding the construct M13-Enh2.

For formic acid mutagenesis, 10 μg of ssM13 DNA was incubated with 18 M formic acid in 100 λ at 20°C for 50 seconds. For hydrazine mutagenesis, 10 μg of ssM13 was incubated with 18 M hydrazine in 100 λ at 20°C for 4.5 minutes. For nitrous acid mutagenesis, 20λ of 1μg/λ ss M13 DNA was mixed with 5λ of NaOAc, pH 4.3 and 25λ of 2.0 M Na nitrite, and incubated at 20°C for 2.5 minutes. All reactions were quenched with 300λ ice cold stop solution (0.83 M NaOAc pH 5.2/0.1mg/ml tRNA) and 1000λ of ice cold ethanol. The samples were incubated at -70°C for five minutes and then precipitated. The samples were resuspended and ethanol precipitated twice more. The samples were then resuspended in 142λ of water, 10λ of 50ng/λ M13 -20 primer, 20λ of 10x reverse transcriptase buffer, and 20λ of 2.5 mM dNTP mix. The primer was hybridized by denaturing at 68°C for five minutes and annealing for 30 minutes at room temperature. MMLV reverse transcriptase (8λ of 200units/λ) was added, and the samples were incubated for one hour at 37°C. The samples were phenol extracted and ethanol precipitated. For Enh2 mutagenesis, an NcoI/XhoI fragment from mutagenized M13-Enh2 was cloned by exchange into pG-BXEX556b. For Zn-finger region mutagenesis, a XhoI/SstI fragment from mutagenized M13-DB/E1-T was cloned by exchange into pG-D. These mutant

libraries were transformed into DH5α, and plasmid DNA was isolated. These plasmid libraries were used to transform yeast to screen for phenotypes. In some cases the supercoiled form of these plasmid libraries was reisolated on an agarose gel to decrease the background from apparently truncated forms.

Yeast Transformations

Yeast strains were transformed by a modification of the LiOAc method (Ito et al., 1983). Briefly, cells grown to stationary phase under selection were diluted into YEPD and grown to OD_{600}=0.6. Fifty ml of cells were pelleted and resuspended in 0.5 ml of LiOAc solution (100 mM LiOAc in TE). To an aliquot of 100λ of cells, 1.0 μg of each plasmid to transform, 50 μg of boiled calf thymus DNA, and 0.6 ml of PEG solution (40% PEG 4000 (w/v) in LiOAc solution) was added. The mixture was incubated at 30°C for 30 minutes and 42°C for 15 minutes. The cells were gently pelleted, resuspended in water, and plated on appropriate selective media.

Yeast Plate and Solution β-galactosidase Assays

For plate assays, yeast colonies were transferred onto nitrocellulose filters, lysed by submerging the filters in liquid nitrogen for ~ five seconds, placed on Whatman filters saturated with 0.3 mg/ml X-gal in Z-buffer, and incubated at 30°C (Schena et al., 1989). β-galactosidase activity was gauged by assessing the intensity of the color produced on a spectrum from white (inactive) to dark blue (wild-type). For quantitative β-galactosidase measurements, solution yeast cultures were used as previously described (Yocum et al., 1984).

Yeast Plasmid Isolation

Briefly, 1.5 ml of a saturated yeast culture was pelleted in a microfuge tube and resuspended in 0.2 ml of 2% Triton X-100, 1% SDS, 100mM NaCl, 10 mM Tris pH 8.0, and 1 mM EDTA. Two ml of phenol/chloroform (1:1) and 0.5 mm glass beads were added. The cells were vortexed for two minutes, spun for five minutes in a microfuge, and 5.0λ of supernatant was used to transform competent E. coli (Hoffman and Winston, 1987).

Sequencing

All mutations were confirmed using Sequenase (Tabor and Richardson, 1987) in the Sanger dideoxy sequencing method (Sanger et al., 1977).

Oligonucleotides

The oligonucleotides used in these studies were synthesized on a Millipore Cyclone Plus DNA Synthesizer.

For in vitro binding experiments, the following oligonucleotides were used. To assess cooperative binding, the oligonucleotide GRE-S3-loop was used: 5'-CTCTCGCCAGAACATCATGTTCTGCGTCGGCCCCCCGCCGACGCAGAACATGATGTTCTGGCGAGAG-3'. To assess half-site affinity, the oligonucleotide GRE-S4-loop was used: CTCTCGCCAGAACATCGATGTTCTGCGTCGGCCCC-CCGCCGACGCAGAACATCGATGTTCTGGCGAGAG-3'. Intramolecular hybridization of these loop oligonucleotides creates a double-stranded DNA molecule that contains a hairpin loop consisting of five cytosine residues. The intramolecular loop creates more stable oligonucleotides without affecting their binding affinities.

For monitoring in vivo activity, the oligonucleotide SX-GRE-S3 was used to create a GRE reporter that contains a GRE identical to the one used for in vitro binding. The sequence is: 5'-TCGACTCTCGCCAGAACATCATGTTCTGCGTC-GGC-3'. The yeast GRE reporter pΔS-1GS3 and the mammalian GRE reporter pΔ(1GS3)DCO were created using this oligonucletide.

Oligonucleotides were ^{32}P end-labeled using T4 polynucleotide kinase and γ-ATP (7000 Ci/mmole).

Wild-type and Mutant T7-X556 expression and purification

Wild-type or mutant T7-X556 vectors were transformed into the bacterial strain BL21. These vectors contain the T7 promoter driving the GR derivative X556. These strains were grown, induced, and harvested following Freedman (Freedman et al., 1988). Briefly, the strain's T7 polymerase was induced with IPTG. After 3 hours, 100 ml of cells was pelleted and frozen at -70°C. The pellets were lysed in 2-3 volumes of lysis buffer. Sodium deoxycholate was added to 0.05% and the lysate was spun at 60,000 x g for one hour. The supernatant was precipitated with polymin P (0.2% final concentration) and the precipitate spun out at 15,000 rpm for 30 minutes in a SS 34 rotor. This supernatant was precipitated by adding 100% ammonium sulfate to a final concentration of 30%. The precipitate was collected by spinning at 10,000 rpm for 10 minutes in a SS 34 rotor. The precipitate was resuspended in 200 λ TGEDZ (50 mM Tris-HCl, pH 7.5; 0.5 mM EDTA; 10% glycerol; 5 mM DTT; 50 mM NaCl; 50 μM $ZnSO_4$. The solution was then dialyzed extensively against TGEDZ and stored in small aliquots at -70°C. The total protein concentration of the extracts was determined by BioRad protein assay (Bradford, 1976), and the concentration of X556 was

determined by quantitative Western analysis using purified T7-X556 protein as a standard.

Western Analysis

Samples were boiled in an equal volume of 2x SDS loading buffer, loaded and run on 15% polyacrylamide gels, transferred to a nitrocellulose membrane, and probed with the BuGR2 monoclonal antibody (Gametchu and Harrison, 1984) directed against GR. GR was detected using alkaline phosphatase conjugated to an anti-IgG secondary antibody and the NBT/BCIP developing system (Towbin et al., 1979).

Wild-type and Mutant T7-X556 Binding Assay

In a 15λ reaction, approximately $5.0x10^{-7}$ M of partially purified wild-type or mutant T7-X556 (~125ng) was mixed with ~$3.3x10^{-10}$ M (~80,000 cpm) of a ^{32}P-labeled GRE oligonucleotide (either GRE-S3-loop or GRE-S4-loop) under the following conditions: 66.7 ng/λ poly dI-dC; 50 mM NaCl; 20 mM Tris-HCl, pH 7.9; 1 mM EDTA; 10% glycerol; 0.1% NP-40; 1 mM DTT; ~100 ng/λ crude E. coli proteins (balanced by adding partially purified E. coli extract from the BL21 strain not expressing T7-X556. The reactions were mixed on ice, and then incubated for 15 minutes at room temperature. Five λ of each binding reaction were assayed by Western analysis with the monoclonal antibody BuGR2. This analysis showed that the wild-type and mutant T7-X556 protein concentrations varied by less than two-fold. To resolve bound from unbound oligonucleotide, five λ of each of the same binding reactions were loaded on a 7.8% (30:1::acryl:bis) gel containing 0.5x TBE and run at 200 volts at room temperature. The gels were dried, and the shifted and unshifted species were quantitated using a Molecular Dynamics Phosphorimager.

Mammalian Cell Culture and Transfections

CV-1 and F9 cells were propagated in Dulbecco's modified Eagle's medium H-16 (Cell Culture Facility, UCSF) supplemented with 10% fetal calf serum (Hyclone; Logan, UT). Cells were grown at 37°C in a humidified incubator (Rh~90%) maintained at 8% CO_2.

For CAT assays, subconfluent cultures ($2x10^5$ cells/60mm dish) treated +/- $1x10^{-7}$ M dexamethasone were cotransfected by the calcium phosphate method (Godowski et al., 1988a). All transfections included 0.2 μg of Δ6RL, an expression vector for β-galactosidase, as an internal control for transfection efficiencies. In addition, to assay simple GRE activation, 2.0 μg of the vector p6R-GR expressing wild-type or mutant GR and 2.0 μg of the CAT reporter pΔ(1GS3)DCO were cotransfected. To assay composite GRE activation, 2.0 μg of p6R-GR expressing wild-type or mutant GR, 1.0 μg of c-Jun expression vector, and 2.0 μg of the CAT reporter plfG3-CAT were cotransfected. To assay composite GRE repression, 2.0 mg of p6R-GR expressing wild-type or mutant GR, 0.2 μg of c-Jun expression vector, 0.2 μg of c-Fos expression vector, and 2.0 μg of the CAT reporter plfG3-CAT were cotransfected.

CAT and β-Galactosidase Assays

For CAT assays, cell cultures +/- $1x10^{-7}$ M dexamethasone were incubated for ~12 hours with $CaPO_4$/DNA precipitates, shocked with 15% glycerol in media for 1.5-2.0 minutes, washed twice with PBS, and incubated another 24 hours +/- $1x10^{-7}$ M dexamethasone. The cells were harvested by washing twice in PBS; then using a rubber policeman, they were scraped in 1.0 ml of PBS and transferred to a 1.5 ml eppendorf tube. The cells were pelleted at 5000xg for one minute and resuspended in 100 l of 0.25 M Tris-HCl pH 7.5. They were lysed by

three cycles of freeze-thaw (-70°C and 37°C) and treated for five minutes at 65°C to inactivate deacetylases. The lysates were spun at 12,000xg for 5 minutes, and the supernatants were used for the CAT assays. Typically, 37.5 λ of extract was assayed for CAT activity using a non-chromatographic method (Sleigh, 1986). Briefly, the extract was incubated at 37°C after adding, 2.5 λ of 14C-Acetyl-CoA, 0.25 λ of 20mM Acetyl-CoA, and 10 λ of 8mM chloramphenicol. The reaction was then extracted with ethyl acetate, and the organic phase was counted with fluor in a liquid scintillation machine. To correct for any transfection efficiency differences, CAT activities were normalized to β-galactosidase activities that were determined by using 2.0 λ of extract in the 4-MUG β-galactosidase assay (Stuart et al., 1984). Briefly, the extract was incubated at 37°C after adding 8.0 λ of 5x b-gal buffer, 0.2 λ of 30 mM 4-MUG, 0.8 λ of 40 mg/ml BSA, and 29 l of water. The reactions were stopped by the addition of 1.0 ml of .25 M glycine pH 10.65. The reactions were quantitated by reading the samples in a Hoefer DNA fluorometer TKO 100.

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APPENDIX I

Mutagenesis of the GR Enhancement Region—Enh2

Abstract

I used random chemical mutagenesis to mutate the GR amino-terminal enhancement region, Enh2 (amino acids 106-318). These mutants, expressed as the GR derivative EX556, were screened in yeast for transcriptional defects. No point mutants were found that effectively inactivated the enhancement potential of the region, implying there may be multiple, redundant activation surfaces. However, this negative result was further weakened by the fact that a high frequency event in the host yeast strain abrogated transcriptional activation by wild-type EX556.

Introduction

Paul Godowski performed a series of experiments in which he transferred regions of the GR amino-terminus onto the Lex DNA binding domain, which is itself incapable of transcriptional activation (Godowski et al., 1988a). Monitoring activation in mammalian cells, he defined Enh2 (amino acids 106-318) as a region capable of conferring most (~70%) of the transcriptional activity of the entire amino-terminus onto the Lex DNA binding domain. Subsequently, this region was cloned onto the GR DNA binding domain (amino acids 407-556) via a synthetic linker to create the constitutive transcriptional activator, EX556. This GR derivative activates transcription in vivo in mammalian cells and yeast and activates transcription in cell free in vitro transcription extracts (Freedman et al., 1989).

It is thought that an activation domain binds a protein target. Through tethering the target close to a linked promoter, the activator can increase the local concentration of the protein target, overcoming a rate-limiting step, and enhancing transcriptional initiation. If an enhancement domain interacts with a protein target, it should be possible to mutate the domain to disrupt the interaction. This strategy has proved successful with the activation region of VP-16. Point mutants have been found that decrease activation potential and disrupt interaction with putative targets.

To define important residues in the GR enhancement domain, Enh2, I have extensively mutagenized the region and screened for loss of transcriptional activation in yeast at a simple GRE.

Results

I mutagenized the enhancement domain Enh2 with three mutagens: formic acid, hydrazine, and sodium nitrite. The mutagenesis was kinetically optimized to yield a ten:one ratio of single hit to multiple hits in the target area. Under these conditions, approximately ten percent of the total population is mutagenized. To screen enough colonies to represent the mutant population, I calculated I needed to screen 30,000 yeast colonies for each mutagen. I actually screened ~30,000 colonies for both formic acid and hydrazine mutant populations, and I screened ~15,000 colonies for the sodium nitrite population.

The screen involved transforming a yeast strain containing an integrated reporter with a yeast expression vector encoding the mutant population of the GR derivative EX556 . This reporter contained three tyrosine amino-transferase simple GREs regulating the minimal CYC1 promoter driving the Lac Z gene. Colony assays for β-galactosidase were performed to monitor transcriptional activity of the population of mutant EX556 derivatives. Functional derivatives

give a blue colony in this assay, colonies defective for β-galactosidase production are white.

I picked a total of approximately 400 white colonies. Western blotting of a representative fraction of this total demonstrated that ~50-60% of the colonies were expressing full length EX556. On Western blots, EX556 typically runs as several discrete bands that are thought to be the result of heterogeneous phosphorylation. Interestingly, in ~50% of the white colonies producing EX556, the derivative ran as a single band at the position of the fastest migrating band seen with wild-type EX556. The rest of the population apparently were not producing EX556 although they still carried the selectable marker for the expression vector. The white colonies containing full length EX556 were confirmed to be clonally down for β-galactosidase production.

The EX556 expression vector was isolated from 16 such strains including derivatives that had the altered Western migration pattern, and the Enh2 region was sequenced. Fourteen of these clones encoded a wild-type Enh2 region. Two had point mutations (D183G and S290G). One of these point mutants (D183G) was reintroduced into a new yeast reporter strain and it was found to be wild-type for transcriptional activation. At this point, no further analysis was pursued.

Discussion

Thus, no point mutant was found that inactivated the activation potential of Enh2 in yeast. About 10% of the white colonies that were producing EX556 had their Enh2 region sequenced. They were found to be either wild-type in sequence or mutant but did not confer a phenotype on a new host strain. One mutant was not further characterized to see if it conferred the phenotype. The implication was that there was some defect in the host strain that occurred at high

frequency to inactivate EX556 enhancement. In some cases, this loss of function may have been correlated with a putative change in phosphorylation.

Marija Krstic (a graduate student in the Yamamoto lab) has seen a similar phenomenon. She had made N795 mutants with multiple lesions at potential phosphorylation sites. When she assayed these mutants in yeast for transcriptional activation potential, she obtained a yeast population with mixed phenotypes. Some strains retained activation potential, while other strains were clonally down for transcriptional activation. This clonal inactivation occurred at a relatively high frequency.

EX556 may be down ten-fold in specific activity for activation relative to wild-type. Krstic's multiple phosphorylation mutants may be similarly compromised for activation. Therefore, with weakened activators, we may have been observing a yeast host event that occurs at relatively high frequency that is normally obscured when using a strongly activating form of GR. Potentially, this host event may be involved in phosphorylation. Thus, pursuing an understanding of this phenomenon may be useful.

However, this phenomenon nullified my attempt to identify point mutants in Enh2 that inactivated its enhancement potential. With such a high background of a host event, I was unable to identify an inactivating point mutant in Enh2 if it existed.

Despite this setback, the mutagenized Enh2 library appears to be good. The region was mutagenized to obtain 10% mutants with a high ratio of single to multiple mutations. With the high strain background, I essentially sequenced 16 random clones. I found two point mutants, which is near the expected frequency.

To pursue the analysis of Enh2, I believe the mutagenized Enh2 region should be cloned back into the N795 or N556 derivatives. These strong activa-

tors may overcome the problem with the high strain background problem and more effectively identify defects in Enh2.

In addition, David Pearce has recently identified the N-terminal region of GR as essential for repression at the proliferin composite GRE. These mutations in Enh2 may prove useful in elucidating this requirement.

APPENDIX II

A Complete List of Zn-Finger Region Mutants and Phenotypes

KEY

Mutant Column

* after the name indicates the wild-type residue at this position is absolutely conserved in the superfamily; ^ after the name means conserved except for one non-conservative change; # after the name indicates that there are only conservative changes found at this position

Putative Function Column

B=Specific Base Contact
C=Coordinating Cysteine
D=Dimerization
G=Growth Defect (+ means causes growth defect, – means fails to cause defect)
H=Hydrophobic Core
P=Phosphate Contact
U=Unknown

Location Column (from X-ray crystal structure, Luisi et al.)

1M=first module=first Zn finger+following α-helix
2M=second module=second Zn finger+following α-helix
Helices R, D, and P are as defined previously

Transcriptional Activation in Yeast Column (% of wild-type)

N=N556 GR derivative (high copy 2μ expression unless otherwise indicated)
F=Full length GR=N795 (high copy 2μ expression unless otherwise indicated)
A=Integrated 3 TAT GRE reporter in strain BJG26.1
B=Episomal single idealized GRE reporter (pΔS-1GS3) in BJ2168
L=GR derivative expressed from a low copy cen-ars vector

Simple GRE Activation by N795 in CV-1 Cells Column (% of wild-type)

1=GMCS reporter (MMTV LTR GREs and promoter driving CAT)
2=pΔ(1GS3)DCO (single idealized GRE and ADH minimal promoter-CAT)

Composite GRE (plfG3 CAT) Activation and Repression by N795 in F9 Cells Column (% of wild-type)

In Vitro DNA Binding Column

3S column:% of wt dimer binding on (a=27 asym oligo; b=GRE-S3-Loop oligo)
4S column:% of wt monomer binding on GRE-S4-Loop oligo
U=undetectable; M=marginal binding, not quantified

Empty boxes indicate the experiment has not been done.

Mutant	Putative Function	Location	Yeast Simple GRE Act.	Mammalian Simple GRE Activation	Mammalian Composite Act.	Mammalian Composite Rep.	In Vitro DNA Binding 4S	In Vitro DNA Binding 3S
C443R *	C	1M	<1% N,A	4%	14%			U a
C460R *	C	1M, Hel. R	<1% N,A					U a
C492W *	C	2M, Hel. P						
D485A	D	2M	1.3% F,B	10%		50%	12%	1% b
R488A	D	2M, Hel. D	1.0% F,B	7%		65%	8%	2% b
R479A	D	2M	49% F,B	30%	<1%	134%	93%	43% b
D481A	D	2M	57% F,B	22%	<1%	50%	73%	22% b
N491A	D	2M, Hel. D	22% F,B	25%	<1%	123%	91%	16% b
R479A/ N491A	D	2M 2M, Hel. D	1.4% F,B	7.7%	<1%	210%	133%	12% b
D481A/ N491A	D	2M 2M, Hel. D	4.1% F,B	3.5%	<1%	119%	122%	8% b
R479D	D	2M	66% N,A					
D481R	D	2M	96% N,A					
R479D/ D481R	D	2M 2M	104% N,A					
R479D/ N491A	D	2M 2M, Hel. D	7% N,A					
D481R/ N491A	D	2M 2M. Hel. D	20% N,A					
R479D/ D481R/ N491A	D	2M 2M 2M, Hel. D	26% N,A					
F463L *	H	1M, Hel. R	<1% N,A	4%		24%		U a
F463V *	H	1M, Hel. R	<1% N,A					
F464V *	H	1M, Hel. R	<1% N,A	6%		<1%		M a
Y497A	H	2M, Hel. P	92% N,A					
Y497L	H	2M, Hel. P	81% N,A					
M505V *	H	2M	<1% N,A	20%		20%		M a
Y452A #	H, P	1M	4% N,A					
R496H #	H, P	2M, Hel. P	<1% N,A	4%		4%		U a
R496S #	H, P	2M, Hel. P	<1% N,A	4%		25%		U a
H451A ^	P	1M	6.2% N,A					
K465A	P	1M, Hel. R	152% N,A					
K465E	P	1M, Hel. R	<1% N,A	5%		17%		U a
R489A *	P	2M, Hel. D	<1% N,A					
K490A	P	2M, Hel. D	67% N,A					

Mutant	Putative Function	Location	Yeast Simple GRE Act.	Mammalian Simple GRE Activation	Mammalian Composite Act.	Mammalian Composite Rep.	In Vitro DNA Binding 4S	In Vitro DNA Binding 3S
K490E	P	2M, Hel. D	<1% N,A	4%		9%		U a
K461E ^	B	1M, Hel. R	<1% N,A	4%		6%		U a
K461I ^	B	1M, Hel. R	<1% N,A	5%		9%		U a
K461A ^	B	1M, Hel. R	<1% N,A			activates 2-3 fold		
V462E	B	1M, Hel. R	<1% N,A	20%		30%		U a
R466A *	B	1M, Hel. R	3% N,A					
S459A	G+	1M, Hel. R	28% F,A 111% F,A,L			wt		
S459R	G–	1M, Hel. R	<1% N,A	23%		15%	75%	7% b
P493A	G–	2M, Hel. P						
P493K	G+	2M, Hel. P						
P493Q	G–	2M, Hel. P						
P493R	G+	2M, Hel. P	35% F,A 114% F,A,L	8%		wt	332%	112% b
K461A/ P493R	B, G+	1M, Hel. R 2M, Hel. P	8.2% F,A					
K461E/ P493R	B, G?	1M, Hel. R 2M, Hel. P						
V462E/ P493R	B, G?	1M, Hel. R 2M, Hel. P						
R466A/ P493R	B, G–	1M, Hel. R 2M, Hel. P	<1% F,A					
K461E/ V462E/ P493R	B, G–	1M, Hel. R 1M, Hel. R 2M, Hel. P						
R479A/ P493R	(as N556) D, G?	2M 2M, Hel. P						
R479A/ N491A/ P493R	(as N556) D, G+	2M 2M, Hel. D 2M, Hel. P						
D485A/ P493R	G– (N556)	2M 2M, Hel. P						
R488A/ P493R	G– (N556)	2M, Hel. D 2M, Hel. P						
V442A #	U	1M	27% N,A					
S444G	U	1M	80% N,A					
D445A #	U	1M	100% N,A	88%				
E446A	U	1M	86% N,A	82%				
L455V	U	1M						
Q471A	U	1M	86% N,A	100%				
N473A	U	1M	97% N,A	73%				

Mutant	Putative Function	Location	Yeast Simple GRE Act.	Mammalian Simple GRE Activation	Mammalian Composite Act.	Mammalian Composite Rep.	In Vitro DNA Binding 4S	In Vitro DNA Binding 3S
L475A	U	1M	74% N,A	66%				
I483A	U	2M	75% N,A	67%				
I483R	U	2M	89% N,A	63%				
K486A	U	2M, Hel. D	95% N,A	91%				
I487A	U	2M, Hel. D	80% N,A	80%				
I487R	U	2M, Hel. D	80% N,A	100%				
R498A	U	2M, Hel. P	89% N,A	80%				
R498Q	U	2M, Hel. P						
K499A #	U	2M, Hel. P	70% N,A	70%				
Q502A	U	2M, Hel. P	77% N,A	100%				
G504A #	U	2M	21% N,A					

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