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by

MARK SCHENA

DISSERTATION

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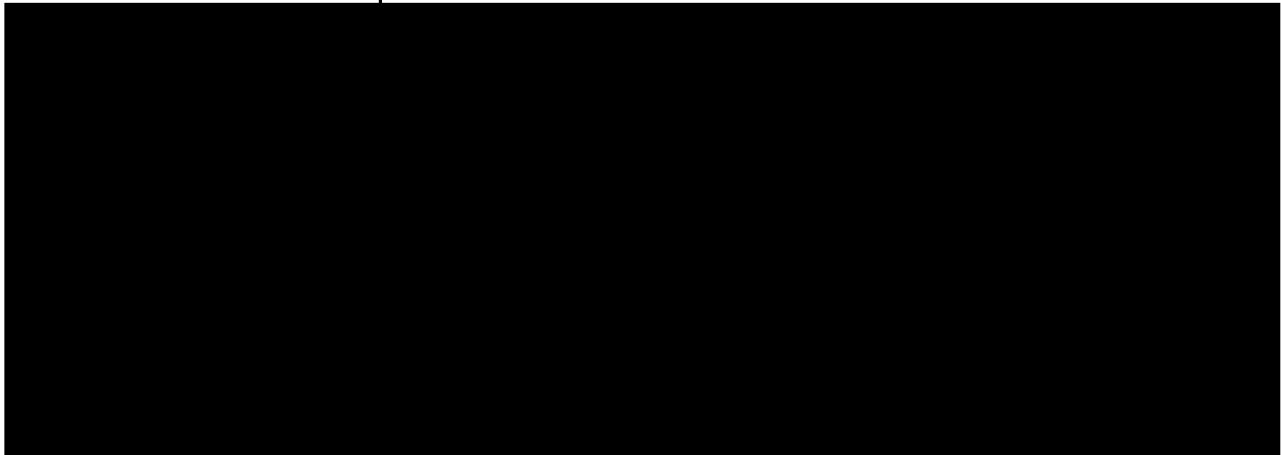
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**This thesis is dedicated to my parents,
to Keith Yamamoto,
and to Mikhail Gorbachev**

MAMMALIAN GLUCOCORTICOID RECEPTOR ACTION IN YEAST

A thesis presented

by

MARK SCHENA

ABSTRACT

The glucocorticoid receptor binds specifically to enhancer DNA sequences and activates transcription from linked promoters in mammalian cells. To investigate the mechanism of this process, I have performed a series of genetic experiments in yeast and present the results of these studies here. I begin by showing that the glucocorticoid receptor activates transcription by a common, highly conserved mechanism in yeast and mammalian cells. The conservation of receptor action in yeast validates the use of this simple organism to genetically analyze receptor functional domains, and to identify interacting cellular factors. I describe the isolation of a large number of receptor zinc finger mutants obtained using random mutagenesis and a genetic screen in yeast. Characterization of these mutants suggests that amino acids just downstream of each finger mediate DNA binding, and that a distinct region of the second receptor finger confers enhancement by contacting a component of the transcription apparatus. In addition, I report the identification of a class of intergenic suppressors in yeast that may define a cellular factor with which receptor interacts to mediate enhancement. Certain practical and theoretical considerations regarding the evolutionary conservation of eukaryotic gene transcription are also discussed.

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Early Studies -- Lessons From Prokaryotes

It was known by the turn of this century that the level of enzymes present in microbial extracts depended on the medium in which organisms were grown. This observation led H. Karstrom, in the course of studying bacterial carbohydrate metabolism, to propose in the 1930's that enzymes be formally classified as *adaptive* or *constitutive*, depending upon whether their levels varied according to growth conditions or remained invariant (see Stent and Calendar, 1978). One adaptive enzyme, β -galactosidase, was found at high levels only in extracts from lactose-grown bacteria. This observation served as the basis for a series of experiments carried out by Jacques Monod and his colleagues who, over a 15 year period starting in 1946, elucidated the basic physiological aspects of adaptive β -galactosidase formation. In the course of this investigation, Monod and co-workers defined the lactose molecule as an *inducer*, while increases in β -galactosidase activity came to be known as *enzyme induction* (Monod et al., 1952). The most important practical aspect of these early experiments was the identification of isopropylthiogalactoside (IPTG) and other non-hydrolyzable analogues of lactose as high potency inducers of β -galactosidase induction. A notable theoretical contribution of this work was the finding that cells respond to environmental stimuli, such as increases in the concentration of soluble ligand, in a definitive manner; clearly, this finding has proven generally relevant to modern biology, particularly in studies of steroid receptors.

Shortly after Monod began his work on the physiological basis of β -galactosidase induction, Joshua Lederberg initiated a complementary series of genetic experiments. By 1948, Lederberg had succeeded in isolating a large number of *E. coli* mutants incapable of utilizing lactose as a carbon source (Lederberg, 1948). In the course of characterizing these Lac- mutants, Lederberg invented among other things a β -galactosidase assay

method that employed the reagent o-nitrophenylgalactoside (ONPG) as a colorimetric substrate. Quantitative measurements of β -galactosidase activity in the Lac- strains indicated that most mutants were defective in either β -galactosidase itself (*lac Z*-) or in the gene encoding the lactose permease (*lac Y*-); interestingly, a third class of mutants constitutively expressed β -galactosidase in the absence of inducer. Analysis of these constitutive or *lac I*- mutants revealed that the *lac I* locus mapped to a region of the genome close to but distinct from the coding sequences for *lac Z* and *lac Y*. The discovery of the *lac I* gene established the genetic basis for enzyme induction and, more importantly, introduced the notion of a gene regulator.

During the 1950's, additional experiments with *lac I*- mutants confirmed and extended the genetic basis for regulation of genes required for lactose metabolism. Mating experiments with *lac I*- and *lac I*+ strains, for example, indicated that the constitutive phenotype of the mutants was recessive, suggesting that the *lac I*- mutations probably constituted loss of function mutations in a negatively acting factor rather than gain of function mutations that cause synthesis of an "internal inducer". Moreover, the isolation of a new class of *lac I* mutants that possessed increased repressor ability (the so-called *lac I^s* alleles, "s" denoting "super"), as well as the identification of temperature sensitive *lac I* alleles substantiated the "repressor" view. In addition, Francois Jacob isolated a class of dominant constitutive mutations that mapped to a DNA site (*lac O*, "O" denoting "operator") between the *lac Z* and *lac I*. These and other experiments led Jacob and Monod to propose, in a classic paper in 1961, that the regulation of the *lac* operon was mediated by a repressor that, in the absence of inducer, exerted a negative effect on gene expression by binding to an operator. The repressor-operator dialectic established the view that a gene regulator was a diffusible molecule [though Jacob and Monod actually thought that the functional repressor was RNA instead of protein] that exerted its effect by

binding to a specific DNA site. This concept has served as a basic paradigm in transcription studies for nearly thirty years.

The next major advance in understanding *lac* control was provided by Walter Gilbert and Benno Müller-Hill who, in 1965, identified and purified the Lac repressor protein from a concentrated *E. coli* preparation by an assay equilibrium dialysis with radiolabeled IPTG (Gilbert and Muller-Hill, 1965). Gilbert and Muller-Hill were able to strongly implicate the LacI protein in IPTG binding by demonstrating that extracts prepared from *lac* I- strains failed to bind radiolabeled inducer; furthermore, it was shown that binding of the purified protein to DNA occurred only in the absence of IPTG and only to phage DNA sequences that contained the *lac* operator (*lac* O). An important practical outcome of these studies was the effectiveness of using radiolabeled ligands in protein purification, an observation that was later exploited to purify mammalian steroid receptors. The work by Gilbert and Muller-Hill, and in parallel by Ptashne (1967), also contributed the theoretical advance that regulatory proteins exert their effects by binding specifically to DNA.

An important general lesson that emerged from these early studies was the utility of combining genetic and biochemical approaches to the study of biological problems. In the case of *lac* regulation, genetic and biochemical experiments were inextricably employed to identify the inducer, the repressor and the structural genes. This combined approach has proven indispensable in modern studies.

Steroid Hormone Action

Steroid hormones mediate a vast array of processes in mammals including

physiological homeostasis, glycogen and mineral metabolism, and the stress response. Steroids have been reported to influence the development and differentiation of the immune system, nervous system, skeletal system, and reproductive organs. So vital are these compounds for vertebrate morphogenesis and so potent their action that Chinese alchemists laboriously purified steroids to near homogeneity as early as the tenth century (Needham, 1983). How do these small cholesterol derivatives exert such profound biological effects? The answer has emerged from molecular studies that span three decades.

In a manner conceptually analogous to studies with the *lac* repressor, the first clues regarding the molecular mechanism of steroid hormone action emerged from physiological studies. It was noticed during the 1960's, for example, that hormone-responsive tissues such as the uterus and anterior pituitary displayed a much higher affinity for estradiol than non-responsive organs (Jensen et al., 1969); these estrogen-binding substances originally termed "estrophiles" were later re-named "estrogen receptors". Samuels and Tomkins (1970) extended the receptor concept by showing that the extent to which a given steroid elicited an assayable response, such as tyrosine aminotransferase (TAT) induction in cultured cells, was highly dependent on the precise stereochemical composition of the ligand. Systematic evaluation of a large number of steroid derivatives led to the establishment of a hierarchy of ligand potency that included strong inducers, weak inducers, non-inducers, and antagonists; consequently, it was postulated that steroids interact selectively with specific receptor proteins that mediate TAT enzyme induction. In addition, as hormone binding to receptor triggered changes in both sedimentation properties and sub-cellular localization, it was hypothesized that allosteric changes govern the transition (transformation) of receptor from the inactive to the active state (Gorski et al., 1968).

The redistribution of the receptor from the cytoplasm to the nucleus upon hormone binding provided the first hint that receptor itself may constitute a transcriptional regulator. Compelling biochemical evidence for this proposal was provided by Yamamoto and Alberts (1972) who showed that the affinity of the estrogen receptor for DNA-cellulose was greatly increased by addition of estradiol prior to column chromatography. The relevance of these *in vitro* studies was bolstered shortly thereafter by somatic cell genetics experiments in which mutant mouse lymphoma lines resistant to the cytolytic effects of glucocorticoids were isolated and characterized (Yamamoto et al., 1974). Hormone-resistance in these lines was found to correlate both with decreases in receptor nuclear affinity and with reduced DNA binding *in vitro*, strongly supporting the postulate that steroid receptors exert their biological effects by directly modulating the expression of specific genes. The apparent paradox arising from the observed non-specificity of receptor DNA binding was reconciled by Yamamoto and Alberts (1975) who, in an impressive theoretical treatise based on studies with the lac repressor, argued convincingly for the existence of specific undetected genomic sites for estrogen receptor binding. The existence of these undetected sites gained additional support in the late 1970's when it was estimated, using two-dimensional gel electrophoresis, that the expression of approximately 50 cellular genes was effected by glucocorticoids (Ivarie and O'Farrell, 1978).

The finding that dexamethasone stimulated the expression of mammary tumor virus (MTV) RNA provided the basis for a definitive test of whether steroid receptors interact selectively with DNA (Ringold et al., 1975). Using cloned MTV sequences, it was shown that DNA elements upstream of the MTV promoter were essential for mediating the glucocorticoid response; moreover, purified glucocorticoid receptor protein

obtained from rat liver preparations (Wrange et al., 1979) exhibited selective binding to five consensus sites present in those upstream sequences (Payvar et al., 1983; Scheidereit et al., 1983). Sequences that bound receptor specifically *in vitro* were also capable of rendering a heterologous promoter hormone-responsive *in vivo* without strict regard for orientation or distance, indicating that glucocorticoid response elements (GREs) constitute receptor-dependent enhancer elements (Chandler et al., 1983). Analysis of mRNA transcripts derived from promoters linked to GREs revealed that receptor stimulated gene expression by increasing promoter utilization, rather than by altering some other aspect of RNA synthesis or processing such as the accuracy of transcription initiation, RNA splicing or polyadenylation (Ucker et al., 1983). By the mid-1980's, theoretical considerations of steroid receptor action served as useful paradigms for understanding the evolution of gene networks and for comprehending how multiple enhancer elements might mediate multifactor gene control (Yamamoto, 1985).

The rat glucocorticoid receptor cDNA was obtained using polysome immunoenrichment with receptor-specific antisera followed by differential hybridization (Miesfeld et al., 1984; 1986). Deletion studies with cloned receptor sequences have delineated regions that impart the various receptor activities including DNA binding (Rusconi and Yamamoto, 1987; Freedman et al., 1988), hormone binding (Rusconi and Yamamoto, 1987), nuclear localization (Picard and Yamamoto, 1987), transcriptional enhancement (Miesfeld et al., 1987; Godowski et al., 1987; Godowski et al., 1988), and transcriptional repression (Sakai et al., 1988; Miesfeld et al., 1988). The DNA binding domain of the receptor resides in the central portion of the coding region and contains two metal binding fingers analogous to those first proposed for TFIIIA (Miller et al., 1985). A small fragment of the receptor encompassing the DNA binding domain is sufficient for sequence specific DNA binding (Freedman et al., 1988), and transcriptional enhancement

(Miesfeld et al., 1987) and repression (Miesfeld et al., 1988). The significance of a second enhancement domain located near the amino terminus of receptor remains unclear but may play a role in the differential regulation of certain promoters (Godowski et al., 1988). The fact that deletions of the hormone binding domain render the receptor constitutive for transcriptional enhancement (Godowski et al., 1987), and that the steroid binding domain can confer hormone-inducibility upon proteins to which it is fused (Picard et al., 1988), suggests that the hormone binding domain encodes a reversible inactivation function whose capacity to repress may require interaction with HSP90 (Sanchez et al., 1985).

The sequence similarity between the glucocorticoid receptor and other steroid receptor proteins indicates that these regulatory molecules comprise a family of related enhancer activators that may have arisen from a common progenitor early in eukaryotic evolution (Evans, 1988; Beato, 1989). Consistent with this view, the glucocorticoid receptor (Schena and Yamamoto, 1988) and the estrogen receptor (Metzger et al., 1988) have been shown to function as transcriptional regulators when expressed in yeast (see below).

Evolutionary Conservation of Eukaryotic Gene Control

A major theme that has emerged from transcription studies during the 1980's is the finding that basic mechanisms of eukaryotic gene control have been conserved over nearly one billion years of evolutionary time - from yeast to man. This discovery arose mainly from a series of experiments in which transcription factors from one organism were expressed and assayed for activity in a foreign host. Early clues that gene control mechanisms had been conserved, however, derived from studies of the *cis*- acting

elements of eukaryotic promoters.

Sequence and deletion analysis of the regulatory regions from various yeast and mammalian genes lead to the identification during the early 1980's of two common cis-acting sites, TATA boxes and upstream elements, found in the vicinity of many RNA polymerase II-specific promoters. TATA boxes were identified as fixed elements of eukaryotic promoters located 60-120 nucleotides upstream (yeast) or 30 nucleotides upstream (mammals and most other eukaryotes) of the transcription start site (Guarente, 1988); deletion of the TATA region was usually accompanied by reduction of the accuracy and efficiency of transcription initiation (Grosschedl and Birnstiel, 1980). Upstream elements, termed upstream activator sequences (UASs) in yeast and enhancers in mammalian cells, were found to reside variable distances up to 10 kilobases upstream and occasionally downstream in the case of enhancers from the TATA box (Banerji et al., 1981; Benoist and Chambon, 1981; Fromm and Berg, 1982; Guarente et al., 1982; Chandler et al., 1983).

Both UASs and enhancers were identified as DNA elements that could potentiate promoter activity without strict regard for spacing, orientation, or promoter composition. In addition, both types of sites apparently served to modulate gene expression according to changes in cell physiology and cell type; thus, rises in glucocorticoid levels in mammalian cells were accompanied by increases in enhancement via GRE-linked promoters (Ringold et al., 1975), just as changes in galactose availability resulted in transcriptional activation of yeast promoters fused to the GAL UASs (Oshima, 1982). UASs and enhancers were both found to modulate transcription in a manner dependent upon the presence of specific regulatory proteins bound to those sequences. The activity of GREs was found to be imparted by binding and enhancement by the glucocorticoid

receptor (Payvar et al., 1983; Scheidereit et al., 1983). Analogously, activation by the upstream activator sequences involved in galactose or amino acid metabolism in yeast was mediated by GAL4 (Oshima, 1982; Johnston and Davis, 1984; Giniger et al., 1985) and GCN4 (Hope and Struhl, 1985; Arndt and Fink, 1986), respectively. On the basis of the similarities between upstream activator sequences and enhancers, it seemed plausible that the regulatory proteins bound to these sites might enhance transcription by a common mechanism.

This hypothesis was first tested by Keith Yamamoto and myself (see chapter two) in 1987. The design of the experiment, in brief, was to express the rat glucocorticoid receptor in yeast using the yeast glyceraldehyde-3-phosphate dehydrogenase promoter, and assay for receptor DNA binding and transcriptional enhancement *in trans* using a reporter plasmid containing GREs fused to a yeast *CYC1* promoter-lacZ gene fusion. Indeed, receptor was found to enhance β -galactosidase expression by more than 100-fold in this assay; moreover, activation occurred via the correct transcription start sites, indicating that receptor was interacting faithfully with the yeast transcription apparatus (Schena and Yamamoto, 1988). During this same period, the estrogen receptor was also shown to function in yeast (Metzger et al., 1988), and conversely the yeast GAL4 protein was demonstrated to activate transcription in mammalian cells (Kakidani and Ptashne, 1988; Webster et al., 1988). Subsequent observations by the late 1980's established that numerous eukaryotic activators function in foreign organisms, a subject that I review extensively in chapter five, indicated that regulatory proteins from yeast and mammalian cells comprised a conserved class of activators (Schena, 1989a).

Given the generality of the enhancer-enhancer binding protein paradigm and the considerable evidence that activators function by contacting a component of the

transcription apparatus (Ptashne, 1988; Yamamoto, 1989), it seemed likely that the conservation of transcriptional control mechanisms might extend beyond the regulatory proteins to the general factors and polymerase. In fact, it is now clear that both TFIID (the TATA binding protein) and TFIIA from yeast can functionally replace, at least in part, the analogous mammalian activities in transcription reactions *in vitro* (Buratowski et al., 1988; Cavallini et al., 1988). Extensive homology has also been observed among the largest subunits of eukaryotic RNA polymerase II (Nonet et al., 1987; Allison et al., 1988).

It appears that the action of enhancers and enhancer binding proteins operating through the general transcription apparatus arose as a paradigm early in eukaryotic evolution, and served as a relatively invariant and restrictive core around which gene control circuits in complex organisms were built. One imagines that the concerted action of enhancers and enhancer binding proteins, reminiscent of the lac repressor-operator system, provided a relatively simple means of evolving and maintaining a gene network. Given the ability of regulatory factors to function *in trans*, only a single transposition event of an enhancer element would have been required to render a gene in a primordial cell enhancer-responsive. Consistent with this view is the finding that, in many cases, binding sites for a common regulatory factor are located upstream of eukaryotic genes required for a common task. The discovery of enhancers and enhancer binding proteins in bacteria (Reitzer and Magasanik, 1986; Popham et al., 1989) indicates that the paradigm of long-range activators binding to specific DNA sites is very old indeed.

Conserved Motifs for DNA binding and Enhancement

Structural and functional analyses of dozens of DNA binding proteins over the past five years indicates that DNA binding domains consist of small autonomous regions containing fewer than 100 amino acids. Nearly all of these discrete domains can be grouped into one of four structurally distinct classes known as the zinc finger, helix-turn-helix, helix-loop-helix, and leucine zipper motifs.

The zinc finger motif has been identified in dozens of eukaryotic transcriptional regulatory proteins including the glucocorticoid receptor and other members of the steroid receptor family (Weinberger et al., 1985), yeast ADR1 (Hartshorne et al., 1986), *Xenopus* transcription factor IIIA (Miller et al., 1985), numerous *Drosophila* factors such as *kruppel* (Rosenberg et al., 1986) and *hunchback* (Tautz et al., 1987), and human Sp1 (Kadonaga et al., 1987). In the case of the rat glucocorticoid receptor, each of two zinc fingers formed via the tetrahedral coordination of metal by four cysteine residues DNA are required for binding activity *in vitro* (Freedman et al., 1988); in addition, I show in chapter three that mutations at these positions render the receptor inactive *in vivo* (Schena et al., 1989b). A second type of zinc binding protein, typified by TFIIIA, employs two cysteines and two histidines for zinc binding. Several other proteins including yeast GAL4 (Pan and Coleman, 1990) and the Tat protein from the human immunodeficiency virus (Frankel et al., 1988) appear to utilize more complicated schemes for metal binding. In each of these cases, however, zinc chelation appears to provide the major structural determinant in the formation and maintenance of the DNA binding domain, and thus is essential for the proper function of these proteins. As I show in chapter three, sequences on the carboxy side of each finger probably mediate DNA binding (Schena et al., 1989a).

The eukaryotic helix-turn-helix proteins, which contain a DNA binding domain putatively formed by two α -helices separated by a β -turn, constitute a diverse class of

regulators that include the yeast MAT α 2 protein (Sauer et al., 1988) and *Drosophila* and vertebrate homeo-box proteins of which there are more than 80 known members (Levine and Hoey, 1988). By analogy to prokaryotic activators and repressors for which crystallographic data is available (Pabo and Sauer, 1984), eukaryotic helix-turn-helix proteins probably recognize specific DNA elements by inserting one of the two α -helices into the major groove of DNA.

Two additional classes of DNA binding proteins include molecules that contain the helix-loop-helix (HLH) and leucine zipper dimerization motifs. The helix-loop-helix proteins which include MyoD (Tapscott et al., 1988), *achaete-scute* and several other *Drosophila* proteins (Cabrera et al., 1987), and κ immunoglobulin enhancer binding proteins E12 and E47 (Murre et al., 1989) contain two potential α -helical regions separated by a linker of variable sequence and length. Sequences corresponding to the HLH motif have been shown to mediate oligimerization of MyoD and E12: in all of these proteins, however, a distinct basic region adjacent to HLH sequences imparts the DNA binding activity (Davis et al., 1990). The leucine zipper proteins, which include the yeast activator GCN4, the Jun and Fos oncoproteins, and C/EBP, all contain four or five dimerization-mediating leucine residues spaced at seven amino acid intervals (Landschultz et al., 1988). It was originally hypothesized that dimerization of these proteins occurred via interdigitation, in a zipper-like fashion, of the hydrophobic substituents from the leucine residues, though more recent evidence suggests that the leucine zipper may constitute a conventional coiled-coil structure (O'Shea et al., 1989). Analogous to the HLH proteins, it is likely that sequences outside the leucine zipper region confer the DNA binding activity.

It was originally believed that eukaryotic DNA binding motifs such as the zinc

finger represented fundamental structural departures from the prokaryotic helix-turn-helix proteins. Recent evidence suggests, however, that the structure of the DNA recognition helices in eukaryotic and prokaryotic proteins may be remarkably similar. Thus, two-dimensional nuclear magnetic resonance studies on the glucocorticoid receptor finger domain (R. Kaptein, pers. comm.) and on the yeast ADR1 protein (Parraga et al., 1988) indicate that in fact α -helices reminiscent of those found in the lambda repressor and cro proteins may mediate DNA binding by these eukaryotic factors. It would be astounding if the α -helix was used as the predominant motif for major groove recognition.

The DNA binding domains of eukaryotic activators serve the common function of tethering the protein to DNA; additional surfaces, however, are required for transcriptional enhancement. The conceptual origins of the separability of binding and enhancement dates back to the early 1980's with the isolation of mutants of lambda repressor (the so-called positive control mutants) that bind DNA normally but fail to activate (Guarente et al., 1982; Hochschild et al., 1983). In the case of several eukaryotic activators including GAL4 (Ma and Ptashne, 1987), GCN4 (Hope and Struhl, 1986) and the glucocorticoid receptor (Godowski et al., 1988), surfaces for binding and activation are obviously distinct in that they reside in separate functional domains. In other cases, such as with one of the enhancement domains of the glucocorticoid receptor that co-localizes to the DNA binding domain, the distinction is less clear. Nonetheless, as I describe in chapter three, point mutations in the receptor finger domain can impair enhancement without affecting binding (Schena et al., 1989b). The isolation of positive control mutations within the DNA binding regions of the yeast HAP1 protein (Kim and Guarente, 1989) and MyoD (Davis et al., 1990) have also been reported recently.

What is the chemical nature of activating surfaces in eukaryotic regulators? The

most striking enhancement motif described to date corresponds to the acidic activating regions found in numerous activators including GAL4 (Ma and Ptashne, 1987), GCN4 (Hope and Struhl, 1986), VP16 (Treizenberg et al., 1988) and E1a (Lillie and Green, 1989), and the steroid receptors (Godowski et al., 1988; Hollenberg and Evans, 1988). These so-called "acid blobs" share no apparent sequence homology; in fact, it appears that random *E. coli* sequences carrying a net negative charge can function as activation domains in yeast (Ma and Ptashne, 1987b). Though acidic protein sequences (Gill and Ptashne, 1987) perhaps with an α -helical structure (Giniger and Ptashne, 1987) confer enhancement in eukaryotic cells, acid blobs do not represent the only enhancement motif. In the case of glucocorticoid receptor in which an enhancement domain maps to the DNA binding region, the activating surface instead may consist of a cluster of basic amino acids, though this hypothesis awaits further testing. A third motif for enhancement, identified by deletion analysis of the human transcription factor Sp1 (Courey and Tjian, 1988), appears to consist of regions rich in glutamine residues. A fourth potential enhancement motif has been identified as a region within the hormone binding domain of the estrogen receptor that lacks obvious clusters of glutamines, basic residues or acidic amino acids (Tora et al., 1989). It will probably be the case that activators contain different types of enhancement domains that confer enhancement by contacting different components of the transcription apparatus (see below).

Targets of Activators

Experiments described here and elsewhere suggest a basic outline for enhancement in which an activator binds specifically to an upstream DNA site, juxtaposes the promoter by looping out intervening DNA, and stimulates promoter utilization by contacting an evolutionarily conserved component of the transcription apparatus (Ptashne, 1988;

Guarente, 1988; Yamamoto, 1989). Which protein(s) is the target of activators? Recent studies implicate each of the obvious candidates including the TATA binding protein (TFIID), RNA polymerase II, and the histones though, to date, an unequivocal answer remains elusive.

The case for TFIID as the target for activators derives from both biochemical and genetic experiments. *In vitro*, various GAL4 derivatives and the mammalian transcription factor ATF have been shown to trigger a change in footprinting by TFIID when both the activator and the TATA-binding protein were bound to the same template (Horikoshi et al., 1988a). In addition, alterations in TFIID binding were observed with derivatives of GAL4 that stimulated *in vitro* transcription, but not with a derivative that failed to activate. The affinity of ATF for DNA was also observed to increase in the presence of TFIID (Horikoshi et al., 1988b). Genetic evidence for the role of the TATA binding protein *in vivo* stems from the finding that SPT15, a gene isolated as a suppressor of a *HIS4* Ty insertion, encodes yeast TFIID (Eisenman et al., 1989).

One apparent shortcoming of models that invoke TFIID as a sole target is that certain promoters, such as those controlling the expression of mammalian "housekeeping" genes, lack a TATA box altogether (Dyran, 1986). Assuming that TFIID is absent from the transcription complexes formed at these TATA-less promoters, one must invoke an alternate mechanism for the Sp1-responsiveness of these genes. Perhaps activators such as GAL4 and ATF interact with TFIID, whereas Sp1 contacts a different factor (see above).

The evidence for RNA polymerase as a target also has both a biochemical and a genetic basis. Brandl and Struhl (1989) showed, for example, that purified Pol II binds to

a GCN4-sepharose column under chromatographic conditions in which most other proteins eluted; conversely, GCN4 produced in *E. coli* bound to a Pol II-sepharose column under equivalent conditions. A disconcerting aspect of these experiments was that a fragment of GCN4 containing only the DNA binding domain, a region that exhibits no detectable enhancement activity *in vivo*, was both necessary and sufficient for this interaction.

A component of the polymerase molecule that has received considerable attention (Sigler, 1988) as a potential site for activator interaction is the unusual heptapeptide repeat structure located on the largest (RPO21) polymerase subunit. Allison and Ingles (1989) have attempted to experimentally determine the relevance of these sequences by comparing the activation activity of various GAL4 derivatives in strains of yeast expressing RPO21 subunits bearing different numbers of heptapeptide repeats. It was found that changing the length of the heptapeptide domain modified the ability of deletion mutants of GAL4 to activate in yeast but did not alter the activity of wild-type GAL4, suggesting that the polymerase tail may interact directly with these activators. In a related set of experiments, Arndt et al. (1989) selected suppressors to a *his4* mutation deleted of all of the upstream regulatory sequences. In these studies, it was found that two of the four suppressor genes corresponded to the two largest subunits of Pol II, indicating that specific mutations in polymerase can lead to increased promoter activity.

Models that invoke histones and chromatin structure in gene regulation are currently less popular (and less intuitive) than other hypotheses, in part, because they generally postulate that activation occurs by removing an inhibitory component (eg. a nucleosome) rather than via recruiting a positive factor (eg. TFIID or Pol II). In the absence of definitive data supporting non-chromatin models, however, studies of

nucleosomes remain valuable. Indeed, several laboratories have shown that changes in histone gene dosage can alter transcription in yeast (Han and Grunstein, 1988; Clark-Adams and Winston, 1988). In one such study, alterations in the transcription of genes adjacent to δ insertions was observed upon either overproduction or underproduction of histones H2A and H2B, indicating that histone stoichiometry is critical *in vivo*. An obvious pitfall of these types of experiments is the extreme pleiotropy of nucleosome loss.

A novel approach to identifying the targets of activators has recently been undertaken by Keith Yamamoto and myself. As described in chapter four, suppressors to glucocorticoid receptor mutants were isolated by selecting revertants of a yeast strain containing a GRE-linked *LEU2* gene. Characterization of these strains indicated that the mutations define a single complementation group of recessive suppressor alleles. Moreover, the allele specificity of some of the mutations suggests that receptor may interact directly with the yeast factor encoded by the wild-type version of this suppressor gene.

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Chapter Two:
Glucocorticoid Receptor Derivatives Enhance Transcription in Yeast

ABSTRACT

In mammalian cells, the glucocorticoid receptor binds specifically to glucocorticoid response element (GRE) DNA sequences and enhances transcription from linked promoters. It is shown here that derivatives of the glucocorticoid receptor also enhance transcription when expressed in yeast. Receptor-mediated enhancement in yeast was observed in fusions of GRE sequences to the yeast cytochrome c1 (*CYC1*) promoter, the *CYC1* upstream activator sequences were not essential, since enhancement was observed in fusions of GREs to mutant *CYC1* promoters retaining only the TATA region and transcription startpoints. We conclude that the receptor appears to operate by a common, highly conserved mechanism in yeast and mammalian cells.

The glucocorticoid receptor selectively regulates gene transcription in animal cells by binding in a steroid-dependent manner to specific DNA sequences termed glucocorticoid response elements (GREs) (1,2). GREs associated with the mouse mammary tumor virus (MTV) promoter, and with other genes that are regulated positively by glucocorticoids, are transcriptional enhancer elements that function only in the presence of bound receptor (2-4). Discrete segments of the 795 amino acid rat glucocorticoid receptor have been defined that mediate nuclear translocation, hormone binding, GRE recognition, and transcriptional regulation (5-9). These studies also revealed that receptor derivatives lacking the hormone-binding domain confer constitutive GRE-dependent enhancement (7); moreover, the DNA-binding domain is sufficient for enhancer activation, although its apparent specific activity is low relative to the intact receptor (8,9).

The phenomenon of transcriptional enhancement has now been documented in

organisms ranging from bacteria to mammals (10). This widespread distribution indicates that enhancement may operate by a common mechanism and that its molecular determinants may have been conserved during evolution. Given the relatively simple physiology and genetic manipulability of *Saccharomyces cerevisiae*, we tested whether the rat glucocorticoid receptor expressed in yeast could enhance transcription from yeast promoters linked to GREs.

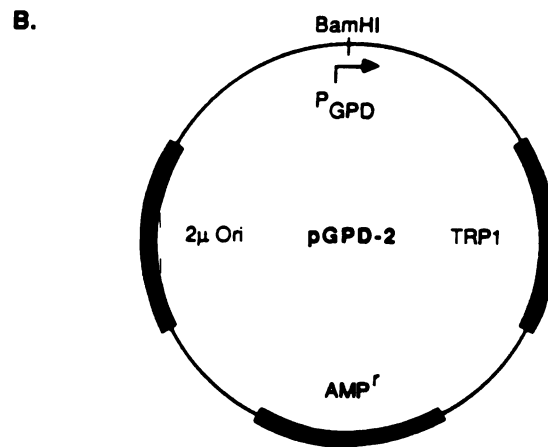
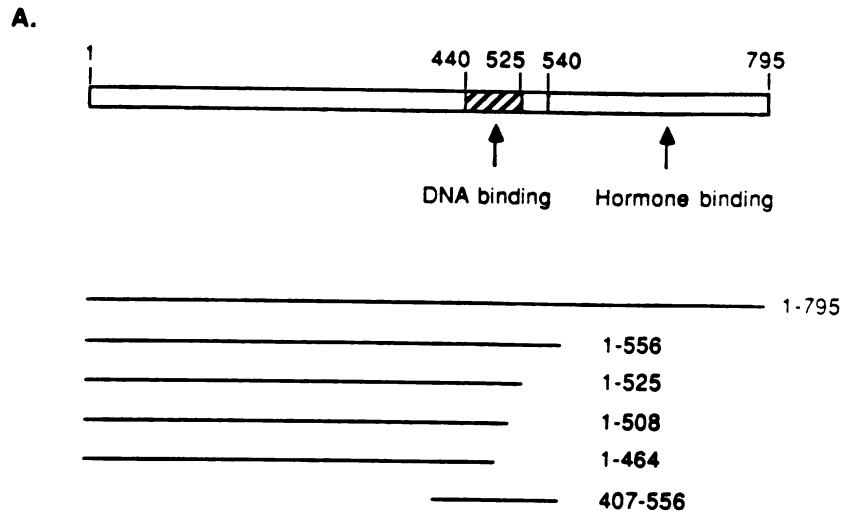
We first expressed in yeast a series of receptor derivatives bearing a deletion of the hormone binding domain, amino acids 557-795. Studies in tissue culture cells showed that receptor derivatives lacking this region confer high-level constitutive GRE-mediated enhancement. As shown in Fig. 1, the receptor derivatives were expressed from the yeast glycerol 3-phosphate dehydrogenase promoter in parent plasmid pGPD-2 (11). All species except X556b, which contains receptor amino acids 407-556, are translated from the normal receptor initiation codon, and each terminates translation in downstream linker or vector sequences, resulting in addition of 4 to 13 nonreceptor amino acids. Comparison of receptor derivatives differing only in these COOH-terminal amino acids (for example, see N556a and N556b in Fig. 2-1) revealed no systematic effects of the short nonreceptor "tails".

Expression and integrity of the various receptor derivatives was assessed by immunoblotting of extracts from strains transformed stably with the receptor expression plasmids. For example, Fig. 2-2A (lanes 1,2) shows accumulation of the predicted 18kD and 65kD proteins, X556b and N556a, respectively, to steady state levels of about 2500 molecules per cell; these intracellular concentrations are comparable to those in mammalian cells. Similar results were obtained with the other receptor constructs shown in Fig. 2-1.

Figure 2-1: Yeast Plasmids Containing Rat Glucocorticoid Receptor Sequences.

The 795-amino acid rat glucocorticoid receptor, denoting the DNA-binding (amino acids 440-525) and hormone-binding domains (amino acids 540-795) (5,8). Receptor segments cloned into yeast plasmids are indicated below the diagram. Receptor sequences were inserted as Bam HI fragments [see refs (5,7)] into the unique Bam HI site 15 bp downstream of the glycerol-3-phosphate dehydrogenase transcription start site in plasmid pGPD-2 (11). The resultant expression plasmid and receptor derivatives are indicated, together with the precise receptor amino acids contained in each derivative and the COOH-terminal amino acids contributed by the polylinker (see text). In each case, translation initiates at the normal receptor NH₂-terminus, except for X556b, which uses a 7-amino acid (sequence: MASWGSP) leader from HSV thymidine kinase (23). The pGPD-2 vector contains the replication origin and ampicillin resistance gene of pBR332, and the TRP1 selectable marker and 2 μ replication origin from yeast.

Figure 2-1.

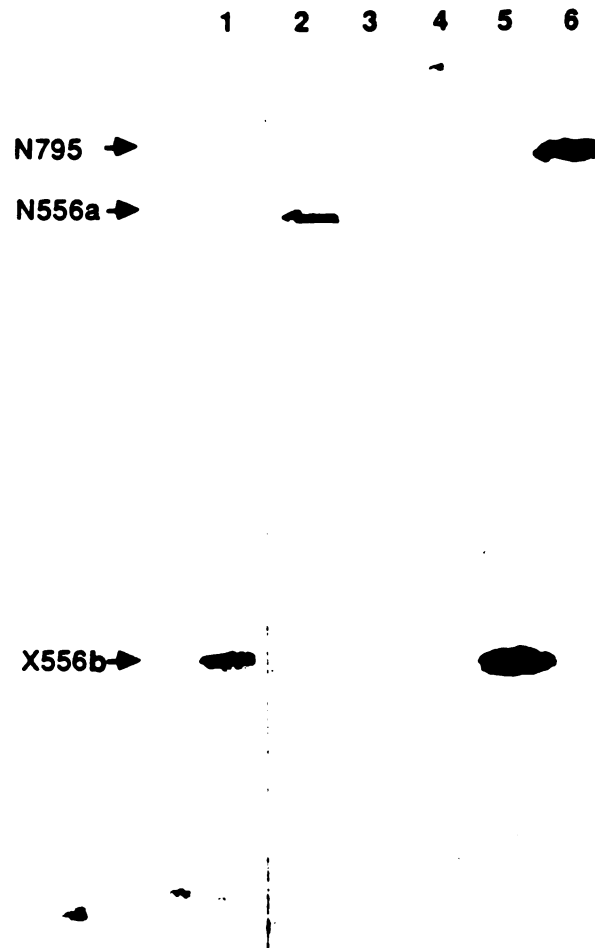


<u>Plasmid</u>	<u>Receptor</u>	<u>Receptor AAs</u>	<u>Nonreceptor C-terminal AAs</u>
pGPD-2	none	none	none
pGPD-795	N795	1-795	none
pGPD-556a	N556a	1-556	GELEFPGLDPST
pGPD-556b	N556b	1-556	GANSRV
pGPD-525	N525	1-525	AGRL
pGPD-508	N508	1-508	AGRL
pGPD-464	N464	1-464	GSRGSVDLDK
pGPD-X556b	X556b	407-556	GANSRV

Figure 2-2: Expression of Glucocorticoid Receptor Derivatives in Yeast.

Shown is an immunoblot of yeast extracts fractionated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with a receptor-specific monoclonal antibody (24) and with goat antibody to mouse IgG horseradish peroxidase-conjugated antibody (Bio Rad). Arrows indicate the intact receptor (N795; 88 kD), and deletion derivatives N556a (65 kD) and X556b (18 kD). Yeast strains were 1205-9B (from A. Mitchell, UCSF; MATa, leu2-3,112, ura3-52, lys2, trp1, ade6) and W303-1B (from R. Rothstein, Columbia Univ.; can1-100, his3-11, 15, leu2-3,112, trp1-1, ura 3-1, ade2-1). Extracts were prepared as described (25); each lane contained 100 μ g of total protein. Lane 1, W303-1B with pGPD-X556b; lane 2, 1205-9B with pGPD-556a; lane 3, W303-1B with pGPD-2; lanes 4, 1205-9B with pGPD-2; lane 5, 1205-9B with pGPD-2 plus 50 ng of pure X556b protein isolated from *E. coli* (26); lane 6, glucocorticoid receptor from HTC cell line 19G11.1 (23).

Figure 2-2.



As an initial test of DNA binding by receptor derivatives *in vivo* in yeast, we inserted GRE sequences between functional elements of the yeast cytochrome c 1 (CYC1) promoter. As shown in Fig. 2-4A, pLG Δ 312S is a plasmid containing the intact CYC1 promoter fused to the *Escherichia coli* β -galactosidase (lac Z) coding sequences (12); two different GRE-containing fragments were inserted at position -178 between the UAS and TATA elements of the promoter (see legend to Fig. 2-4A). Others have shown that insertions of nonspecific DNA fragments as large as 350 bp at this site have only modest effects on promoter function (12), but that specifically bound proteins at this position could strongly inhibit transcription (13). Paradoxically, we discovered that insertion of either of the GRE fragments reduced promoter activity 10-100-fold in the absence of receptor, and that expression of the N556a receptor derivative fully restored promoter activity (14). As expected, initiation from the CYC1 promoter lacking a GRE was unaffected by receptor (Fig. 2-4A). The striking decrease in CYC1 promoter activity brought about by the GRE fragments is not understood, but it implies that a yeast protein may bind tightly to the inserted sequences. In any case, the recovery of activity in the presence of N556a indicates that this receptor derivative indeed interacts with GREs in yeast.

These results suggest either that N556a directly activates transcription in yeast, or that it relieves inhibition of UAS activity by displacing a putative yeast protein bound to the GRE fragment without itself blocking UAS function. We examined these possibilities by testing the effects of GRE sequences inserted at position -178 in the absence of CYC1 UAS elements (see legend to Fig. 2-4B). Thus, pSXG carries a 340 bp fragment of the MTV long terminal repeat (LTR) sequence containing a GRE; pSX26.1 and pSX26.2 include three copies and one copy, respectively, of a 26-bp GRE fragment from rat tyrosine aminotransferase, and pXX46 contains a synthetic 46-bp

Figure 2-3: Primer Extension Analysis of CYC1-lacZ Transcripts.

Total RNA was isolated from transformed yeast strains (27) and subjected to primer extension (28) using a 20 nucleotide primer (5'-TCACCAGTGAGACGGGCAAC-3'; gift of Sandy Johnson, UCSF) complementary to lacZ sense strand sequences 17 bp downstream of the Bam HI site (29). Extension products from 25 µg RNA were fractionated on a sequencing gel adjacent to ³²P-labelled Hae III fragments from pBR322; the 67 and 110 nucleotide fragments are noted. Lane 1, untransformed host strain 1205-9B; all others are 1205-9B transformants containing: lane 2, pLGΔ312S and pGPD-2; lane 3, p312XG.2 and pGPD-2; lane 4, p312XG.2 and pGPD-556a; lane 5, pSX26.1 and pGPD-2; lane 6, pSX26.1 and pGPD-556a. Expression and reporter plasmids are described in Fig. 2-1 and 2-4; for clarity, the presence or absence of N556a, of a GRE, and of the CYC UASs are indicated.

Figure 2-3.

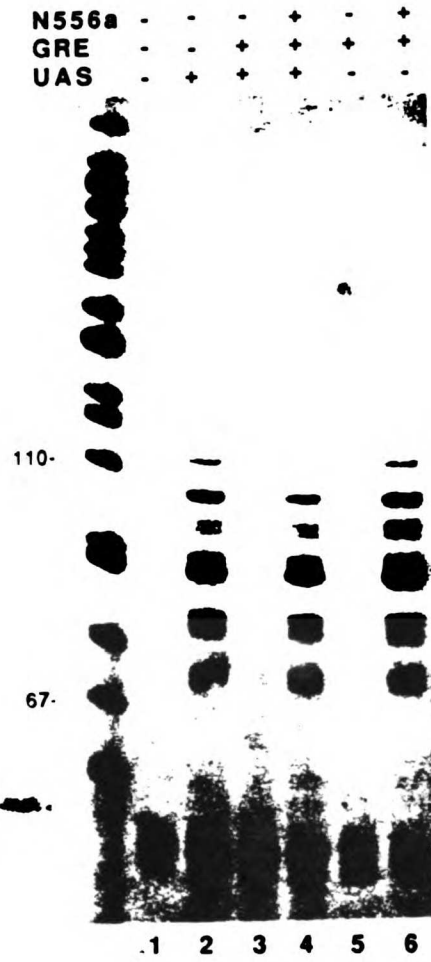


Figure 2-4: Receptor-Mediated Enhancement of GRE-Linked CYC1 Promoter Constructs.

Receptor derivative N556a increases GRE-linked CYC promoter activity. Co-transformants of strain 1205-9B were obtained using the method of Ito (30) and were selected and propagated in minimal medium deficient in uracil and tryptophan (31). Transformed strains expressing N556a or containing the vector alone are indicated as +N556a and -N556a, respectively. Reporter plasmids were constructed from pLGΔ312S (ref. 12) by inserting GREs at the Xho I site at position -178 between the UAS (hatched box) and TATA elements of the CYC1 promoter. GRE340 is a 340bp Sau3A fragment from the MTV LTR GRE (1), and GRE46 is a 46bp palindromic oligonucleotide derived from that fragment that displays strong GRE activity (15); arrows above GRE340 indicate orientation relative to that within the LTR. Results represent the average of at least three independent assays, which varied by less than 20%; β-galactosidase assays were performed according to Yocum et al. (32) and units are defined as 1000 times the change in OD420 due to hydrolysis of o-nitrophenyl-β-D-galactoside divided by the product of the assay time (min) times the culture volume (ml) times OD600 of the culture.

Receptor-mediated enhancement of CYC1 promoter activity is UAS-independent. Strains, plasmids and assays are as in (A), except that the reporter plasmids carry deletions of the CYC1 UAS: pSS was constructed by deleting a 140 bp Sal I to Sma I fragment from pLGΔ312S (12), plasmid pXX arose by deletion of a 390 bp Xho I to Xho I fragment from RY52 (29); pXX contains about 1.5 kb of upstream CYC1 DNA not present in pSS. GRE26 is a synthetic 26bp oligonucleotide derived from the tyrosine aminotransferase GRE (33).

Figure 2-4.

Receptor-mediated enhancement of GRE-linked CYC1 promoter constructs				
Reporter Plasmid	Diagram	β-galactosidase		
		-N556a	+N556a	
A. pLGΔ312S		863	639	
p312XG.1		6.9	509	
p312XG.2		11.1	442	
p312X46		108	493	
B. pSS		0.7	0.6	
pSXG		1.0	48	
pSX26.1		1.4	392	
pSX26.2		1.1	35	
pXX		4.9	3.5	
pXX46		1.5	213	

oligonucleotide with strong GRE activity (15). Introduction of these constructs into yeast together with plasmids expressing N556a yielded β -galactosidase levels 30 to 150 times higher than controls without receptor.

The β -galactosidase assays imply that the receptor derivatives stimulate transcription initiation from the bona fide CYC1 promoter for all GRE-containing reporter plasmids tested (Fig. 2-4B). Indeed, as measured by primer extension, insertion of GREs between the CYC1 UAS and TATA motifs drastically reduced promoter activity, and this effect was reversed upon expression of N556a (Fig. 2-3, lanes 3 and 4). Similarly, N556a-dependent activation of pSX26.1, which contains three GREs but lacks UASs, reflected increased initiation at the normal start sites.

Using these same approaches, we compared the activities of various glucocorticoid receptor derivatives (see Fig. 2-1) in yeast. As shown in Table 1, N525, which contains the first 525 amino acids of the receptor, is as active as N556a or N556b. However, deletion of 17 additional C-terminal amino acids yielded N508, which retains only about 10% the activity of N525; N464 is only about 1% active relative to N525, and X556b, which specifies receptor amino acids 407-556, displays similar low activity. These results parallel closely those obtained with expression of these receptor derivatives in animal cells (7,8).

In contrast, expression of the full length receptor (N795; Fig. 2-1) in yeast elicited no detectable stimulation of β -galactosidase activity in the presence of dexamethasone (Table 2-1). We have recently discovered, however, that several related corticosteroids strongly stimulate β -galactosidase expression via the intact receptor (16). The cause of this apparent altered ligand specificity is not understood, but these results

Table 2-1: Transcriptional Enhancement by Various Glucocorticoid Receptor Derivatives.

Shown are β -galactosidase data from strain 1205-9B double transformants carrying receptor derivatives and either pSX26.1 (+GRE26) or the same plasmid lacking the GREs, pSS (-GRE26) (see Fig. 2-4B). Receptor derivatives are described in Figure 2-1. Enzyme assays were performed as in Fig. 2-4A, except for the addition of 1 μ M dexamethasone to cultures expressing N795. Shown is the average of at least three independent assays, which varied by <20%.

Table 2-1.

Transcriptional Regulatory Activity Of Glucocorticoid Receptor Derivatives		
Receptor Derivative	β-galactosidase	
	-GRE26	+GRE26
N795	0.7	1.3
N556a	0.6	392
N556b	0.7	584
N525	0.6	420
N508	0.7	64
N464	0.8	5.7
X556b	1.1	3.4

imply that steroids can enter yeast cells, and that the receptor is functional for signal transduction in yeast.

Taken together, our results suggest that the glucocorticoid receptor enhances transcription in yeast and mammalian cells by a similar mechanism. That is, the action of a series of receptor constructs on three distinct GREs (MTV, TAT, and a synthetic oligomer) is closely correlated in yeast and animal cells. Results similar to those obtained with GRE-CYC1 fusions have been observed in strains containing a chromosomal GRE-LEU2 gene (16). The reduced activity of the N508 receptor relative to N525 is consistent with previous findings from DNA binding studies in vitro (5) and from assays in transfected animal cells. Likewise, N464, which retains only one of the two "zinc fingers" of the DNA-binding domain, displays low levels of activity in animal cells (17), as does X556, a small receptor fragment that encompasses the complete DNA binding domain (8). Finally, Godowski et al. (18) have defined two distinct segments of the receptor that confer enhancement in animal cells; we find that deletions within either of these regions reduce the extent of enhancement in yeast (Table 2-1).

Unlike the case in animal cells, we have so far failed to detect enhancer activity from GREs residing downstream of promoters. This observation is reminiscent of the lack of UAS activity when those elements are inserted downstream of promoters (12). Interestingly, UASs can function from downstream positions in animal cells that are expressing the corresponding UAS binding protein (19). This implies that the capacity of regulatory elements and their cognate binding proteins to act downstream of a promoter may reflect structural or functional distinctions between yeast and animal cell transcription initiation complexes, rather than differences in the regulatory mechanisms per se.

Our results establish that a metazoan DNA-binding transcriptional regulatory factor can function in an organism as distantly related as yeast. This finding complements reports that appeared after completion of this work showing that in cultured animal cells the yeast regulatory factor GAL4 constitutively activates animal cell promoters fused to GAL4 binding sites (19,20); analogously, a derivative of the animal cell fos protein stimulates yeast transcription when tethered adjacent to a yeast promoter through a bacterial repressor DNA binding site (21). These and other recent studies (22) may indicate strong conservation of protein-protein contacts between regulatory factors and components of the transcription initiation apparatus.

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14. **Plasmid copy numbers in the transfected yeast strains was monitored by southern blotting and by DNA transformation of E. coli. Both methods revealed that copy numbers varied by <2-fold between clones. Thus, the 10-100 fold effects of GREs and N556 cannot merely reflect copy number fluctuations.**

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Chapter Three:
**Mutants of the Glucocorticoid Receptor That Uncouple DNA Binding and Transcriptional
Enhancement**

SUMMARY

Mammalian glucocorticoid receptors bind specifically to glucocorticoid response element (GRE) DNA sequences and enhance transcription from GRE-linked promoters in mammalian cells and in yeast. We randomly mutagenized a segment of the receptor encompassing sequences responsible for DNA binding and transcriptional regulation, and screened in yeast for receptor defects. The mutations all mapped to a 66 amino acid subregion that includes two zinc fingers; in general, parallel phenotypes were observed in yeast and animal cells. Mutants defective for DNA binding also failed either to enhance or to repress transcription. However, several mutations in the second finger selectively impaired enhancement; we suggest that such "positive control" mutants may alter protein-protein contacts required for transcriptional activation.

INTRODUCTION

In the presence of bound hormone, the glucocorticoid receptor protein regulates transcription initiation from specific animal cell promoters utilized by RNA polymerase II. The receptor enhances transcription by associating selectively with DNA sequences termed glucocorticoid response elements (GREs) (Chandler et al. 1983; Payvar et al. 1983; Scheidereit et al. 1983). In addition, the receptor represses transcription by binding to a distinct class of sequences termed "negative GREs" (nGREs) (Sakai et al. 1988). In association with the receptor, both GREs and nGREs act at a distance to modulate the activity of linked promoters, thereby serving as enhancers and long range operators, respectively (Chandler et al. 1983; Sakai et al. 1988).

Functional regions of the glucocorticoid receptor and other members of the nuclear receptor gene superfamily have been characterized in some detail (Evans 1988; Green and Chambon 1988; Beato 1989). Studies of the 795 amino acid rat glucocorticoid receptor demonstrated, for example, that a 150 amino acid fragment, residues 407-556, is sufficient for GRE and nGRE binding (Rusconi and Yamamoto 1987; Mordacq and Linzer 1989), nuclear localization (Picard and Yamamoto 1987), transcriptional enhancement (Miesfeld et al. 1987; Godowski et al. 1988) and transcriptional repression (Miesfeld et al. 1988). This segment of the receptor includes a 61 amino acid subregion containing two "zinc finger" motifs (Weinberger et al. 1985; Miller et al. 1985; Berg 1986); Freedman et al. (1988) showed that the segment indeed binds two zinc ions, each coordinated tetrahedrally by four cysteine sulfur atoms, and that metal coordination is essential for proper folding and DNA binding. In addition, a 28 amino acid subfragment has been identified, which lies downstream of the fingers, that exhibits nuclear localization activity, but fails to bind DNA or to modulate

transcription (Picard and Yamamoto 1987; Rusconi and Yamamoto 1987). Thus, of the four activities resident within the 407-556 region (denoted here as the "finger domain") only nuclear localization has been uncoupled from the others.

What is the relationship of the DNA binding and transcriptional regulatory activities within the finger domain? A commonly held view is that "long range regulators" such as the receptor bind to DNA sequences in the vicinity of promoters that they regulate and then confer regulation via protein-protein interactions with a component of the transcription initiation machinery (Ptashne 1986, 1988; Yamamoto 1985, 1989). According to this view, DNA binding is essential for regulation, and the individual residues that mediate DNA binding and regulation are at least in part distinct; this has been shown clearly to be the case for proteins containing regulatory domains that are widely separated from their cognate DNA binding domains (Hope and Struhl 1986; Ma and Ptashne 1987; Godowski et al. 1988).

To pursue the functional complexity of the finger domain, we sought to develop a rapid and efficient genetic approach. Recently, it has been demonstrated that various transcriptional regulators and initiation factors can function in both yeast and mammalian cells (Kakidani and Ptashne 1988; Webster et al. 1988; Chodosh et al. 1988; Lech et al. 1988; Struhl 1988). In particular, the glucocorticoid receptor (Schena and Yamamoto 1988) and the estrogen receptor (Metzger et al. 1988) confer transcriptional enhancement upon promoters in yeast that are linked to GREs and EREs, respectively. These findings suggested that we might be able to exploit the genetic capabilities of yeast to screen a large number of random point mutations within the glucocorticoid receptor as a step toward developing a broad genetic approach to dissecting functional receptor domains and identifying interacting cellular factors. We therefore mutagenized a fragment of

receptor cDNA encoding the finger domain, inserted those segments into a wild type receptor recipient, and screened in yeast for mutants defective in the expression of a GRE-linked reporter gene. We report here the isolation and preliminary characterization, in yeast, in animal cells, and in vitro, of a series of such point mutations.

RESULTS

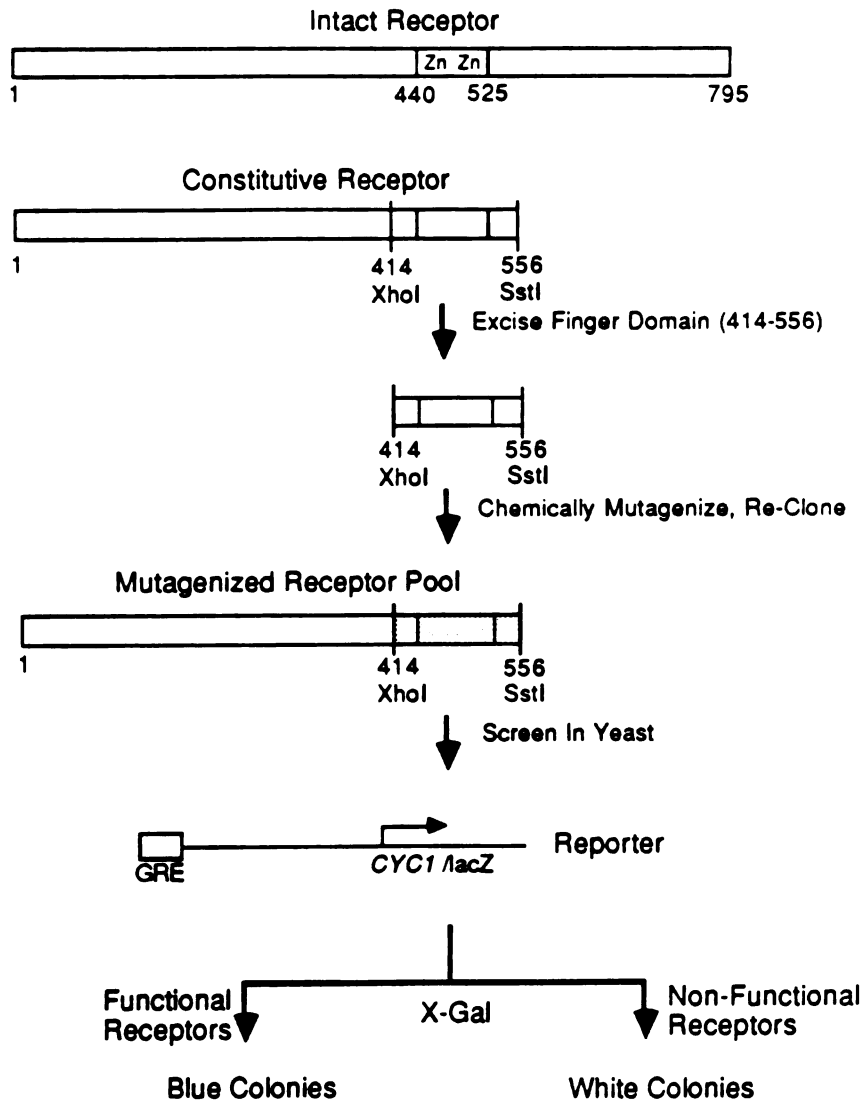
Mutagenesis and Screening

To isolate mutants defective in the DNA binding and transcriptional regulatory functions of the receptor "finger domain", we employed random mutagenesis coupled with a genetic screen in yeast (Figure 4-1). A 430 bp Xho I - Sst I fragment that encodes 143 amino acids (residues 414-556) encompassing the finger domain was mutagenized *in vitro* with sodium nitrite (see Experimental Procedures). The fragments were then reinserted into an unmutagenized receptor expression vector cleaved with Xho I and Sst I to generate a pool of 10^4 derivatives bearing a high frequency of point mutations within the 414-556 region. To simplify our manipulations, the recipient vector plasmid encoded N556a, a truncated receptor derivative that lacks the hormone binding domain, amino acids 557-795 (Rusconi and Yamamoto 1988), and is constitutively active in animal cells (Godowski et al. 1987) and in yeast (Skena and Yamamoto 1988). The mutagenized plasmids were transformed into a yeast strain containing an integrated, GRE-linked *E. coli* β -galactosidase (*lacZ*) reporter gene driven by the yeast *CYC1* promoter (Guarante and Hoar, 1984). Colonies expressing wild type N556a are dark blue on Xgal indicator plates under these conditions. White colonies (putative *lacZ*⁻) appeared at a rate of 0.4%, and light blue colonies (reduced *lacZ* expression) were observed at 0.04%. Fifty colonies displaying these phenotypes were isolated and subcloned; those defective in β -galactosidase expression were commonly larger than those expressing normal levels, consistent with the finding that functional receptor derivatives reduce slightly the growth rate of yeast (M. Skena unpublished).

Figure 3-1: Screen for Glucocorticoid Receptor Zinc Finger Mutants.

A 430 bp Xho I-Sst I fragment was excised from a cDNA encoding a constitutive glucocorticoid receptor derivative, N556; the fragment, which encompasses the zinc finger region, was transferred to a single-strand vector and mutagenized with sodium nitrite (see Experimental procedures). Mutagenized inserts were reinserted into the wild type receptor backbone in a yeast expression plasmid, and these species were transformed into yeast strain BJ-G26.1 which bears an integrated GRE-linked reporter gene (Schena and Yamamoto 1988) consisting of the yeast CYC1 promoter fused to lacZ (Guarante and Hoar 1984). Yeast transformants expressing low levels of β -galactosidase were detected as white or light blue colonies on indicator plates.

Figure 3-1.



Sequence Alterations and Protein Stability of the Mutants

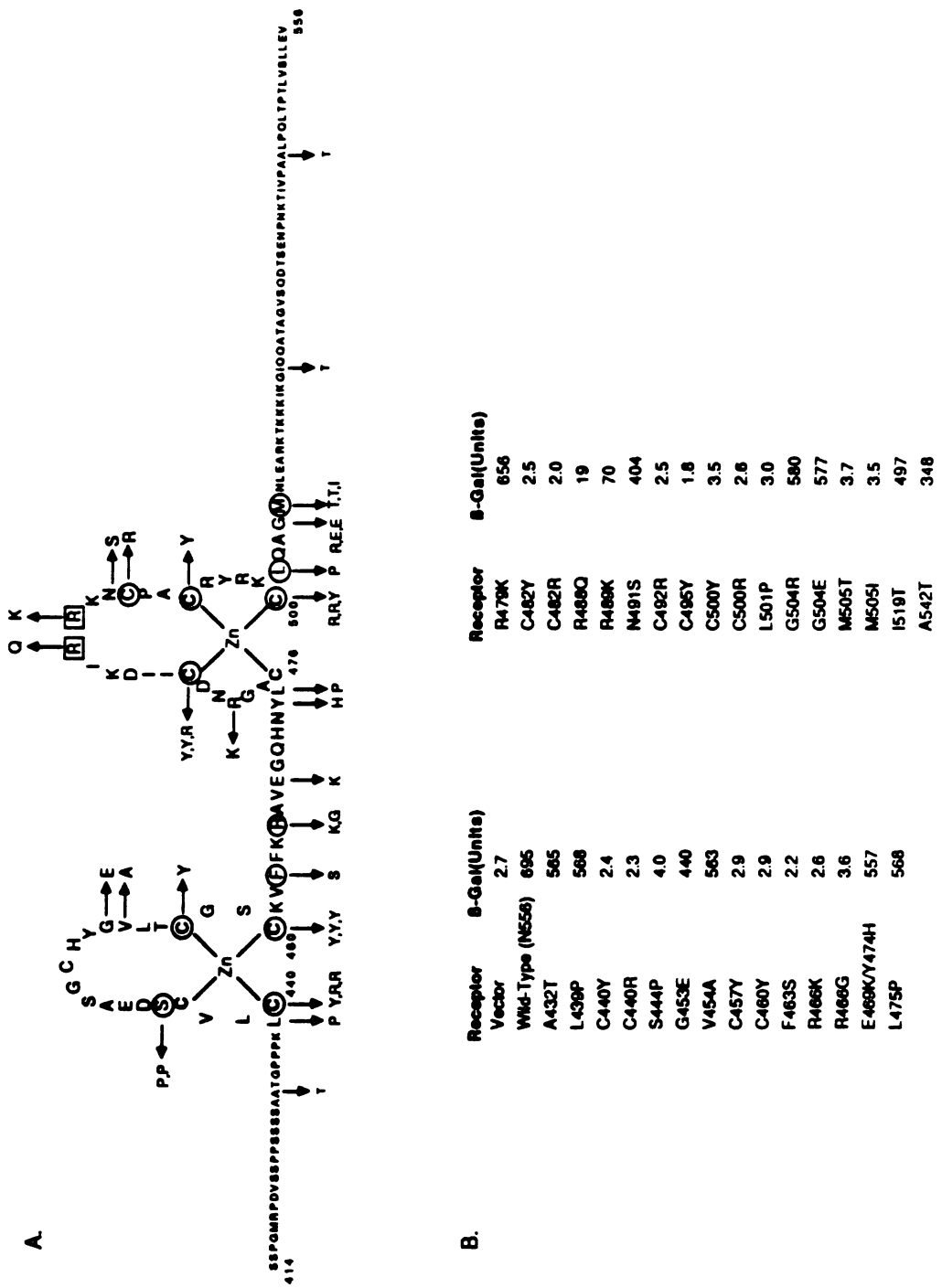
The receptor expression plasmids from 50 yeast isolates deficient in β -galactosidase expression were transformed into *E. coli* for efficient propagation, and the mutagenized segments were sequenced. Single, double or triple point mutations (in a ratio of 5:2:1) were detected in 41 plasmids, and all but three of the base changes were G to A or T to C transitions, as expected for sodium nitrite mutagenesis (Myers et al. 1985). The remaining nine plasmids lacked mutations and exhibited normal β -galactosidase production upon retesting in yeast, suggesting that they were false positives in the initial screen. The lesions within the 25 plasmids that carried single point mutations were all clustered in a 66 amino acid segment (residues 440 to 505) that corresponds closely to the 61 amino acids proposed to form the two zinc fingers (Figure 2A). Moreover, of the 16 plasmids containing double or triple mutations, each contained at least one alteration within the 66 amino acid region. The defects in 10 such multiple mutants were separated to generate 14 additional single mutants and 1 double mutant. This revealed a series of "neutral" mutations (e.g., N491S; see Figure 2B) -- amino acid alterations that did not impair significantly receptor function in our assays. Notably, the neutral mutations were distributed throughout the mutagenized fragment (e.g., A542T; see Figure 3-2B). For each of the 10 multiple mutants, however, the mutant phenotype could be attributed to a lesion within the 66 amino acid segment.

The double mutant and 38 of the single mutants were individually re-introduced into the yeast strain harboring a GRE-linked reporter plasmid; together, these represent 32 amino acid substitutions at 26 positions within the mutagenized fragment. [Mutant receptors are named by the single letter designation of a wild type amino acid and its

Figure 3-2: Point Mutations within the Glucocorticoid Receptor Zinc Finger Domain.

Receptor residues 414-556 are depicted as two zinc fingers. Enlarged segment shows a 66 amino acid subregion that includes all point mutations isolated within this region that impair receptor function. Circled residues indicate point mutations that each abolish receptor function in yeast; residues enclosed by squares indicate amino acid changes that partially impair receptor activity. Amino acids marked with an arrow only indicate "neutral" alterations with little or no phenotype effect in yeast. Multiple isolates were obtained at some positions as shown; in most cases, however, we could not determine whether multiple isolates of the same mutation represented independent mutational events. Each point mutant shown in Figure 3-2A was assayed for β -galactosidase activity as described in Experimental Procedures. Nomenclature for mutant receptor species includes the single letter amino acid designation for a wild type residue, followed by its position within the protein, and the single letter code for the mutant residue; thus, L439P contains a leucine to proline substitution at receptor residue 439. As a negative control, the same strain was transformed with an expression plasmid lacking receptor sequences (vector). All values represent the average of at least three independent measurements; individual determinations varied by less than 20%.

Figure 3-2.



position within the receptor, followed by the identity of the mutant residue; thus, C440Y contains a tyrosine substitution for cysteine at position 440.] Quantitative β -galactosidase measurements (Figure 2B) confirmed the phenotypes ascribed originally by screening. We conclude that residues within and very close to the receptor zinc fingers are essential for GRE-mediated enhancement in yeast; in contrast, point mutations in the flanking residues (amino acids 414-439 and 506-556) are apparently not sufficient to alter enhancement activity. Mutations that completely abolished enhancement (Figure 3-2A, circled residues) were located in particular at cysteines proposed to coordinate zinc ions, and at residues immediately carboxy terminal to each finger. Mutations in the "coordinating cysteines" likely alter the structure of the fingers (Freedman et al. 1988), whereas the lesions carboxy terminal to the fingers may directly impair a receptor function, such as DNA recognition (e.g., see Mader et al. 1989).

We recovered several mutations that affected enhancement but did not reside at the coordinating cysteines (see Discussion) or at the carboxy side of a finger. For example, S444P and C492R were fully defective. In addition, enhancement activity was reduced 10-30 fold by mutations in either of the arginine residues at the "tip" of the second finger (Figure 3-2A, residues enclosed in squares; Figure 3-2B). Consistent with these partial defects, R488Q and R489K were initially isolated as "light blue" colonies on indicator plates. While the mutations at these positions cannot be interpreted without protein structural information, it is notable that these lesions also reside within the 66 amino acid segment encompassing the zinc fingers. In contrast, neutral mutations mapped throughout the 143 amino acid mutagenized receptor segment (Figure 3-2A, residues marked only with arrows; Figure 3-2B).

The observed mutant phenotypes might simply have reflected low level

expression or rapid degradation of the altered receptor derivatives. We therefore examined each of the mutants by immunoblotting of yeast extracts with a receptor-specific monoclonal antibody. Remarkably, the expression, solubility and integrity of the mutant receptors was in every case similar to that of the wild-type N556a receptor derivative (Figure 3-3, compare lanes 4 through 20 with lane 3). Thus, the failure of a mutant receptor to activate the GRE-linked reporter gene in yeast cells in no case reflected receptor underproduction or instability.

DNA Binding

Freedman et al. (1988) used a bacteriophage T7 promoter and T7 polymerase system (Studier and Moffatt 1986) to overproduce and purify the receptor derivative X556 from E. coli; we adopted this same approach to overproduce the mutant X556 derivatives for DNA binding studies (see Experimental Procedures). Following expression and partial purification, wild type and mutant receptor derivatives were tested for specific DNA binding in a gel retardation assay. With the wild type X556 product, we detected two discrete retarded bands (complexes 1 and 2), which correspond to the binding of one and two receptors, respectively, to the labeled GRE-containing DNA fragment tested (J. La Baer and K.R. Yamamoto unpublished) (Figure 3-4A, lanes 1 and 2). No binding was detected with 13 mutants that were fully defective for enhancement (Figure 3-4A, lanes 3-11 and 15-20); titration experiments (data not shown) imply that GRE binding by these mutants is reduced by at least 50-fold relative to that of wild type N556a. In contrast to these results, R489K, which confers about 10% of wild type enhancement, displayed readily detectable DNA binding (Figure 3-4A, lane 13); titration experiments indicated a 10-20 fold reduced affinity for the GRE-containing DNA fragment (data not shown). In addition, two mutants, R488Q and N491S, exhibited

Figure 3-3: Stability of Receptor Finger Domain Mutants in Yeast.

Shown is an immunoblot of a mammalian extract and yeast extracts from the parental (BJ-G26.1) strain and from transformants producing various receptor derivatives; the mammalian N795 extract was prepared from HTC cell line 19G11.1 (Miesfeld et al. 1986). The extracts were fractionated by SDS polyacrylamide gel electrophoresis and probed with a receptor-specific monoclonal antibody (Gametchu and Harrison 1984). Arrows indicate migration positions of the intact receptor N795 (88kD) and the N556 constitutive receptor derivatives (65kD).

Figure 3-3.

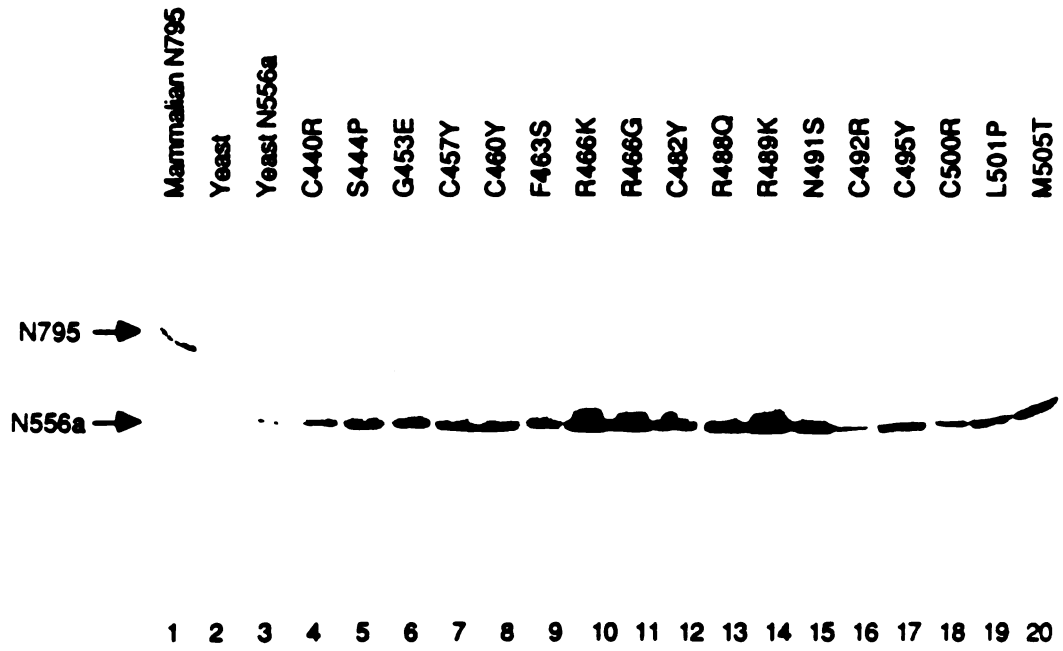
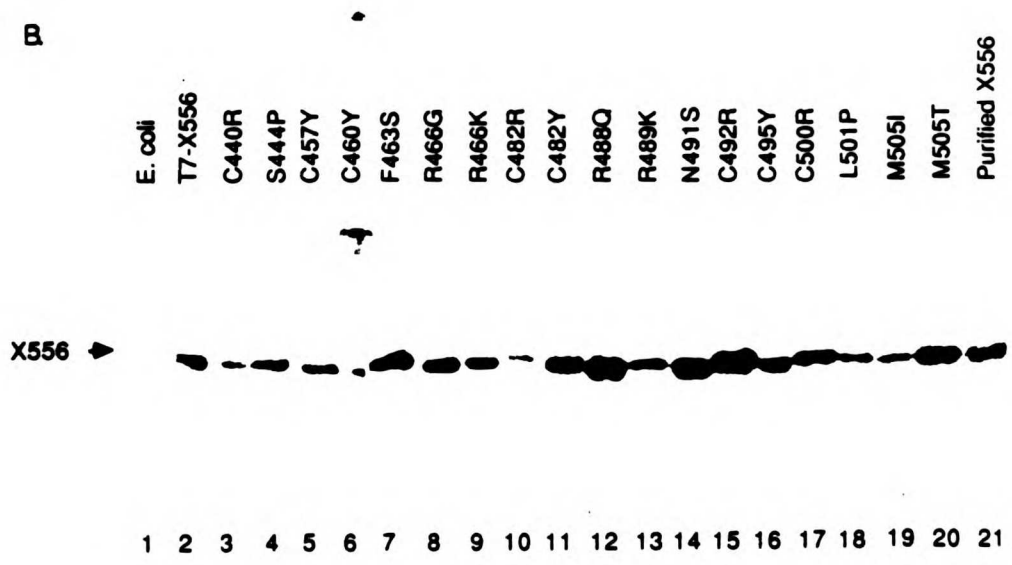
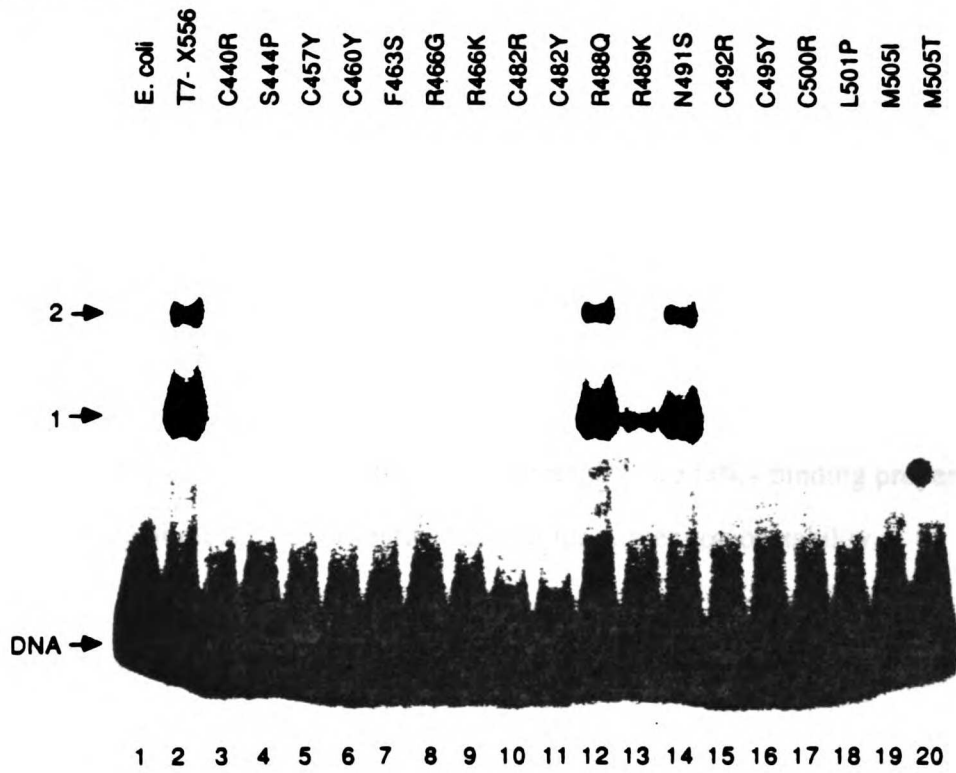


Figure 3-4: DNA Binding Activities of the Receptor Finger Domain in vitro.

(A) Mutant receptor species were assayed for DNA binding in a gel mobility assay. *E. coli* extracts containing wild type and mutant receptor derivatives were incubated with a labeled GRE-containing DNA fragment and electrophoresed in nondenaturing polyacrylamide gels as described in Experimental Procedures. Arrows indicate free DNA (DNA), and protein-DNA complexes with one (1) or two (2) molecules of receptor bound per molecule of DNA (J. LaBaer and K.R. Yamamoto unpublished).

(B) *E. coli* extracts used for the gel mobility assay were fractionated by SDS-polyacrylamide gel electrophoresis and probed with a receptor-specific monoclonal antibody. The arrow indicates the mobility position of the X556a (19kD) receptor derivative. Relative receptor content in each extract was estimated from staining intensity, and minor differences were normalized prior to gel mobility analysis.

Figure 3-4.



normal DNA binding (Figure 3-4, compare lanes 12 and 14 with lane 2). Interestingly, N491S is nearly fully active in yeast with respect to enhancement, whereas R488Q produces less than 3% the enhancement activity of N556a (Fig. 3-2B; see also Table 3-2 and Figure 3-6).

An immunoblot of the protein fractions used for the DNA binding experiments confirmed that the expected 19kD X556a receptor derivatives were produced at similar levels after induction of the appropriate bacterial cultures and that degradation was negligible (Figure 3-4B); thus, differences in the *in vitro* DNA binding properties of the various receptor mutants do not reflect differential expression or stability.

A Cold Sensitive Receptor Mutant

Cold sensitive mutations have commonly been associated with defects in protein-protein interactions (see Discussion). It seemed conceivable that some of the mutant receptors might be conditionally defective at elevated or reduced temperatures in yeast. We therefore replica plated yeast strains expressing receptor mutants that displayed normal or partial activity at the standard (30°C) temperature and assessed β -galactosidase activity on indicator plates at 19°, 30° and 37°C. One mutant, R489K, displayed a cold-sensitive phenotype; quantitation of β -galactosidase activity in liquid cultures confirmed that R489K was virtually inactive at 19°C, whereas modest activity was observed at higher temperatures (Table 3-1). In contrast, the other receptors exhibited similar β -galactosidase induction levels at all three temperatures (Table 3-1 and data not shown).

Enhancement in Mammalian Cells

We next characterized the activities of the receptor mutants in animal cells. For these experiments, the mutant receptor inserts were transferred from yeast plasmids into mammalian expression vectors to reconstruct full-length receptor derivatives. We tested each species for enhancement of a GRE-linked chloramphenicol acetyltransferase (CAT) reporter gene in transient cotransfections of CV-1 monkey cells, which lack endogenous receptor. Previous studies in both yeast and CV-1 cells established that the hormone-treated intact receptor (denoted N795) exhibits about twice the activity of the constitutive N556a derivative (see Table 3-2). Given the similar activities of these two receptor species in yeast and animal cells, we compared a series of mutants in these molecular and cellular backgrounds. As shown in Figure 3-5A, the wild type receptor (N795) strongly enhanced CAT expression in a hormone dependent manner, as did G453E, a neutral mutant in yeast (Figure 3-5A and Table 3-2; see also Figure 3-2B). Indeed, six additional neutral mutants in yeast (L439P, E469K/Y474H, L475P, R479K, G504E, G504R) also displayed full activity in animal cells (data not shown). In contrast, most of the receptor mutants that failed to function in yeast were similarly inactive in CV-1 cells (Figures 3-5A and 3-2B; C440R, S444P, C460Y, F463Y and C482Y and C492R). Thus, despite the fact that the yeast and animal cell reporter plasmids employ different reporter genes, different promoters and different GRE sequences (see Experimental Procedures), the activities of receptor point mutants in yeast were closely paralleled in animal cells; this supports strongly the view that the receptor functions by a common mechanism in yeast and mammals.

Table 3-1: A Cold-Sensitive Glucocorticoid Receptor Mutant.

Yeast strain BJ-G26.1 was stably transformed with yeast expression plasmids encoding wild type (N556a) or the R489K mutant receptor derivatives. Cultures were propagated at 19°C, 30°C or 37°C, and β -galactosidase activities were measured as described in the Experimental Procedures. A background activity of 2 units (obtained from a transformant lacking receptor sequences) was subtracted from the values shown, which represent the average of at least three independent measurements; individual determinations varied by less than 20%. No other cs or ts phenotypes were detected among the 10 mutants that have been tested to date (data not shown).

Table 3-1.

Receptor	β -Galactosidase (Units)		
	19°C	30°C	37°C
N556a	433	496	554
R489K	3	34	42

Interestingly, relative enhancement in yeast and animal cells differed rather dramatically in four cases (Table 3-2). Of these four, R466K was somewhat difficult to evaluate, as its activity is low in both cell types; it was inactive in yeast, but displayed 6% of wild type N556a activity in CV-1 cells (Table 3-2). More striking departures from parallel behavior in yeast and animal cells were observed with the other three mutants, all located in the tip of the second zinc finger. R489K displayed 12% of wild type activity in yeast at 30°C and was cold sensitive, whereas it conferred 92% activity in animal cells; similarly, R488Q yielded 3% of full activity in yeast and 58% in animal cells (Figures 3-2B and 3-5A). In contrast, N491S was obtained in yeast as a neutral mutant, displaying 58% of full activity (Figure 3-2B), and bound DNA normally *in vitro* (Figure 3-4A), yet it conferred no detectable enhancement in mammalian cells (Figure 3-5A). Conceivably, this cluster of mutations conferring nonparallel phenotypes in different species may identify a region of protein-protein contact (see Discussion).

Repression in Mammalian Cells

The glucocorticoid receptor represses transcription by binding specifically at nGRE sequences (Sakai et al. 1988); the finger domain alone is sufficient to confer nGRE-mediated repression (Miesfeld et al. 1988). We therefore tested our point mutants for transcriptional repression by transient cotransfections of CV-1 cells with receptor expression plasmids together with a reporter plasmid containing the bovine prolactin promoter and nGRE (Camper et al. 1985; Sakai et al. 1988). As with certain animal cell lines (Sakai et al. 1988), the prolactin nGRE is nonfunctional in yeast (data not shown). In CV-1 cells, however, hormone-dependent repression was observed with the wild type receptor and with mutants that retained full or partial enhancement activity (Figure 3-5B; see N795, G453E, R489K). Conversely, mutants that failed to enhance transcription in

yeast, and were defective for DNA binding *in vitro*, lacked repression activity in animal cells (Figure 3-5B, C440R, S444P, C460Y, F463S, C482Y and C492R). Thus, many of the same amino acids that are essential for GRE binding and enhancement are also required for receptor-mediated repression at nGREs. Unexpectedly, two mutants, F463S and C482Y, produced lower basal activities (Figure 3-5B); whether this indicates that these altered receptors can bind to nGREs even in the absence of hormone has not been directly tested. Finally, N491S, which bound DNA normally *in vitro* and enhanced transcription in yeast but not in animal cells, retained modest but reproducible repression activity at the prolactin nGRE (Figure 3-5B).

Figure 3-5: Enhancement and Repression by Receptor Finger Mutants in Mammalian Cells.

(A) Enhancement activities of the mutants were assayed in CV-1 cells. Reporter plasmid GMCS (DeFranco and Yamamoto 1986) contains the chloramphenicol acetyltransferase (CAT) gene driven by the MTV promoter and flanked by GREs from the MTV and MoMSV LTRs. Extracts were prepared from CV-1 cultures co-transfected transiently with GMCS DNA and with the intact receptor (N795) or with the mutant receptor derivatives shown, and propagated in the (-) absence or (+) presence of 0.1 μ M dexamethasone. Upper signals are the reaction products.

(B) Repression activities of mutants were assayed in CV-1 cells as above, except that reporter plasmid PPCV was used in place of GMCS. PPCV contains the bovine prolactin nGREs and promoter (-586 PRL) driving the CAT gene fused upstream of the SV40 enhancer (Sakai et al. 1988).

DISCUSSION

By coupling random mutagenesis *in vitro* and phenotypic screening in yeast, we have recovered a novel series of point mutations in a 143 amino acid ("finger domain") segment of the rat glucocorticoid receptor. Characterization of these mutants in yeast, in animal cells and *in vitro* revealed that: (1) point mutations that reduce enhancement activity are restricted to a 66 amino acid subregion that encompasses the zinc fingers; (2) all of the mutant derivatives tested are soluble and accumulate to intracellular levels similar to the wild type species; (3) in general (but see below), the mutants are phenotypically similar in yeast and animal cells, supporting further the notion that the receptor acts by a common mechanism in these diverse eukaryotes (Schena and Yamamoto 1988); (4) mutations that abolish GRE DNA binding are distributed across both fingers, especially at the cysteines thought to coordinate zinc ions, and in the five amino acids just downstream of each finger; (5) a series of mutations that selectively affect enhancement ("pc-like"; see below), and a cold sensitive mutation, are tightly clustered in a portion of the second finger; (6) certain of the mutants, particularly those with pc-like behavior, exhibit striking phenotypic differences in yeast and animal cells, consistent with the view that residues at these positions may be involved in protein-protein interactions.

It is worth noting that several transcription initiation factors and regulators from yeast and animal cells can function in cells from non-homologous species (Buratowski et al. 1988; Kakidani and Ptashne 1988; Chodosh et al. 1988; Struhl 1988; Lambert et al. 1989); this implies that our strategy may provide a general approach for fine structure analysis of other gene products from organisms with complex or inaccessible genetics.

Table 3-2: Enhancement by Receptor Mutants in Yeast and Mammalian Cells.

Relative enhancement activities (normalized to N556a activity) of various receptor point mutants in yeast strain BJ-G26.1 were computed from β -galactosidase activities; values represent the average of at least three independent assays that varied by less than 20%. Values from dexamethasone-treated mammalian CV-1 cells were calculated from CAT activities of reporter gene GMCS normalized to the intact receptor N795; to facilitate comparison with the yeast data, the CV-1 results are also normalized to N556a; values represent the average of the three independent cotransfection experiments. Recent studies (data not shown) confirm that the mutant phenotypes observed in yeast in the N556a backbone are unchanged when the same mutants are assayed in hormone-treated yeast cultures in an N795 backbone. The three mutants that display pc-like phenotypes in yeast or animal cells are R488Q, R489K and N491S. Note that the DNA-binding activity of R489K is reduced in vitro, and that in no case have we yet proven that the mutant proteins actually bind GREs in vivo; such occupancy tests will be essential to assess the pc-like characteristics unequivocally.

Table 3-2.

Receptor	Yeast (% Activity)	CV-1	
		(% Activity)	(% N556a)
N795	212	100	208
N556a	100	48	100
G453E	63	27	56
R466K	<0.5	3	6
R488Q	3	28	56
R489K	12	44	88
N491S	58	<0.5	<0.5

The procedure appears particularly well suited to the facile isolation and preliminary characterization of a large number of mutants; indeed, the mutants described here were all obtained after treatment of only one DNA strand with a single mutagen.

It is striking that every mutation that impaired receptor function was located in a 66 amino acid segment coinciding precisely with the 61 residues of the zinc fingers plus five amino acids downstream of the second finger. Point mutations within this region were roughly equally distributed across the two fingers, showing clearly that each is essential for receptor function. Thus, our results support strongly the proposed zinc coordination pattern (Weinberger et al. 1985; Miesfeld et al. 1986) shown in Figure 3-2. We have not, however, ruled out an alternative scheme (Severne et al. 1988) suggesting that cysteine 492 may be involved in zinc binding, as mutation of this residue also abolished DNA binding. Direct biochemical and spectroscopic measurements comparing purified mutant and wild type proteins will be necessary to determine unequivocally the correct coordination pattern. It is also notable that point mutations in the nuclear localization signal within the finger domain (residues 497-524) (Picard and Yamamoto, 1987) were not recovered in our screen, implying that single amino acid changes in those sequences are insufficient to produce a phenotype.

Hollenberg and Evans (1988) employed a site-directed mutagenesis approach to this same region of the glucocorticoid receptor, substituting glycine residues for individual conserved amino acids within the two zinc finger motifs. At several positions, mutants that we isolated by screening in yeast were phenotypically similar to those observed by targeted mutagenesis of the same amino acids; moreover, we extend the conclusions of Hollenberg and Evans (1988) by establishing that the finger motifs themselves are the essential functional sequences within the finger domain.

Mader et al. (1989) swapped segments of the estrogen and glucocorticoid receptors and constructed site-directed mutants to search for residues involved in distinguishing ERE and GRE DNA sequences; that study identified a cluster of three amino acids at the downstream side of the first finger that effect sequence specificity. Our results in turn emphasize the notion that both fingers in their entirety may be essential for forming a specific structure that facilitates sequence recognition by a small subset of amino acids.

The mutants that we analyzed were screened solely for defects in GRE-mediated positive regulation; in fact, we have been unable to demonstrate activity of the prolactin nGRE in yeast (M. Schena unpublished). We found that many of the resultant point mutants were severely deficient in GRE binding in vitro, and that all of these DNA binding mutants were also defective in nGRE-mediated repression when tested in CV-1 cells. Conversely, mutants that were competent to bind to GREs were also competent for repression via nGREs. Thus, although we have not identified residues that appear to distinguish GRE and nGRE sequences, our results support strongly the notion that DNA binding by the receptor is essential for repression. This conclusion disagrees with that of Adler et al. (1988), who studied estrogen and glucocorticoid inhibition of rat prolactin transcription and suggested from transient transfection experiments that repression is independent of the DNA binding domains of the receptors. It may be relevant that Adler et al. (1988) used a recipient cell line that expresses endogenous estrogen and glucocorticoid receptors, perhaps resulting in competition or negative complementation between the wild type endogenous and mutant transfected receptor derivatives.

The most interesting class of mutants that we obtained were those that

distinguished sequences essential for transcriptional enhancement from those sequences in repression or DNA binding. This phenotypic class is analogous to the "positive control" (pc) mutants of the lambda repressor, which fail to activate transcription while maintaining DNA binding and repression activities (Guarente et al. 1982; Hochschild et al. 1983). The pc-type mutants that we isolated have three striking characteristics. First, they are tightly linked to the second finger (R488Q, R489K, N491S; Figure 3-6; see also Figures 3-2, 3-5 and Table 3-2). Notably, Godowski et al. (1989) independently constructed a linker scanning mutation, LS-7, that displays a pc phenotype; remarkably, LS-7 is a double point mutant, P493R and A494S, in the same region of the second finger. Second, the only conditional mutant that we recovered is a cold sensitive lesion that affects the severity of the R489K pc mutant; cold sensitive mutants commonly reflect defects in protein-protein interactions (Guthrie et al. 1969; Jarvik and Botstein 1975). Third, only the three pc mutants exhibit strongly discordant phenotypes in yeast and animal cells, perhaps implying subtle differences in protein-protein contacts between receptor and homologous (but nonidentical) factors in yeast and animal cells. According to this view, such protein-protein interactions may be essential for receptor-mediated enhancement.

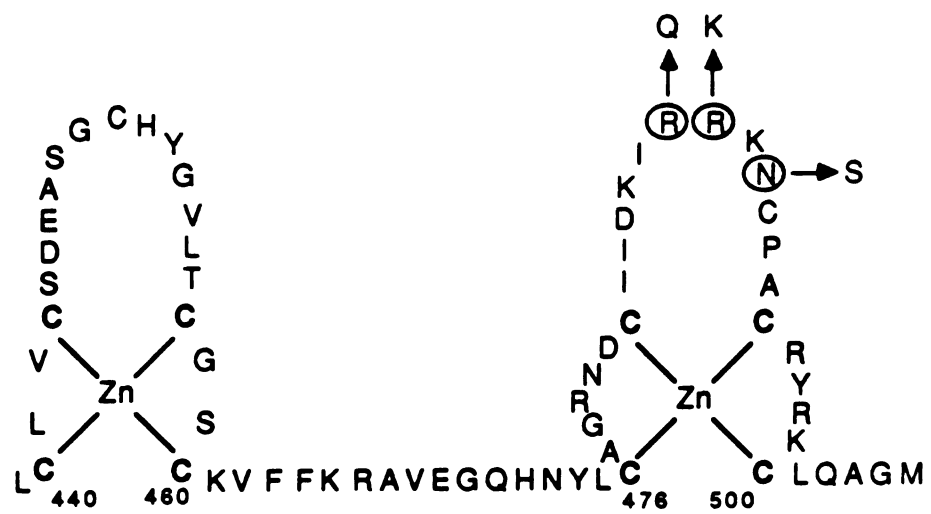
The cluster of pc mutations within the second finger maps genetically an enhancement region (enh1) within the finger domain, extending the findings that this portion of the receptor alone is sufficient for enhancement, albeit with substantially reduced activity, both in vivo (Miesfeld et al. 1987) and in vitro (Freedman et al. 1989). In this regard, it is particularly intriguing that deletion mutagenesis (Giguere et al. 1986), as well as direct activity assays (Godowski et al. 1988), identified a second enhancement region, enh2, near the N-terminus of the receptor, and other work has inferred yet another distinct enhancement region within the receptor (Hollenberg and Evans 1988; see

also Godowski et al. 1988). Since certain point mutations within enh1 can abolish enhancement by the otherwise wild type receptor, multiple enhancement regions may be required in concert to enhance transcription, despite their capacities to function independently in certain contexts (Miesfeld et al. 1987; Hollenberg et al. 1987; Godowski et al. 1988). It may be possible to use mutations in enh1 to obtain suppressor mutants that identify factors with which enh1 interacts to give rise to specific regulation of gene transcription.

Figure 3-6: "Positive-Control" Mutants of the Glucocorticoid Receptor.

A tight "cluster" of receptor point mutations includes positive control (pc) and cold sensitive (cs) lesions, and exhibits discordant phenotypes in yeast and animal cells. None of these three characteristics was associated with any other mutation identified in the yeast screening procedure described here. Using linker scanning mutagenesis, Godowski et al. (1989) identified another pc-like mutation that mapped to two amino acids in the same region (P493R and A494S). See text for details.

Figure 3-6.



	<u>Yeast</u>	<u>Mammals</u>
R488Q	pc	+
R489K	pc(cs)	+
N491S	+	pc

EXPERIMENTAL PROCEDURES

Yeast Strains and Media

The parent yeast strain, BJ2168 (*pep4-3*, *prc1-407*, *prb1-1122*, *ura3-52*, *trp-1*, *leu2*) (Jones 1977; Sorger and Pelham 1987), carries defects in three protease genes. Strain BJ-G26.1 was constructed by integration of plasmid pl-G26.1, which contains a GRE-CYC1/*lacZ* fusion and *URA3*, at *leu2* of BJ2163. Cultures were propagated in standard yeast media (Sherman et al. 1986).

Plasmid Constructions

To facilitate mutagenesis of the receptor finger region, we constructed yeast shuttle plasmid pG-D, a pGPD-556a (Bitter and Egan 1984; Schena and Yamamoto 1988) derivative in which the pBR322 and the 2 μ flanking sequences between the Bgl II and Eco RI were substituted with the pUC-18 origin of replication and ampicillin resistance gene. The plasmid thus includes receptor sequences encoding residues 1-556 driven by the yeast glycerol-3-phosphate dehydrogenase promoter, together with the yeast 2 μ origin of replication and TRP1 gene.

The unique Xho I site in pG-D at receptor amino acid 414 was introduced by oligonucleotide-directed mutagenesis (Kunkel et al. 1987). An Apa I to Sst I fragment of the receptor cDNA (residues 317-556) was inserted into Bluescript M13+ (Messing 1983; Stratagene) and single-stranded DNA was isolated and hybridized (Schena 1989) to an oligonucleotide primer, 5'-GGGTACTCGAGCCCTGG-3'; mismatch repair produced a conservative, single base change in the serine codon at receptor amino acid

414, thereby creating an Xho I site. The Apa I to Sst I receptor fragment was then re-introduced into the parent plasmid yielding unique restriction sites flanking the receptor finger domain. The Sst I site in pG-D is located six nucleotides downstream of receptor amino acid 556 in a polylinker that encodes 13 nonreceptor amino acids (GELEFPGLDPST) prior to translation termination. Plasmid pl-G26.1, used to construct yeast strain BJ-G26.1, was made by digesting pHR35 (gift of R. Rothstein) with Nco I and Bgl II in the URA3 gene and Ty1-17 element, respectively, and inserting an Nco I to Bgl II fragment from pS-G26.1 (Schena and Yamamoto 1988) containing the URA3 gene and a GRE-linked CYC1/lacZ gene. Plasmid pl-G26.1 contains the yeast URA3 gene, a GRE-CYC1/lacZ fusion, a 760 bp fragment of the LEU2 gene and the pBR322 E. coli origin of replication and ampicillin resistance gene.

E. coli expression plasmid pT7X556 (Freedman et al. 1988) contains receptor residues 407 to 556 inserted downstream of the inducible bacteriophage T7 promoter. An Xho I site was generated at receptor residue 414 by subcloning a Bam HI fragment encompassing receptor sequences into Bluescript M13+ and performing oligonucleotide-directed mutagenesis (as above) to give pT7X556X. E. coli expression plasmids were constructed by shuttling Xho I to Sst I mutant receptor inserts from yeast plasmids into phosphatase-treated pT7X556X digested with Xho I and Sst I.

To transfer the receptor point mutations into mammalian vectors, we first deleted wild type receptor sequences from the expression vector (here denoted pVARO) used for N795 expression by Picard and Yamamoto (1987) by cleaving at Bam HI sites that encompass the receptor coding sequences. Mutant receptor sequences were then introduced by triple ligations of the Bam HI to Sph I (receptor residues 1-494) and Sph I to Bam HI (receptor residues 494-795) fragments into Bam HI digested, phosphatase

treated pVARO. The final expression vectors contained: the SV40 enhancer, the human α -globin promoter driving the intact glucocorticoid receptor coding region (residues 1-795), and the rabbit β -globin splice and polyA addition sites, cloned into SP64. Reporter plasmid GMCS (DeFranco and Yamamoto 1986) contains: the MTV LTR, the CAT gene, the SV40 polyA addition site, and the MoMSV enhancer, cloned into pSP64. Reporter plasmid PPCV (Sakai et al. 1988, Miesfeld et al. 1988) contains: the bovine prolactin nGRE and promoter, the CAT gene, and the SV40 enhancer, cloned into pUC9.

Chemical Mutagenesis

A fragment of yeast shuttle plasmid pG-D (encoding receptor residues 414-556) was excised using Xho I and Sst I and inserted into Xho I and Sst I polylinker sites in Bluescript M13+. Single-stranded (sense strand) DNA (7 μ g) was treated for 20 min with sodium nitrite (Myers et al. 1985), and reverse transcriptase (BRL) was used to extend through the mutagenized region from a T7 primer hybridized to the Bluescript M13+ polylinker. Mutagenized, double-stranded receptor inserts were excised with Xho I and Sst I, purified from low melt agarose (Vogelstein and Gillespie 1979) and ligated to Xho I and Sst I cleaved, unmutagenized, phosphatase-treated Bluescript M13+ DNA. The ligation mixture was transformed into *E. coli* and plasmid DNA was prepared from a pool of 10⁴ bacterial transformants. The mutagenized receptor inserts were liberated with Xho I and Sst I, purified from low melt agarose and inserted into phosphatase-treated pG-D digested with Xho I and Sst I. Purified pG-D DNA was prepared from about 10⁴ *E. coli* transformants to give the mutagenized receptor pool (figure 3-1).

Yeast Screen and β -Galactosidase Assays

Strain BJ-G26.1 was made competent with lithium acetate (Ito et al. 1983) and aliquots of 2×10^7 cells (2 OD600 units) were transformed with 300 ng of pG-D DNA from the mutagenized receptor pool. Transformants were selected on minimal plates deficient for uracil and tryptophan, and colonies (about 300 per plate) were transferred to nitrocellulose filters, and lysed in liquid nitrogen and scored for β -galactosidase production with 0.3mg/ml 5-bromo-4-chloro-indolyl- β -D-galactopyranoside (Xgal); about 0.4% of the colonies were white or light blue. Quantitative β -galactosidase measurements were made in triplicate on two separate occasions using yeast liquid cultures (Yocum et al. 1984); duplicate assays varied by less than 20%. β -galactosidase units were defined as 10^3 times the change in optical density (OD) at 420 nm (due to hydrolysis of o-nitrophenyl- β -galactoside) divided by the product of the assay duration (min) times the culture volume (ml) times the OD at 600 nm of the culture.

Yeast Plasmid Isolation and DNA Sequencing

Cells from 1.5 ml of stationary yeast cultures were collected by centrifugation, washed in 1ml of 1M sorbitol and incubated in 250 μ l 1M sorbitol, 50 mM Tris (pH 7.5), 20 mM β -mercaptoethanol and 2 mg/ml zymolase (20T) at 37°C for 20 min. Spheroplasts were pelleted, resuspended in 125 μ l of 50 mM EDTA, 0.3% SDS and incubated at 68°C for 20 min. After removal of cell debris by ammonium acetate precipitation, plasmid DNA was phenol extracted, ethanol precipitated, and dissolved in 25 μ l 10 mM Tris-HCl, pH 7.5, 1 mM EDTA containing 100 μ g/ml RNase A; 1 μ l was used to transform E. coli strain DH5. Double-stranded miniprep DNA was subjected to DNA sequencing (Chen and Seeburg 1985) using oligonucleotide primers (Biomolecular Resource Center, UCSF) to receptor sequences encompassing the mutagenized region

(amino acids 414-556).

DNA Binding

The Xho I - Sst I receptor segments from various mutants were shuttled into the corresponding sites of a T7X556a expression plasmid, in which the receptor sequences are expressed from a bacteriophage T7 promoter; *E. coli* transformants were induced to overexpress the wild type or mutant derivatives as described previously (Freedman et al. 1988). The receptor species were precipitated from 30% ammonium sulfate (Freedman et al. 1988), and were redissolved in TEGDZ50 buffer (50 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 10% glycerol, 2.5 mM DTT, 50 μ M ZnCl₂, 50 mM NaCl) and dialyzed against the same buffer; extract protein concentrations were approximately 10 mg/ml, with the receptor derivative comprising about 10% of the total protein. Gel mobility assays (Fried and Crothers 1981) were performed by pre-incubating 40 ng of extract protein with 1 μ g poly(dI:dC) for 10 min at room temperature, followed by addition of a ³²P-labeled 256 bp fragment containing a 27 bp GRE (J. La Baer and K.R. Yamamoto unpublished); receptor protein-DNA complexes were separated from free DNA on a non-denaturing 7.8% polyacrylamide gel at 4°C.

Cell Culture and DNA Transfection

Cells were propagated in Dulbecco's modified Eagle's medium (Cell Culture Facility, UCSF) supplemented with 5% defined calf serum (HyClone). Mixtures of pVARO expression vectors (2 μ g) and GMCS or PPCV reporter plasmids (0.5 μ g) were cotransfected (Graham and van der Eb, 1973) into subconfluent cultures of CV-1 cells in 60mm dishes. Cells were incubated with the calcium phosphate precipitate for 16 hr,

then transferred to fresh medium with or without 0.2 μ M dexamethasone for an additional 24 hr. Extracts were prepared by three rounds of freezing (-70°C) and thawing (68°C), followed by centrifugation at 15,000 $\times g$ for 15 min. The CAT activity in 7 μ g of soluble protein from each extract was determined as described (Gorman et al. 1982); extracts prepared from cells transfected with GMCS or PPCV were incubated with substrate at 37°C for 5 hr or 20 hr, respectively.

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Chapter Four:
**Suppressors of Glucocorticoid Receptor Mutations that Potentiate Transcriptional
Enhancement**

ABSTRACT

The glucocorticoid receptor enhances transcription by a common mechanism in yeast and mammalian cells, apparently by contacting one or more evolutionarily conserved components of the eukaryotic transcriptional apparatus. In an attempt to identify cellular factor(s) with which receptor interacts, we have undertaken a suppressor analysis in yeast in which intergenic revertants were isolated to a weak DNA binding mutant of the glucocorticoid receptor defective in the induction of a GRE-regulated *LEU2* gene. Genetic analysis of 22 Leu⁺ revertants indicates that all of the mutants comprise a single complementation group of recessive suppressor alleles that potentiate the activity of receptor. Certain of the revertants that potentiate receptor action in an allele-selective manner also stimulate the activity of other activators in yeast, suggesting that the protein encoded by this suppressor gene may play a direct and general role in transcriptional enhancement.

INTRODUCTION

In the presence of bound hormone, the glucocorticoid receptor protein associates selectively with enhancer DNA sequences termed glucocorticoid response elements (GREs) (Chandler et al., 1983; Payvar et al., 1983; Scheidereit et al., 1983) and enhances transcription initiation from specific animal cell promoters utilized by RNA polymerase II. Functional regions of the glucocorticoid receptor and other members of the nuclear receptor gene superfamily have been previously characterized in detail (Evans, 1988; Green and Chambon, 1988; Beato, 1989). In the case of the 795 amino acid rat glucocorticoid receptor, deletion studies have identified sequences that impart DNA binding (Rusconi and Yamamoto, 1987; Freedman et al., 1988), hormone binding (Rusconi and Yamamoto, 1987), nuclear localization (Picard and Yamamoto, 1987) and transcriptional enhancement (Miesfeld et al., 1987; Godowski et al., 1987; Godowski et al., 1988).

It has also been demonstrated that the glucocorticoid receptor (Schena and Yamamoto, 1988), estrogen receptor (Metzger et al., 1988), and numerous other mammalian transcriptional regulators and initiation factors can function in yeast (Kakidani and Ptashne, 1988; Webster et al., 1988; Chodosh et al., 1988; Lech et al., 1988; Struhl, 1988). Moreover, genetic and biochemical experiments indicate that these factors function by a common mechanism in yeast and mammalian cells, implying that the strategies for eukaryotic gene control have been highly conserved in evolution (Schena, 1989a).

The evolutionary conservation of eukaryotic gene transcription validates genetic approaches in yeast to study regulatory proteins from more complex organisms. In fact,

Schena et al. (1989b) have utilized this approach to identify a class of glucocorticoid receptor zinc finger mutants that retain DNA binding capability but fail to enhance transcription. The identification of these "positive control" mutants of the glucocorticoid receptor, first described for bacterial λ repressor protein (Guarente et al., 1982; Hochschild et al., 1983), have subsequently been isolated in several other eukaryotic regulators including the yeast HAP1 protein (Kim and Guarente, 1989) and MyoD (Davis et al., 1990). Thus, it appears that transcriptional activation requires distinct DNA binding and transcriptional enhancement activities, the latter probably occurring via protein-protein contacts between a given enhancer binding protein and a component of the transcriptional apparatus (Ptashne, 1986, 1988; Yamamoto, 1985, 1989).

What are the cellular factors with which activators such as the glucocorticoid receptor interact to bring about transcriptional enhancement? Genetic and biochemical experiments with the yeast activators GAL4 and GCN4 have implicated the TATA-binding protein TFIID (Horikoshi et al., 1988) and RNA polymerase (Brandl and Struhl, 1989; Allison and Ingles, 1989) as potential target proteins, although conclusive evidence will require further experimentation. Although certain experiments suggest that the 90 kD heat shock protein may play a role in regulating glucocorticoid receptor signal transduction (Sanchez et al., 1985; Picard et al., 1988, 1990; Garabedian et al., 1990), the unequivocal participation of cellular factors in any aspect of steroid receptor function has not been demonstrated.

In an attempt to identify cellular factors that interact with the glucocorticoid receptor, we have undertaken a genetic suppressor analysis in yeast. We began this study by constructing a yeast strain in which the upstream regulatory region of the *LEU2* gene was replaced with glucocorticoid response element (GRE) sequences. Cells

containing this *GRE-LEU2* fusion are thus rendered receptor-dependent for growth; conversely, transformants of this strain that express receptor mutants that fail to activate the *LEU2* gene are phenotypically LEU- and thus provide the basis for a genetic selection and screen. This work describes the isolation and genetic characterization of intergenic suppressors that potentiate the activity of glucocorticoid receptor derivatives.

RESULTS

Isolation of Suppressors

As a first step towards isolating intergenic suppressors, we sought to create a yeast strain in which the expression of a selectable yeast gene was dependent upon enhancement by the glucocorticoid receptor. To this end, we employed homologous recombination to replace the upstream regulatory sequences of the *LEU2* gene in strain U-457 with glucocorticoid response element (GRE) DNA sequences. We then transformed this new strain (U-12.38) with a GRE-linked *CYC1-lacZ* reporter plasmid (pSX26.1; Schena and Yamamoto, 1988; Schena et al., 1990) to create a strain in which prototrophy and β -galactosidase expression was receptor-dependent.

Transformants of strain U-12.38 that contain the β -galactosidase reporter plasmid and express the intact glucocorticoid receptor (N795) in the presence of hormone or a constitutive derivative of receptor deleted of the hormone binding domain (N556) were strongly *LEU*⁺ and *lacZ*⁺ as expected (Table 4-1). Two point mutants of N556 (N556R488Q and N556R489K) impaired in transcriptional enhancement (Schena and Yamamoto, 1989b), showed reduced β -galactosidase activity and displayed correspondingly weaker *Leu*⁺ phenotypes (Table 4-1). A pair of mutants (N556M505I and N556M505T) bearing chemically similar point mutations at the same position that impair DNA binding each failed to induce any β -galactosidase expression; interestingly, cells expressing N556M505I and N556M505T were phenotypically *LEU*⁺ and *LEU*⁻, respectively, suggesting that the level of *LEU2* induction by N556M505T was just below the minimal amount required for prototrophy (Table 4-1) and thus was suitable for use in suppressor studies.

Table 4-1: Activation of a GRE-LEU2 Gene Fusion by Glucocorticoid Receptor Derivatives.

Yeast strain U-12.38, which contains an integrated GRE-*LEU2* fusion (see Methods), was transformed (Ito et al., 1983) with a GRE-linked *CYC1-lacZ* reporter (pSX26.1; Schena and Yamamoto, 1988) and various 2 μ -based glucocorticoid receptor expression plasmids (Schena et al., 1989). Expression plasmids encode the wild-type receptor (N795), a constitutive mutant deleted of the hormone binding domain (N556), two point mutants impaired in transcriptional enhancement (N556R488Q and N556R489K), two point mutants impaired in DNA binding (N556M505I and N556M505T), and an N556 derivative that contains a point mutation in a cysteine residue required for tetrahedral zinc coordination (N556C460Y). The pG-1 expression plasmid (see Methods) lacks a receptor insert (None). All transformants except those expressing N795 were tested for their ability to form 2mm colonies on minimal plates lacking leucine after a period of 2 days (+++), 3 days (++) , 4 days (+). Transformants expressing N795 were grown and tested as above except that the minimal plates were supplemented with 10 μ M deoxycorticosterone. In several cases, transformants were unable to grow in the absence of leucine (-). β -galactosidase activity (β -gal) was determined (see Methods) using triplicate cultures grown on two separate occasions; values varied by less than 20% and were consistent from day to day. The LacZ phenotype (Plate) refers the colorimetric results of transformants tested in the β -galactosidase plate assay (see Methods).

Table 4-1

Receptor	Receptor Alteration	LEU	β-gal	Plate
N795	Wild Type	+++	2261	Dark Blue
N556	Constitutive	+++	865	Dark Blue
N556R488Q	Enhancement	++	31	Light Blue
N556R489K	Enhancement	++	116	Light Blue
N556M505I	DNA Binding	+	2.4	White
N555M505T	DNA Binding	-	2.2	White
N556C460Y	Structure	-	2.3	White
None	NA	-	2.3	White

Cultures from ten independent clones of U-12.38 containing the CYC1-lacZ fusion plasmid and the N556M505T receptor expression vector were each plated on a minimal plate lacking leucine (5×10^7 cells per plate) and incubated for 4 days at 30°C to allow the appearance of revertants (Figure 4-1). Approximately 50 LEU+ revertants were recovered from each plate (frequency: 10^{-6}), and of these about 10% also displayed elevated levels of β -galactosidase (frequency: 10^{-7}). Ten LEU+/lacZ- revertants and fourteen LEU+/lacZ+ revertants were chosen at random from the ten plates, subcloned, and characterized in detail.

Genetic Analysis of Suppressors

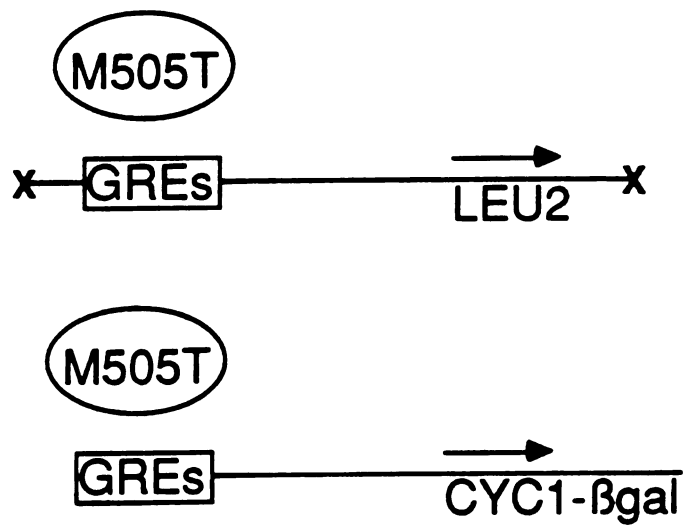
All of the twenty-four revertants (designated Sup1-24) retained the original LEU+ phenotype upon rescreening; in addition, the fourteen revertants that scored lacZ+ on the plate assay correspondingly displayed elevated β -galactosidase levels in assays of liquid-grown cultures (Table 4-2). Crosses with each of the haploid revertants to a LEU- yeast strain (1784, see Methods) revealed that, with the exception of Sup12 and Sup19 which were not characterized further, all of the suppressor strains mated efficiently (Table 4-2). All of the diploid strains scored LEU- and lacZ-, indicating that the Sup mutations were all recessive (Table 4-2 and data not shown).

Dissection of 10 tetrads from each strain yielded approximately 35 viable spores per strain. Spores that retained the receptor expression plasmid (TRP+ clones) constituted 25% to 80% of the viable spores. Analysis of the TRP+ spores revealed a LEU+/TRP+ ratio of approximately 25%, consistent with the Mendelian segregation of the suppressor gene and the GRE-LEU2 fusion during meiosis (Table 4-2).

Figure 4-1: Scheme for Isolating Yeast Mutants that Suppress a Glucocorticoid Receptor Mutation.

A haploid yeast strain (U457, see Methods) was rendered glucocorticoid receptor-dependent for growth by replacing the upstream regulatory region of the *LEU2* gene with glucocorticoid response element (GRE) DNA sequences (U-12.38, see Methods). When transformed with a plasmid containing GREs fused to a *CYC1-lacZ* gene fusion (pSX26.1; Schena and Yamamoto, 1988; Schena et al., 1990) and a receptor expression plasmid encoding the N556M505T DNA binding mutant, strain U-12.38 is phenotypically LEU⁻ and lacZ⁻ (White). Selecting and screening for LEU⁺ and lacZ⁺ (light blue), respectively, allows the isolation of intergenic suppressors.

Figure 4-1



LEU-

White



Select and Screen

LEU+

Light Blue

Table 4-2: Genetic Characterization of Yeast Suppressor Mutants.

The parental LEU⁻ haploid yeast strain U-12.38 (Wild-Type) and 24 LEU⁺ revertants (Sup1-24) were tested for β -galactosidase activity as in the legend to Table 4-1; asterisks denote clones that also showed elevated β -galactosidase activity in the plate assay. All but two of the revertants mated as efficiently to strain 1784 as U-12.38 (+++). Mating was undetectable for Sup12 and Sup19. The number of LEU⁺ spores divided by the total number of spores (LEU⁺/TRP⁺) reflects the segregation of the suppressor locus and the GRE-linked *LEU2* gene. Diploids formed by the mating of each of the Sup strains to strain 1784 all exhibited LEU⁻ phenotypes indicating a recessive (R) suppressor mutation. The LEU⁺ phenotype of Sup21 and Sup 23 was complemented by U-12.38 (Yes) but not by any of the Sup strains (No) indicating a single complementation group.

Table 4-2.

Strain	LEU	β -gal	Mating	LEU+/TRP+	D/R	Sup21,23 Comp.
Wild Type	-	2.2	+++	0/20	NA	Yes
Sup1	+	2.3	+++	6/15	R	No
Sup2	+	1.9	+++	6/23	R	No
Sup3	+	4.6*	+++	4/17	R	No
Sup4	+	5.9*	+++	4/17	R	No
Sup5	+	3.6*	+++	6/22	R	No
Sup6	+	2.2	+++	7/24	R	No
Sup7	+	1.6	+++	3/19	R	No
Sup8	+	2.7	+++	2/14	R	No
Sup9	+	2.0	+++	5/19	R	No
Sup10	+	2.1	+++	5/25	R	No
Sup11	+	2.3	+++	4/19	R	No
Sup12	+	2.2	-/+	0/30	R	ND
Sup13	+	2.2	+++	4/15	R	No
Sup14	+	6.5*	+++	8/27	R	No
Sup15	+	7.9*	+++	5/21	R	No
Sup16	+	9.6*	+++	3/10	R	No
Sup17	+	7.0*	+++	6/24	R	No
Sup18	+	6.3*	+++	5/29	R	No
Sup19	+	2.9*	-	ND	ND	ND
Sup20	+	5.6*	+++	3/9	R	No
Sup21	+	5.1*	+++	3/12	R	No
Sup22	+	5.2*	+++	3/12	R	No
Sup23	+	6.4*	+++	6/22	R	No
Sup24	+	5.8*	+++	4/12	R	No

The inability of any of the Sup strains to complement either Sup21 or Sup23 indicted a single complementation group (Table 4-2). In all cases suppression was receptor-dependent, as strains cured of the N556M505T receptor expression vector were phenotypically lacZ- (Table 4-4), though each retained a weak LEU+ phenotype (data not shown). None of the strains revealed a growth phenotype at elevated or reduced temperatures, though transformation efficiencies of all of the Sup mutants were reduced 5- to 10-fold relative to parent strain U-12.38.

Receptor Levels in Suppressors

To determine whether the suppression was due to elevated receptor levels, extracts prepared from the wild-type strain (U-12.38) and several suppressors (Sup1,5,16,18,23) were subjected to immunoblotting with a receptor-specific monoclonal antibody (Gametchu and Harrison, 1984). The level of the N556M505T receptor protein in each of the Sup strains was similar to the level of N556M505T in the parental strain (Figure 4-2).

Suppression of Other Receptor Derivatives

To test whether the suppressor mutations displayed allele specificity in their effects on receptor action, various Sup mutants were cured of the pSX26.1 β -galactosidase reporter plasmid and the N556M505T receptor expression plasmid [by growing strains under non-selective conditions (see Methods)] and retransformed with pSX26.1 and one of several receptor expression plasmids. β -galactosidase assays of liquid-grown cultures indicated that the activity of the intact receptor (N795) and two positive control mutants of receptor (N556R488Q and N556R489K) were also increased

in many of the suppressor strains (Table 4-3); interestingly, the degree of suppression of the various receptor derivatives was not identical. For example, N795 protein in the absence of hormone was suppressed by as much as 15-fold, whereas the N556R489K protein was only marginally more active in the Sup strains (Table 4-3). Several of the suppressor strains also manifested differential effects on various receptor derivatives; thus, Sup1 N795 and N556M505T were suppressed 15-fold and 1-fold, respectively, while suppression of the same receptor proteins in Sup18 was 2.6-fold and 2.9-fold, respectively. The lack of significant suppression of N795 in the presence of hormone probably implies that transcription initiation from the *CYC1* promoter under these conditions is operating at near-maximal efficiency (see Discussion). These data clearly indicate that the potentiation of various receptor derivatives in these strains is allele-selective and thus may indicate a direct interaction between the factor encoded by the sup gene and receptor.

Potentiation of Other Activators

To assess whether the suppressor strains could increase the activity of other activators, fusion proteins containing portions of the glucocorticoid receptor fused to the DNA binding moiety of the bacterial LexA protein were tested for enhancement of a β -galactosidase reporter gene containing a LexA operator fused upstream of the *CYC1* promoter. Indeed, the activity of fusion constructs containing either the amino terminus of receptor fused to LexA (NLx) or both the amino- and carboxy-termini fused to LexA (NLxC) were elevated significantly in the Sup18 background (Table 4-4). Similarly, the activity of the *CYC1* upstream activator sequences was also enhanced in Sup18, implying potentiation of the yeast HAP1-4 proteins (Table 4-4).

Figure 4-2: Glucocorticoid Receptor Levels in Suppressor Strains.

Extracts were prepared from the parental yeast strain U-12.38 (Wild-Type) or from various suppressor strains (Sup1, 5, 16 and 23) expressing the N556M505T glucocorticoid receptor derivative, fractionated by SDS-polyacrylamide gel electrophoresis, and subjected to immunoblotting using a receptor-specific monoclonal antibody (Gametchu and Harrison, 1984), followed by an alkaline phosphatase conjugated goat anti-mouse antibody (Biorad). Values correspond to the molecular masses (in kilodaltons) of molecular weight (MW) standards. The arrow indicates the position of the N556M505T (65kD) receptor derivative.

Figure 4-2

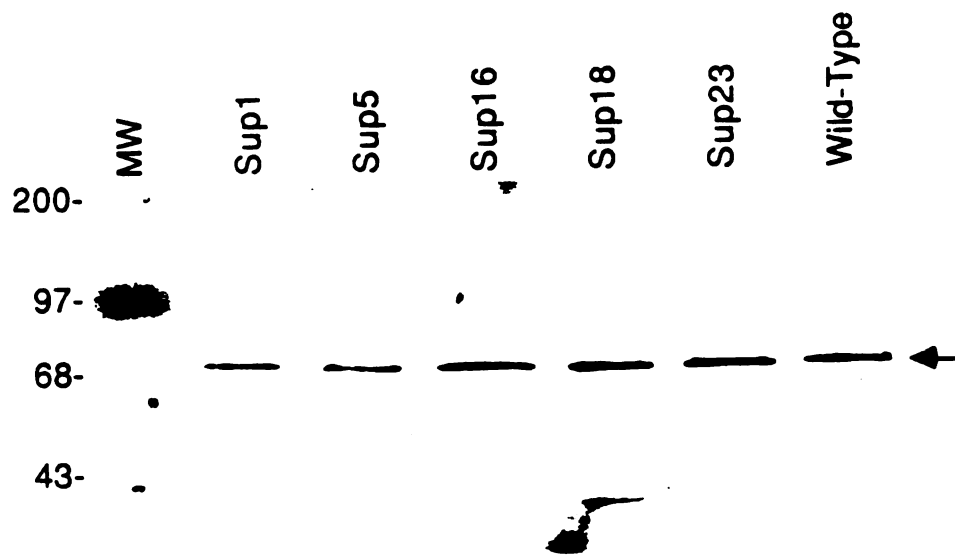


Table 4-3: Allele-Selective Suppression of Glucocorticoid Receptor Mutations.

The parental yeast strain U-12.38 (Wild-Type) and four suppressor strains (Sup1, 5, 16, 18) were transformed (Ito et al., 1983) with a GRE-linked *CYC1-lacZ* reporter (pSX26.1; Schena and Yamamoto, 1988) and receptor expression plasmids (see Methods) encoding the intact glucocorticoid receptor (N795), or three N556 derivatives (Schena et al., 1989) bearing point mutations in the finger domain that impair transcriptional enhancement (N556R488Q and N556R489K) or DNA binding (N556M505T). β -galactosidase assays (see legend to Table 4-1) were performed on cultures grown in minimal media lacking uracil and tryptophan supplemented with 1 μ M deoxycorticosterone (+ hormone) or with .1% ethanol (- hormone). The suppression ratio was calculated as the β -galactosidase activity of each of the Sup strains divided by the β -galactosidase activity of the wild-type.

Table 4-3.

Strain	Receptor	Hormone	β -gal	Suppression
Wild-Type	N795	-	12	1.0
Sup1			169	15
Sup5			97	8.4
Sup16			112	10
Sup18			30	2.6
Wild-Type	N795	+	2261	1.0
Sup1			2938	1.3
Sup5			2726	1.2
Sup16			2246	1.0
Sup18			3287	1.4
Wild-Type	N556	-	896	1.0
Sup1			2451	2.7
Sup5			2231	2.5
Sup16			2678	3.0
Sup18			2790	3.1
Wild-Type	N556R488Q	-	31	1.0
Sup1			320	10
Sup5			64	2.1
Sup16			70	2.3
Sup18			132	4.3
Wild-Type	N556R489K	-	116	1.0
Sup1			354	3.1
Sup5			101	.9
Sup16			164	1.4
Sup18			178	1.5
Wild-Type	N556M505T	-	2.2	1.0
Sup1			2.3	1.0
Sup5			3.6	1.6
Sup16			9.6	4.4
Sup18			2.9	2.9

Table 4-4: Potentiation of Other Activators in Yeast.

Parental yeast strain U-12.38 (Wild-Type) and a suppressor strain (Sup18) were transformed (Ito et al., 1983) with *CYC1-lacZ* reporter constructs containing the following upstream elements: a LexA operator located at -178 (Brent and Ptashne, 1985), the *CYC1* UASs at -178 (pLGΔ312S; Schena and Yamamoto, 1988), the *CYC1* UASs at -248 (p312X46; Schena and Yamamoto, 1988), or three copies of a 26 bp GRE at -178 (pSX26.1; Schena and Yamamoto, 1988). Activators NLx and NLxC contain the amino-terminal or both the amino and carboxy terminal portions of the glucocorticoid receptor, respectively, fused to the DNA binding domain of the LexA protein (Godowski et al., 1988). The four known activators of the *CYC1* UASs (HAP1-4) are endogenous yeast proteins (Hahn et al., 1988) presumed to be present in strains U-12.38 and Sup18. Transformants containing the parent expression plasmid pG-1 (see Methods) are shown (None). Hormone treatments, β-galactosidase assays and suppression ratios were performed and calculated as in the legend to Table 4-3.

Table 4-4.

Strain	Activator	Upstream Element	Hormone	β -gal	Potentiation
Wild-Type Sup18	NLx	-178 Lex op.	-	2066 3153	1.5
Wild-Type Sup18	NLxC	-178 Lex op.	-	7 27	4.0
Wild-Type Sup18			+	1653 2614	1.6
Wild-Type Sup18	HAP1-4	-178 UAS _{CYC1}	-	557 1140	2.0
Wild-Type Sup18	HAP1-4	-248 UAS _{CYC1}	-	57 144	2.5
Wild-Type Sup18	None	-178 GREs	-	7 7	1.0

DISCUSSION

In an attempt to identify cellular factors involved in glucocorticoid receptor action, we have undertaken a suppressor analysis in yeast. Phenotypic revertants to weak DNA binding mutants of the glucocorticoid receptor were isolated as intergenic suppressors capable of restoring activation to a *GRE-LEU2* test gene. Characterization of 22 *LEU+* strains indicated that all of the revertants comprised a single complementation group. Various of these revertants potentiated receptor action in an allele-selective manner and stimulated the activity of other activators in yeast, suggesting that the protein encoded by the suppressor gene identified in these experiments may play a direct and general role in transcriptional enhancement.

Receptor mutants completely defective in β -galactosidase induction were sufficient to render strain U-12.38 weakly *LEU+*, indicating that the threshold of the *LEU2* product required for viability in these cells is extremely low. The low *LEU2* threshold seemed particularly advantageous in a suppressor analysis in that only subtle changes in the activity or structure of an essential transcription factor would be required to generate a selectable phenotype. Though the strength of the *LEU+* phenotype of the various Sup strains was indistinguishable, the differential suppression of β -galactosidase expression suggests that the mutations in various of the revertants probably correspond to distinct alleles of the suppressor gene.

A challenging aspect of this work will be to understand the level at which transcription is potentiated in the various suppressor strains. Formally, the mutations could act either at the level of receptor DNA binding or transcriptional enhancement, though all models must account for the potentiation of both receptor-LexA fusion proteins

and the yeast HAP activators that act through the *CYC1* upstream activator sequences. Potential candidates include mutations that increase activator binding by increasing DNA accessibility, such as lesions that weaken chromatin structure. Consistent with this hypothesis, several studies indeed indicate that certain histone mutations can alter gene expression (Han and Grunstein, 1988; Clark-Adams and Winston, 1988).

Models invoking direct stimulation of DNA binding, though sufficient to explain the suppression of DNA binding mutants such as N556M505T employed in our study, are somewhat more difficult to rationalize for the positive-control mutants and for the receptor-LexA fusion proteins. It is difficult to understand, for example, how suppressor mutations leading to better DNA binding would increase the activity of receptor derivatives such as N556R488Q that presumably fully occupy the GRE *in vivo*. In addition, the NLx and NLxC molecules are probably expressed at levels well in excess of those required for maximal occupancy of the single Lex operator (Brent and Ptashne, 1984), implying that suppression of these molecules more likely occurs at the level of transcriptional enhancement. The allele-specificity and the saturability of suppression appear to exclude other less interesting models for suppression such as those affecting message stability or translation efficiency.

The fact that all of the revertants displayed recessive phenotypes indicates that the alleles represent either loss of function or altered function mutations. One model is that the suppressors constitute loss of function mutations in a gene whose product negatively regulates the enhancement activity of receptor such as a protein kinase. One could imagine, for example, that receptor activity is decreased by specific phosphorylation events catalyzed by a suppressor-encoded protein kinase; according to this model, mutations that impaired its kinase activity would correspondingly lead to increased

receptor activity. Though it has been claimed from biochemical studies that phosphorylation inhibits receptor function (Auricchio, 1989), the role of kinases in receptor action remains to be established.

An alternative view of the genetic recessivity of the mutants is that the alleles represent mutations that alter the specificity of a factor required for transcriptional enhancement. According to this model, the suppressor mutations might relax the kinetic barriers required for the formation of a multi-protein structure such as the transcription initiation complex at the *LEU2* promoter. Such mutations might potentiate the activity of receptor (and other activators) by increasing the rate of association of a rate-limiting factor with the transcription apparatus, while decreasing the affinity of such a factor for the complex relative to the wild-type protein. It is interesting to note that in a series of experiments aimed at generating HIS⁺ revertants of the his4-912 δ mutation, a large number of recessive suppressors were also recovered (Clark-Adams et al., 1988). We have recently attempted to isolate dominant alleles of the suppressor gene by selecting for LEU⁺ revertants in a diploid strain containing a GRE-LEU2 fusion. Though LEU⁺ clones were recovered at a frequency of approximately 10⁻⁷, none of the mutations in the 10 strains analyzed displayed Mendelian segregation upon tetrad analysis (data not shown). These experiments do not exclude the existence of dominant alleles of this gene whose spontaneous rate of appearance is below 10⁻⁸.

It is striking to also note that only a single complementation group of suppressors was identified. This probably partly owes to the use in the selection scheme of a receptor derivative deleted of the hormone binding domain; thus, while the N556M505T receptor averted the potential recovery of a large number of steroid uptake mutants, the recovery of factors that might interact exclusively with the receptor steroid binding domain was

precluded. Experiments utilizing intact derivatives of the receptor bearing point mutations in the finger domain may help to identify additional cellular genes, such as the members of the heat shock protein family which have been implicated in steroid receptor function (Sanchez et al., 1985; Picard et al., 1988). Selections in strains containing GREs fused to other essential yeast genes may also serve to identify promoter-specific factors. In addition, revertants to strains expressing one of the positive control mutants of receptor may assist in broadening the repertoire of genes whose products augment receptor action.

The immediate goal of future experiments focuses on cloning the suppressor gene. As no selection against the product of the *LEU2* gene is available, the recessive phenotype of the Sup alleles will require complementation of the LEU+ phenotype by the wild-type Sup gene followed by screening for a LEU- transformant. Sequence analysis of the suppressor gene should provide immediate clues as to the mechanism of suppression, and further genetic and biochemical experiments will determine whether the product of the Sup gene interacts directly with receptor and other activators.

METHODS

Yeast Strains and Media

The parent yeast strain U-12.38 was derived from strain U-457 (gift of Rodney Rothstein; MATa, ade2-1^o, can1-100^o, trp1-1^a, ura3-1, SUP53-a/LEU2) by substituting a GRE-linked LEU2 gene for the SUP53a-linked LEU2 gene using gene replacement (Rothstein, 1983). In particular, strain U457 was transformed with pHR-(G26.1)₅ DNA linearized at the KpnI site and URA⁺ transformants were screened for homologous integration of the plasmid by scoring for LEU⁻. Strains were cured of plasmid sequences by plating cells on 5-FOA (Boeke et al., 1984) and the correct excision event was scored as the corresponding loss of the TRP⁺ phenotype. Yeast strain 1784 (gift of Paul Siliciano; MATa, leu2-3, leu2-112, ura3-52, trp1, his4) was used in mating experiments and in tetrad analysis. Cultures were propagated using standard techniques (Sherman et al., 1986).

Suppressor Isolation

Strain U-12.38 was transformed (Ito et al., 1983) with plasmids pSX26.1 (Schena and Yamamoto, 1988) and pG-N556M505T (Schena et al., 1989) and selected on minimal medium lacking uracil and tryptophan. Ten independent isolates were grown in 2 ml minimal medium lacking tryptophan and uracil to stationary phase and a 1.5 ml aliquot (5×10^7 cells) was spread on minimal plates lacking uracil, tryptophan and leucine. Plates were incubated at 30°C until revertants reached a size of 1-2 mm; after 4 days, each plate contained approximately 50 LEU⁺ colonies of roughly equal size. Colonies were then transferred by replica-plating onto nitrocellulose filters and tested for β -galactosidase

activity. From the 10 plates analyzed, 10 LEU+ revertants and 14 LEU+/lacZ+ clones were chosen at random for further analysis. Strains were "cured" of the 2 μ plasmids by continuous propagation in YEPD medium for 4 days, followed plating on YEPD plates to allow single colonies to form, then replica-plated onto selective plates to score URA and TRP phenotypes. Under these conditions, approximately 10% of the clones scored URA- and TRP- (ie. had lost both plasmids).

Tetrad Analysis and Complementation Tests

Suppressor strains of U-12.38 were crossed to yeast strain 1784 by co-incubation on YEPD plates for 16 hr at 30°C and replica plated onto minimal plates lacking uracil, tryptophan and adenine to select diploids, and onto minimal plates lacking uracil, tryptophan, adenine and leucine to determine dominance or recessivity. Tetrad analysis was carried out on diploid cells incubated on sporulation plates for 5 days at 30°C. Aliquots of approximately 10⁶ sporulated cells were resuspended in .5 ml of 1M sorbitol containing 2 mg per ml zymolase (Seikagaku Kogyo-20T) and treated for 10 min at 37°C. Tetrads were dissected by micromanipulation, germinated on YEPD plates at 30°C for 2 days, and replica plated onto minimal plates lacking either tryptophan or both tryptophan and leucine to score spores for the presence of the N556M505T receptor expression plasmid and for the segregation of the LEU+ phenotype, respectively. Complementation analysis was performed by crossing Sup21 and Sup23 to spores of each of the other suppressors and assaying the LEU phenotype on minimal plates lacking leucine and other appropriate amino acids.

β -Galactosidase Assays

Quantitative β -galactosidase measurements were made in triplicate on two separate occasions using yeast liquid cultures (Yocum et al., 1984); duplicate assays varied by less than 20%. β -galactosidase units were defined as 10^3 times the change in optical density (OD) at 420 nm (due to hydrolysis of O-nitrophenyl- β -galactoside) divided by the product of the assay duration (min) times the culture volume (ml) times the OD at 600 nm of the culture. β -galactosidase plate assays were performed by transferring colonies from minimal plates onto nitrocellulose filters by replica plating, then submerging filters in liquid nitrogen for 5 sec to allow cell lysis. Immediately following treatment in liquid nitrogen, filters were incubated for 30 min at 30°C on Whatman circles containing 2 ml of Z buffer (Yocum et al., 1984) supplemented with 0.3mg/ml 5-bromo-4-chloro-indolyl- β -D-galactopyranoside (Xgal).

Western Analysis

Cultures of strain U-12.38 containing the pSX26.1 reporter plasmid and the pG-N556M505T expression vector were propagated to near stationary phase in minimal media lacking uracil and tryptophan, and 1.5 ml (5×10^7 cells) aliquots were harvested by centrifugation for 1 min at 13,000 x g in a microfuge. Cell pellets were washed once with 1 ml of H₂O, and resuspended and incubated in 0.5 ml of 1M sorbitol containing 19mM β -mercaptoethanol and 2 mg per ml zymolase (20T) at 30°C for 30 min. Spheroplasts were recovered by centrifugation at 3,000xg for 5 min, resuspended in 100 μ l of SDS sample buffer, and vortexed for 3 min in the presence of 1 pellet volume of glass beads. Samples were incubated in a boiling water bath for 5 min, clarified by centrifugation 13,000xg for 5 min in a microfuge, fractionated by 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Nitrocellulose filters were subjected to western blotting by primary and secondary incubations with a

receptor-specific monoclonal antibody (Gametchu and Harrison, 1984) and an alkaline phosphatase conjugated goat anti-mouse antibody (Biorad), respectively, and reacted with substrate for 10 min.

Plasmid Constructions

Plasmid pHR-(G26.1)₅ was derived from pHR-35 (Sчена et al., 1989b) by inserting 5 copies of a BamHI-linked 26 bp GRE from the tyrosine aminotransferase gene (Jantzen et al., 1987) into the BglIII site of pHR-35. All receptor expression plasmids, derived by inserting BamHI fragments encoding N795, N556R488Q, N556R489K and N556M505T (Sचना et al., 1989) or NLx and NLxC (Godowski et al., 1988) into the BamHI site of pG-1 (Sचना et al., 1990), contain the following DNA elements: the yeast GPD promoter, PGK terminator, TRP1 gene, and 2 μ origin of replication; and the bacterial origin of replication and ampicillin resistance gene from pUC-18. β -galactosidase reporter plasmids, all derived from plasmid p Δ SS (Sचना, 1988), contain the following sequences: the yeast *URA3* gene, 2 μ origin of replication, and the CYC1 promoter region fused to the bacterial *lacZ* gene; and the bacterial origin of replication and ampicillin resistance genes from pBR322. The reporter plasmids, which differ only in the DNA sequences located upstream of the CYC1 promoter, are as follows: pSX26.1 contains 3 TAT GREs at -178 (Sचना and Yamamoto, 1988; Sचना et al., 1990), pSXLex contains a single Lex operator at -178 (Brent and Ptashne, 1985), pLG Δ 312S and p312X46 contain CYC1 UASs at -178 and -248, respectively (Sचना and Yamamoto, 1988).

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SUMMARY

The basic components required for eukaryotic gene transcription have been highly conserved in evolution. Structural and functional homology has now been documented among promoters, promoter factors, regulatory proteins, and RNA polymerases from eukaryotes as diverse as yeast and mammals. The ability of these proteins and DNA sequences to function across phylogenetic boundaries demonstrates that common molecular mechanisms underlie gene control in all eukaryotic cells and provides the basis for powerful new approaches to the study of eukaryotic gene transcription.

INTRODUCTION

Transcription is the cellular process by which RNA is synthesized from a DNA template. Like other cellular processes such as DNA replication and RNA splicing, gene transcription is mediated by both protein and nucleic acid constituents. The basic components required for accurate, efficient and regulable eukaryotic transcription initiation include two types of DNA elements known as promoters and upstream regulatory sequences, two sets of proteins known as general promoter factors and regulatory proteins, and the RNA synthesizing enzyme RNA polymerase. Recent experiments indicate that many of these basic components have been structurally and functionally conserved in eukaryotes as diverse as yeast and mammals, indicating that similar molecular mechanisms probably underlie gene transcription in all eukaryotes.

The purpose of this review is to summarize the topic of the evolutionary conservation of eukaryotic gene transcription. I shall discuss, in turn, the functional conservation of promoters, general promoter factors, RNA polymerase, activator proteins, and the regulation of activator proteins. I shall then present an overview of some of the conserved molecular motifs employed for DNA binding and transcriptional enhancement. Finally, I present a speculative discussion on new experimental approaches that exploit the function of transcriptional regulators across phylogenetic boundaries to study the mechanism of eukaryotic gene regulation. As a starting point, let us consider the basic components of the eukaryotic transcriptional apparatus.

The Eukaryotic Transcriptional Apparatus

Eukaryotic RNA polymerase II, a complex enzyme consisting of 10 protein

subunits, synthesizes messenger RNA (mRNA) corresponding to the protein-coding genes in eukaryotic cells (98). Polymerase initiates transcription at specific chromosomal sites known as promoters, which are short DNA sequences (<80bp) located at the 5' end of transcribed genes (Fig. 5-1A). Eukaryotic polymerase does not recognize promoter sequences directly; rather, additional proteins known as general promoter factors bind first to promoter sequences and mediate promoter binding by polymerase (20, 38, 88, 93).

The general promoter factors are a set of four transcription factors IID, IIA, IIE and IIB. These proteins are thought to bind promoters in a stepwise manner (10, 88) and are apparently required for accurate and efficient transcription initiation at all promoters transcribed by polymerase II. Transcription studies *in vitro* have shown, for example, that TFIID binds directly to the promoter element known as the TATA-box (Fig. 5-1A) (88). The TATA-box, which is found in nearly all eukaryotic promoters, is located about 30 bp upstream of the transcription start site in mammalian promoters (30) and somewhat further and more variably upstream (40 to 120 bp) of the start site in yeast promoters (105). TFIID, together with TFIIA, form a stable protein-DNA complex known as the pre-initiation complex on the promoter (Fig. 5-1A) Subsequent binding by TFIIIB and TFIIE, which appear to interact with each other and with polymerase, yields a complete initiation complex (Fig. 5-1A) (10, 88). When bound by these general promoter factors and polymerase, promoters are able to direct basal level gene expression *in vitro* and *in vivo*.

Though promoters themselves are sufficient to direct basal level gene expression, vigorous and regulable transcription requires the assistance of a second set of proteins known as regulatory proteins (68, 85). One of the two classes of regulatory proteins,

known as activators, stimulate transcription by binding to specific DNA sequences known as upstream regulatory elements. Upstream regulatory elements that confer positive promoter regulation (ie bind activators) are known as upstream promoter elements or enhancers in mammalian cells, and as upstream activator sequences (UASs) in yeast (31). Upstream promoter elements and UASs are usually located within several hundred base pairs of linked promoters (Fig. 5-1B), whereas mammalian enhancers may reside as far as several thousand bases upstream or downstream from a given promoter. Though these DNA sequences may function slightly differently, one may collectively view them as members of a single class of upstream regulatory elements that function in the context of a bound activator to stimulate promoter activity (Fig. 5-1B) (86, 87). Activator proteins, bound to upstream regulatory elements, are believed to stimulate the TATA-based reaction by interacting with a protein component of the transcriptional apparatus such a general promoter factor (TFIID, A, B, E) or with RNA polymerase, although the mechanistic details of transcriptional enhancement remain to be elucidated.

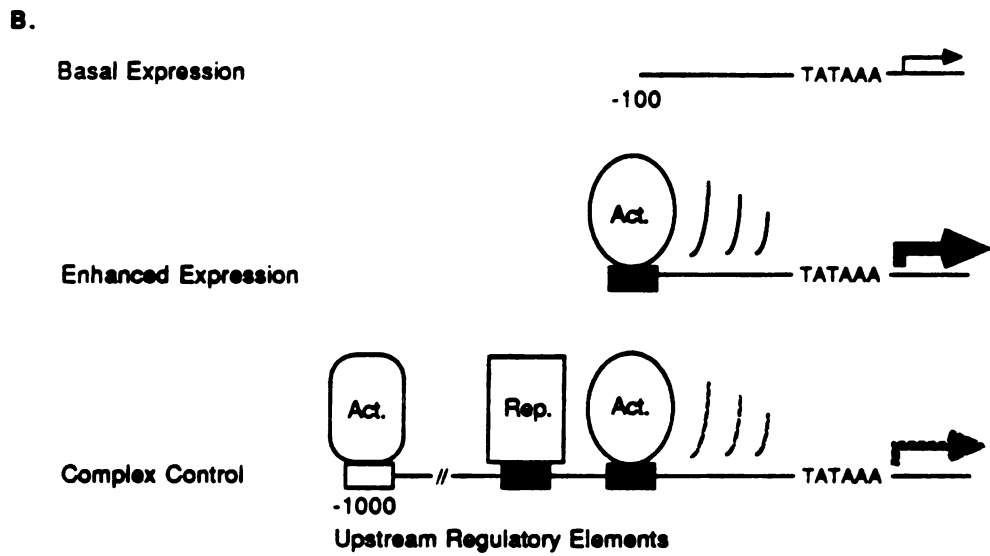
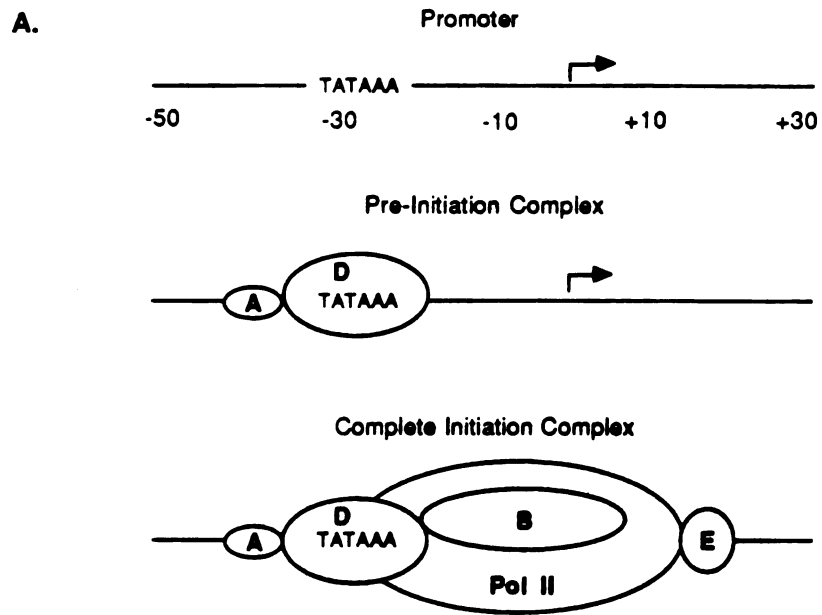
A single upstream regulatory element is sufficient for promoter stimulation although, in most cases, multiple types of elements (bound by distinct activators) are found upstream of eukaryotic genes. Moreover, certain promoter regions contain a distinct class of upstream regulatory elements, known as operators, that function to decrease the rate of gene transcription. The activity of operators is mediated by regulatory proteins known as repressors, which appear to exert their effects either by antagonizing the efficacy of an activator or by decreasing the TATA-based reaction directly (44). Complex control in eukaryotes is afforded by combining multiple binding sites for activators and repressors upstream (or downstream) of a single promoter (Fig. 5-1B) (115). In general, a typical eukaryotic promoter region is best viewed as a series of discrete DNA elements to which multiple proteins bind; poorly understood interactions

Figure 5-1: Proteins and DNA Sequences Required for Eukaryotic Gene Expression.

(A) Shown are the basic components of a eukaryotic promoter based on in vitro studies with the adenovirus major late promoter (10). The AT-rich TATA-Box (TATAAA) is located about 30 bp upstream of the transcription start site (arrow). Binding of the general promoter factor TFIID (D) to the TATA-box, perhaps in concert with TFIIA (A), constitutes the formation of a pre-initiation complex. Stepwise addition of TFIIB (B), TFIIE (E) and RNA polymerase (Pol II) affords a complete initiation complex. The general transcription factors and polymerase schematized here are believed to be required for accurate and efficient expression of all genes transcribed by RNA polymerase II (Buratowski et al., 1989).

(B) Represented here are three promoters linked to various upstream regulatory sequences. The binding of an activator protein (Act.) to its cognate upstream regulatory element confers enhanced expression (bold arrow) upon the linked promoter that otherwise mediates only basal level expression (light arrow). Many eukaryotic promoters are subject to complex control (stipled arrow), depicted here by the combination of two different activators and a repressor (Rep.) bound to multiple sites present upstream. Activators and repressors alter promoter efficiency by modulating the TATA-based promoter reaction, although all three promoters are shown without bound promoter factors and polymerase to emphasize the fact that the mechanistic details of enhancement and repression remain obscure.

Figure 5-1.



Conservation of Promoters, Promoter Factors and Polymerase

Promoters. It has been known for nearly a decade that promoter regions from widely divergent eukaryotes contain similar DNA elements such as the TATA box and upstream regulatory sequences (31). The common architecture of eukaryotic promoters and the presence of enhancer elements in both yeast and mammals provided early clues that the sequences and proteins that mediated promoter function have been conserved during evolution. The earliest demonstration of promoter function across species boundaries came from studies on RNA splicing. Beggs et al. (4) sought to determine whether splicing of a mammalian transcript could occur in a simple eukaryote by introducing a segment of chromosomal DNA encompassing the rabbit β -globin gene into yeast. Though no splicing of the primary transcript was detected in yeast cells, expression of β -globin gene was observed, indicating that the rabbit β -globin promoter was functional in yeast. In an analogous series of experiments, it was demonstrated that the *Drosophila* alcohol dehydrogenase gene was also functional in yeast (109).

Recent studies on promoter conservation have shown that both the ADR2 promoter from *Saccharomyces cerevisiae* (budding yeast) (90) and the early promoter from the SV40 animal cell virus function in *Schizosaccharomyces pombe* (fission yeast) (51). In addition, the estrogen-inducible *Xenopus* (toad) vitellogenin A2 promoter has been shown to function in human cells (52). Since high level promoter function requires the concerted action of proteins bound to both promoter and upstream elements (Fig. 5-1B), detectable promoter function across species boundaries implies that both promoter factors and regulatory factors have been conserved during evolution. Indeed, the estrogen-responsive element from the *Xenopus* vitellogenin A2 gene has been shown to function as an enhancer element in human cells, demonstrating the conservation of

estrogen receptors in toads and humans (52). Moreover, recent genetic studies with the adenovirus E3 promoter in yeast have revealed that mutations in either the TATA box or in the upstream regulatory elements, impaired the binding of yeast factors to these sequences in vitro and reduced adenovirus E3 promoter function in vivo (54).

These data suggest that many of the proteins and DNA sequences involved in promoter function have been evolutionarily conserved, but should not be taken to mean that all aspects of promoter function are identical in eukaryotes or that every eukaryotic promoter will function across phylogenetic boundaries. In many cases, for example, the start sites for transcription initiation from a given promoter differ in yeast and animal cells. In fact, transcription initiation from nearly all of the promoters described above was found to occur further downstream of the TATA box in yeast compared to higher cells. Abberancies observed in the transcription start sites of mRNAs from mammalian promoters in yeast cells probably reflect the fact that the TATA box in higher eukaryotic promoters is usually closer to the start site than in yeast; perhaps subtle differences exist in the molecular device that "measures" the distance between the TATA box and the transcription start site in yeast and mammals.

TATA-Binding Protein. Extracts prepared from mammalian cells (93), *Drosophila* embryos (100) and from yeast (60) have been shown to yield accurate transcription initiation by polymerase II from a minimal TATA-containing DNA template in vitro. The competency of extracts from several eukaryotes to support in vitro transcription, coupled with the presence of the TATA element in nearly all eukaryotic promoters has led to the investigation of TFIID conservation.

Functional conservation of TFIID was examined directly by determining whether

the yeast TATA-binding protein could substitute for mammalian TFIID in an in vitro transcription reaction containing RNA polymerase and the three other required mammalian core factors (TFIIA, B and E). It was found that TFIID strongly supported accurate in vitro transcription from the adenovirus promoter in extracts lacking mammalian TFIID (11, 13). The yeast protein also appeared to promote formation of the pre-initiation complex as agents that prevent mammalian TFIID DNA binding also blocked yeast TFIID interaction with the DNA template. The binding of yeast TFIID was stimulated by mammalian TFIIA in a manner similar to that observed for mammalian TATA-binding protein, suggesting a conservation of protein-protein contacts between yeast TFIID and mammalian TFIIA. The functional conservation of the TATA-binding protein provides strong evidence that at least one general promoter factor has been evolutionarily conserved from yeast to mammals.

RNA Polymerase. The activity of RNA polymerase II is potently inhibited by a compound known as α -amanitin. The sensitivity of polymerase to α -amanitin has facilitated the isolation of α -amanitin resistant variants of polymerase in *Drosophila* (28) and other organisms, subsequently providing the basis for the isolation of a molecular clone of the α -amanitin resistant polymerase subunit (29). Molecular analysis of the α -amanitin resistant polymerase clone revealed that α -amanitin sensitivity maps to the largest of the 10 polymerase II subunits. The *Drosophila* polymerase clone has allowed the isolation of homologs from yeast, mouse, hamster and human, providing direct evidence that the largest polymerase subunit has been conserved throughout eukaryotic evolution (92). Each of the eukaryotic clones also displayed homology to the β' subunit of bacterial RNA polymerase, indicating that sequences constituting eukaryotic polymerase may have been acquired from the prokaryotic enzyme (2).

Two notable features of the largest polymerase subunit are common to all known eukaryotic isolates. The first of these is a highly basic 30 amino acid segment located approximately 350 residues from the amino terminus (92). This basic region may act to contribute general DNA affinity to polymerase, an electrostatic property presumed to be essential for juxtaposition of the enzyme with the negatively charged DNA phosphate backbone during mRNA synthesis. In fact, biochemists have long exploited the affinity of RNA polymerase for anionic resins such as phosphocellulose as a step in the purification of the enzyme.

A second common feature is a seven amino acid repeat that comprises the carboxy terminus and whose consensus sequence (Tyr-Ser-Pro-Thr-Ser-Pro-Ser) is repeated 26 times in yeast, 44 times in hamster and 52 times in human (1, 72). The presence of the heptapeptide "tail" was unexpected since repeated stretches of amino acid sequences are most often associated with structural proteins (eg. keratin, tropomyosin and myosin), though subsequent experiments have clearly established its functional importance. First, specific transcription initiation by polymerase in vitro is inhibited either by deletions of the heptapeptide or by pre-incubating polymerase with a heptapeptide-specific monoclonal antibody (17). More compelling, deletions in the yeast polymerase that reduce the tail by more than 40% result in cold sensitive strains that grow slowly, and deletions that shorten the tail by more than 60% are lethal (1, 72).

Although the mechanistic role of the heptapeptide repeat remains a mystery, one hypothesis postulates that it functions as a molecular "plow", acting to remove nucleosomes during mRNA synthesis. Alternatively, the heptapeptide repeat may interact with either a general promoter factor or with a regulatory protein, and thus may play a direct role in transcriptional regulation (1). In any case, the functional conservation of the

heptapeptide during evolution was dramatized by the finding that a yeast strain carrying a deletion in the gene encoding the largest polymerase subunit could be complementated by a chimeric yeast polymerase gene fused to sequences encoding the heptapeptide from hamster (1). The extent to which the remaining nine polymerase subunits display functional conservation awaits sequence information.

Conservation of Activators

Eukaryotic transcriptional activators including steroid receptors, CCAAT-binding proteins, the products of oncogenes, animal virus gene products, heat shock transcription factors and many yeast activators bind to upstream regulatory sequences and enhance transcription from linked promoters (Fig. 5-1B). Recent evidence indicates that all of these activators can function across phylogenetic boundaries .

Steroid Receptors. Steroid hormones coordinate complex events in mammals including development, differentiation and physiological responses to diverse stimuli. Steroid hormone action is mediated by soluble intracellular proteins known as steroid receptors (114) which, in the presence of the hormone, bind enhancer elements (14, 76, 95) located near regulated promoters and stimulate promoter activity by increasing the rate of transcription initiation. Molecular clones encoding glucocorticoid, mineralocorticoid, progesterone, estrogen, vitamin D, thyroid, retinoic acid and related receptors have been isolated (3, 19, 27). The overall genetic composition of all of these receptors is similar, implying that steroid receptors constitute a related family of hormone-inducible regulatory proteins. The apparent relatedness of these molecules suggests that each may have arisen from a common progenitor early in the evolution of eukaryotic cells. In support of

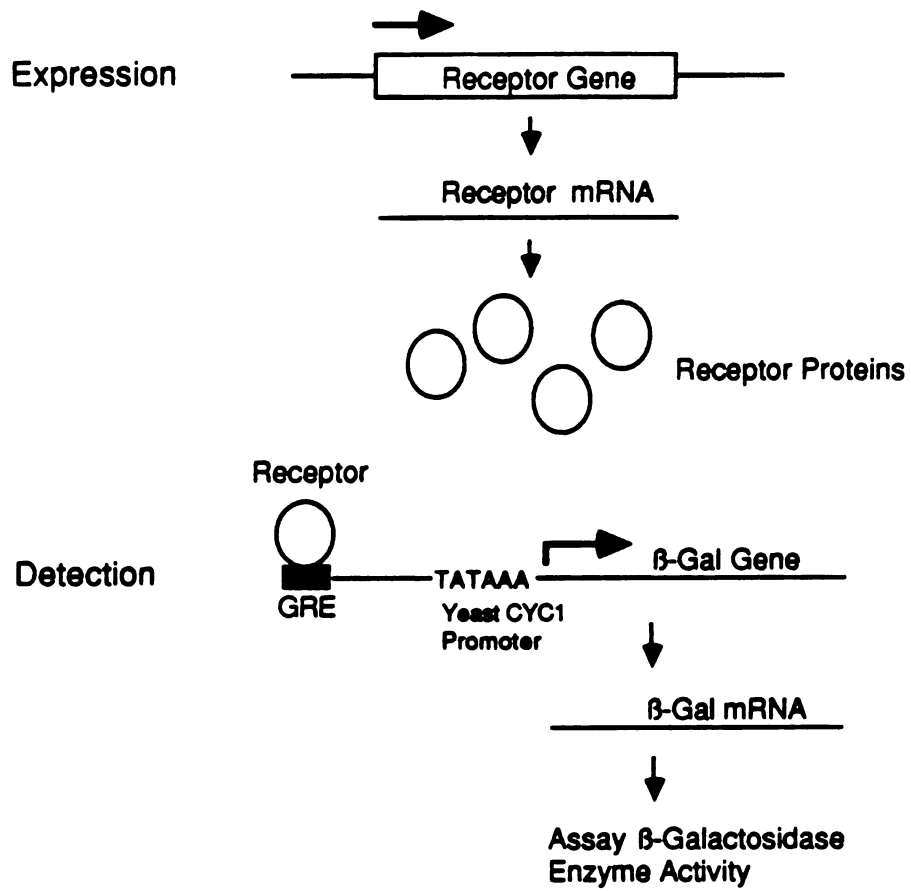
this notion, investigators have identified steroid binding activities in extracts prepared from various fungi (12).

The presence of putative steroid receptors in yeast as well as the similar architecture of yeast and animal cell promoters led two groups to investigate whether mammalian steroid receptors could function in fungi. To test this hypothesis, DNA encoding the glucocorticoid or the estrogen receptor was introduced into yeast cells to allow expression of the steroid receptor protein. The ability of the steroid receptor to bind DNA and enhance transcription was easily assayed since the enhancer element had been placed upstream of a well characterized yeast promoter linked to the lacZ gene. Thus, receptor-dependent increases in lacZ (β -galactosidase) expression would provide the experimental evidence (Fig. 5-2).

Figure 5-2: A Simple Assay for Mammalian Glucocorticoid Receptor Action in Yeast.

The cDNA encoding the rat glucocorticoid receptor (receptor gene) is introduced into yeast cells, and expressed using a suitable yeast promoter. Translation of the receptor messenger RNA (receptor mRNA) affords the glucocorticoid receptor protein (shown as spheres). Receptor function is easily quantitated by measuring the expression of the lacZ gene fused to the yeast CYC1 promoter, upstream of which is the glucocorticoid receptor enhancer element (shown as a filled square). Transcriptional activation by receptor causes an increase in β -Galactosidase mRNA which, in turn, leads to an increase in the assayable β -Galactosidase enzyme. Similar versions of this assay have been employed to detect enhancement by several other mammalian activators in yeast.

Figure 5-2.



Both the glucocorticoid receptor (96) and the estrogen receptor (70) were shown to enhance transcription from yeast promoters linked to enhancer sequences; moreover, transcriptional activation occurred in a hormone-dependent manner. The start sites for mRNA synthesis were identical when compared to yeast promoters under the control of yeast activator proteins, indicating that mammalian steroid receptors function in yeast by faithfully interacting with a component of the yeast transcriptional machinery. The observation that mammalian steroid receptors function in yeast and in *Drosophila* (S.K. Yoshinaga and K.R. Yamamoto, manuscript in preparation) argues that the molecular mechanisms that underlie steroid-mediated enhancement arose early in evolution and imply that the family of mammalian steroid receptors may have diverged from a primordial steroid receptor. These results also suggest that steroid receptors enhance transcription by a similar mechanism in yeast and mammalian cells.

CCAAT-Binding Proteins. Activators that bind to upstream promoter elements containing the pentanucleotide CCAAT sequence (pronounced "cat sequence") constitute a family of transcription factors known as the CCAAT-binding proteins (5, 68). Members of this family include C/EBP, CTF (NF-1), CP1 and CP2 and others. The CCAAT sequence element has been found upstream of a diverse set of mammalian promoters including human α -globin, mouse α -globin, human hsp70, HSV thymidine kinase, H-2Kb, adenovirus major late promoter and origin, MSV LTR, rat g-fibrinogen and others (16). Although each of these factors recognizes a similar DNA element, sequence analysis of molecular clones has confirmed that separate genes encode C/EBP and CTF (57). Molecular clones have not yet been isolated for CP-1 and CP-2, but biochemical data demonstrates that these proteins are also distinct from each other and from C/EBP and CTF. Interestingly, biochemical experiments have shown that one of the CCAAT-binding proteins, CP1, recognizes DNA as complex composed of at least

two heterologous subunits; that is, maximal DNA binding by CP-1 requires two components, CP-1A and CP-1B, that form a stable CP-1A,B protein complex in solution (16).

Regulatory elements containing the CCAAT consensus have also been identified upstream of yeast genes. The CCAAT sequence located in the CYC1 UASs matches perfectly the consensus sequence recognized by mammalian CP-1 (34). Two yeast proteins, HAP2 and HAP3, bind to the CYC1 UAS CCAAT element and activate the CYC1 promoter when yeast cells are grown on non-fermentable carbon sources. Genetic and biochemical studies have shown that HAP2 and HAP3 bind DNA as a heteromeric protein complex (36). Furthermore, yeast HAP2 and HAP3 make the same DNA contacts as the CP-1A,B complex. The existence of CCAAT regulatory sequences in yeast and mammalian cells and the heteromeric composition and similar DNA binding properties of yeast and mammalian CCAAT-binding proteins, has led investigators to examine whether yeast HAP2 and HkAP3 and human CP-1A and CP-1B are functionally related.

The functional interchangeability of subunits from the HAP2,3 and CP-1A,B proteins was investigated by measuring the ability of mixed chromatographic fractions from yeast and human cells to bind a DNA element containing a CCAAT consensus sequence. Remarkably, heteromeric complexes of CP-1A and HAP2 or CP-1B and HAP3 exhibited an affinity for the CCAAT sequence similar to complexes consisting of yeast HAP2,3 and mammalian CP-1A,B (15). Thus, a CCAAT binding protein from yeast and human cells consists of heterologous protein subunits that are functionally interchangeable. No obvious physiological connection exists, however, between yeast cytochrome genes and mammalian promoters under CP-1A,B regulation. Nonetheless,

the functional homology of CCAAT-binding protein subunits in yeast and mammals argues that the CCAAT pentanucleotide sequence arose as an upstream regulatory element early in eukaryotic evolution and that subunit contacts between the factors that recognize this sequence have apparently been highly conserved as well.

Oncogenic Activators. Several dozen oncogenes (cancer causing genes) have been identified over the past ten years (7). The products of these viral and cellular genes constitute a diverse class of proteins that include growth factors and growth factor receptors, G proteins, protein kinases and nuclear proteins. Oncogene products that are secreted, membrane bound or localized to the cytoplasm apparently induce neoplasia by altering normal cellular signal transduction and metabolism. In contrast, several oncogenes that express nuclear proteins are believed to induce abnormal cell growth by altering gene expression directly.

A provocative link between transcriptional activation and neoplasia was established by the finding that v-jun, the presumed oncogene of the avian sarcoma virus, apparently arose by transduction of the cellular gene that encodes a human transcriptional activator protein termed AP-1 (8). Promoters under AP-1 regulation, including the human metallothionein gene and the SV-40 early region, contain binding sites for AP-1. Purified Jun protein binds to the same DNA sequence as AP-1 and, unexpectedly, the AP-1 binding site was found to be nearly identical to the core consensus DNA sequence recognized by GCN4, a yeast transcriptional activator involved in the coordinate regulation of amino acid biosynthetic genes (59). Furthermore, the DNA binding domains of mammalian AP-1 and yeast GCN4 show significant protein homology suggesting that mammalian Jun may have arisen from primordial regulators whose DNA binding and transcriptional enhancement functions were conserved from yeast to

mammals (108).

The DNA binding domain of Jun, when fused to the activation region of GCN4, was found to activate the expression of yeast amino acid biosynthetic genes normally under GCN4 control, indicating that Jun is capable of recognizing GCN4 binding sites upstream of yeast amino acid biosynthetic genes (104). Mammalian Jun is also capable of activating transcription in yeast. A recombinant molecule consisting of Jun fused to the DNA binding moiety of bacterial Lex-A protein was found to stimulate expression of the yeast CYC1 promoter fused to lexA binding sites (103). Moreover, the AP-1 DNA regulatory element from the SV40 early promoter was shown to function as an enhancer element both in budding yeast (37) and in fission yeast (48). Interestingly, a second yeast factor related to GCN4 also appears to recognize this site suggesting the existence of a family of AP-1/Jun-like molecules in fungi. The function of the products of mammalian oncogenes as activators in yeast appears to be a fairly general phenomenon since two other nuclear oncoproteins, Myc and Fos, also activate the CYC1 promoter as fusions to LexA (58). These findings suggest that one cause of neoplasia in mammals is the aberrant expression of cellular genes; moreover, the products of mammalian oncogenes may have arisen from ancestral transcriptional activators that mediate critical regulatory functions in simple eukaryotes.

Virus-Encoded Activators. Many animal cell viruses, like bacterial viruses, utilize the enzymes of infected cells to carry out various aspects of viral DNA replication, gene transcription and protein biosynthesis. All viruses, however, encode a small number of viral proteins needed for specialized aspects of the viral lifecycle. Virus-encoded transcriptional activators are frequently required, in concert with host cell transcription factors and RNA polymerase, to activate the expression of viral genes (49,

68).

Two viral activators, E2 from the bovine papilloma virus (BPV) and VP16 from the herpes simplex virus (HSV), have been analyzed in some detail at the molecular level. The E2 protein of BPV contains functional domains typical of gene activators including sequences that mediate DNA binding and transcriptional activation (Fig. 5-1B) (66). E2 binds to specific sites near several BPV promoters and increases transcription of viral genes whose products are required in early aspects of the BPV lifecycle. Similar to the E2 protein, VP16 also contributes an enhancement domain that acts to stimulate early viral gene expression. Unlike the E2 protein, however, VP16 does not bind to enhancer elements directly, but rather interacts in a poorly understood manner with a host enhancer binding protein that occupies regulatory elements upstream of herpes virus genes under VP-16 control (107). Nonetheless, both E2 and VP-16 are viral-encoded transcriptional activators that have co-evolved with mammalian cells to insure efficient interaction with the host transcriptional apparatus. Might the potent activators encoded by animal cell viruses activate transcription across phylogenetic boundaries?

The E2 protein was tested for enhancement activity in yeast by expressing the mammalian protein in yeast cells containing a reporter gene consisting of E2 binding sites positioned upstream of the CYC1 promoter (as in Fig. 5-2). It was found that E2 activated transcription of the CYC1 promoter and that enhancement was cooperative as induction was much more efficient with two E2 sites than with a single site (55). These experiments demonstrate that a virus-encoded activator can stimulate transcription in yeast; furthermore, E2 appears to interact with a similar protein in fungi and mammals since cooperativity of E2 action (but not E2 DNA binding) was observed in both cell types. The herpes VP16 protein was also tested for enhancement in yeast as a fusion

molecule containing VP-16 tethered to the DNA binding domain of the yeast GAL4 protein. The VP-16 molecule fused to GAL4 was found to activate the yeast CYC1 promoter when GAL4 binding sites were present as upstream elements (91; I. Sadowski, personal communication).

Heat Shock Transcription Factor. The heat shock response in eukaryotic organisms is the process whereby the expression of a group of genes, the heat shock genes, is coordinately induced in response to elevated temperatures (77). In general, thermal elevations exceeding the ambient growth temperature by more than 15-20% are sufficient to trigger the heat shock response. In mammals, yeast, and *Drosophila*, thermal increases to 42°C, 37°C and 30°C, respectively, elicit the heat shock response in these organisms which are normally grown at 37°C, 30°C and 25°C, respectively.

The products of heat shock genes (the heat shock proteins) are believed to protect cells from elevated temperatures, in part, by binding to cellular proteins and preventing their heat-induced denaturation (62). The coordinate induction of heat shock genes is mediated by a phylogenetically conserved DNA element located upstream of heat shock gene promoters. The heat shock element (HSE) from mammals, yeast and *Drosophila* exhibits classical enhancer qualities, conferring heat-inducibility upon heterologous promoters (79). The activity of HSEs is imparted by the heat shock transcription factor (HSTF), which binds specifically to the HSE consensus sequence and induces transcription from heat shock promoters in a temperature-dependent manner (75, 102). The universal existence of the heat shock response in eukaryotes and the remarkable similarity of HSEs and heat shock proteins, suggests that strong selective pressures have maintained primordial mechanisms of thermal defense.

Consistent with this view, investigators have shown that a consensus HSE functions in a diverse class of eukaryotes including mammals, frogs, *Drosophila*, and yeast, implying the presence of a highly conserved heat shock transcription factor in these organisms (77). In fact, purified yeast HSTF is identical in electrophoretic mobility to the *Drosophila* protein and binds specifically to the HSE upstream of the *Drosophila* hsp 70 gene; similarly, purified *Drosophila* HSTF binds specifically to the yeast hsp 70 heat shock element (112). The essential cellular function of HSTF was dramatized by the recent demonstration that yeast cells carrying mutations in this transcription factor are inviable (101, 111). These and other experiments indicate that the heat shock element, heat shock transcription factor and heat shock proteins are critical in homeostasis and have been remarkably conserved during evolution.

Yeast Regulatory Proteins. Much is known about the proteins that regulate gene expression in yeast (32). Perhaps the best studied of these regulatory factors is GAL4, a protein that induces the expression a set of genes whose products are required for galactose utilization by yeast cells (45). The functional domains of the GAL4 protein have been dissected and include sequences that mediate DNA binding, transcriptional activation and GAL80 association (47, 65). The GAL4 protein is active only in the presence of galactose which binds to GAL80 and is thought to trigger the dissociation of the inactive GAL4/GAL80 complex. Yeast genes under GAL4 control contain binding sites for the GAL4 protein; these upstream activator sequences can confer galactose regulability upon heterologous promoters, function without strict regard to spacing and thus display the properties of mammalian enhancers (35). The similarities between yeast GAL4 upstream activator sequences and mammalian enhancers and the common architecture of eukaryotic promoters lead several groups to determine whether GAL4 could function in higher eukaryotic cells.

The function of GAL4 in mammalian cells was assayed by co-transfecting appropriate tissue culture lines with the yeast GAL4 gene and a suitable reporter plasmid. Reporter plasmids contained GAL4 binding sites positioned upstream of various mammalian promoters fused to the bacterial gene encoding chloramphenicol acetyl transferase (CAT) enzyme. Significant increases in CAT activity were observed in cells co-transfected with the GAL4 protein and a GAL4 UAS-linked mammalian promoter (50, 110). Enhancement was observed from the mouse mammary tumor virus, herpes simplex virus thymidine kinase gene, rabbit β -globin gene and the adenovirus major late promoters, indicating that GAL4 interacts with a component of the general transcription apparatus required for the expression of a diverse set of mammalian and viral genes. Furthermore, GAL4 acted synergistically with several mammalian regulatory proteins such as the glucocorticoid receptor and the SV40 enhancer binding proteins, suggesting that yeast and mammalian activators probably contact a common component of the general transcriptional apparatus. The GAL4 activator has also been shown to function in plant cells (63) and in *Drosophila* (21), thus providing the best evidence that gene activation is mechanistically similar in all eukaryotes.

Conservation of Activator Protein Regulation

The gratuitous presence of a gene activator in the nucleus of a cell can lead to a dampening or "squenching" of gene expression that presumably results from the titration of an essential component of the transcriptional apparatus (24). Regulating the activity of gene activators is thus critical to homeostasis. Regulatory protein regulation is also essential during development. In the *Drosophila*, for example, tissue formation is known to involve a delicate hierarchy of spatially and temporally restricted patterns of gene

expression; mutations in presumed gene activators can lead to profound developmental defects (97).

The potency of a given activator protein can be modulated either by varying its cellular concentration or by altering its specific activity. The specific activity of a regulatory protein can be altered by covalent modifications such as phosphorylation, or by noncovalent associations such as the binding of a ligand. Covalent modifications and noncovalent associations can be direct as in the phosphorylation of HSTF (101), or indirect as in GAL4 activation by galactose binding to GAL80 (45). In general, it appears that nature has utilized a relatively small number mechanisms to regulate the activity of eukaryotic activators (116). The presence of similar mechanisms of activator protein regulation in eukaryotes suggests that regulatory factors such as kinases and inhibitory proteins may have been conserved during evolution.

Mammalian steroid receptor action in yeast provides the best example of the conservation of regulatory protein regulation across phylogenetic boundaries (70, 96). Recall that receptor-dependent transcriptional enhancement in yeast and animal cells occurs only in the presence of bound steroid. It is known that hormone binding triggers glucocorticoid receptor activation or "transformation" leading to rapid nuclear localization, DNA binding and transcriptional enhancement (115). The unliganded, cytoplasmic form of the glucocorticoid receptor is believed to exist as a heteromeric complex with the Hsp90 protein (80, 83). According to this model, hormone binding triggers dissociation of the receptor-Hsp90 complex leading to the unmasking of receptor transcriptional regulatory activities.

If the Hsp90 model for steroid receptor regulation is correct, yeast must contain

an Hsp90 homologue whose conservation is sufficient to permit formation of an inhibitory complex with the mammalian glucocorticoid receptor. In fact, studies have shown that *S. cerevisiae* expresses two Hsp90 homologues known as Hsp82 and Hsc82 (S. Lindquist, personal communication). More compelling, the mammalian hsp90 gene can complement a yeast strain doubly deleted in hsp82 and hsc82, suggesting a high degree of functional homology between the mammalian Hsp 90 and the yeast homologues (D. Picard, K.R. Yamamoto and S. Lindquist, unpublished results). Thus, it appears that the noncovalent regulation of the receptor by steroid ligand is mediated by a cellular component (Hsp90) that has have been conserved over a long period of eukaryotic evolution.

Conserved Motifs for Binding and Activation

What is the molecular basis for the functional conservation of activator proteins across phylogenetic boundaries? The answer, in part, is that the molecular motifs for DNA binding and transcriptional activation have been conserved in evolution.

DNA Binding. Though a large number of activators have been identified, all available data indicates that protein-DNA interaction is mediated by three chemical attractions that include hydrogen bonding, electrostatic interactions and van der Waals forces. Primary sequence and biochemical data suggests that the structures of the DNA binding domains of all known eukaryotic activators fall into three classes; these include the helix-turn-helix, the zinc finger and the leucine-zipper motifs (Table 5-1) (106).

The helix-turn-helix proteins (74), which contain a DNA binding domain formed by two α -helices separated by a β -turn, constitute a diverse class of regulators that

include the yeast MAT α -2 protein (92) and Drosophila and vertebrate homeo-box proteins of which there are more than 80 known members (61). By analogy to prokaryotic activators and repressors for which crystallographic data are available, eukaryotic helix-turn-helix proteins probably recognize specific DNA elements by inserting one of the two helices into the DNA major groove. The zinc finger proteins, for which zinc coordination constitutes the major structural determinant, include the *Xenopus* transcription factor IIIA (71), the yeast GAL4 protein (46) and the mammalian steroid receptors (Table 5-1) (22, 113). Residues within or adjacent to the loops or "fingers" formed by cysteine and histidine binding to zinc are believed to mediate DNA binding (6, 53). The leucine-zipper class, first identified in the C/EBP CAAT-binding protein (56), includes several other mammalian transcription factors such as Jun and AP-1, the yeast activator GCN4, and the oncoproteins Fos and Myc (Table 5-1). Leucine-zipper proteins are known to recognize DNA as dimers. Dimers of leucine-zipper proteins are believed to form through interdigitations of an evenly spaced series of leucine residues that act as a molecular "zipper" (56).

Given that a small number of chemical forces and structural motifs are probably employed for DNA binding by all eukaryotic activator proteins, the functional conservation of the protein-DNA interactions across phylogenetic boundaries is likely explained by the similarities in these basic biophysical and structural properties.

Transcriptional Enhancement. Activator proteins bind to DNA elements located near promoters and enhance transcription probably by interacting with some protein component of the transcriptional machinery (39, 86, 116). Obvious candidates for the "targets" of eukaryotic activators include histones, the general promoter factors and RNA polymerase. One study has shown that the GAL4 activator alters the DNA binding

properties of TFIID, suggesting that the mechanism of enhancement by this protein may involve direct interaction with the TATA-binding protein (42). In other studies, GCN4 has been shown to interact with RNA polymerase in vitro, possibly indicating that this protein stimulates transcription by recruiting polymerase to the promoter (9). In no case, however, has the mechanism of transcriptional enhancement been unequivocally established.

Though the protein(s) with which activators interact remain unknown, the regions of activators that mediate these presumed protein-protein contacts have been identified and studied in detail. Surprisingly, the activation domains of some eukaryotic regulators appear simply to consist of short polypeptide sequences that carry a net negative charge (23, 26, 40, 41, 65). Acidic activation domains ("acid blobs"), rich in aspartic and glutamic residues, share no apparent sequence homology; in fact, random *E. coli* sequences that carry a net negative charge have also been shown to function as activation domains in yeast (64). Thus, it appears that acidic protein sequences, perhaps with α -helical secondary structure (25), can mediate enhancement in yeast, plants, *Drosophila*, and mammals. Acid blobs do not appear to represent the only enhancement motif; in fact, several lines of evidence indicate that other sequences contribute selectively to transcriptional enhancement (9, 18, 69). Acidic enhancement domains do, however, play a major role in transcription; moreover, the apparent plasticity of these acidic regions certainly accounts for some of the promiscuity of eukaryotic activators (Table 5-1).

Table 5-1.

Activator	Source	DNA Binding	Acidic Domain	Functions In
ER	human	Zn finger	?	yeast
CP-1A,B	human	?	?	yeast ^a
Myc	human	leucine zipper	+	yeast ^c
AP-1	human	leucine zipper	+	yeast ^a
GR	rat	Zn finger	+	yeast, Drosophila
Fos	mouse	leucine zipper	+	yeast ^c
VP-16	HSV	-	+	yeast ^c , hamster ^c
E2	BPV	?	+	yeast
Jun	ASV	leucine zipper	+	yeast
HSTF	yeast, Drosophila	?	+ ^b	Drosophila ^a , yeast ^a
GAL4	yeast	Zn finger	+	plants, Drosophila, human
HAP2,3	yeast	?	?	human ^a
GCN4	yeast	leucine zipper	+	human ^c

Table 5-1: Evolutionary Conservation of Activator Protein Structure and Function.

Shown are activator proteins from various organisms that function across phylogenetic boundaries. The estrogen receptor, glucocorticoid receptor and heat shock transcription factor are designated ER, GR and HSTF, respectively. The herpes simplex, bovine papilloma, and avian sarcoma viruses are designated as HSV, BPV and ASV, respectively. In most cases, the presence of a zinc finger or leucine zipper motif is inferred from primary sequence and biochemical data, not from structural information. Proteins for which acidic domains are thought to contribute to enhancement are indicated by a plus (+), except for HSTF in which acidity contribute by protein phosphorylation is thought to be critical (b). The function of activators in foreign organisms was assessed by enhancement in vivo, except in cases where truncated molecules were tested in vivo (c) or where DNA binding in vitro was used as the functional criterion (a). References for each of the activators can be found in the text.

Perspectives--Exploiting the Conservation

The evolutionary conservation of all of the basic components required for eukaryotic transcription initiation suggests gene control in multicellular organisms arose by exploiting, in increasingly complex ways, the basic molecular machinery present in simple eukaryotes, rather than by discarding primordial cellular mechanisms and adopting fundamentally novel methods of mRNA synthesis. The functional conservation of these components across organismic boundaries provides the basis for new experimental strategies for the study of eukaryotic gene transcription.

Genetic experiments with mammalian transcription factors in yeast, for example, circumvent the prohibitive nature of similar approaches in tissue culture cells. In principle, random mutagenesis coupled with a genetic screen or selection should provide an efficient means by which to study the structure and function of any mammalian regulator whose function is assayable in yeast. In fact, Schena et al (manuscript submitted) have recently used chemical mutagenesis and a yeast plate assay to genetically dissect the zinc finger region of the mammalian glucocorticoid receptor. These studies have led to the identification of a class of apparent "positive control" mutants that retain DNA binding activity but fail to enhance transcription.

Yeast genetics could also be useful in dissecting consensus DNA binding sites for regulatory proteins from higher cells. One could, for example, insert DNA fragments containing random mutants of a consensus binding site upstream of a yeast reporter gene and screen yeast cells expressing the mammalian factor for inserts that fail to mediate enhancement. A similar type of experiment could be used to identify a binding site for a cloned mammalian factor for which a consensus sequence has yet to be identified such in

the case of mammalian oncogenes. An elegant experiment designed to determine the residues of a regulatory protein that mediate DNA contact involves generating second site suppressor mutants that display altered sequence recognition. It might be possible to isolate a mutant protein that recognizes altered DNA site.

Experiments across species boundaries also holds promise for identifying additional cellular factors required for transcriptional control including proteins that act directly in enhancement such as general promoter factors and RNA polymerase, and proteins involved indirectly such as kinases and inhibitors that regulate the activity of activators.

Yeast genetics might facilitate the identification of mammalian transcription factors. It might be possible, for example, to isolate cDNAs encoding a desired mammalian homologue either by complementing a yeast strain bearing a mutation in a similar factor or by isolating a yeast mutant that selectively requires the expression of a mammalian protein for viability. This latter approach, recently termed "cloning by complementation", embodies the distinct advantage of allowing one to clone functionally homologous proteins that show no primary sequence homology. In cases where a binding site (but no factor) is available, one should also be able to devise assays in yeast to identify these novel regulatory proteins.

In vitro transcription experiments using mammalian and Drosophila extracts are much more advanced than similar experiments using yeast extracts. Since it is clear that TFIID is conserved in yeast and mammals, it should be possible to combine biochemical and genetic approaches to study and purify transcription factors. Cloned yeast transcription factors will be useful in establishing the in vivo role of a given protein via

gene replacement strategies, and may serve as a useful hybridization probes for isolating mammalian homologues.

All of these approaches assume and require a high degree of conservation of transcriptional regulatory proteins in eukaryotic cells. This discussion is not intended to suggest, however, that all aspects of transcriptional regulation are identical in all eukaryotic organisms. It is already clear from mammalian steroid receptor experiments in yeast, for example, that subtle differences in receptor function exist between yeast and mammals. Furthermore, the apparent ability of random sequences bearing a net negative charge to function as enhancement domains provides the basis for potential artifacts in interpreting the physiological relevance of a given mammalian protein to activate yeast gene transcription. Nonetheless, cautious genetic and biochemical experimentation across phylogenetic boundaries provides the basis for powerful new approaches to the study of eukaryotic gene transcription.

I have largely excluded discussions of the prokaryotic transcription literature in this review. The reader should be aware, however, that many the conceptual themes presented here derive from experiments with bacterial promoters and activators (43, 67, 74, 82, 84, 89, 117) . Furthermore, while fundamental differences clearly exist between prokaryotic and eukaryotic transcription, the general chemical principles that appear to govern regulation in complex organisms probably apply to all living cells.

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In mammalian cells, the glucocorticoid receptor binds specifically to enhancer DNA sequences and activates transcription from linked promoters. To study the mechanism of enhancement by receptor in genetic detail, I undertook the experiments described in this thesis.

I began by developing a powerful genetic system in which to study receptor action. Based on the similarities of factors and sequences required for eukaryotic transcription, Keith Yamamoto and I hypothesized several years ago that the mammalian glucocorticoid receptor might function as an enhancer activating protein when expressed in yeast. Indeed, as I show in chapter two, derivatives of the glucocorticoid receptor enhanced transcription when expressed in yeast cells containing fusions of GRE sequences to the *CYCI* promoter. Since enhancement was observed in fusions of GREs to mutant *CYCI* promoters retaining only the TATA region and transcription startpoints, it is demonstrated that receptor requires only yeast promoter sequences to activate transcription. In addition, I show that GREs function without strict regard for spacing or orientation relative to the *CYCI* promoter and thus act as enhancers in yeast. I also show that the activity of a series of receptor deletion mutants expressed at physiological levels, possess a similar level of enhancement activity in yeast and mammalian cells. Together, these findings indicate that receptor operates by a common, highly conserved mechanism in yeast and mammalian cells.

The mechanistic conservation of receptor action in yeast validated further genetic studies aimed, for example, at dissecting in detail the functions encoded in the receptor zinc finger region. The finger domain was of particular interest as it encodes both DNA binding and transcriptional regulatory activities; thus, analysis of a small region of receptor would shed light on two important aspects of receptor function. To analyze this

region in genetic detail, I randomly mutagenized a segment of the receptor encompassing these sequences, and screened in yeast for receptor mutants that failed to induce β -galactosidase expression from an integrated GRE-linked *CYC1*-lacZ reporter gene. In chapter three, I report the isolation and preliminary characterization, in yeast, in animal cells, and *in vitro*, of a series of such receptor point mutations. In particular, I show that point mutations that impaired receptor function mapped to a 66 amino acid subregion of the finger domain that tightly encompassed the zinc fingers; moreover, all of the mutant derivatives tested were soluble and accumulated to intracellular levels similar to the wild type species. In most cases, the mutants were phenotypically similar in yeast and animal cells, supporting further the notion that the receptor acts by a common mechanism in these diverse eukaryotes. Mutations that abolished GRE DNA binding were distributed across both fingers, especially at the cysteines known to coordinate zinc ions, and in the five amino acids just downstream of each finger. I hypothesized that the mutations that mapped just downstream of each finger define receptor sequences involved in DNA recognition.

I also isolated a class of mutations tightly clustered on a portion of the second finger that impaired enhancement but not DNA binding. The identification of mutants that displayed a positive control phenotype confirmed that DNA binding and transcriptional enhancement were separable receptor functions. Certain of the positive-control mutants exhibited striking phenotypic differences in yeast and animal cells, suggesting that residues at these positions may mediate protein-protein interactions between receptor and an incompletely conserved component of the eukaryotic transcription apparatus.

How might one identify cellular factors with which receptor interacts? An

approach that seemed particularly powerful involved the isolation of genetic suppressors in yeast. I began these experiments by constructing a yeast strain in which the upstream regulatory region of the *LEU2* gene was replaced with GRE sequences. Cells containing this GRE-*LEU2* fusion were thus rendered receptor-dependent for growth; conversely, transformants of this strain that expressed receptor mutants defective in *LEU2* activation were phenotypically LEU⁻, and thus provided the basis for a genetic selection. Using this selection scheme, I succeeded in isolating revertants that restored the ability of a defective glucocorticoid receptor point mutant to activate *LEU2* expression. The work in chapter four describes the genetic characterization of these revertants. In brief, I show that all 22 of the LEU⁺ revertants analyzed comprised a single complementation group and thus define a single yeast gene. In addition, all of the alleles were recessive. It was also found that certain of the revertants potentiated receptor action in an allele-specific manner, implying that the product of the suppressor gene may interact directly with receptor. Suppression in these strains was observed with other activators acting through different promoters, consistent with the idea that the protein encoded by the suppressor gene may play a general role in transcriptional enhancement in yeast.

Remaining Questions

The work described here constitutes important progress in understanding the mechanism of glucocorticoid receptor action. Nonetheless, basic questions remain regarding all of the major aspects of receptor function including DNA binding, transcriptional enhancement and signal transduction.

Based on genetic evidence and recent structural information (Hard et al., 1990a,b), it appears that amino acids on the carboxy side of each finger comprise α -

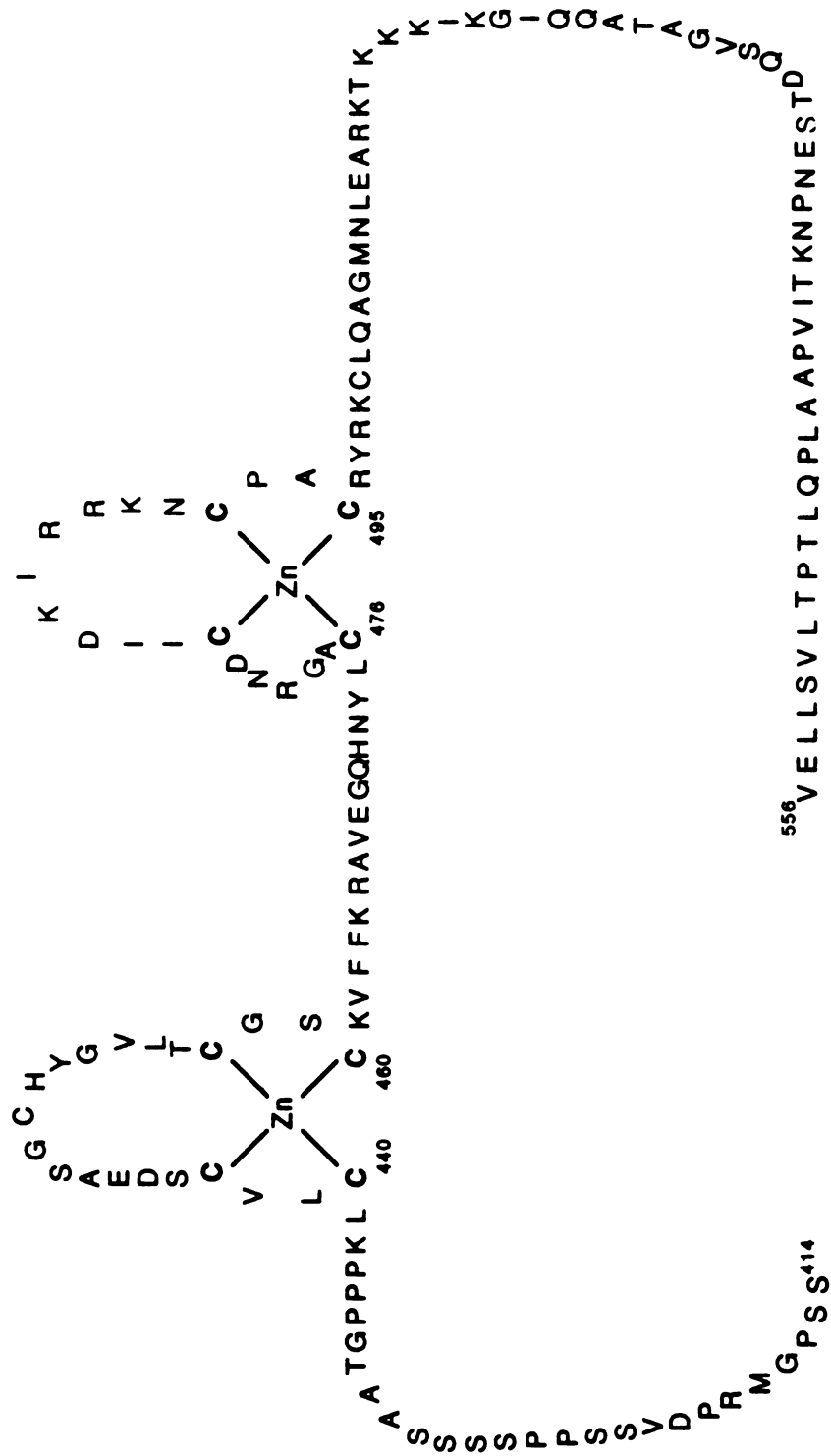
helical segments that impart the DNA binding function of receptor. Neither of these approaches, however, have determined the individual residues involved in making specific contacts to DNA. One way of approaching this question involves isolating mutants of receptor capable of recognizing mutant GREs; the precedent for this type of experiment derives from studies of the Mnt repressor of bacteriophage P22 (Youderian et al., 1983) and the CAP protein from *E. coli* (Ebright et al., 1984). In the case of receptor, these experiments are most readily approached in yeast by linking point mutants of the GRE to a dominant selectable marker such as the *LEU2* gene, then selecting LEU⁺ revertants. Strategies for maximizing the chances of recovering the mutants of choice might include pre-mutagenesis of receptor sequences and the use of diploid yeast strains to ensure the recovery of dominant mutations.

Recent NMR evidence also suggests that residues from the first receptor zinc finger make hydrophobic and electrostatic contacts with second finger amino acids (Hard et al., 1990a,b). Are unique "interfinger contacts" required for maintenance of the globular structure of the finger domain, or will other pairs of interacting amino acids at these positions confer function? One way to test this model genetically would be to ask whether mutations at these first finger positions are suppressed by compensating mutations at the corresponding positions in the second finger. This experiment is best approached by randomly mutagenizing the region of interest in the second receptor finger using doped oligonucleotide mutagenesis, and screening on β -galactosidase indicator plates for mutants that intragenically suppress point mutations in the first finger. [As the thesis was being completed, NMR data were obtained (Hard et al., 1990a,b) that suggest a zinc coordination scheme for the second receptor finger that differs from of the one presented in chapter three (Figure 6-1).]

Figure 6-1: Alternate Coordination Scheme of the Second Receptor Zinc Finger.

Shown is the coordination scheme of the second receptor finger as determined by recent structural studies using two-dimensional nuclear magnetic resonance spectroscopy.

Figure 6-1.



Genetic and biochemical information also suggests that dimerization contacts between finger domain monomers may be made between sequences located at the amino-terminal base of the second receptor finger (Dahlman-Wright et al., 1990; Hard et al., 1990a). Residues believed to mediate dimerization of receptor could be randomly mutagenized and mutants could then be screened in yeast. Biochemical experiments with purified mutant proteins could then be used to assay dimerization defects.

Basic questions regarding the mechanism of enhancement by receptor in yeast include understanding (1) additional aspects of the genetic function of receptor enhancement domains and (2) elucidating the role of nonreceptor factors involved in this process. The identification of multiple receptor enhancement domains (Enh1 and Enh2), coupled with the finding that point mutations in one of these regions (Enh1) can fully abrogate activation suggests that both Enh1 and Enh2 are required for enhancement, though each can apparently function independently in certain contexts. It is conceivable that, within the context of the intact receptor, Enh1 and Enh2 interact directly. One way to address this model would be to test whether pc mutants could be isolated in Enh2. Obviously only a positive result would be meaningful in this case; a negative result could simply indicate that sequences within Enh2 are functionally redundant, for example, and thus recalcitrant to inactivation by single missense mutations. In addition, the isolation of mutations in Enh 2 that intragenically suppress Enh1 lesions would be very informative.

The presence of multiple enhancement domains within receptor implies that Enh1 and Enh2 may function by interacting with multiple targets. One of these targets may have already been identified as a suppressor to N556M505T protein (Chapter 5). Additional suppressor experiments using the Enh1 positive control mutants might lead to the identification of new alleles of the previously identified suppressor gene, or to the

isolation of new genes. It might also be informative to utilize Enh2 mutants as substrates in suppressor experiments. Definitive tests of a direct interaction between any of the factors identified in these experiments and receptor will require biochemical analyses such as affinity chromatography, chemical cross-linking, and *in vitro* transcription approaches.

Analogous to understanding enhancement, comprehension of signal transduction will require a detailed analysis both of receptor sequences and nonreceptor factors required for this process. Studies with the intact receptor indicate that the mechanism of receptor signal transduction has been functionally conserved in yeast, suggesting that yeast genetics offers an opportunity to genetically dissect this pathway. In fact, random mutagenesis of the receptor hormone binding domain has facilitated the isolation of point mutants in this region that impair receptor function. Characterization of these receptor molecules in yeast, in animal cells, and *in vitro* indicates that the mutants comprise two broad classes (Garabedian et al., 1990). Mutants of the first class bind hormone with reduced affinity and are thus defective in activation of the β -galactosidase reporter gene because they fail carry out this early step in the signal transduction process; interestingly, several of these mutants possess a restricted pattern of ligand specificity. Mutants of the second class bind hormone normally *in vitro* but fail to induce β -galactosidase expression *in vivo*, suggesting that these mutants are defective in some later step in the signal transduction pathway such as nuclear localization or transcriptional activation. Analysis of both classes of hormone binding mutants will provide a detailed genetic understanding of the receptor sequences required to carry out these processes. Moreover, the isolation of revertants to receptor proteins altered in the functions of the C-terminal segment provides a means by which to identify nonreceptor factors that interact with these sequences. Current data suggest that such factors might include proteins involved in both signal transduction and transcriptional regulation such as HSP90 (Sanchez et al., 1985;

Picard et al., 1990; Garabedian et al., 1990), a protein kinase (Auricchio, 1989), or a component of the yeast transcription apparatus (Webster et al., 1988).

Shortcomings

Recapitulation of steroid hormone action in yeast constitutes a major technological advance in studying the basic mechanistic aspects of glucocorticoid receptor function; nonetheless, certain technical and theoretical limitations of this system continue to impede progress.

One technical shortcoming of the yeast system derives from the fact that the level of expression of receptor via the GPD promoter can vary by more than 10-fold depending on the yeast strain. In every case examined, levels of receptor were found to inversely correlate with strain doubling times such that faster growing cells produced a higher level of receptor protein. Quantitation of N795 expression in yeast indicates that levels of this protein ($\approx 0.1\%$ of the soluble yeast protein) in strain BJ2168 using the 2μ -based pG-N795 vector are close to saturating for reporter plasmid pSX26.1. For this reason, care must be taken to ensure that a given genetic background is compatible with a particular experiment. Complications such as reduced assay sensitivity can arise if experiments are performed in cells producing levels of receptor that exceed the linear range, particularly in cases involving the isolation of receptor mutants or in genetic suppressor experiments.

A second technical shortcoming of receptor experiments in yeast pertains to the low expression threshold of selectable markers required to impart strain viability. In strain U-12.38, for example, which contains an integrated reporter gene containing 5

GREs fused to *LEU2*, receptor mutants possessing as little as 1% activity are sufficient to give a LEU+ phenotype, excluding the usefulness of this strain in suppressor studies with the *pc* mutants. To broaden the applicability of the GRE-*LEU2* selection system, steps must be taken to weaken the expression of this test gene. In one case, this has been accomplished by using a centromeric plasmid to reduce receptor expression (J. Austin and K.R. Yamamoto, unpublished data). Additional approaches include altering the growth temperature of cells, or linking the *LEU2* gene to fewer copies of the GRE. Development of a GRE-*URA3* selection system would also be extremely useful as both positive and negative selections exist for the product of the *URA3* gene.

Characterizing large numbers of randomly introduced point mutations in receptor by DNA sequencing is cumbersome at present, particularly in large domains of the molecule such as the ~250 amino acid steroid binding domain. Thus, the development of a gene mapping technique such as UV-induced mitotic recombination (Johnston and Dover, 1988) would be extremely useful. Future experiments should focus on perfecting this technology, particularly if one wishes to isolate point mutants randomly introduced into the intact receptor cDNA.

Though yeast has provided a powerful genetic system in which to study receptor action, biochemical approaches for analyzing receptor in yeast remain relatively primitive. The development of an *in vitro* transcription system (Dignam et al., 1983; Fire and Sharp, 1984; Soeller et al., 1988; Lue et al., 1989; Freedman et al., 1989), for example, would provide an excellent means in which to assay the activity of factors isolated in genetic selections, and provide a critical link between receptor studies in yeast, *Drosophila*, and cultured cells.

In addition to the current technical limitations of the yeast system, several theoretical limitations plague receptor studies in this organism. One shortcoming derives from the fact that yeast lacks the physiological context of a mammalian cell. In cultured cells, receptor activates a large number of endogenous mammalian genes and thus mediates the expression of a gene network; in contrast, many of these genes are probably absent or have yet to be identified in yeast. Nonetheless, yeast could prove useful in the identification of hormone responsive mammalian genes. One could imagine transforming a yeast strain expressing the glucocorticoid receptor with a reporter plasmid containing an essential yeast gene fused to random mammalian genomic fragments. Inserts conferring enhancement by receptor could then be easily selected in yeast, and used as hybridization probes for identifying hormone-responsive mammalian genes.

All of the experiments described above exploit the fact that receptor action has been highly conserved in evolution. In the case of the DNA binding and transcriptional enhancement activities, all available evidence suggests a mechanistic commonality in yeast and mammalian cells. In studying less characterized aspects of receptor action in yeast such as signal transduction and negative regulation (see below), care must be taken to conduct parallel studies in yeast and animal cells to avoid misinformation arising from species differences. Discordant phenotypes should be analyzed critically, as they may provide important mechanistic clues particularly in cases involving protein-protein interactions.

Puzzles

Several aspects of the work described here remain enigmatic, including the facts that (1) levels of receptor in yeast exceeding 0.1% are strikingly growth inhibitory (2)

GREs are enhancers in yeast but fail to function at downstream positions (3) negative regulation by receptor in yeast has not yet been recapitulated and (4) steroid ligand specificity is altered in yeast.

The expression of constitutive derivatives of receptor (eg. N556) in yeast is growth inhibitory at levels as low as 0.1% of the total soluble protein, and is lethal at several-fold higher levels. Transformants of strain BJ2168 expressing N556 grow slowly on minimal plates, whereas transformants of W303-1B expressing N556 fail to form colonies at all. Growth inhibition in these strains was not observed with either the intact receptor in the absence of hormone, the X556 derivative, or with any of several N556 derivatives containing point mutations that impair DNA binding; taken together, these results suggest that DNA binding, Enh1, and Enh2 are required for this inhibitory effect. It remains unclear whether growth inhibition by receptor reflects a squelching phenomenon, the inappropriate dimerization of receptor with a yeast factor, or some other mechanism. In any case, the isolation and characterization of revertants in yeast resistant to high levels of N556 may provide a powerful genetic approach to addressing this interesting question.

A second puzzle pertains to the inability of GREs to function at positions downstream of linked promoters in yeast, despite the fact that these same sequences function at variable distances in both orientations from upstream positions. In fact, enhancer activation from downstream positions has not in any case been demonstrated in yeast, despite the finding that many enhancers function 3' of promoters in mammalian cells. Given that yeast activators can function through UASs located downstream of mammalian promoters in mammalian cells, the distinction between yeast and mammalian cells probably reflects a difference in some component of the general transcription

apparatus. It could be that evolutionary pressures have led to an alteration in the geometry or composition of the transcription complex in yeast, preventing enhancement from downstream positions. One way to address this proposal would be to isolate and characterize revertants in yeast that allow downstream activation; alternatively, yeast might provide an appropriate genetic environment in which to clone the mammalian factor that putatively mediates this activity.

Preliminary experiments with complex GREs (cGREs) in yeast such as those associated with the prolactin or proliferin genes, suggest that these elements neither enhance or repress transcription in yeast in the presence of receptor. It is now clear that the activity conferred by the proliferin cGRE in mammalian cells is determined by the binding of both receptor and other factors to this element (Diamond et al., 1990); the most likely candidates for these ancillary proteins include members of the AP-1 family. Complex GREs can act as either positive or negative elements apparently depending on the stoichiometry of receptor and these other factors. Both in the absence and presence of receptor cGREs lack activity in yeast, perhaps reflecting the nonexistence of these molecules in simple organisms. One way to test this model would be to express Jun and Fos in yeast, and test whether function is restored to cGREs in the presence of receptor.

Extensive *in vivo* experimentation of the receptor signal transduction pathway in yeast, coupled with a large number of hormone binding studies *in vitro*, indicate that both the affinity and selectivity of receptor for ligand is altered in yeast. Minimally, these results indicate that receptor sequences alone are not the sole determinants of steroid binding, and it seems likely that either post-translational modifications or binding of the unliganded form of receptor to other proteins may modulate ligand binding. These models can be addressed using yeast genetics.

Broader Significance

The practical ramifications of these findings are widespread. The fact that the glucocorticoid receptor (Schena and Yamamoto, 1988) and many other proteins from higher cells (for a review, see Chapter Five) can function in yeast suggests that nearly any protein with an assayable activity can now be subjected to detailed genetic analysis. The use of yeast genetics is particularly attractive in the study of factors from organisms such as mammals or higher plants which possess complex genetics; in these cases, approaches in yeast may provide the only efficient experimental way to dissect genetic function. Obviously, the usefulness of this strategy to the study of factors from higher cells possesses certain limitations; for example, the presence of a functionally analogous activity in yeast or the absence of an ancillary factor required for the function of a protein of interest would complicate this general approach. Nonetheless, studies during the next decade are likely to reflect the realization that yeast is an excellent genetic system in which to study proteins from higher cells.

The theoretical relevance of this work pertains mainly to the evolution of transcriptional regulatory mechanisms in eukaryotes. The fact that both DNA binding and transcriptional regulatory activities of receptor have been functionally conserved in yeast indicates that common mechanisms underlie both protein-DNA and protein-protein contacts in eukaryotes. Mechanistic conservation of the activation function in yeast and mammals argues that activators contact a conserved surface of the eukaryotic transcription apparatus. Based on these findings and other work, it is now clear that in addition to the conservation of biomolecular structure (e.g. proteins, RNA, DNA), entire regulatory pathways (e.g. gene regulation, RNA splicing, heat shock response) have been

conserved, providing a relatively invariant core around which these processes evolved in multicellular organisms. Our growing knowledge of the unity of the molecular biology in eukaryotic cells indicates that selective pressures to perpetuate these deeply rooted mechanisms exceeded the forces at work to topple them. Thus, complex organisms apparently arose not by discarding these primordial mechanisms, but mainly by utilizing these fundamental processes in more complex ways.

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Appendix One:

Activity of Glucocorticoid Receptor Deletion and Point Mutants in Yeast

Table A1-1: Activity of Glucocorticoid Receptor Deletion Mutants in Yeast.

Yeast strain BJ2168 (See Chapter 3, Experimental Procedures) containing a GRE-linked *CYC1* -lacZ reporter plasmid (pSX26.1; Schena and Yamamoto, 1988) was transformed (Ito, 1983) with expression plasmids containing fragments of the glucocorticoid receptor cDNA inserted into either pGPD-2 (Schena and Yamamoto, 1988) or pG-1 (Schena et al., 1990). In certain cases, receptor proteins contain carboxy-terminal amino acids (a = GELEFPGLEDPST; b = GANSRV) contributed by polylinker sequences (Schena and Yamamoto, 1988). Liquid β -galactosidase assays were performed in triplicate on cultures treated with 1 μ M deoxycorticosterone (+DOC) or with ethanol as a control (-DOC). The endpoints of amino acid sequences removed from the intact receptor (N795) are shown numerically (Deletion). Solid lines below the receptor cDNA diagram denote DNA sequences present that encode the corresponding receptor proteins. The dashed segment of N556 Δ 4B1 denotes a receptor cDNA sequence in which a frameshift mutation (asterisk) at amino acid 300 leads to translation termination at residue 301.

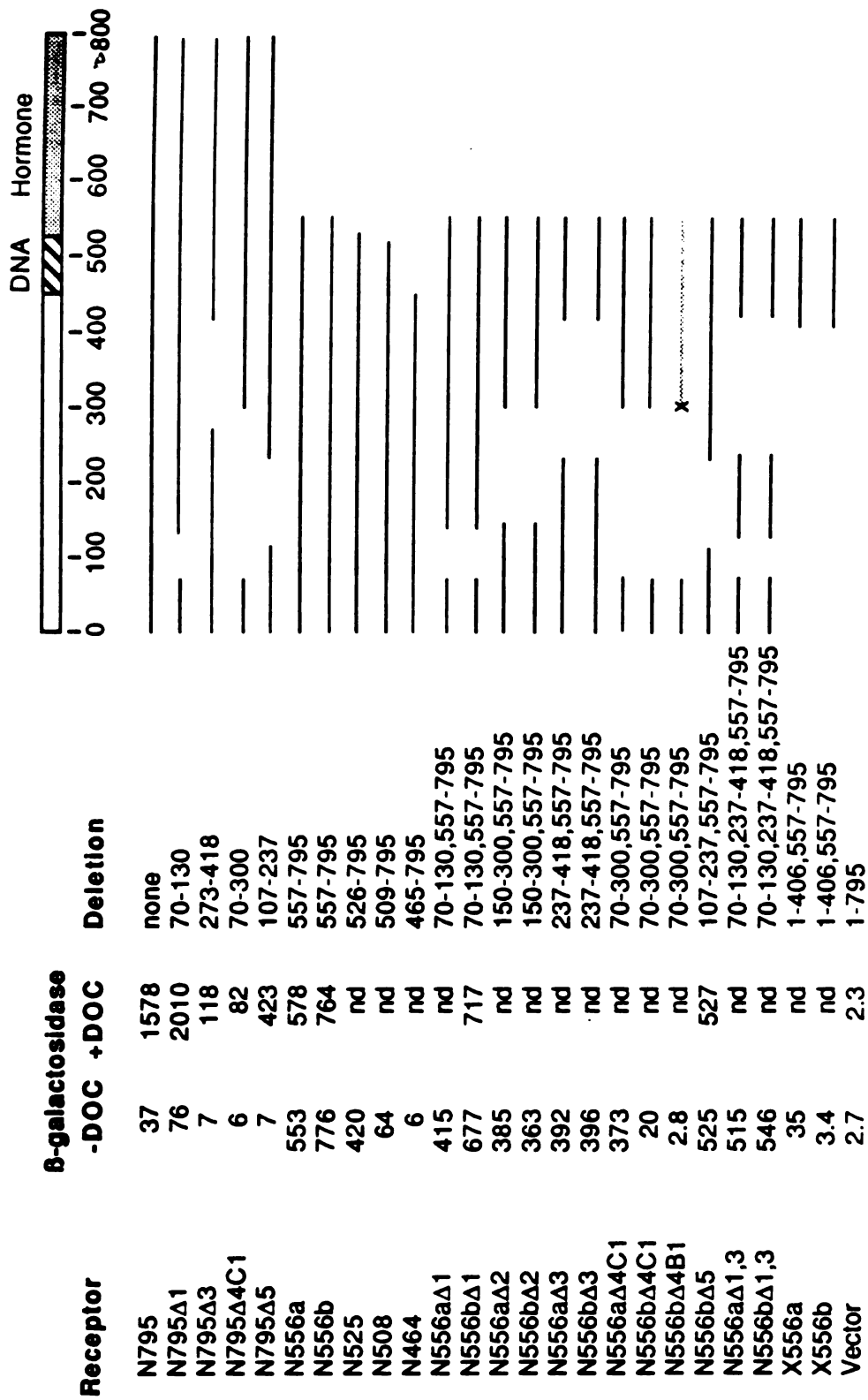


Table A1-2: Enhancement Activity of Glucocorticoid Receptor Positive Control Mutants in Yeast.

Shown is the transcriptional regulatory activity of glucocorticoid receptor derivatives in yeast strain YLT (derived from strain W303-1B; MAT α ; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1; ade2-1; RPO21 containing 38 "repeats"; Allison and Ingles, 1989) transformed with plasmid pSX26.1 (see figure 2-4). Cultures growing in minimal medium deficient of tryptophan and uracil were incubated for 8hr with either 2 μ M deoxycorticosterone (+DOC), 5 μ M diacylcortidizole (+DAC), or with ethanol as a control (-). β -galactosidase(β -gal) enzyme assays were performed as in figure 2-4. Similar results were obtained with strain W303-1B (data not shown).

Table A1-2.

Receptor	Hormone	β-gal
N795	-	18
	+ DOC	1130
	+ DAC	1632
N795R488Q	-	3
	+ DOC	10
	+ DAC	8
N795R489K	-	2
	+ DOC	3
	+ DAC	18

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Appendix Two:

High Efficiency Oligonucleotide-Directed Mutagenesis Using Sequenase

SUMMARY

We have employed oligo-directed mutagenesis to introduce a single base change in the rat glucocorticoid receptor. T4 polymerase was replaced by SequenaseTM in the standard complementary DNA strand synthesis reaction. We find that the use of SequenaseTM accurately increased the mutagenesis efficiency of this template by about 1000-fold compared with the use of T4 DNA polymerase.

Our goal was to create a unique XhoI restriction site at amino acid 414 in the rat glucocorticoid receptor cDNA. We employed oligonucleotide-directed mutagenesis (Kunkel, 1985) to create a conservative single base change as shown:

Wild-Type Receptor Sequence: 5'- GGGTA CTCAAG CCCTGG - 3'

Mutated Receptor Sequence: 5'- GGGTA CTCGAG CCCTGG - 3'

A 1.7kb fragment of the receptor cDNA (amino acids 1 to 556) was cloned into the Bluescript (-) plasmid (Stratagene). Strain CJ236 (dut-ung-) was transformed with the Bluescript plasmid containing the cDNA insert. Uracil-containing single stranded DNA was obtained by VCSM13 helper phage rescue (Kunkel et al., 1987).

A template competent for DNA synthesis was prepared by annealing 20ng of the 17-mer (shown above) with 200ng of uracil-containing template in a 10 μ l volume containing 10mM Tris-HCl(pH 7.5), 2mM MgCl₂ and 50mM NaCl. Reactions were heated to 75°C in a beaker, allowed to cool to room temperature (25°C) over a 60 min period and placed on ice.

The synthesis reaction was carried out by adding the following components to the 10 μ l annealed reactions: 1 μ l of 10X synthesis buffer (0.4mM dNTPs, 1mM ATP, 20mM Tris-HCl (pH 7.5), 4mM MgCl₂ and 20mM DTT), 1 μ l (5 units) of T4 DNA ligase and 1 μ l (5 units) of either T4 DNA polymerase or SequenaseTM. Reactions were incubated on ice for 5 minutes, at 25°C for 5 min and at 37°C for 60 min. Reactions were then precipitated directly with ethanol after adding 5 μ g carrier tRNA and TE buffer to 100 μ l. DNA pellets were washed with 70% ethanol, dried *in vacuo*, resuspended in

20 μ l H₂O and ligated with T4 DNA ligase for an additional 60 min at room temperature. One-tenth of the ligated template was transformed into E. coli strain DH5 (dut+ ung+). The data in Table A2-1 summarizes the transformation and mutagenesis efficiencies using T4 DNA polymerase versus SequenaseTM on this template.

Ten clones from the SequenaseTM transformants containing a XhoI site were subjected to restriction analysis and all contained only a single A to G change at amino acid 414.

Table A2-1: Use of Sequenase in Site-Directed Mutagenesis.

Shown is the DNA synthesizing capability of Sequenase versus T4 DNA polymerase in the site-directed mutagenesis reaction based on the number of ampicillin resistant colonies (transformants) obtained from *E. coli* strain DH5 using 20 ng of newly synthesized template. The percentage of clones containing the desired site-directed mutation was deduced from the presence of a new restriction site (XhoI site) in the template and from direct sequence analysis (see text).

Table A2-1.

Enzyme	Transformants	XhoI Site
T4 DNA Polymerase	36	8
Sequenase™	>5,000	59

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Appendix Three:
Chemical Mutagenesis of Glucocorticoid Receptor Sequences

The following protocol describes the methodology employed to chemically mutagenize sequences encoding the zinc finger domain of the glucocorticoid receptor (Scheda et al., 1989).

A fragment of the N556a-containing yeast shuttle plasmid pG-D (encoding receptor residues 414-556) was excised using Xho I and Sst I and inserted into Xho I and Sst I polylinker sites in Bluescript M13+. Chemical mutagenesis was performed in a 50 μ l volume in an eppendorf tube containing the following reagents: 20 μ l of 1 μ g per μ l single-stranded (sense strand) DNA, 5 μ l of 2.5 M sodium acetate (pH 4.3), and 25 μ l of 2M sodium nitrite (added at T=0 min). Aliquots of 16 μ l were removed at three mutagenesis intervals corresponding to T=2 min, T=6 min and T=20 min and to each was immediately added a 35 μ l solution of 0.35 M sodium acetate (pH 5.4) containing 20 μ g of carrier tRNA, followed by the addition of 100 μ l of ice cold 100% ethanol. The mutagenized DNA was recovered by centrifugation in a microfuge for 5 min, and purified away from mutagen by two additional rounds of ethanol precipitation in fresh eppendorf tubes. The mutagenized templates (approximately 7 μ g) were resuspended in a final volume of 20 μ l of TE (pH 8.0).

The primer annealing step was performed in a 30 μ l reaction containing the following reagents: 20 μ l of mutagenized single-stranded template in TE (pH 8.0), 3 μ l of 10X KS buffer (200 mM Tris-HCl pH 7.4, 20 mM MgCl₂ and 500 mM NaCl), 3 μ l (75 ng) T7 primer, and 4 μ l H₂O. The 30 μ l sample was heated to 75°C for 5 min and annealed for 15 min at 40°C. The DNA synthesis reaction was performed by adding 5 μ l of 2 mM dNTPs and 20 units of AMV reverse transcriptase (Life Sciences) to the 30 μ l annealed reaction mix, followed by a 60 min incubation at 40°C. The synthesis reactions

were extracted with phenol/chloroform, precipitated with ethanol, and resuspended in 20 μ l of TE (pH 8.0).

Mutagenized, double-stranded receptor inserts were excised with Xho I and Sst I, purified from low melt agarose (Vogelstein and Gillespie, 1979) and ligated to Xho I and Sst I cleaved, unmutagenized, phosphatase-treated Bluescript M13+ DNA. The ligation mixture was transformed into *E. coli* and plasmid DNA was prepared from a pool of 10^4 bacterial transformants. The mutagenized receptor inserts were liberated with Xho I and Sst I, purified from low melt agarose, and inserted into phosphatase-treated pG-D digested with Xho I and Sst I. Purified pG-D DNA was prepared from about 10^4 *E. coli* transformants to give a mutagenized receptor pool.

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Appendix Four:

New Vectors for Constitutive and Inducible Gene Expression in Yeast

INTRODUCTION

The development of plasmid vectors for expression of cloned DNA sequences in yeast has aided in establishing *Saccharomyces cerevisiae* as a model system for cellular, molecular and genetic studies. Vectors that mediate high-level constitutive gene expression in yeast commonly employ the upstream activator sequences (UASs) and promoters from yeast genes encoding metabolic enzymes such as alcohol dehydrogenase I (ADH1) (1) and 3-phosphoglycerate kinase (PGK) (2). Other plasmids have been described that allow inducible gene expression in yeast. For example, the UAS and promoter region from the yeast GAL10 gene (3-5) permits selective expression of fused sequences when cells are grown on non-fermentable carbon sources supplemented with galactose. In a second example, the UAS and promoter of the yeast PHO5 gene confers strongly induced expression of linked sequences in media depleted of inorganic phosphate (6,7). These vectors are maintained at a high copy number in yeast by the 2 μ origin of replication and by the presence of dominant selectable markers such as URA3, LEU2, or TRP1 (5).

Many of the available expression systems, however, possess certain limitations. Thus, although the ADH1 and PGK vectors are generally considered to be "constitutive", expression from these promoters is actually repressed as much as 10-fold (8) and 30-fold (2), respectively, on non-fermentable carbon sources. In the case of inducible expression vectors, induction generally involves drastic alterations in growth conditions, such as carbon source changes, which have highly pleiotropic effects on cellular metabolism; moreover, the levels of expression achieved upon induction are often lower than those obtained with constitutive promoters. Finally, many of the available yeast expression

vectors lack convenient cloning sites and replicate poorly in *E. coli*.

Here we describe two novel vector systems for constitutive and inducible gene expression in *Saccharomyces cerevisiae*, that appear to remedy some of these problems. In particular, we have constructed a series of glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter-based vectors that allow convenient, high level constitutive gene expression in yeast. In addition, we have developed a hormone-inducible expression vector whose low basal promoter activity is strongly enhanced by the addition of glucocorticoids to yeast cells expressing the glucocorticoid receptor; importantly, steroid hormones are gratuitous inducers of gene expression in yeast having little or no effect on the expression of endogenous genes. The purpose of this chapter is to provide a practical discussion on the features and use of these plasmids.

Constitutive Expression

We have constructed three new plasmids (pG-1, pG-2 and pG-3) that direct high level constitutive gene expression in yeast. These vectors, derived from plasmids originally developed for expression of the rat glucocorticoid receptor cDNA in yeast (9,10), contain the very efficient yeast glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter (11,12). In addition, each plasmid contains the yeast TRP1 gene and 2 μ origin of replication, and the ampicillin resistance gene and prokaryotic origin of replication from pUC18.

Using unique restriction sites present in a polylinker in each construct, cloned genes or cDNAs can be readily inserted downstream of the GPD promoter (Fig. A4-1); transcripts initiate approximately 20 nucleotides upstream of the proximal BamHI site in each vector (13,14). As this "leader sequence" does not introduce an upstream AUG codon, translation initiation depends upon the presence of a start codon within the inserted sequences; translation then proceeds either to a termination codon within the insert, or to a cluster of termination codons in all three reading frames immediately distal to the polylinker. Polyadenylation and termination of the transcripts are conferred by sequences from the 3' untranslated region of the yeast phosphoglycerate kinase (PGK) gene that resides downstream of the polylinker.

Plasmids pG-1 and pG-2 (Fig. A4-1A,B) are identical except that pG-2 lacks a BglII site located downstream of the TRP1 gene in pG-1, thus enabling introduction of a unique BglII site into the pG-2 polylinker if needed. Plasmid pG-3 is also similar to pG-1, except that it contains a 1.7 kb insert at the BamHI site in the pG-1 polylinker, which provides additional novel cloning sites (Fig. A4-1A, C). Table A4-1 lists restriction sites

Figure A4-1: Constitutive Yeast Expression Vectors.

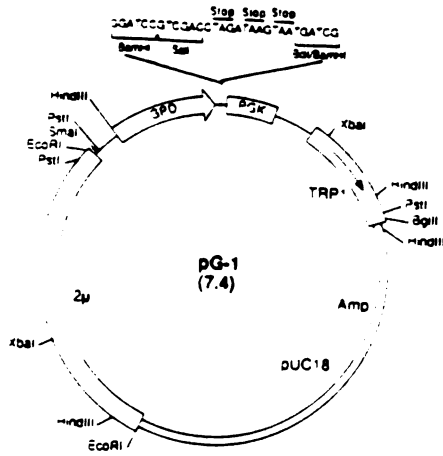
(A) Plasmid pG-1. Thick boxes represent the yeast GPD promoter, the yeast PGK transcription terminator and polyadenylation signal, the yeast TRP1 gene, and the yeast 2 μ origin of replication. The thin box indicates bacterial origin of replication and ampicillin resistance gene from pUC18. Thin lines between the 2 μ region and the GPD promoter, and between the PGK terminator and the TRP1 gene denote pBR322 sequences. The nucleotide sequence of the polylinker is shown, including restriction sites and translation termination codons. Arrows indicate the direction of transcription from the GPD promoter and of the TRP1 gene, respectively. The approximate size of the plasmid is given in kilobases.

(B) Plasmid pG-2. Plasmid pG-2 is identical to pG-1 except that the BglII site downstream of the TRP1 gene has been eliminated (see text).

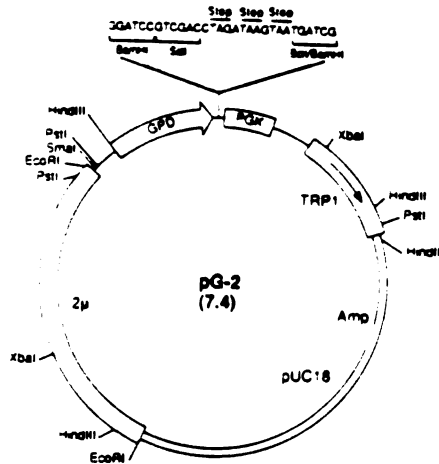
(C) Plasmid pG-3. Plasmid pG-3 is identical to pG-1, except that a fragment containing additional restriction sites has been inserted into the pG-1 polylinker at the BamHI site. The dashed line between the EcoRI sites in the polylinker denotes 1.7 kb of spacer DNA from the lac operon that has been inserted into the BamHI site (see text).

Figure A4-1.

A



B



C

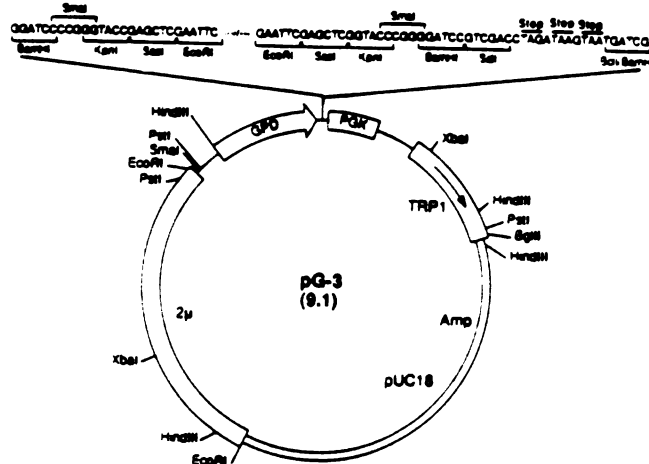


Table A4-1: Restriction Sites Present in the Constitutive and Inducible Yeast Expression Vectors.

Shown is the number of restriction sites present in each vector for twelve commonly used restriction enzymes. See Fig. A4-1 and Fig. A4-3 for map locations.

Table A4-1.

Enzyme	pG-1	pG-2	pG-3	p2UG
BamHI	1	1	2	1
BglII	1	0	1	0
EcoRI	2	2	4	3
HindIII	4	4	4	2
KpnI	0	0	2	1
NcoI	0	0	0	1
PstI	3	3	3	2
SacI	0	0	2	1
Sall	1	1	1	0
SmaI	1	1	1	1
XbaI	2	2	2	1
XhoI	0	0	0	1

present in each vector for 12 commonly used restriction enzymes.

To compare the levels of expression obtained with these constructs to standard yeast expression systems, a 2.8 kb cDNA fragment encoding the 795 amino acid rat glucocorticoid receptor (15) was inserted into the BamHI site in plasmid pG-1, and into the BamHI site of plasmid pLGSD5 (5), to yield plasmids pG-N795 and pSD-N795, respectively. Expression plasmid pLGSD5 contains the GAL10 UAS fused to the CYC1 TATA region, as well as the yeast URA3 gene and 2 μ origin of replication, and thus directs high-level expression of inserted sequences when transformants are grown on minimal galactose medium. Yeast strain BJ2168 (16,17) was transformed with pG-N795, pG-1 or pSD-N795 according to the method of Ito et al. (18), and transformants were selected and propagated on minimal glucose or galactose medium lacking tryptophan or uracil (19). Extracts prepared from these cultures were assessed for expression of the glucocorticoid receptor protein (denoted N795) by immunoblotting, using a receptor-specific monoclonal antibody.

The N795 protein was detected in extracts from both glucose- and galactose-grown transformants carrying pG-N795, and in extracts from galactose-grown transformants containing pSD-N795 (Fig A4-2, lanes 3-5). Importantly, expression from pG-N795 in cells grown on glucose or galactose was 5-fold and 2-fold higher, respectively, relative to that observed in galactose-induced cells containing pSD-N795 (Fig. A4-2, compare lanes 4 and 5 to lane 3). Reconstitution experiments with purified glucocorticoid receptor indicate that the N795 product constitutes approximately 0.1% of the soluble protein in BJ2168 transformants propagated on minimal glucose media (data not shown). Levels of N795 approaching 1% of the soluble protein have been achieved with pG-N795 in yeast strains with shorter generation times than BJ2168; in general,

Figure A4-2: Levels of Expression in Yeast by Constitutive Expression Vectors.

Shown is an immunoblot of yeast extracts fractionated by polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with a glucocorticoid receptor-specific monoclonal antibody (32) and with a goat anti-mouse IgG conjugated to alkaline phosphatase (Biorad). Expression plasmids pG-1, pG-N795 and pSD5-N795 (see text) were transformed into yeast strain BJ2168 (from E. Jones, MATa, pep4-3, prc1-407, prb1-1122, ura3-52, trp1, leu2) according to Ito et al. (18), and extracts were prepared as described (10) from cells grown in minimal yeast medium (19). Each lane contains 10 μ g of total yeast protein prepared from logarithmic BJ2168 cultures transformed with: pG-1 (lane 1), pSD5-N795 (lanes 2,3) or pG-N795 (lanes 4,5), grown for 36 hr in minimal glucose medium (lanes 1,2 and 4) or in minimal galactose medium (2% galactose, 3% glycerol, 2% ethanol) (lanes 3 and 5). The arrow indicates the migration position of the N795 (88 kD) receptor protein.

Figure A4-2.

N795 →



1 2 3 4 5

both strain background and insert characteristics appear to influence expression level (data not shown).

Growth of pG-N795 transformants in rich media allows strains to be "cured" of the expression plasmids at a rate of about 0.1% per generation. The pUC18 origin of replication facilitates efficient shuttling of the plasmids between yeast and *E. coli* (10); plasmid yields from minilysate preparations are approximately 10 µg per ml of bacterial culture.

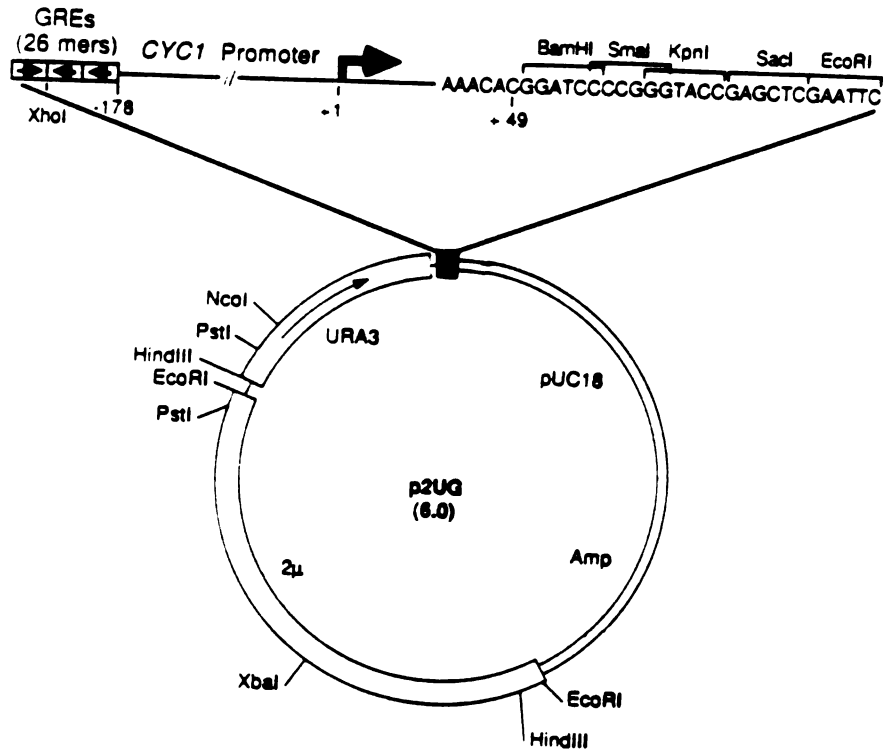
Inducible Expression

Mammalian steroid receptors maintain their activities as conditional transcriptional regulators when expressed in heterologous species such as *Saccharomyces cerevisiae* (9,20) and in *Schizosaccharomyces pombe* (21). These findings prompted the development of a glucocorticoid-inducible yeast expression vector termed p2UG (21). Plasmid p2UG contains three tandem 26 bp glucocorticoid response elements (GREs) fused upstream of the yeast CYC1 promoter region; in cells containing p2UG and a second plasmid (pG-N795) that encodes the glucocorticoid receptor, the addition of glucocorticoids results in specific binding of receptor to GREs within p2UG and transcriptional enhancement of the CYC1 promoter. Thus, as in mammalian cells (22), expression of sequences inserted into a polylinker downstream of a glucocorticoid-regulated promoter in yeast is strongly dependent upon expression of the glucocorticoid receptor, and upon addition of steroid hormone to the yeast culture medium. Plasmid p2UG also contains the yeast URA3 gene and 2µ origin of replication (Fig. A4-3); selected restriction sites in p2UG are listed in Table A4-1.

Figure A4-3: A Hormone-Inducible Yeast Expression Vector.

Shown is a diagram of plasmid p2UG (21). Thick boxes represent the yeast 2 μ origin of replication and the yeast URA3 gene. The arrow indicates the direction of transcription of the URA3 gene. The thin box indicates the bacterial origin of replication and the ampicillin resistance gene from pUC18. The solid box (detailed above the plasmid diagram) depicts the yeast CYC1 TATA region (33) fused to three copies of a 26 bp GRE oligonucleotide in the designated orientations (9,21) derived from the rat tyrosine aminotransferase gene (34), and positioned 178 bp upstream of the 5'-most cap site (35). The nucleotide sequence of the polylinker containing the designated restriction sites is also shown.

Figure A4-3.



To determine the levels of expression from p2UG, the bacterial chloramphenicol acetyltransferase (CAT) gene was inserted at the BamHI site within the polylinker to generate p2UGCAT (21). Plasmids p2UGCAT and pG-N795 (see above) were co-transformed into yeast strain BJ2168 and selected and propagated on minimal glucose media deficient of uracil and tryptophan (19). Treatment of these cultures with the glucocorticoid deoxycorticosterone at a level of 10 μ M, resulted in a 50 to 100-fold increase in CAT expression compared to uninduced cultures (Table A4-2; see also 21). This level of enhancement agrees well with the magnitude of β -galactosidase induction observed using a closely related reporter plasmid (9); moreover, the level of CAT expression obtained upon induction of p2UGCAT was equivalent to that observed when CAT was expressed from the GPD promoter in the constitutive pG-CAT construct (Table A4-2).

An important feature of the p2UG/pG-N795 inducible expression system is that steroid hormones are gratuitous inducers in yeast; they have little or no effect on the expression of endogenous genes. Thus, GRE-linked sequences can be specifically and strongly induced without general metabolic perturbations. Moreover, induction kinetics are rapid (studies in mammalian cells reveal promoter activation with a t_{1/2} of 7-9 min after hormone addition; 23), and intermediate levels of induction can be achieved simply by titrating the levels of hormone in the culture medium between 1nM and 10 μ M (21; M.J. Garabedian and KRY, unpublished results). In principle, any gene or cDNA that contains a translation initiation sequence and a stop codon can be inserted into p2UG and faithfully expressed in yeast in a hormone-dependent manner.

Table A4-2: Hormone-Inducible CAT Expression in Yeast.

Yeast strain BJ2168 (see legend to Fig. A4-2) was transformed (18) with plasmids p2UGCAT , pG-1 or pG-N795 (see text), together with plasmid pG-CAT, which was constructed by inserting the CAT gene from p2UGCAT into pG-1. Yeast transformants were selected and propagated on minimal media deficient of the appropriate amino acids (19). Extracts were prepared according to Jones et al. (30) from cells grown in the absence (-) or presence (+) of 10 μ M deoxycorticosterone (DOC) (Sigma D-6875; prepared as a 10 mM stock in 100% ethanol and stored at -20° C). CAT activities were measured according to Sleight (31) using 10 to 70 ng of extract protein incubated with substrate for one hour at 37°C. Results from two experiments are shown; experiment 2 presents data from three independent transformants. CAT activity is expressed as: ^3H -acetylated chloramphenicol (CPM) divided by the product of Extract protein (μ g) \times Reaction time (min).

Table A4-2.

Experiment	Plasmids	CAT Activity		Induction Ratio
		-	+ DOC	
1	pG-1 + p2UGCAT	300	n.d.	-
	pG-N795 + p2UGCAT	1,100	57,000	52
	pG-CAT	60,000	n.d.	-
2	pG-N795 + p2UGCAT	450	30,000	67
	pG-N795 + p2UGCAT	300	31,000	103
	pG-N795 + p2UGCAT	400	30,000	75

Vector Constructions

Plasmid pG-1 was derived from pG-D (10) by deleting the 1.7 kb BamHI fragment encompassing a glucocorticoid receptor cDNA insert. Plasmid pG-D was constructed from pGPD-556a (9) by deleting the 5.5 kb EcoRI fragment of yeast 2 μ DNA, followed by replacement of the pBR322 sequences with pUC18. These deletions were accomplished by a quadruple ligation of the following pGPD-556a and pUC18 fragments: a BglII to EcoRI fragment encompassing pUC18; an EcoRI to XbaI fragment of yeast 2 μ plasmid; an XbaI to SacI fragment encompassing the remainder of 2 μ , the GPD promoter and the glucocorticoid receptor cDNA; and a SacI to BglII fragment encompassing the PGK terminator and the yeast TRP1 gene. The BglII site was introduced into pUC18 by adding a BglII linker to the PstI polylinker site rendered blunt-ended with T4 DNA polymerase.

Plasmid pG-1 thus contains the following sequences: the 650 bp TaqI fragment of the GPD promoter (13) whose 3' border lies at position -24 relative to the +1 ATG initiation codon (11); the 38 bp BamHI to BclI polylinker sequence from pSV7d (15); the 380 bp BglII to HindIII fragment of the transcription termination and polyadenylation region of the yeast PGK gene (24); the 850 bp EcoRI to BglII fragment of the yeast TRP1 gene (25), the 2700 bp BglII to EcoRI fragment of pUC18 (26); and the 2246 bp EcoRI to EcoRI fragment of B-form of the yeast 2 μ plasmid (27,28). The nucleotide sequence of the GPD promoter and additional cloning details are given by Bitter (11).

Plasmid pG-2 was generated by the blunt-end ligation of plasmid pG-1 linearized with BglII. Plasmid pG-3 was generated by inserting a 1.7 kb fragment into the BamHI site of pG-1; this fragment corresponds to a HincII fragment of the lac operon

(29) that was inserted via EcoRI linkers into a plasmid bearing inverted pUC19 polylinkers (Bob DuBridge, Genetech) , and liberated by digestion with BamHI.

Plasmid p2UG (21) was derived from parent plasmid pSX26.1 (9) by substituting a polylinker for CYC1 sequences downstream of position +49 as well as the β -galactosidase coding region; furthermore, the pBR322 sequences were replaced with pUC18.

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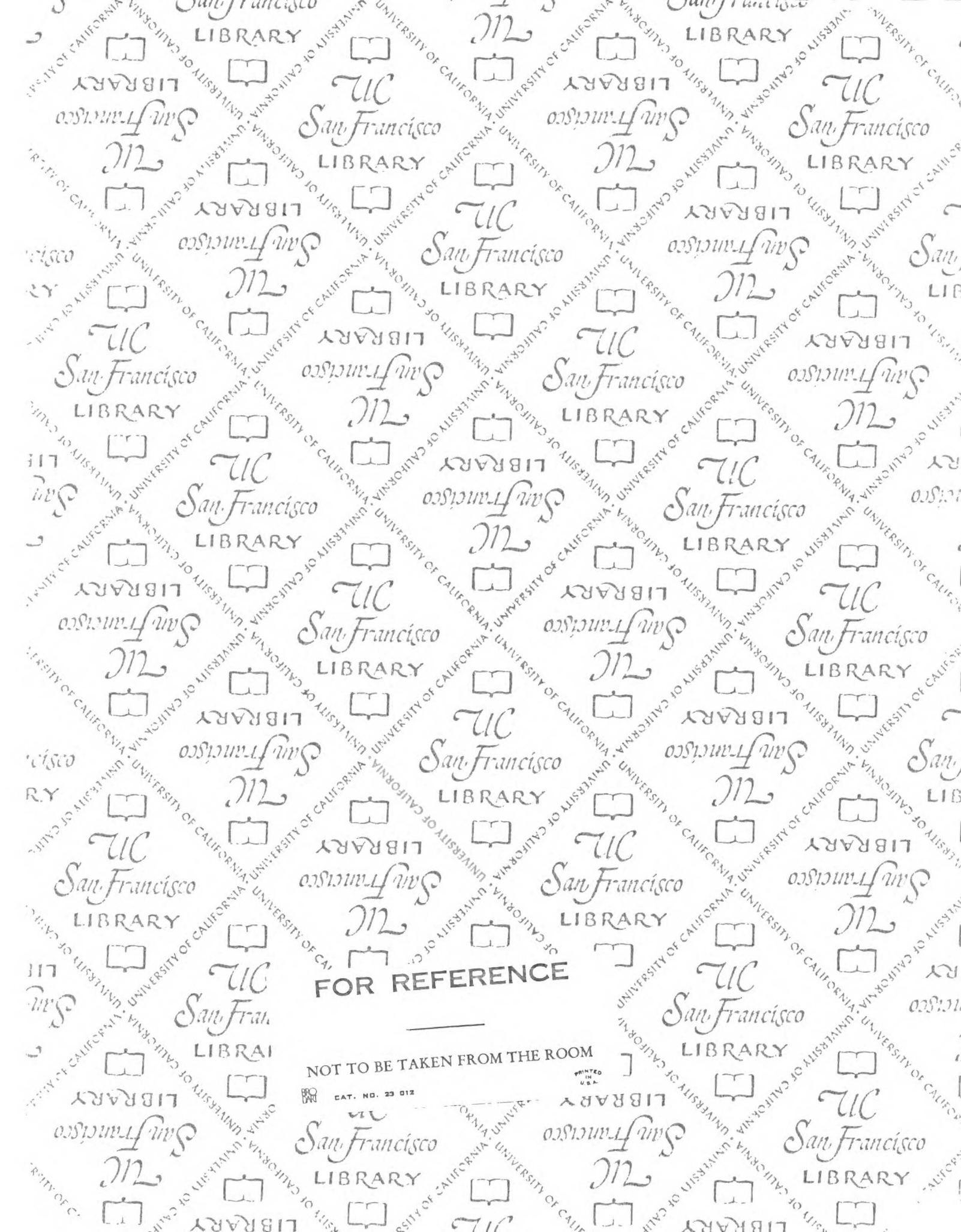
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