Three Amino Acid Substitutions Selectively Disrupt the Activation but Not the Repression Function of the Glucocorticoid Receptor N Terminus*

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A 210-amino acid region, termed enh2, near the N terminus of the rat glucocorticoid receptor, is necessary for both transcriptional activation and repression. The mechanism(s) of transcriptional regulation conferred by this region, however, are poorly understood. We screened in Saccharomyces cerevisiae a library of random mutants in the enh2 region of a constitutive glucocorticoid receptor derivative and isolated a series of multiply substituted receptors that are specifically defective in transcriptional activation. Although many substitutions in this area were tolerated, three amino acid substitutions (E219K, F220L, and W234R) within a 16-amino acid region were sufficient to disrupt the enh2 transcriptional activation function both in yeast and in mammalian cells. Although this region is rich in acidic residues, the conserved tryptophan at position 234 appears to be a more critical feature for enh2 activity; hydrophobic but not charged residues were tolerated at this position. Notably, the mutants uncoupled the activation and repression functions of enh2, as the activation defective isolates remained competent for repression of AP-1 at the composite response element p17G.

In animal cells, the effects of steroid hormones on the genome are mediated by members of the intracellular receptor superfamily, a vast collection of proteins endowed with the capacity to regulate the transcription of an equally diverse array of target genes during development and in response to specific physiological and pathological cues (1, 2). The glucocorticoid receptor (GR) is responsible for the effects of glucocorticoids and constitutes a prototype for this family of transcriptional regulators (3, 4). Upon ligand binding to its C-terminal region, GR is recruited to gene enhancers and promoters via a central zinc-binding region that is capable of recognizing specific DNA sequences termed glucocorticoid response elements (GREs). Once in the vicinity of a promoter, the receptor can mediate either stimulatory or inhibitory influences on transcription (5–7). The direction of the effect appears to be dictated by the nature of the response element recognized by the receptor and by interactions with other sequence-specific transcription factors (8–10).

In contrast to the relatively high amino acid sequence conservation in the zinc-binding and ligand-binding domains, the N-terminal regions of intracellular receptors are divergent both in size and sequence (11, 12). This implies that the N-terminal regions may contribute strongly to the class specificity of otherwise closely related receptors. Deletion of the C-terminal ligand-binding regions of steroid receptors yields constitutive (hormone-independent) transcriptional activators, implying that the N-terminal regions harbor autonomous transcriptional activation functions (13). Insertion and deletion analysis (14, 15) as well as fusions to heterologous DNA-binding domains (16) circumscribed the activation function (termed enh2 or enh1) to a region flanked by amino acids 108 and 317 in the rat GR. A prevalence of negatively charged residues, together with cross synergy and interference assays, led to the classification of enh2 as a so-called “acidic activation domain” (17). As with other activators, however, the features of this region essential for transcriptional activation have been difficult to define (16, 18, 19).

In addition to its transcriptional stimulation activity, GR has the potential to inhibit transcription driven by other activators, such as AP-1 (6, 7, 20, 21). For example, at p17G, a composite GRE from the proliferin gene, transcriptional activation by the oJun-oFos heterodimeric form of AP-1 is repressed by the hormone-bound GR (5, 6, 8). Exploiting the inability of the closely related mineralocorticoid receptor to repress in this context, Pearce and Yamamoto (22) generated receptor chimeras that demonstrated a requirement for the N-terminal region of GR for repression from p17G. Thus, a single region of GR harbors determinants for transcriptional activation and repression.

The mechanisms by which GR achieves activation or repression are unknown. It has been suggested, however, that both chromatin-dependent and -independent mechanisms of activation are at work. That is, transcriptional activation by GR expressed in Saccharomyces cerevisiae requires the Swi1, Swi2, and Swi3 proteins (23), part of a multiprotein Swi/Snf complex that may be involved in chromatin remodeling (24). Similarly, in human cells, GR activity is potentiated by a mammalian Swi2 homolog (25). Moreover, a screen for genomic mutations causing loss of GR function in yeast yielded swp73, an additional member the Swi/Snf complex (26). On the other hand, in vitro studies with “naked” DNA templates suggest that the enh2 region of GR may stimulate transcription by a mechanism independent of chromatin (27). Studies with various activators suggest that activation may involve interactions with components of the basal transcription machinery either directly or via accessory factors or coactivators (28–30). Biochemical- and interaction-based assays have yielded several molecules that

The abbreviations used are: GR, glucocorticoid receptor; GRE, glucocorticoid response element; WT, wild type; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate; MMTV, mouse mammary tumor virus; LTR, long terminal repeat; RSV, Rous sarcoma virus; VP16, herpes simplex viral protein 16.
physically interact with particular steroid receptors, including GR (31–41). Most of those factors interact with the receptor C-terminal region, which appear also to carry transcriptional regulatory activities. The functional significance and role in transcriptional activation for most of these interactions, however, remain largely unknown.

Efforts to elucidate the transcriptional regulatory mechanisms of the N-terminal region of GR would be aided by point mutants that distinguish activation from repression "surfaces," and that could be used to test the functional significance of physical interactions with potential targets. One approach to the characterization of activation domains has been to mutate frequently represented amino acid residues, such as glutamine, proline, or those with acidic side chains. This strategy, however, may fail to identify residues important for function (42–44). In this report, we describe a genetic approach in *S. cerevisiae* in which we screened a large set of rat GR derivatives carrying multiple substitutions in enh 2 for mutants that are specifically defective in transcriptional activation.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Media, and Plasmids**—The yeast strains W303–1a (MAT a ade2-1 trp1-1 ura3-1 leu2-3, 112, his3-11, 15 can1-100) and its isogenic MAT a counterpart (W303–1b) were used for receptor activity and screening. Yeast strains were grown in minimal medium with amino acids and 2% glucose or galactose (45). Plasmid selection was maintained by growing in medium lacking the appropriate amino acid(s). The CEN ARS, galactose-inducible expression plasmid, pRS114(G) consists of a 670-bp pair EcoRI (blunted) BamHI fragment containing the yeast Gal 1–10 promoter region ligated into the *SpeI* (blunted)/BamHI sites of the plasmid pRS314 (46). To generate pRS114(G)/N525, the coding region of rat GR amino acids 1 to 525 (13) was placed downstream of the Gal 1 promoter as a BamHI fragment, and some restriction sites in the 3’ polylinker region were eliminated by digesting with *EcORI* and Kpnl, treating with T4 DNA polymerase, and re-ligating. Digestion of pRS114(G)/N525 with NcoI and Bsp120I, repair of the termini to blunt ends with T4 DNA polymerase, and religation produced a precise deletion of amino acids 108 to 317. The CEN ARS plasmid pHCA N795 expresses wild-type full-length GR from the constitutive yeast glyceraldehyde-3-phosphate dehydrogenase promoter. The 2 μ reporter plasmid pα282X contains a minimal CYC1 promoter linked to three copies of a GRE derived from the tyrosine aminotransferase gene driving the expression of the E. coli Luc Z gene (47).

**Library Construction and Screening**—A library of mutants targeted to the region between amino acids 108 and 317 of rat GR was constructed by the polymerase chain reaction. The oligonucleotide primers 5’-ATCAAGACGCCTTCACTGTCC-3’ and 5’-TGTCCTCCAGAGG-TACTAGTCTC-3’ were used to amplify an NcoI-Bsp120I fragment of GR under mutagenic conditions: 67 mM Tris-HCl, pH 8.8, 16 mM (NH₄)₂SO₄, 6.1 mM MgCl₂, 0.17 mg/ml bovine serum albumin, 10 mM β-mercaptoethanol, 10% (v/v) dimethyl sulfoxide, 0.5 mM MnCl₂, 0.2 mM dATP, and 1 mM restricting deoxyribonucleotides phosphates. The resulting product was digested with NcoI and Apal (residues 108 to 317), ligated to pRS314(G)/N525 cut with the same enzymes, and transformed into DH1α cells. After plating, an estimated 6 × 10⁶ transformants were harvested for plasmid preparation. Limited sequence analysis of random clones indicated an overall mutation rate of approximately 2% at the nucleotide level.

For screening, W303–1b cells harboring the full-length receptor expression plasmid pHCA N795 and the reporter plasmid pα282X were transformed with the mutant library and plated in selective medium. Transformants (~1.7 × 10⁶) were replated-plated three times onto selective media containing: (a) 2% galactose; (b) 2% glucose and 10 mM deoxyxcorticosterone; or (c) galactose and deoxyxcorticosterone. Receptor activity was assessed by an agar overlay procedure as described (48). Colonies displaying a decreased constitutive receptor activity compared to the WT in galactose plates (light blue color) but maintaining WT activity upon receptor treatment were isolated and characterized. The isolates were then tested for the level of expression of the mutant receptor by immunoblotting using the monoclonal antibody BuGR-2, which recognizes a nonmutagenized epitope on the receptor (49). Plasmids rescued from 16 isolates displaying GR expression levels similar to the WT protein were sequenced across the mutagenized region and further characterized. In all cases, the phenotype was confirmed to be plasmid-borne by retransformation and retesting. To segregate the mutations present in individual mutants, plasmids were digested with different pairs of enzymes (NcoI, SalI, NsiI, EcoN1, BglII, and Apal). The appropriate fragments containing the desired region were then inserted into pRS314(G)/N525 cleaved with the same enzyme pairs. In some cases, individual amino acid changes were introduced by site-directed mutagenesis using the method of Kunkel (50) and confirmed by sequencing.

**β-Galactosidase Assay**—Yeast cultures were grown to saturation in glucose-selective media (200 μl) in 96-well microtiter plates under constant agitation at 30 °C. To prevent evaporation, the plates were maintained in a humidified environment. Cultures were diluted 1:40 in fresh galactose (2%) selective medium and grown for an additional 14–16 h. Cell density was determined as absorbance at 650 nm. Cells were permeabilized in microtiter plates by mixing 10 μl of each culture with an equal volume of twice concentrated reaction buffer (120 mM sodium phosphate, pH 7.0, 10 mM KCl, 1 mM MgSO₄, and 20 mM β-mercaptoethanol) supplemented with 5% CHAPS and incubated 5 min at 25 °C under constant agitation. Reactions were initiated by the addition of 180 μl of 0.5 mM chlorophenol red-β-D-galactopyranoside (Boehringer Mannheim) in reaction buffer prewarmed at 37 °C. Progress of the reaction was monitored at 37 °C in a temperature-controlled microplate reader (Molecular Devices, Sunnyvale, CA) by measuring the difference in the absorbances at 550 nm (test) and 650 nm (reference) at 2-min intervals. Activity units are defined as shown in Equation 1.

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A = \frac{\Delta OD_{550-650} \times cv}{OD_{650} \times sv} \quad \text{(Eq. 1)}
\]

\(\Delta OD_{550-650}\) is the rate of change in absorbance difference between 550 and 650 nm (in OD650 units), \(OD_{650}\) is the absorbance of the cultures at 650 nm in the microtiter plate, \(cv\) is the volume of culture used to determine \(OD_{650}\) (200 μl standard), and \(sv\) is the volume of culture used for the assay (10-μl standard). This simple and high throughput assay is similar in sensitivity and reproducibility to the standard Miller assay (51).

**Mammalian Cell Culture, Plasmids, and Transfections**—F9 mouse embryonic carcinoma cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Inc.) supplemented with 5% fetal bovine serum. The reporter plasmids pCAT-T-Luc and pGFP-Luc contain three copies of the tyrosine aminotransferase (52) and proliferin (6) GREs, respectively, upstream of a minimal Drosophila alcohol dehydrogenase promoter (ads ~33 to +53) and the luciferase gene. The reporter plasmid pMMTV-LTR-Luc was constructed by ligating a 501-bp XhoI fragment from MMTV LTR (13) from +1183 to +256) that had been rendered blunt with Klenow fragment of DNA polymerase at the Smal site of pG-L3 Basic (Promega Corp.). The plasmids p6RGR (16), p6GR Δ108–317 (9), and p6RGR N525 (16) allow the expression of full-length GR, and deletions of amino acids 108 to 317 and 526 to 795, respectively. To construct mammalian expression vectors for the mutants identified in yeast, the mutant region was transferred to a yeast 5’-end pair-revert plasmid (pN525 fragment to pRGR N525, Deletion of amino acids 108–317 was achieved in the same way as for the yeast vector (see above). Full-length versions of these plasmids were obtained by ligating a 1501-base pair Pstl/NcoI fragment from pRGR containing the remaining GR coding sequence into the same sites of the corresponding N525 derivatives. The above plasmids as well as the empty (pRSB), c-Jun (pRSV-Jun), cFos (pRSV-Fos/53), and β-galactosidase (pβR-gal/22) expression vectors are derivatives of p65 in which the RSV promoter drives the expression of the corresponding gene. Cells from subconfluent 100-mm dishes were diluted 10-fold and seeded in 60-mm dishes 24 h prior to transfection by the calcium phosphate precipitation method (9). Following overnight exposure of the cells to the DNA-calcium phosphate mixture, cells were glycerol-shocked (15% glycerol in DMEM H16 for 2 min), washed once in phosphate-buffered saline lacking calcium and magnesium, and incubated an additional 24 h in fresh medium. In the case of experiments involving hormone treatment, the final 24-h incubation was in medium supplemented with 5% charcoal-stripped fetal bovine serum in the presence of dexamethasone (0.1 μM) or vehicle (0.1% ethanol). In addition to the expression plasmids indicated in the figure legends, all transfections included 2 μg of reporter plasmid and 3 μg of the ligand control plasmid. For extract preparation, phosphate-buffered saline washed cells were lysed (15 min at 25 °C) in 100 μl of reporter lysis buffer (Promega), and cellular debris was removed by centrifugation (5 min, 1000 × g, 4 °C). Luciferase activity was determined as described (9). Values were normalized to the β-galactosidase activity present in the extract, as assessed by the rate of hydrolysis of chlorophenol red-β-D-galactopyranosidase (47).
side under the same conditions described above for yeast cells. On the basis of the β-galactosidase activities observed, no significant alterations in the activity of the RSV promoter were detected under the conditions used.

RESULTS

Isolation of Transcriptionally Defective N-terminal Mutants—Glucocorticoid receptor derivatives lacking the C-terminal hormone-binding region function as ligand-independent constitutive activators in mammalian cells (13). We took advantage of the ability of N525, a truncated rat GR lacking the C-terminal 270 amino acids, to activate transcription in *S. cerevisiae* (47) to screen for substitution mutants between amino acids 108 and 317 that are defective in transcriptional activation. Given the apparent functional redundancy of most activation domains and prior difficulties in further delineating the transcriptional activation function(s) present within this region, the library was designed to contain multiple base pair substitutions per molecule (12 on average). Using this library, mutants with defects in transcriptional activity (~50–70% of WT) appeared at a high frequency (~20%). None appeared to interfere with the WT full-length receptor.

To avoid inactive derivatives truncated at nonsense mutations, we chose to analyze 50 mutants displaying low but detectable levels of activity. Such mutants were still abundant but less frequent (~2.5%). After confirming that the phenotype was plasmid-borne, 16 mutants that are expressed at levels comparable to WT (see “Experimental Procedures”; data not shown) were sequenced over the entire mutagenized region. As shown in Fig. 1A, these mutants display activation defects ranging from mild (70% of WT) to severe (~5% of WT). Analysis of the activity of these mutants using various reporter constructs indicated that the phenotype is independent of the exact sequence, location with respect to the transcription start site, or whether they were episomal or chromosomal (data not shown). When these mutants were analyzed in mammalian cells (Fig. 1B), their phenotypes appeared to be somewhat accentuated, whereas their rank order was similar, implying that activation was similarly affected in mammalian cells and in yeast. Interestingly, certain mutants were disproportionately defective in mammalian cells (e.g. see mutant enh2.9). This effect does not appear to reflect the higher temperature at which mammalian cells are maintained (37°C *versus* 30°C), because the pattern of activity in yeast was similar at the two temperatures (data not shown).

Sequence analysis revealed that the mutation frequency in this panel of mutants is 2.5 and 4.9% at the nucleotide and amino acid levels, respectively, yielding an average of 10.3 amino acid changes per molecule. The spatial distribution of the mutations revealed no apparent clustering, either by visual inspection or by comparison to a theoretical set of random mutants computer generated to have the same overall mutation frequency as the actual mutant set (data not shown). An inverse relationship was observed between transcriptional activation and the number of amino acid changes (* insets* of Fig. 1). On average, about four mutations were required for a 2-fold decrease in activity, and an order of magnitude decrease was associated with derivatives carrying perhaps 10 or more mutations. Although this may imply that the genetic target is large or that the underlying function may be redundant within enh2, it is also possible that the phenotype observed in these highly mutagenized isolates might be conferred by only a subset of the amino acid changes that they incurred.

The Activation Defect Maps to the Central Region of enh2—As a first step to determine which mutations were responsible for the phenotype, we transferred subregions from five of the mutants, each carrying 12–16 mutations, into an otherwise wild-type N525 background. As seen in Fig. 2, the phenotypes of the analyzed mutants appeared to be contributed by mutations in enh2 subregion II, between amino acids 180 and 237. Notably, the 10–20-fold activation defects of mutants enh2.20 and enh2.30 could be fully accounted for by five mutations within their respective subregion II. In contrast, subregions I and III of each mutant contained similar densities and types of changes, but neither contributed to the phenotype. Subregion II overlaps a segment of human GR (corresponding to amino acids 208–248 of rat GR) that has been proposed to contain a deletion spanning the mutagenized region (Δ enh2) as well as individual mutants identified in the screen. Cells were cultured and assayed as described under “Experimental Procedures.” Data represent the averages (bars, S.E.) of at least six independent transformants and is expressed as the percentage of the wild-type activity, 1230 ± 81 units. No detectable activity above background was observed in uninduced cultures. B, F9 embryonal carcinoma cells were transiently transfected as indicated under “Experimental Procedures” with the reporter plasmid pΔTAT-Luc (3X TAT GRE-adh-Luciferase) and 1 μg of the empty vector (pRS68), or vectors for the expression of the indicated N525 receptor derivatives. Data represent the averages (bars, S.E.) of three to five independent transfections performed in triplicate and are expressed as a percentage of wild-type activity, 3.24 × 10^3 ± 7.17 × 10^2 units. * Insets* of the activities of the mutants in yeast (A) and mammalian cells (B) are plotted as a function of the number of amino acid changes in each mutant.

![Fig. 1. Transcriptional activity of N525 harboring enh2 mutants in yeast and mammalian cells.](image)

A

![S. cerevisiae](image)

B

![F9 carcinoma](image)
deduced amino acid sequences of subregion II in eight strongly affected mutants (Fig. 3) revealed that a unique tryptophan residue, Trp²³⁴, was mutated to arginine in five cases, whereas it was unaltered in eight mutants with weaker phenotypes (data not shown). We infer that Trp²³⁴ might be a particularly important residue for enh2-mediated activation.

Three Substitutions Disrupt the Activation Function of enh2—To identify precisely the mutations that abrogate activation by enh2, we bisected subregion II of mutant enh2.30 and analyzed their separate activities in yeast and mammalian cells. We found that enh2.30I A, which contains two mutations (Q194R and V202A), retained a nearly wild-type activation function, whereas enh2.30IB, which harbors three carboxy-terminal mutations within subregion II (E219K, F220L, and W234R), was severely defective (Fig. 4). Interestingly, all three residues are conserved in other species (bold and underlined in Fig. 3). All pairwise combinations of the three mutations within 30IB yielded only mild phenotypes in yeast, and the individual mutations displayed modest or undetectable effects (Fig. 4, left). In murine F9 cells, the patterns were similar, but as in previous experiments (Fig. 1), the mutant phenotypes were slightly stronger. Notably, the W234R point mutant exhibited a 4-fold activation defect in the F9 cell background, and the E219K/W234R double mutant was nearly as defective as 30IB itself (Fig. 4, right). Taken together, these results indicate that many amino acid substitutions in enh2 have little or no effect on the transcription activation function within this domain, but that mutation of two or three critical residues largely abrogates this activity.

Further characterization of the 30IB mutant revealed that enh2 transcriptional activation is similarly defective in simian CV-1 cells, and that the defect could not be overcome by overexpression of the mutant receptor (data not shown). In addition, the 30IB mutant phenotype was independent of the response element, because the activation defect was maintained in yeast constructs in which the GR zinc-binding region was replaced by the DNA-binding domain of the yeast regulator Gal4 and tested on a yeast promoter bearing Gal4-binding sites (30IB activity in that context was 13.9% ± 0.7 of WT; n = 16). Taken together, these results are consistent with the idea that the 30IB mutations directly disrupt the transcriptional activation function of enh2.

Hydrophobic but Not Charged Residues Are Tolerated at Position 234—To explore in more detail the role of Trp²³⁴, we examined in yeast the effects of other amino acid substitutions at this position, in isolation or in the presence of the accompanying mutations found in 30IB. Within this region rich in
acidic residues, the replacement of Trp234 by the strongly basic arginine residue might conceivably compromise transcription by reducing the overall acidic character of the region. As seen in Fig. 5, however, substitution of Trp234 by glutamic acid produced a phenotype similar to that of W234R, either alone or in combination with E219K, or both E219K and F220L. Only when combined with F220L did we observe a milder phenotype. Thus, regardless of the polarity, it appears that charged residues are not well tolerated at position 234. In contrast, the W234F mutant had no effect on enh2-mediated transcription. Thus, reminiscent of the importance of hydrophobic residues in the activators VP16 and p53 (55, 56), activation by enh2 may be more dependent on specific hydrophobic residues than on its overall acidic character. This is consistent with the observation that alanine substitutions of multiple negatively charged residues in this area, such as E219A, had only modest effects on transcriptional activation (57).

Effect in Full-length GR—The truncated N525 derivative of GR facilitated isolation of activation-defective enh2 mutants in our screen. We then introduced the mutations into the full-length GR, which includes a potential activation function in its C-terminal region (31, 34), and re-assessed in transfected F9 cells the mutant phenotypes in two different GRE and promoter contexts. In the case of a simple GRE, TAT3, activation by full-length GR was unaffected by deletion of enh2 (Fig. 6), implying that regulation in this context can be conferred by the C-terminal activation function (31). Consistent with this observation, enh2 mutations in the N525 derivative that compromised transcription activation from the TAT3 element had only modest effects in full-length GR (data not shown).

In contrast, the Δ enh2 full-length GR failed to activate transcription from the MMTV LTR (Fig. 6), suggesting that the N-terminal activation function is necessary for transcriptional activity in this context and that other activation function(s) do not suffice. Moreover, the enh2 mutants that were activation-
defective in the N525 backbone produced similar phenotypes as full-length derivatives (compare Figs. 7A and 1B). Parallel findings were obtained with a different GRE that requires enh2 for activation (a derivative of plfG9 that behaves as a simple GRE; data not shown). Thus, even within a single cell type, GR uses different activation surfaces in different GRE and promoter contexts. Furthermore, the functional alterations produced by the enh2 mutations affect similarly the constitutive and the full-length GR derivatives, implying that our strategy identifies nonsite activation surfaces used during the normal operation of the intact receptor.

**Activation-defective Mutants Are Competent for Repression**

Confirming and extending a previous report that N-terminal sequences of GR are required for transcriptional repression (22), we found that deletion of enh2 rendered GR unable to repress AP-1 activation from the plfG composite element (Fig. 7B). This is unlikely to reflect either global misfolding of the deletion derivative, or lack of expression, because the Δ enh2 GR remains competent for ligand-dependent activation from the TAT3 GRE (see above). Remarkably, all of the activation-defective mutants retained full repression activity at plfG (Fig. 7B). These results suggest that enh2 supports activation and repression via distinct determinants.

An alternative interpretation of these findings is that the mutant phenotypes actually reflect acquisition by the mutants of a spurious repression function that is codominant with the activation function and thus presents as a loss of activation. If this were the case, however, the mutants would be expected to display increased repression activity in circumstances where the receptor normally represses. On the contrary, a dye-response study of 30IB receptors over a wide range of transfected DNA (Fig. 8), together with a survey of 16 mutants (Fig. 7B), revealed no significant differences in repression activity between the wild-type and mutant derivatives. Taken together, our results support strongly the view that the contributions of enh2 to transcriptional activation and repression are through independent functional surfaces.

**DISCUSSION**

**Disruption of the N-terminal Activation Function of GR**—As with numerous other transcriptional regulators, GR can either activate or repress transcription depending on the DNA or protein context at the response element and promoter. Thus, GR may selectively expose or use different regulatory surfaces. The selection of functional surfaces appears to be governed by factors that interact with the receptor (3, 5), including not only other sequence-specific transcriptional regulators like AP-1 (6–8) but also the precise DNA sequences recognized by GR (9, 10). In certain contexts, activation by GR relies on determinants present in enh2. It appears then, that the mutants we have isolated fail under those same conditions to establish a functional enh2 activation surface.

The initial analysis of the mutants we have identified implied that a large number of mutations in enh2 might be required to achieve an activation-defective phenotype. Further
characterization revealed, however, that a modest number of mutations between amino acids 180 and 236 severely compromised the activity of the whole 210 amino acid enh2 domain, and that three amino acid substitutions within a 16-amino acid segment of that subregion were sufficient to preclude enh2-mediated activation. A corollary of those findings is that enh2 can tolerate a substantial number of alterations without apparent effect on its transcriptional activation function. Thus, the original correlation between activity and number of mutations appears to reflect the need for concordant alteration of specific residues in a small area, rather than a simple requirement for a large number of mutations per se. Interestingly, the three lesions in 30IIB (E219K, F220L, and W234R) appeared to cooperate to yield the final phenotype, because the individual mutations had little or no effect. The W234R mutation may be the more significant, because it had a substantial effect in mammalian cells, and was recovered multiple times in the screen in combination with various additional mutations.

Role of Hydrophobic Versus Acidic Residues—The enh2 activation function has been categorized as a so-called acidic activation domain, based on a modest prevalence of acidic residues, and on cross-squelching and synergy studies that suggest a similarity to the prototypic acidic activator VP16 (17). The substitutions in 30IIB resulted in the loss of one negative similarity to the prototypic acidic activator VP16 (17). The substitutions in 30IIB might alter a contact surface for a cellular target. The mutations in 30IIB are in close proximity to Ser224 and Ser232, which are targets for phosphorylation in mammalian cells (60) and yeast.2 This supports the idea that this region is accessible to interaction with other polypeptides. The functional role of the phosphorylation events, however, remains poorly understood. Alanine substitutions at these positions only modestly affect transcriptional activation by GR, and only in certain contexts.3 Thus, the strong phenotype of 30IIB is unlikely to be due solely to changes in GR phosphorylation.

Numerous studies have suggested that strong activation domains commonly can be dissected into multiple partially functional subdomains (44). One interpretation is that an activation surface depends on the cooperative summation of individually weak local contacts. In this view, mutations that compromise individual contacts may have only small effects on the overall activity. If this is the case, the phenotypes of 30IIB or other mutants that strongly reduce activation may reflect dominant effects in which unfavorable steric clashes have been introduced into a contact surface, thereby precluding the cooperative formation of the remaining local contacts. A full understanding of these mutations will require the identification of targets and structural characterization of the interacting surfaces.

Studies of activation domains, including enh2, have generally failed to indicate well-defined structures under physiological conditions (44), perhaps suggesting that these domains may achieve stable structures only upon interaction with their targets. For example, a recent report suggests that the VP16 activation domain is conformationally constrained upon interaction with TBP (61). In the absence of a target, Dahlman-Wright et al. (62) found that a peptide from human GR that encompasses the mutant region in 30IIB adopts an α-helical configuration in a nonpolar environment and proposed that two α-helices separated by a loop occupy that region under those conditions. Consistent with that scheme, replacement of two hydrophobic residues in the first helical segment (corresponding to Leu215 and Leu216 in rat GR) by prolines reduced activation activity (63). In this model, Glu219 and Phe220 would lie at the end of the first helix and Trp234 within the loop. Interestingly, L214P was recovered in three of our most affected mutants (see Fig. 3), twice in combination with W234R. Whether the effect of L214P reflects helix disruption by the proline or loss of hydrophobicity has not been examined.

Dissociation of the N-terminal Contributions to Activation and Repression—Deletion of enh2 compromised the N-terminal contributions both to repression and activation in the contexts examined, suggesting that enh2 is necessary for both functions. Surprisingly, all of the activation-defective mutants remained fully active for repression, despite the fact that we introduced no intentional bias for retention of this function in the screen. This implies that distinct functional surfaces confer enh2-dependent repression and activation under our conditions, and that substitution mutations that strongly affect activation commonly leave intact the repression surface. In this regard, it is interesting to consider the recent findings of Kamei et al. (64), who suggested that GR inhibits AP-1 activity at simple AP-1 sites by competing for a common factor required for activation by both GR and AP-1. At pIgR, where GR also interacts with AP-1 and alters its activity positively or negatively (5, 6), our findings show that the contributions of enh2 to composite repression are independent of the activation function affected by the mutations. The receptor must, therefore, repress the activating effects of AP-1 using an independent functional surface. Thus, as indicated also by Kamei et al. (64), repression by GR at pIgR and at simple AP-1 sites may use different mechanisms. In this context, it would be interesting to test at a simple AP-1 site the repression activity of our activation-defective mutants.

Conclusions and Perspectives—We isolated a collection of GR derivatives specifically defective in enh2-mediated transcriptional activation and showed that although the mutants remained fully competent for repression, at least at pIgR, the

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2 M. D. Krestic and K. R. Yamamoto, unpublished observations.

3 C. Jamieson and K. R. Yamamoto, unpublished observations.
activation defect was quite independent of cell and response element/promoter context. The close correspondence of the mutant phenotypes in yeast and mammalian cells validates our strategy of exploiting facile genetic manipulations in yeast to probe the mechanisms of action of a mammalian regulatory protein.

Our present studies show that the relative contributions of enh2 and C-terminal GR sequences to overall activation differ by B. D. Darimont, R. G. Grosschedl, I. Herskowitz, E. O’Shea, D. B. Starr, R. M. Krstic and C. Jamieson for the communication of results, and the members of the Yamamoto laboratory for discussion and assistance. We also appreciate helpful comments on the manuscript by B. D. Darimont, R. Grosschedl, I. Herskowitz, E. O’Shea, D. B. Starr, R. Tjian, and M. d. M. Vivanco-Ruiz.

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REFERENCES
1. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) Cell 83, 835–839
2. Tsai, M.-J., and O’Malley, B. W. (1994) Mol. Endocrinol. 8, 2241–2246
3. Yamamoto, K. R. (1996) The Harvey Lectures, in press
4. Beato, M., Herrlich, P., and Schütz, G. (1995) Cell 83, 851–857
5. Yamamoto, K. R., Pearce, D., Thomas, J., and Miner, J. N. (1992) in Transcriptional Regulation (McKnight, S. L., and Yamamoto, K. R., ed.) pp. 1189–1192, Cold Spring Harbor Press, Cold Spring Harbor, NY
6. Diamond, M. I., Miner, J. N., Yoshinaga, S. K., and Yamamoto, K. R. (1990) Science 249, 1266–1272
7. Jonat, C., Rahmsdorf, H. J., Herrlich, P., Park, K.-K., Cato, A. C. B., Gebel, S., Penta, H., and Herrlich, P. (1990) Cell 62, 1189–1204
8. Miner, J. N., and Yamamoto, K. R. (1992) Genes Dev. 12, 2491–2501
9. Starr, D. B., Matsu, W., Thomas, J. R., and Yamamoto, K. R. (1996) Genes Dev. 10, 1271–1283
10. Leffist, J. A., Thomas, J. R., and Yamamoto, K. R. (1994) Genes Dev. 8, 2842–2856
11. Segraves, W. A. (1991) Cell 67, 225–228
12. Deter-Wuldeigh, S. D., and Fanning, T. G. (1994) Mol. Phylogenet. Evol. 3, 192–205
13. Godowski, P. J., Rusconi, S., Miesfeld, R., and Yamamoto, K. R. (1987) Nature 325, 365–368
14. Hollenberg, S. M., and Evans, R. M. (1988) Cell 55, 899–906
15. Giguère, V., Hollenberg, S. M., Rosenfeld, M. G., and Evans, R. M. (1986) Cell 46, 645–652
16. Godowski, P. J., Picard, D., and Yamamoto, K. R. (1988) Science 241, 812–816
17. Tasset, D., Tora, L., Fromental, C., Scheer, E., and Chambon, P. (1990) Cell 62, 1177–1187
18. Miesfeld, R., Sako, D., Inoue, A., Schena, M., Godowski, P. J., and Yamamoto, K. R. (1988) in Steroid Hormone Action, UCLA Symposium on Molecular and Cellular Biology (Ringold, G., ed.) Vol. 75, pp. 193–200, Alan R. Liss, Inc., New York
19. Miesfeld, R., Godowski, P. J., Maler, B. A., and Yamamoto, K. R. (1987) Science 236, 423–427