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Genetic Dissection of Thyroid Hormone Receptor β: Identification of Mutations That Separate Hormone Binding and Transcriptional Activation

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The thyroid hormone receptors (TR) are members of the nuclear receptor family of ligand-mediated transcription factors. The large region of TR that lies C-terminal to its DNA-binding domain subserves functions of ligand binding, dimerization, and transactivation. Little is known regarding the structural or functional determinants of these processes. We have utilized genetic screening in the yeast *Saccharomyces cerevisiae* to identify residues involved in these functions. Random mutations of the rat TRβ1 isoform between amino acid residues 179 and 456 were screened, and mutants with reduced hormone-dependent activation of reporter gene activity were isolated. In this paper we describe the characterization of a class of mutants that exhibit a dissociation between hormone binding and transcriptional activation. These mutants retained hormone binding (>15% of the wild-type level) yet failed to transactivate a reporter gene. A number of these mutations occurred within the D region, which links the DNA-binding and ligand-binding domains of the receptor. One subset of these mutations abrogated DNA binding, supporting a role of the D region in this process. The remainder retain DNA binding and thus highlight residues critical for receptor activation. In addition, an unexpected group of "superactivator" mutations that led to enhanced hormone-dependent activation in *S. cerevisiae* were found. These mutations localized to the carboxy-terminal portion of the receptor in a region which contains elements conserved across the superfamily of nuclear receptors. The hormone-dependent phenotype of these superactivator mutations suggests an important role of this segment in ligand-mediated transcriptional activation.

Thyroid hormone receptors (TR) are members of the nuclear receptor family of ligand-mediated transcription factors (17). TR interact with DNA recognition elements known as thyroid hormone response elements (TRE) to regulate transcription of specific genes and thereby influence diverse aspects of development and homeostasis. TR may function as either homodimers or heterodimers with the retinoid X receptor (RXR) in binding to TRE sequences. By analogy to the steroid receptors (30), the TR sequence has been divided into five regions designated A/B (rat TRβ1 [TRβ] residues 1 to 101), C (102 to 169), D (170 to 237), and E (238 to 456) (throughout this paper, we have used the numbering system of Murray et al. [43] for amino acid positions). The C segment comprises the DNA-binding domain, a 68-amino-acid region containing two zinc finger elements that are highly conserved among family members. This domain confers DNA-binding specificity on various receptors in the family (40, 63). Other functions of TR, including ligand binding, dimerization, and transcriptional activation, have been attributed to the poorly defined D and E regions (33). However, attempts to identify the critical amino acid residues responsible for these various functions have met with limited success, suggesting considerable overlap in these domains. Our goal in initiating these studies was to define mutations of TR that would distinguish residues important for hormone binding and transcriptional activation.

Attempts to define domains of TR important for its various functions have been based on mutagenesis experiments that created deletions or amino acid substitutions within the coding sequences. Deletion of the A/B region of TRβ had no effect on its activity in cotransfection experiments, suggesting that this region was not critical to the functioning of the receptor (61). On the basis of a region of homology between TR and the steroid receptors, residues 281 to 300 of the TRβ isoform were found to be important for heterodimerization with RXR (45, 46). Further information has been gained from analyses of mutant receptors from individuals afflicted with the syndrome of generalized resistance to thyroid hormone (GRTH). In this autosomal dominant genetic disorder, TRβ gene mutations result in the production of receptors deficient in their responses to thyroid hormone. GRTH mutations highlight two regions of human TRβ—amino acids 310 to 347 and 417 to 453 (42, 67). The resulting receptors behave as dominant repressors of TR-specific gene transcription.

Limitations in the analysis of in vitro and GRTH mutations may have precluded the identification of important residues of TR. For studies using site-directed mutagenesis, the time-consuming nature of testing each mutation necessarily limits the number of mutations that can be evaluated. GRTH mutations are limited by the size of the patient population and the requirement that these mutations give a dominant negative phenotype. Thus, we sought to establish a scheme whereby the entire D and E regions could be investigated in an unbiased and expedient fashion.

The expression and functional analysis of various mammalian transcription factors in the yeast *Saccharomyces cerevisiae* have revealed a basic conservation in the processes of transcription and transcriptional activation in eucaryotes. This genetically malleable system has proven useful for studying the mechanism of action of steroid receptors. For example, Yamamoto and colleagues defined important residues in the DNA- and ligand-binding domains of the glucocorticoid recep-

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tor (19, 56). Vegeto et al. have described an altered-specificity mutant of the progesterone receptor that activates transcription in response to the antagonist RU486 (65). Wilson et al. (69) have described the binding site for the NGFI-B factor and have identified amino acids important for this function. Ince et al. (26) and Wrenn and Katzenellenbogen (70) have used yeast phenotypic screens to identify dominant negative estrogen receptors and have highlighted residues of the estrogen receptor important for hormone binding. All of these studies have been possible only in light of the fact that genetic and phenotypic screens can be performed in *S. cerevisiae* with relative ease.

Human TRβ and rat TRβ have also been shown to function in *S. cerevisiae* (47, 49). We describe in this paper a yeast system for the functional characterization of rat TRβ. We performed a mutational analysis of the D and E regions of TRβ to identify mutations that led to defects in transcriptional activation. In particular, we have identified mutations that retain DNA and hormone binding but are deficient in activation. These mutations identify several specific regions of TR that are critical to its function as a ligand-dependent transcriptional factor.

MATERIALS AND METHODS

Plasmids. All subcloning and transformations were done essentially as described by Sambrook et al. (54). Constructions of individual plasmids were as follows.

(i) pG2M. pG2M is a modified version of the yeast expression vector pG2 described by Schena et al. (57). *Bam*HI-digested pG2 was blunt ended by using the Klenow fragment of DNA polymerase I, and an *Mlu*I site was created by insertion of an *Mlu*I linker. This vector contains the yeast glycerol-3-phosphate dehydrogenase promoter to drive expression of exogenous DNA and a *TRP1* gene for selection.

(ii) pG2M/TRβ1. Rat TRβ1 sequences were amplified by PCR with four primers, designated 1 to 4 (see Fig. 2). Primer 1 contains an *Mlu*I site, primer 4 contains a *Sal*I site, and the overlapping primers 2 and 3 contain *Bgl*II sites. In separate reactions, regions spanned by primers 1 and 2 or by primers 3 and 4 were amplified with Vent DNA polymerase (New England Biolabs). Subsequently, these products were digested with the requisite enzymes (either *Mlu*I and *Bgl*II or *Bgl*II and *Sal*I). These fragments were then ligated into an *Mlu*I-*Sal*I-digested pG2M vector.

(iii) TRβ truncations. Carboxyl-terminal truncations were performed to create receptors of 200, 320, and 410 amino acids. The TRβ200 and TRβ320 truncations were created by amplification with primer 3 and appropriately positioned 3′ primers. The TRβ410 truncation was created by amplifying a clone that had a termination codon inserted at amino acid 410. All of these fragments were cut with *Bgl*II and *Sal*I and inserted into *Bgl*II-*Sal*I-cut pG2M/TRβ1.

(iv) GAL4-TRβ fusions. GAL4-TRβ fusion constructs were prepared in a three-step fashion. First, a *Bam*HI-*Cla*I fragment, encompassing the coding region for GAL4 amino acids 1 to 147, was isolated and cloned into *Bam*HI-*Cla*I-digested plasmid pSP73 (Promega). This construct was then digested with *Acc*I, which cuts in the polylinker of pSP73 and at position −54 relative to the GAL4 initiation codon. An *Mlu*I site was then introduced at position −54 via linker addition. Finally, an *Mlu*I-*Bgl*II fragment from this construct, encompassing the GAL4 coding region for amino acids 1 to 147, was exchanged into the corresponding region in pG2M/TRβ1. These manipulations result in the fusion of GAL4 residues 1 to 147 with TRβ residues 172 to 456. Three amino acids (Met-Ile-Ser) were inserted at the junction of the fusion. This same strategy was used to create fusions with specific TRβ mutants.

(v) pTRE-cyc1/lacZ. The yeast reporter vector, which contained two copies of the TRE_{pal} element upstream of the yeast *cyc1* promoter, was obtained from M. Privalsky, University of California, Davis (49).

(vi) pLGSD5. The construct to test the function of GAL4 fusions contained the upstream activating sequence of the *GAL1/GAL10* gene located 5′ to a *CYC1* promoter/β-galactosidase cassette (23).

(vii) CMVS4. CMVS4 is a derivative of the mammalian expression vector CMV4 (2). Since the *Sal*I site located in the polylinker is not unique in the plasmid, CMV4 was partially digested with *Sal*I, and the singly digested linear fragment was isolated. This DNA was blunt ended with Klenow DNA polymerase and resealed. Plasmids with a unique *Sal*I site in the polylinker were then identified by restriction enzyme mapping.

(viii) CMVS4/TRβ. TRβ and all mutants were introduced into CMVS4 as *Mlu*I-*Sal*I fragments.

(ix) 4XTREpal/TKCAT. 4XTREpal/TKCAT contains four copies of the TRE_{pal} element upstream of the thymidine kinase promoter, which drives expression of the bacterial chloramphenicol acetyltransferase coding region (39).

PCR. (i) Normal PCR. Reactions were performed according to the manufacturer's directions with Pfu polymerase (Stratagene) or Vent polymerase (New England Biolabs). Denaturation was at 94°C for 1 min, annealing was at 45°C for 1 min, and extension was at 72°C for 2 min, for 25 cycles.

(ii) Mutagenic PCR. The technique used for mutagenic PCR was a minor modification of that described by Leung et al. (38). Reaction mixtures contained 1 ng of template to be mutagenized, 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 6.1 mM $MgCl_2$, 0.5 mM $MnCl_2$, 6.7 μM EDTA, 170 μg of bovine serum albumin per ml, 10 mM β-mercaptoethanol, primers at 1 μM, 1 mM each dGTP, dTTP, and dCTP, 400 μM dATP, and 2.5 U of *Taq* DNA polymerase (Cetus). Cycling was performed as for normal PCR.

Creation of mutant library. Rat TRβ1 sequences were amplified with primers 3 and 4 by using mutagenic PCR conditions. Mutant fragments were isolated by electrophoresis on a 1% agarose gel and cleaved with *Bgl*II and *Sal*I. These fragments were then inserted into *Bgl*II-*Sal*I-digested pG2M/TRβ1. Ligations were transformed into SURE competent *E. coli* (Stratagene). A pool of all transformants (>2,000 individual colonies) was created, and plasmid DNA was isolated.

Yeast strains and media. Strain SSL204 (*MAT*α *his3 leu2 trp1 ade2 ura3*) was used throughout this work (1). For testing of GAL4 fusions, YM706 (*MAT*α *ura3 his3 ade2 lys2 trp1 tyr1 gal4*) was used. This strain has a deletion of all GAL4 coding sequences. Methods for growth and manipulation of yeast strains were as described previously (3). Yeast transformations were performed by electroporation by the method of Becker and Guarente (8).

Generation of antibody to rat TRβ. The receptor was overexpressed in *Escherichia coli* [BL21(DE3)/pLysS] by using a T7 RNA polymerase system. Cells were disrupted, and insoluble material was collected by centrifugation. This material was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and the receptor was isolated by electroelution. The preparation of polyclonal TRβ antibodies was performed by Babco (Houston, Tex.). Antibodies were then affinity purified from serum as described by Cama et al. (12).

SDS-polyacrylamide gel electrophoresis and Western blotting (immunoblotting). Gels were run according to the method of Laemmli (32). Two sources of extract were used. Either 400 μl of a yeast culture grown to an A_{600} of 1 or 12.5 μg of whole-cell extract (see below) was loaded per lane. Proteins were transferred to nitrocellulose filters (Costar), and immunoblotting was performed with anti-rat TRβ antibodies at a dilution of 1:750 and the ECL detection system (Amersham).

Preparation of yeast extracts. Extracts were prepared as described by Olesen et al. (48) and were used for electrophoretic mobility shift assays (EMSA) and immunoblotting analysis. Yeast cells were grown in 100 ml of minimal medium to an A_{600} of 1. Cells were harvested and washed in 5 ml of EB [0.2 M Tris-HCl (pH 8), 400 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, and 7 mM β-mercaptoethanol). The pellets were transferred to microcentrifuge tubes in 1 ml of EB and centrifuged to collect cells. After resuspension in 200 μl of EB with 1 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, and 1 μg of pepstatin per ml, a one-half volume of glass beads was added. The suspension was frozen in a dry-ice–ethanol bath and thawed on ice. Cells were then vortexed at 4°C for 20 min. A further 100 μl of EB was added, and cells were left on ice for 30 min. The suspension was centrifuged for 5 min, and the supernatant was transferred to a new tube and centrifuged for 1 h in a microcentrifuge. The supernatant was then made to 40% with $(NH_4)_2SO_4$ and gently rocked for 30 min. After a 10-min centrifugation, the pellet was resuspended in 300 μl of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 8.0)–5 mM EDTA–7 mM β-mercaptoethanol–1 mM phenylmethylsulfonyl fluoride–1 μg of leupeptin per ml–1 μg of pepstatin per ml–20% glycerol. Dialysis was then performed against the same buffer, and aliquots were stored at −70°C.

Analysis of T_3 binding. A 5-ml culture of *S. cerevisiae* was grown to an A_{600} of 1. Cells were pelleted, washed with 0.5 ml of ice-cold BB (20 mM Tris-HCl [pH 7.5], 50 mM NaCl, 2 mM EDTA, 5 mM β-mercaptoethanol, 10% glycerol, and 1 mM $MgCl_2$) and transferred to a microcentrifuge tube. After centrifugation, the pellets were resuspended in 100 μl of BB containing 0.4 M KCl and an equal volume of glass beads. The samples were then frozen at −70°C and thawed on ice prior to disruption. Phenylmethylsulfonyl fluoride was added to 1 mM, and the tubes were vortexed for 5 min at 4°C. Fifty microliters of BB was added, and samples were vortexed for 10 s and centrifuged for 5 min. The hormone binding assays were performed with 25 μl of extract and 0.5 nM ^{125}I-labeled 3,5,3′-triiodothyronine (^{125}I-T_3) (Dupont/NEN) in a volume of 250 μl, and assay mixtures were incubated for 16 h at 4°C. Nonspecific binding was assessed by including a 1,000-fold excess of unlabeled T_3. Bound counts were determined by the nitrocellulose filter binding assay of Inoue et al. (27). All assays were performed in duplicate on extracts of yeast colonies from two different transformations. Results are expressed relative to that for wild-type TR, which is set at 100%.

β-Galactosidase assays. In situ assays were performed on yeast colonies that had been replica plated and grown on minimal plates in the absence or presence of 1 μM 3,5,3′-triiodothyroacetic acid (Triac) at 25°C for 3 to 4 days. Ten milliliters of a molten solution containing 0.5 M potassium phosphate (pH 7), 0.1% SDS, 2% dimethylformamide, 0.2% X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and 0.5% agarose was poured over the plate. After the agarose hardened, the plates were incubated at 37°C for 4 to 5 h and then stored overnight at 4°C. Yeast colonies with decreased β-galactosidase activity were

easily distinguished. Quantitative assays were done as described by Himmelfarb et al. (25). All assays were repeated at least twice on yeast colonies from separate transformations. β-Galactosidase units were calculated as $(1{,}000 \times A_{420})/(\text{minutes} \times \text{milligrams of protein})$, and duplicates varied by <20% in this assay. Since the GAL4-TRβ fusions were expressed at a lower level than wild-type TRβ, Galacto-Light (Tropix Inc., Bedford, Mass.), a more sensitive luminescent substrate for β-galactosidase, was used. Yeast extracts were prepared in 100 mM potassium phosphate (pH 7.8)–0.2% Triton X-100–1 mM dithiothreitol and were assayed according to the manufacturer's directions in a Berthold luminometer. Activity was defined as follows: units = [luminescence units/(minutes × milligrams of protein)]/100.

Recovery of pG2M/TRβ1 plasmids from *S. cerevisiae*. Selected yeast colonies were streaked on plates containing 5.7 mM 5-fluoroorotic acid and grown for 3 days. Plasmid DNA was isolated by the spheroplast lysis method of Boeke et al. (10). Digestion with *Hin*dIII was performed to assess yields and to ensure that no rearrangements of the plasmid occurred in *S. cerevisiae*.

DNA sequencing. Sequencing was performed by the Sanger dideoxynucleotide method as adapted for modified T7 DNA polymerase (U.S. Biochemicals).

Separation of multiple mutations. In certain cases, multiple mutations that were present within a single mutant receptor were separated by swapping restriction fragments with the normal TRβ vector. *Bst*XI cuts at amino acid 280 in TRβ and once in pG2M. Consequently, plasmids were digested with *Bst*XI, and the appropriate fragments were exchanged with wild-type *Bst*XI fragment. Clones were sequenced to ensure the presence of the mutation. Mutations that were separated are designated with a lowercase letter (a or b) following the number of the mutant.

EMSA. Binding of wild-type and mutant TR in yeast extracts (2.5 μg) to a single-copy TRE_{pal} probe was assessed by using the binding and electrophoresis conditions described by Bodenner et al. (9), except with 2 μg of poly(dI · dC) and without hormone in the binding buffer.

Cell culture and transient transfection. CV-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. On the day prior to transfection, 10^6 cells were plated per 60-mm-diameter plate in medium supplemented with 100 nM dexamethasone and 10% fetal bovine serum stripped of endogenous thyroid hormones (55). Cells were transfected via calcium phosphate coprecipitation as described by Zilz et al. (73), except precipitate was left on cells for 12 to 14 h and dimethyl sulfoxide shock was not performed. Each plate received 3 μg of 4XTREpal/TKCAT, 0.5 μg of Rous sarcoma virus long terminal repeat-driven luciferase internal control plasmid, 50 ng of receptor expression vector, and 0.95 μg of CMVS4. Cells were then washed, and medium without or with 50 nM T_3 was added. After a 24-h culture, cells were harvested in Reporter Lysis Buffer (Promega, Madison, Wis.) according to the manufacturer's directions. Chloramphenicol acetyltransferase (CAT) assays were then performed as described previously (28), and results are expressed normalized to luciferase activity.

RESULTS

Rat TRβ function in *S. cerevisiae*. In order to achieve expression and transcriptional competence of rat TRβ in *S. cerevisiae*, we utilized a system described by Privalsky et al. (49). In this system yeast cells are cotransformed with two plasmids. The first plasmid contains the coding sequences of TRβ driven by a constitutive promoter. The second plasmid has a basal yeast (*CYC1*) promoter upstream of the *E. coli* β-galactosidase gene. Two copies of the palindromic TRE (22) are located upstream of the basal promoter. In this fashion, expression of the β-galactosidase reporter gene was coupled to binding of functional TR to its DNA recognition site.

To demonstrate expression of the rat TR in *S. cerevisiae*, we measured T_3 binding in an extract from cells transformed with the TRβ expression plasmid. Scatchard analysis showed a binding affinity for T_3 indistinguishable from that demonstrated in mammalian cells (data not shown). In addition, extracts were analyzed by Western blotting. A rabbit polyclonal antibody for TRβ recognized a 50-kDa band in extracts from *S. cerevisiae* transformed with the TR expression vector (Fig. 1B). No bands were observed in extracts from *S. cerevisiae* containing the expression vector without TR coding sequences (see Fig. 4A). Functional activity was assessed by monitoring reporter gene expression in the absence and presence of thyroid hormone. As observed in *S. cerevisiae* by others (47, 49), the expression of the reporter gene was elevated in a receptor- and TRE-dependent fashion by rat TRβ (Fig. 1A). This activity contrasts to the

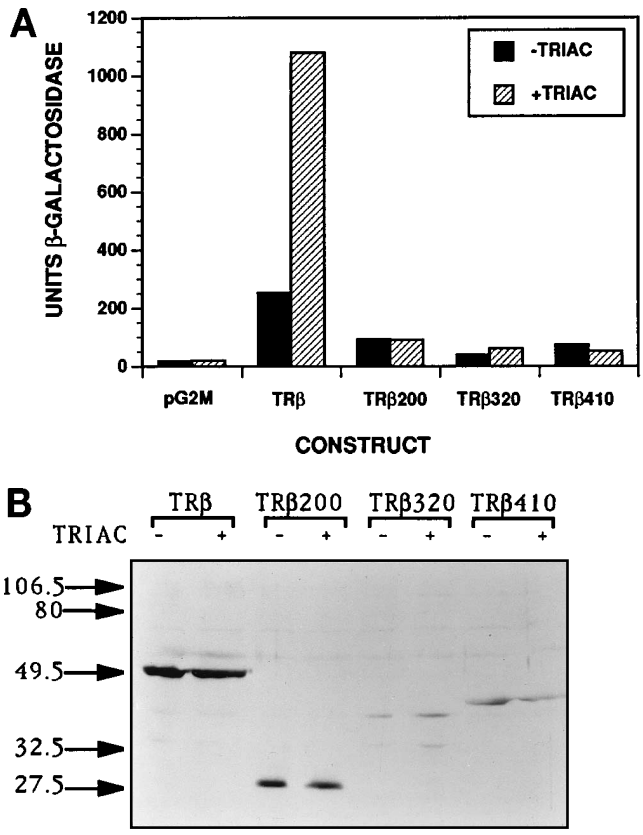

FIG. 1. (A) Activation of the TRE_{pal} reporter by wild-type TR and various C-terminally truncated forms. Truncated TR were created to contain the first 200, 320, or 410 amino acids of TRβ. Expression vectors containing these constructs were cotransformed with the TRE_{pal} reporter plasmid into *S. cerevisiae*. Transformants were grown without or with 1 μM Triac to an A_{600} of 1 and assayed for β-galactosidase activity. pG2M represents activity from the TRE_{pal} reporter in the absence of TRβ coding sequences. The β-galactosidase activity is expressed as units per milligram of protein and represent the average of duplicates that varied by <20%. (B) Western blot analysis of extracts from *S. cerevisiae* containing wild-type and truncated versions of TRβ. Yeast cells from the same cultures grown for the β-galactosidase assay were harvested by a brief centrifugation. The cell pellet was resuspended directly in sample buffer, boiled, and loaded on an SDS–10% polyacrylamide gel. After electrophoresis and transfer to nitrocellulose, the filter was probed with a polyclonal TRβ antibody and bands were visualized with the ECL detection system. The approximate molecular weights (in thousands) of prestained markers (Bio-Rad Laboratories) are indicated on the left.

observation that in mammalian cells unliganded receptor often causes an inhibition of basal transcriptional activity (11, 15). To test the hormone-dependent activity of TR in *S. cerevisiae*, we used the acetic acid analog of T_3, Triac, since it permeates *S. cerevisiae* more effectively than T_3 (49). In the presence of Triac, TRβ acts as a ligand-dependent transcriptional activator, giving a three- to fivefold increase in reporter gene activity.

To confirm that the D and E regions of TR are necessary for ligand-mediated transcriptional activation, we prepared a series of C-terminal truncations of TR and expressed these in *S. cerevisiae*. The truncations retain the first 200, 320, or 410 amino acids of rat TRβ. Expression of these constructs was confirmed by Western blotting (Fig. 1B). The TRβ truncation containing residues 1 to 200 is expressed at levels comparable to those of the wild-type TR, whereas the other two truncated TR are present at reduced levels. As shown in Fig. 1A, all of the truncated TR forms retain some hormone-independent activity, albeit reduced compared with that of the full-length

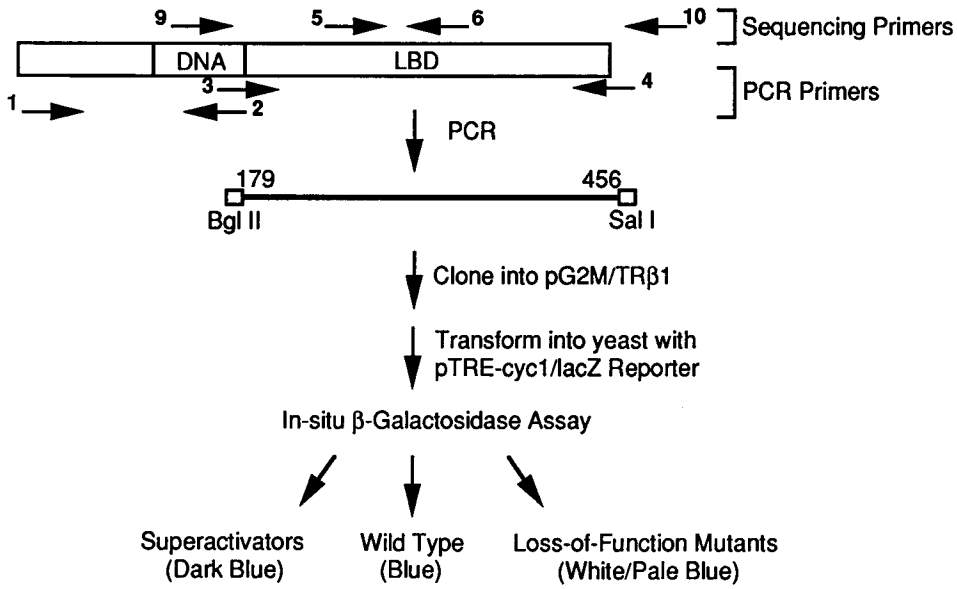

FIG. 2. Strategy for generating and screening TRβ mutations in the extended (D/E) ligand-binding domain. Primers 3 and 4 were used under mutagenic PCR conditions to amplify nucleotides 510 to 1383 of TRβ1. After digestion with *Bgl*II and *Sal*I, amplification products were cloned into *Bgl*II-*Sal*I-digested pG2M/TRβ. A library of mutants was created by passaging the ligated DNA through *E. coli*, pooling the cells, and isolating plasmid DNA. Yeast cells were then transformed with the library and the TRE_{pal} reporter. Transformants were replica plated without and with 1 μM Triac. After growth for 3 days, an in situ β-galactosidase assay was performed. The phenotypes observed and subsequent classification are as indicated.

receptor. This observation suggests that these proteins retain some ability to interact with TRE_{pal}. Thus, a portion of the constitutive activity seen in *S. cerevisiae* is encoded by the A/B or C domain of TRβ. Upon addition of hormone, the truncated receptors did not stimulate reporter gene activity. Thus, as expected, all hormone-dependent activity is abrogated by truncations of the C-terminal region of TRβ.

Design of the genetic screen. Our primary interest in pursuing a yeast system for TR action was to use the genetic capabilities of *S. cerevisiae* to define regions of the receptor critical to its function. Specifically, we were interested in defining important amino acids located in the D and E regions, comprising the 277 amino acid residues downstream of the DNA-binding domain. To this end, we devised a mutant screening strategy which is summarized in Fig. 2. The basic features of this yeast screen have been described by Garabedian and Yamamoto (19).

In the first stage of this screen, we created a cassette version of the TRβ cDNA clone that contained a *Bgl*II site at nucleotides 513 to 518 and a *Sal*I site adjacent to the termination codon. These modifications did not alter the amino acid sequence of TRβ, and this clone was functionally indistinguishable from the original TRβ when expressed in *S. cerevisiae* (data not shown). Primers that flanked amino acids 179 and 456 were then used in a mutagenic PCR amplification reaction under conditions described by Leung et al. (38). Conditions were optimized to achieve a mutation rate of two or three nucleotides for the 831-bp region (see Materials and Methods). The PCR products were digested with *Bgl*II and *Sal*I and reintroduced into the yeast expression vector in place of the wild-type sequences. Passage through *E. coli* resulted in a library of mutants containing >2,000 individual TR expression plasmids. These mutant TR sequences were then cotransformed with the reporter plasmid containing TRE_{pal} sequences into *S. cerevisiae*.

Transformed yeast colonies were isolated and replicated to plates without and with 1 μM Triac. This concentration of hormone is higher than that required for saturation of the receptor and thus eliminated the identification of mutants that had minor reductions in ligand-binding affinity. An in situ β-galactosidase assay was used to screen approximately 600 colonies. Since each plasmid had an average of two amino acid substitutions, roughly 1,200 mutations were present in the pool of mutants screened. Thus, each amino acid in the 277-residue region should be mutated, on average, four times in the colonies screened. Putative mutants (164 colonies) were either white or light blue relative to a wild-type control. Fortuitously, we identified a set of 18 colonies that appeared to give higher β-galactosidase activities than normal TRβ (see below).

Several additional screening steps were performed to eliminate mutations that were not of interest to us. First, Western blotting allowed us to identify and eliminate unstable or truncated versions of TRβ. Next, the TRβ expression plasmids were isolated and separately retransformed into *S. cerevisiae* along with the TRE_{pal} reporter plasmid. Extracts from the transformed yeast cells grown with or without hormone were prepared and assayed for β-galactosidase activity by a quantitative in vitro technique. This step ensured that the deficient phenotype was due to mutation of the TR expression vector. This assay also helped to eliminate a small number of wild-type receptors which scored as false positives in the in situ assay. On the basis of the β-galactosidase assay, mutant receptors were divided into two groups: ones that retained some level of hormone-independent activity (70 mutants) and ones that had lost all hormone-independent activity (44 mutants). Hormone-independent activity requires nuclear localization and DNA binding. Since we were most interested in identifying residues that affected the transactivation function of TR, only mutants that retained hormone-independent activity were further characterized.

We next prepared cell extracts from the mutants that retained hormone-independent activity to assess their T_3-binding abilities. This assay divided the mutants into two major classes of approximately equal size: those that retained T_3 binding and

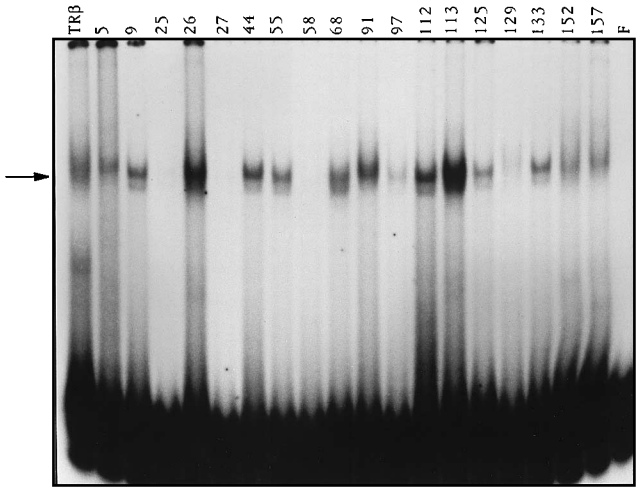

FIG. 3. EMSA of TRβ mutants with the TRE_{pal} probe. Whole-cell extracts (25 μg) containing the receptor mutants indicated above the lanes were assessed for DNA binding by using the TRE_{pal} sequence. Lane F (free) contains probe without any protein. The arrow indicates the position of the homodimer complex formed with the TRE_{pal} sequence.

those that did not. We arbitrarily chose a binding activity of >15% of that of the wild-type receptor as the criterion for inclusion in the T_3-binding class. The focus of this paper is on 18 mutants that fell into this class and on the properties of 14 mutants which gave transactivation higher than that of the normal receptor. The characterization of the remaining mutants with alterations in hormone-binding properties will be discussed elsewhere (64).

Analysis of DNA binding of activation-deficient, T_3-binding-positive mutants. We next sought to determine whether the defects in activation in the mutants that retained T_3 binding were due to an inability to bind to the DNA response element. DNA binding was assessed with the TRE_{pal} oligonucleotide in the EMSA. No bands were observed with an extract prepared from *S. cerevisiae* lacking TR coding sequences (data not shown). Extracts from *S. cerevisiae* bearing the wild-type TR gave a single complex that could be inhibited by unlabeled TRE_{pal} oligonucleotide but not a control oligonucleotide. The migration of this band was coincident with that observed with TRE_{lap} as a probe (data not shown). TRβ has been shown to bind preferentially as a homodimer to this latter sequence (5, 71). Thus, we conclude that the wild-type TRβ band observed with the TRE_{pal} sequence represents a homodimer.

Since mutagenesis was targeted to regions outside the DNA-binding domain of TR, we did not expect to find mutations that directly affected this function. To corroborate this assumption, extracts from *S. cerevisiae* with various mutant TR were prepared and tested for binding to the TRE_{pal} probe. Most of the mutants (13 of 18) bound to an extent similar to that of the wild-type receptor (Fig. 3). Minor changes in the mobilities of certain mutant forms can be seen and may represent alterations in the conformation or charge of these proteins. These mutants, being normal in both hormone binding and DNA binding, represent alterations that affect the transactivation function of TR. Surprisingly, 3 of the 18 mutants (25a, 27a, and 58a) did not detectably bind TRE_{pal}. Two other mutants (97 and 129a) bound weakly to the labeled probe. For these five mutants, the defect in binding to TRE_{pal} can account for the deficiency in hormone-dependent transactivation. Thus, there are at least two subsets of mutant TR: those that bind and those that do not bind TRE_{pal}.

Sequence analysis of mutant TR. To examine the locations of mutations giving rise to mutant phenotypes, the receptor sequences were determined by using four primers that allowed complete sequencing of the 831-bp region subjected to mutagenesis (Fig. 2). Many of the mutant TR contained more than one mutation. Several of these multiple mutations were separated, and each mutation was tested individually. In every case, the defective phenotype segregated with a single amino acid change.

The distribution of these mutations was nonrandom. Eleven of the 18 mutants contain mutations in the D region between amino acids 179 and 206. This "hinge" region, denoted such since it connects the DNA-binding and ligand-binding domains (30), contains a high concentration of positively and negatively charged amino acids. Fourteen of 24 residues in this segment are charged. Six of the mutations found in this region resulted in a conversion from a charged to a neutral amino acid residue. Eight of the mutations did not affect the net charge of the receptor, and one change (N-190 to D [N190D]) resulted in a region with more charge. Thus, mutations were not preferentially found at charged residues. Amino acids changed in more than one mutant include K-184, N-190, and L-199. Table 1 summarizes the mutations, levels of hormone binding, and reporter gene activities for these 11 D region mutants.

Interestingly, all of the mutants defective in DNA binding had mutations that mapped to the hinge region. For example, two different mutations that abrogated DNA binding were found at residue N-190 (25a and 27a). This region of the receptor has recently been suggested to regulate the rotational flexibility between the C and E domains of TR that is important for DNA binding (31). The remaining six mutants with mutations in the hinge region bound normally to the TRE_{pal} probe. In addition, all of these hinge region mutants bound hormone comparably to the wild-type receptor. Indeed, Scatchard analysis of mutant 9 demonstrated that its T_3-binding affinity was unchanged relative to that of wild-type TRβ (data not shown). Thus, the D region plays a dual role in DNA binding and transactivation of TR.

The properties of the seven remaining mutants which fail to transactivate are summarized in Table 2. These mutations are not clustered but are dispersed widely throughout the E region. Mutant 5, which has a P448S mutation, is coincident with a mutant from the GRTH syndrome. The properties of the P448H mutant have been described by Nagaya and Jameson (44). Mutant 113 contains two substitutions in a region corresponding to inactivating mutations introduced into rat TRα, as described by Lee and Mahdavi (36). Mutants 152 and 157 contain mutations that are 6 amino acids apart and immediately downstream from the heterodimerization domain defined by O'Donnell et al. (45, 46). The locations of changes in mutants 26, 112, and 125 have not been implicated in previous work. However, mutants 26 and 68 both contain changes of L-261, in mutant 26 to a proline and in mutant 68 to a glutamine.

The 18 mutants exhibited a spectrum of hormone-independent and hormone-dependent activities. Several of the mutants (e.g., mutants 26, 55, 97, 133, 152, and 157) retained a modest degree of response to hormone but much less than that observed with the wild-type receptor. Others showed no hormonal induction of reporter gene activity. A range of activities in the absence of hormone was also noted. Many receptors showed a hormone-independent activity comparable to that of the wild-type receptor, indicating that the defect was limited to the hormone-activated response. Others showed a decrease in hormone-independent activity as well as a depression in the hormone-stimulated level. Given these differences, a compar-

TABLE 1. TR mutants with mutations within the D (hinge) region[a]

TR	β-Galactosidase (U)		% T3 binding	Mutation(s)
	−TH	+TH		
TRβ (wild type)	353	1,141	100	
Mutants that do not bind TRE_{pal}				
25a	50	55	46	N190D
27a	100	177	114	N190S
58a	250	333	110	K179R, R184G
97	104	256	119	K206R
129a	108	161	102	R194G, L199P
Mutants that bind TRE_{pal}				
9	91	104	100	I203T
44a	163	130	124	L199P
55a	206	515	167	I187T
68	374	418	93	E192G, E198G, L261Q
91	125	198	119	R184G, E215G
133	170	467	139	R191H

[a] Wild-type or mutant TR expression vectors were introduced into *S. cerevisiae* together with the TRE_{pal} reporter vector and grown in the absence (−TH) or presence (+TH) of 1 μM Triac. β-Galactosidase activity in extracts of these yeast cells was measured. Hormone binding was estimated by incubating extracts in the presence of 0.5 nM ^{125}I-T_3 for 16 h and then separating bound hormone from free hormone by filtration on nitrocelluose. All values for T3 binding are expressed relative to that for wild-type TRβ, which was set at 100%. All measurements were repeated with yeast cultures from a separate transformation and gave comparable results. β-Galactosidase activity in yeast colonies that were transformed with the reporter vector and the parental TR expression vector was 15 to 20 U.

ison of mutant TR activities as fold inductions has little meaning. Some of the variation in reporter gene activity may reflect modest differences in levels of mutant receptors expressed in *S. cerevisiae*. However, as can be seen from Fig. 4A, most of the mutant forms were expressed at levels similar to that of the wild-type receptor. Thus, the differences in activities of these mutants likely reflect changes in their transcriptional activation potentials.

TR mutants that superactivate. Although our screen was designed for isolating loss-of-function mutants, we serendipitously recovered a set of TR mutants with enhanced transactivation ability. These colonies appeared dark blue relative to colonies with wild-type TR in the in situ screen. These mutant forms have been designated superactivators. Because these mutants may modify the transactivation function of the receptor, we have pursued the characterization of this unexpected group of mutants. The hormone binding, reporter gene activities, and mutational changes of these receptors are summarized in Table 3.

Most of these superactivators have increased transcriptional potencies only in the presence of hormone. However, some show increased hormone-independent activity as well. Only one, mutant S20, does not show any hormone-dependent increase in reporter gene activity. Although some mutants demonstrate only modest increases in hormone-dependent activation, mutants S4 and S15 have strikingly increased hormone-dependent activities. These mutants show a 10-fold increase in activation in response to hormone. Interestingly, both contain the same K419E change. Mutant S3 also contains this mutation, but it activates only three- to fourfold more than wild-type TR. Presumably the impact of the K419 change is blunted by the presence of the two other changes present in this protein.

A possible explanation for the superactivator phenotype is that these mutants have increased levels of protein expression. However, this did not appear likely in light of the fact that the hormone-independent activity was unchanged in the majority of these TR. To formally exclude this possibility, we performed Western analysis on extracts of *S. cerevisiae* that contained these proteins (Fig. 4B). With the exception of mutant S17, all

TABLE 2. TR mutants with mutations within the E region[a]

TR	β-Galactosidase (U)		% T3 binding	Mutation(s)
	−TH	+TH		
TRβ (wild type)	353	1,141	100	
5	333	399	37	P448S
26a	322	484	43	L261P
112	296	441	142	N359S
113	345	480	27	Q236P, F240S, N326D S356T
125	346	440	15	T322A, F394L
152	214	697	60	L300P, A431V
157	237	795	51	A226V, E306G

[a] See Table 1, footnote *a*.

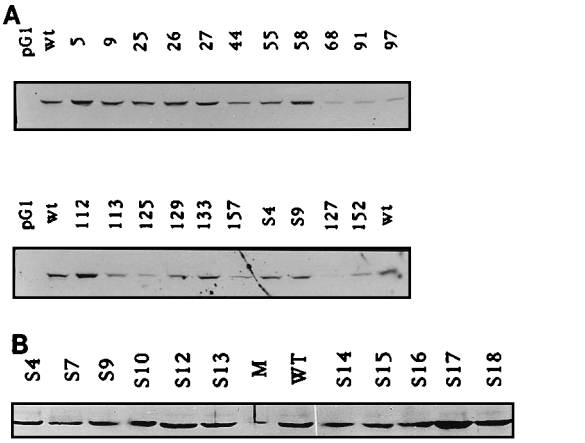

FIG. 4. (A) Western blot of TR mutants that lost wild-type activation function but retained T_3 binding. Yeast whole-cell extracts were prepared from cells containing the mutant TRs indicated above the lanes. pG1, extract from *S. cerevisiae* containing expression vector without TRβ coding sequences; wt, extract from *S. cerevisiae* containing TRβ. Each lane was loaded with 25 μg of extract, and a Western blot was performed as described in Materials and Methods. (B) Western blot of superactivator TRβ mutants. These extracts were prepared as described for Fig. 1B. M, markers; WT, wild type. Mutants S3, S19, and S20 are not included in this Western blot.

TABLE 3. TR mutants that superactivate transcription[a]

TR	β-Galactosidase (U)		% T3 binding	Mutation(s)
	−TH	+TH		
TRβ (wild type)	444	1,132	100	
S3	206	4,384	ND[b]	G246R, R277G, K419E
S4	631	12,149	42	K419E
S7	80	2,039	21	D211G, Y401C, E455G
S9	317	2,883	97	E452A
S10	1,099	3,340	61	K415E
S12	931	2,242	56	K179T, I275T, E452G
S13	585	2,629	174	F412S, R433H
S14	102	2,004	40	E364G
S15	1,200	11,906	9	K419E, F450S
S16	380	2,927	166	E198G, R424Q
S17	585	3,682	196	K218R, H436R
S18	515	2,079	172	T421M
S19	212	2,169	ND	F288L, C293R
S20	6,663	2,559	ND	V279E, K283R, K301Q

[a] See Table 1, footnote *a*.
[b] ND, not determined.

of these mutants are expressed at levels less than or equivalent to that of wild-type TR. In fact, the strongly active S4 is actually expressed at a level slightly less than that of the normal receptor.

The T_3-binding abilities of these receptors were also analyzed. Most of the mutant TR in this group bound T_3 comparably to the wild-type TR. Surprisingly, one of these mutants (S15) showed little T_3 binding, despite the fact that it gave a strong induction of reporter gene activity in the presence of hormone. We presumed that this mutant retained a reduced affinity for hormone. This was confirmed by analysis of reporter gene activities of mutants S4 and S15 and wild-type TR grown with various amounts of Triac. The hormone-dependent activation by S15 required a significantly higher concentration of Triac than that by wild-type TR or S4 (data not shown). Whereas wild-type TR and S4 responded well at 10 nM Triac, S15 displayed no activity at this hormone concentration.

The most intriguing feature of the superactivators is the distribution of the mutations that give rise to the phenotype. Eleven of the 14 superactivators have mutations between amino acid residues 415 and 456 at the C-terminal end of the receptor. This region in the steroid/thyroid receptor family of proteins has been implicated in transactivation (7, 16, 52).

Effects of TRβ mutations on activities of GAL4-TR fusion proteins. To verify that the mutations identified affected the transactivation function of TR, several mutants were tested in the context of a heterologous DNA-binding and dimerization domain. For this purpose, we chose the well-characterized DNA-binding and dimerization domain of the yeast GAL4 transcriptional activator. The domain spanning amino acids 1 to 147 of GAL4 has been shown to function when fused to a wide variety of transactivating proteins, including members of the steroid receptor family (66). In addition, GAL4-TR fusion proteins have been shown to exhibit both transactivation and repression functions when introduced into mammalian cells (6). In a GAL4-TRβ fusion, the GAL4 portion of the fusion should not require elements outside amino acids 1 to 147 for efficient DNA binding. This strategy thus allows us to directly test effects of mutations on transactivation.

A fusion protein was prepared by joining the coding sequences for GAL4 amino acids 1 to 147 to the region coding for amino acids 172 to 456 of TRβ in the yeast expression

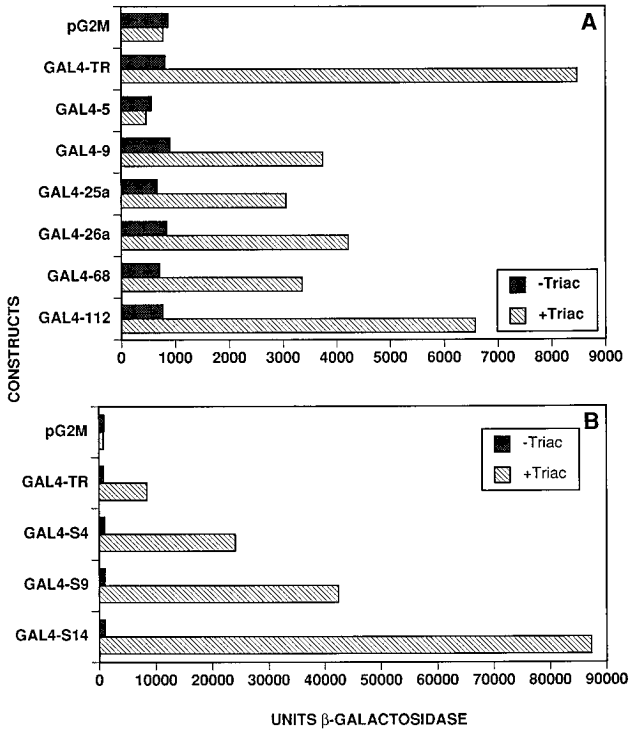

FIG. 5. Activities of GAL4-TRβ fusions. (A) T_3-binding, transactivation-deficient class. (B) Superactivator class. All constructs fused GAL4 amino acids 1 to 147 with TRβ (wild type or mutant) amino acids 172 to 456. The indicated constructs were cotransformed with pLGSD5 into the GAL4-deficient yeast strain YM706. Transformants were grown without or with 1 μM Triac to an A_{600} of 1, and β-galactosidase activity was determined. pG2M represents activity of the pLGSD5 reporter in the absence of any fusion protein. Units are expressed per milligram of protein and are representative of two independent experiments.

vector. This fusion construct was introduced into *S. cerevisiae* together with a β-galactosidase reporter construct containing binding sites for GAL4. Since these fusions were expressed at a level lower than that of full-length TRβ (data not shown), a more sensitive assay for β-galactosidase was utilized (see Materials and Methods). The fusion protein with wild-type TRβ sequences was indeed found to function as a hormone-dependent activator in *S. cerevisiae* (Fig. 5A). The degree of hormone-dependent induction with this fusion protein was about 10-fold, which is somewhat more than that observed for the wild-type receptor. This difference may reflect the fact that the GAL4-TR fusion protein shows little hormone-independent activity, which suggests that this activity is a product of the A/B or C region of TRβ. These data emphasize that the C-terminal region of TRβ contains a hormone-dependent activation domain that can function in *S. cerevisiae* independent of the A/B and C regions.

Several loss-of-function TR mutants were subcloned into the GAL4-TR fusion vector and tested for their abilities to support hormone-dependent activation (Fig. 5A). All forms showed decreased activity relative to the wild-type receptor sequences, although the extent of the effect varied with different mutants. For example, the fusion containing mutant 5 sequences is devoid of activity, whereas the fusion with mutant 112 retains about 75% of the wild-type activity. Several individual mutations that occur in the D region of the receptor displayed between 30 and 50% of the activity of the fusion containing wild-type sequences. Because the 25a TR mutant was also defective in TRE_{pal} binding, the reduced activity observed with

GAL4-25a suggests that this residue plays a role in both DNA binding and transactivation. Western blots of extracts from the fusion constructs indicated no significant differences in the levels of proteins expressed relative to that of the wild-type GAL4-TRβ fusion (data not shown). The diminished hormone induction supported by the mutant fusion proteins strongly implicates the D and E domains in the process of transcriptional activation by TRβ.

Several mutants from the class of superactivators were also tested as fusion proteins with GAL4 (Fig. 5B). All of these fusions displayed hormone-dependent activations that were greater than the activity of the wild-type fusion protein. Interestingly, the strongest superactivator in this context appeared to be S14 rather than S4. One explanation for the differences in activation between full-length TRβ mutants and fusion proteins of these mutants may be the absence of the A/B/C domains of TRβ. The fact that each superactivator behaves as such in the contexts of both the full-length product and the fusion construct again argues that the mutations specifically affect the transactivation potentials of these molecules.

Mammalian cell activity of mutants. To test whether TR mutations that led to defective transcriptional activation in *S. cerevisiae* effected a loss of function in mammalian cells, we tested the activities of the full-length mutant TR in CV-1 cells. This fibroblastic cell line does not express significant levels of TR. A CAT reporter plasmid containing four copies of the TRE_{pal} element was introduced into CV-1 cells along with a set of TRβ mutants. As shown in Fig. 6A, this reporter is not activated in the presence of hormone when the expression vector CMVS4 alone is cotransfected. With TRβ, a strong hormone-dependent induction of CAT activity is observed. All mutants tested showed a reduced response to hormone. However, the magnitude of these effects did not correlate with those observed in *S. cerevisiae*. In general, the mutant TR were less affected in CV-1 cells than in *S. cerevisiae*. However, none of these constructs yielded more than half the induction in mammalian cells compared with the wild-type TR control. For example, mutant 5 had no induction in *S. cerevisiae* yet showed a seven- to eightfold induction in CV-1 cells. To eliminate the possibility that these data reflect unequal levels of the different receptors in mammalian cells, we tested the levels of mutants 5, 9, 26, and 112 expressed and found no major discrepancies relative to the wild-type receptor (data not shown).

We also tested several superactivator mutants (S4, S10, and S13) in CV-1 cells (Fig. 6B). Surprisingly, none of these mutants were superactivators in this context. Indeed, all effected a reduced level of activation in CV-1 cells. Mutant S4, the strongest hormone-dependent activator in *S. cerevisiae*, actually was the weakest activator in CV-1 cells. These data demonstrate that the alteration that results in a more powerful activation domain in *S. cerevisiae* results in an opposite phenotype in mammalian cells.

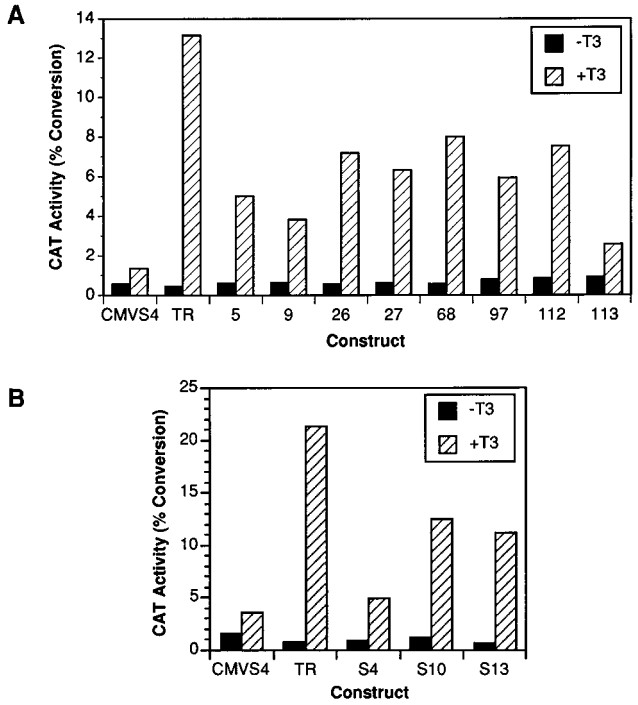

FIG. 6. (A) Activities of T_3-binding-competent, transactivation-deficient mutants in CV-1 cells. Wild-type TRβ and the indicated mutants were transferred to the CMVS4 expression vector and transfected into CV-1 cells along with the 4XTREpal/TKCAT reporter. Cells were grown in the absence or presence of 50 nM T_3, and CAT assays were performed. CAT activity was normalized to the amount of luciferase activity expressed from a Rous sarcoma virus-luciferase internal control plasmid. (B) Activities of some superactivator mutants in CV-1 cells. Superactivators S4, S10, and S13 were transferred to CMVS4, and transfections were performed as described for panel A.

DISCUSSION

Transcriptional activation by TR in *S. cerevisiae*. The process of transcriptional activation involves the formation of protein-protein contacts between specific domains of the transcriptional activator and components of the transcriptional machinery. The specific domains of several transcriptional activators involved in this process have been identified (for a review, see reference 41). These domains are classified largely on the basis of the amino acid content (i.e., acidic, glutamine rich, or proline rich), since no structural information about these regions is presently available. For TR and many members of the steroid receptor family, little is known regarding the nature of the transactivation domain. In part, this lack of information stems from the intimate association between residues important for hormone binding and transcriptional activation. The extensive D and E regions of TR not only contain dispersed residues involved in hormone binding but also contain residues important for transactivation. Thus, attempts to dissociate hormone binding and transactivation have been largely unsuccessful. We undertook an unbiased screen for transactivation mutations of *S. cerevisiae*. Our mutant collection shows unambigiously that select changes in the D and E regions can affect transactivation without altering hormone binding.

An understanding of the meaning of mutant phenotypes generated in *S. cerevisiae* requires a discussion of the ways in which the behavior of TR in *S. cerevisiae* either mimics or does not mimic the function of TR in its normal setting. The basic features of TR activation are retained in *S. cerevisiae*. Induction of reporter gene activity is dependent on both TR expression and the presence of a TRE_{pal} sequence. Similar responses to hormone are observed with reporter vectors containing two other TREs known to be active in mammalian cells: a direct repeat of the AGGTCA motif with a 4-bp spacer or an inverted repeat separated by 6 bp (data not shown). Furthermore, induction occurs in the presence of active thyromimetic analogs, including T_3, T_4, and Triac, but not in the presence of inactive analogs such as reverse T_3 (reference 49 and data not shown). Thus, the abilities of TR to bind to response elements and activate when bound to ligand are maintained in *S. cerevisiae*. Hormone-dependent activation in *S. cerevisiae* requires se-

quences from the C-terminal region of the receptor, as shown by the loss of activity in C-terminally truncated forms of TR. This conclusion was confirmed by the observation that the D/E region confers hormone-dependent transcriptional activation on a heterologous DNA-binding and dimerization domain. Again, this property has been found for TR activity in mammalian cells as well. Finally, several of the T_3-binding-competent, transactivation-defective mutants identified in the yeast system behave as mutant TR in mammalian cells. Thus, the process of hormone-activated transcription likely involves conserved determinants of the receptor in both yeast and mammalian cells.

A number of features, some of known origin and some of unknown origin, distinguish TR action in *S. cerevisiae* from TR action in mammalian cells. The most obvious difference is the transactivation that occurs in *S. cerevisiae* when hormone is not present. Recent data suggest that TR possesses a discrete repressing or silencing function in its D/E region (13, 14). This repressing activity can function on a heterologous promoter in transfected cells or in a cell-free transcription assay (18) and may be mediated by a direct interaction of TR and the basal transcription factor TFIIB (4). We suggest that the repressing activity of TR does not function in *S. cerevisiae*. This difference could represent the deficiency in *S. cerevisiae* of a soluble repressor protein that recognizes TR or a lack of evolutionary conservation between the repressing domain of TR and the basal yeast transcription apparatus. In the absence of this repressing function, the receptor which binds DNA in the unliganded state displays a low level of transcriptional activation. The nature of this activity remains undefined to date. A truncated form of TRβ containing amino acids 1 to 200 retained approximately 30% of the hormone-independent activity of the full-length receptor. Thus, at least a portion of the hormone-independent activity arises from the A/B or C region. Further evidence supporting this idea comes from experiments with GAL4 fusion proteins. The GAL4-TR chimeric protein, which lacks the A/B and C regions, was inactive in the absence of hormone. The TRα1 isoform has been reported to contain a hormone-independent N-terminal activation domain that functions only on a modified TRE sequence in mammalian cells (53). However, no such function has been reported for TRβ. Thompson and Evans (61) deleted the N terminus of human TRβ and showed activity equivalent to that of the wild-type receptor in CV-1 cells. Since some activation domains are cell specific, the presence of an activation region in the N terminus of TR that functions in certain mammalian cell types cannot be excluded.

Another difference between yeast and mammalian cells is the behavior of TR mutants. Although the transactivation-deficient mutants selected in *S. cerevisiae* were also defective in CV-1 cells, there were quantitative differences in the impact of these mutations. All of the mutants tested in mammalian cells retained a modest ability to induce the reporter in the presence of hormone. In addition, superactivator mutants in *S. cerevisiae* were transactivation deficient in mammalian cells. Two major factors may account for the differences in behavior of TR mutants in yeast and mammalian cells. First, because the yeast transcriptional machinery has not evolved to respond to TR, it is only reasonable that the yeast does not reflect every feature of TR in the exact manner with which mammalian cells respond. Consequently, although our screen for mutant TR in *S. cerevisiae* will detect certain residues critical for function, others not conserved in their action will be undetected. Second, and more importantly, the nuclear milieus of the two cells are different. *S. cerevisiae* does not possess RXR. As will be described below, the absence of RXR in *S. cerevisiae* might be responsible for many of the differences we observe in the behavior of mutant TR in the two cells.

The use of the yeast genetic screen allowed us to rapidly recover amino acid substitution mutations that were defective in either hormone binding or transcriptional activation. In this study we focused on a group of defective TR mutants that retained T_3 binding. Given the high concentration of hormone used in the screening procedure, a receptor with a reduced affinity for T_3 and a functional activation domain would still be expected to activate the TRE_{pal} reporter. This, in fact, was borne out by the properties of the superactivator mutant S15, which bound T_3 only weakly but activated in a hormone-dependent fashion. Privalsky et al. (49) have also shown that v-ErbA, which has 7% of wild-type hormone-binding activity, displays hormone-dependent activity in *S. cerevisiae*. Thus, the mutants selected in our screen likely do not result from changes in hormone-binding affinity but rather represent defects in some other step in the process of transcriptional activation.

Mutations in the D (hinge) region. The major group of mutations leading to a defect in hormone-dependent transcriptional activation localize to a 37-amino-acid region (amino acids 179 to 215) located immediately C terminal to the DNA-binding domain. These mutations occur within the D or hinge region, a segment which is poorly conserved in the family of steroid/thyroid hormone receptors. Two distinct phenotypes in mutants with mutations in the D region can be distinguished. The first group result in a loss of binding of mutant receptors to the TRE_{pal} sequence. Mutation of four different amino acids (K-179, N-190, R-194, and K-206) resulted in a loss of DNA binding (Fig. 7). The designation of K179R as the inactivating mutation for DNA binding in the double mutant 58a is based on the finding that the second mutation in this mutant, R184G, is also found in mutant 91, which binds to the TRE_{pal} sequence. Likewise, the L199P mutation of the double mutant 129a is also found in mutant 44a, which binds to DNA, suggesting that R194G is the mutation leading to defective TRE_{pal} binding. The finding that mutations in the D region would alter DNA binding by TR was not anticipated at the outset of this work, since this function had been attributed entirely to residues of the zinc finger-containing C domain. However, recent work suggests that the D region may play a role in this process in the thyroid/retinoid receptor subfamily.

For this group of mutations, several possibilities could explain the defect in DNA binding. The mutated TR residues could make direct DNA contacts with bases of the TRE_{pal} oligonucleotide. This possibility is suggested by work on the NGFI-B orphan receptor. Wilson et al. (69) found that amino acids of NGFI-B corresponding to TRβ residues 182 to 190, which were designated the A box, influence DNA binding. This orphan receptor, unlike most members of the family, binds as a monomer to an extended octamer sequence. Residues of the A box were shown to contact the upstream bases of this response element (68, 69). One of the DNA-binding-defective mutations (at N-190) occurs within the A box, whereas the other three occur at positions surrounding it. Recently, two groups have shown that TRα has the capability of binding to DNA as a monomer (29, 58). In doing so, the DNA-binding site preference is extended in the 5′ direction by two bases from the AGGTCA core motif, suggesting that residues in the A box may contact these upstream bases. Zechel et al. (72) have confirmed that binding of TRα as a monomer required residues of the A box and positions immediately upstream of the A box in the D region. It should be pointed out, however, that in our studies, the TRE sequence used for mutant screening did not contain the optimized monomer-binding site. Fur-

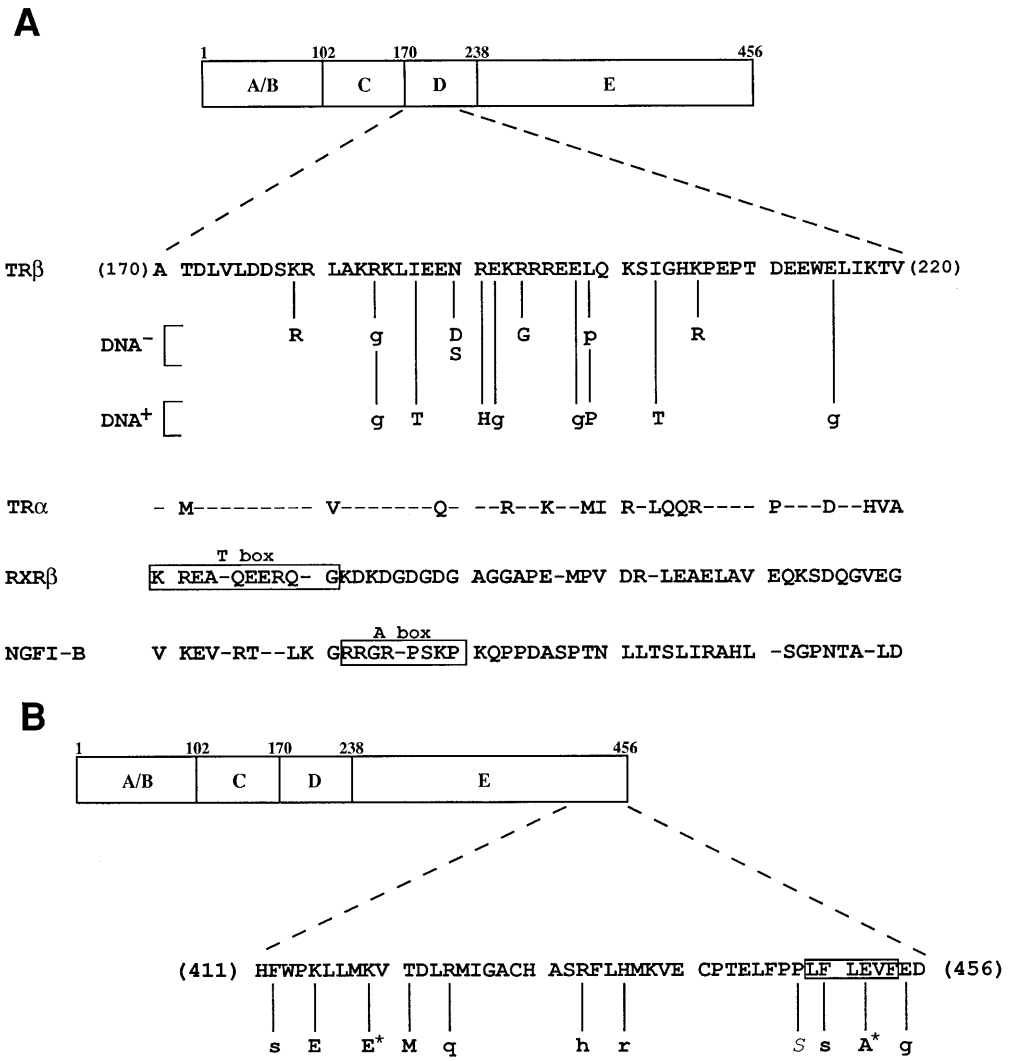

FIG. 7. (A) Sequence of the D region of TRβ and mutations in the D region leading to inactivation of receptor. Mutations are divided into those that disrupt binding to the TRE_{pal} (DNA^-) and those that bind TRE_{pal} comparably to wild-type receptor (DNA^+). Uppercase letters indicate mutations that can unambiguously be defined as causing the phenotype, while lowercase letters indicate mutations that are present as double mutations, for which assignment of the inactivating mutation cannot be unambiguously determined. Shown for comparison are the amino acid sequences of rat TRα, rat RXRβ, and rat NGFI-B over the homologous region. Dashes indicate residues that are identical to those in TRβ. Residues designated the T box of RXRβ and the A box of NGFI-B are indicated (69). (B) Sequence of the C-terminal end of TRβ and mutations in this region altering transcriptional activation properties of receptor. Upper- and lowercase letters are as described for panel A. Asterisks indicate residues altered in more than one mutant. Italics indicate an inactivating mutation. The box indicates a conserved region, ΦΦXEΦΦ (where Φ indicates a hydrophobic residue), in the steroid/thyroid family of receptors, as pointed out previously (7, 16).

thermore, the binding complex detected in the EMSA with the TRE_{pal} probe migrates as expected for a homodimer. The question of whether the role of the A box in contacting DNA extends to homodimer binding of TR is unanswered.

A second explanation for defective DNA binding is that mutated residues could be critical for dimerization interactions in the region adjacent to the zinc fingers. This possibility was suggested by work on RXR. Residues of the hinge region were found to be critical in the binding of RXR to an RXR-specific response element (35, 69) or binding of RXR as a heterodimer with RAR to a DR+2 response element. These residues, which correspond to TRβ residues 170 to 181, were designated the T box and were suggested to play a role in dimerization. Again, we find that one of the TR mutations resulting in defective DNA binding (at K-179) is within the region corresponding to the T box.

A third possibility is that these TR mutations affect the ability of the receptor to undergo a conformational change to bring the E domains of adjacent receptors into the proper configuration for dimerization, as suggested by Kurokawa et al. (31). These workers suggested that the D region confers rotational flexibility between the C and D/E domains of TR. This rotational flexibility was postulated to account for the ability of TR to activate from TREs containing binding motifs arranged as direct repeats, palindromes, or inverted palindromes. These investigators did not test the functional activity of mutations in this region in transfection assays. However, they found that mutations at residues corresponding to the A box (181 to 190) blocked the ability of TRβ to bind to either direct repeat or inverted palindromic response elements as homodimers. At present, it is not possible to distinguish between these explanations.

In addition to the mutations in the D region that affect DNA binding, some mutations in this region also confer binding to the TRE_{pal} comparable to that of the wild-type receptor yet are incapable of transactivation. Mutations in this group span the region from R-184 to E-215 and thus overlap with mutations affecting DNA binding (Fig. 7). These mutants also bind T_3 equivalently to the normal receptor. Thus, because both DNA binding and hormone binding are near normal, the D region must play an independent role in transcriptional activation. This conclusion is supported by the properties of GAL4 fusion proteins containing mutants in this class. Both mutants 9 and 68 diminish the activation observed with the wild-type GAL4-TR fusion protein. Since the GAL4 domain provides both DNA-binding and dimerization functions, the defects in these mutants must result from a distinct process in transcriptional activation. It is interesting that the mutant 25a, which was defective in DNA binding, also showed reduced activity as a GAL4 fusion. This result implies that this mutation affects not only DNA binding but transcriptional activation as well. Again, this result emphasizes the highly overlapping nature of the functions imparted by the D domain.

These D region mutants could affect a transactivation domain of the receptor directly involved in contacting a downstream component of the transcriptional machinery. In the glucocorticoid receptor, a hormone-independent transactivation domain, $\tau 2$, has been localized to the D region (21). However, similarly located transactivation domains have not been found in other members of the steroid receptor family. Alternatively, this region of the receptor could be important for a conformational change necessary to position the transactivation domain of TR for proper interaction with the transcriptional machinery. Several lines of evidence support the concept that hormone binding results in an alteration in receptor conformation. Toney et al. (62) have shown an altered pattern of circular dichroism associated with hormone binding. Leng et al. (37) used partial proteolytic cleavage to demonstrate that hormone binding induced a change in TR to a more protease-resistant conformation. We currently favor the possibility that these mutations in the D region interfere with this conformational change, since it may explain the overlapping nature of mutations in this region that affect DNA binding and transactivation. Both of these processes may require conformational flexibility of the D region. For DNA binding, this flexibility is necessary to properly orient the E region dimerization domains to stabilize binding, and for transactivation to allow exposure of the critical residues involved in interaction with the basal transcriptional machinery.

Mutations dispersed in the TRβ E region. Several of the mutants that were defective in hormone-dependent transcriptional activation contained mutations broadly dispersed in the middle of the E region. All of these mutants bound the TRE_{pal} oligonucleotide comparably to the normal receptor. Several of these showed some impairment in their ability to bind T_3. However, we suggest that the major defect in these mutants is in their ability to transactivate.

Several mutations in this group emphasize the critical nature and overlapping role of amino acid residues between positions 280 and 310. Mutants 152 and 157 contain mutations that are close together and near a 20-amino-acid region (amino acids 281 to 300) suggested by Koenig and colleagues to be important for heterodimerization with RXR (45, 46, 51). Mutant 157 contains two changes, A226V and E306G. The latter of these is directly adjacent to this heterodimerization domain. EMSA with mutant 157 indicates that it binds with high affinity to the TRE_{pal} sequence but only weakly as a heterodimer to this sequence (data not shown). This observation suggests that the heterodimerization domain may extend to at least residue 306. The properties of this mutant parallel those described for an R311H mutation in human TRβ that gives rise to a selective pituitary resistance to thyroid hormone (20). On the other hand, mutant 152 contains an L300P change and yet binds comparably to the wild-type receptor as a homodimer or RXR heterodimer (data not shown). Two of the superactivators, S19 and S20, also have mutations that are localized in this area of the receptor around residue 300. For S20, the addition of hormone actually reduces its activity. Perhaps in *S. cerevisiae* the protein is folded to expose the transactivation domain of the mutant receptor. In addition to these mutations, mutations affecting hormone binding have also been found to map to residues 304 and 308 (64). In the syndrome of GRTH, one of two hot spots for mutations is between residues 305 and 342 (50). These mutations also generally reduce hormone binding of TR. Thus, this complex region is clearly critical to multiple overlapping functions of the receptor.

Lee and Mahdavi (36) demonstrated that mutations in residues 188 to 190 of TRα1 (corresponding to residues 237 to 239 of TRβ) led to defects in transactivation but did not affect nuclear localization, T_3 binding, or DNA binding. Mutant 113 contains two changes (Q236P and F240S) flanking this segment and thus may alter the conformation of TR in this region. In addition, mutant 26 (L261P) is located relatively close to these residues and may influence its activity. The role of this segment in transactivation is unknown.

Mutations in the C-terminal part of the E domain. Several mutants that affect transcriptional activity contain changes in the last 45 amino acids of TRβ. In particular, the class of superactivator mutants includes those with mutations that are concentrated between residues F-412 and E-455 of TRβ (Fig. 7B). The most striking change occurs in mutants containing a K419E mutation, which display a 10-fold-enhanced ability to activate in the presence of hormone. Other mutants of interest in this class are S7 and S9, which contain changes of E455G and E452A, respectively. These two glutamate residues, especially E-452, are highly conserved among the steroid/thyroid receptor family, as noted by Danielian et al. (16). That mutations in this region give rise to a superactivation phenotype in *S. cerevisiae* is underscored by the fact that only a single mutant of the T_3-binding-competent, transactivation-deficient class with a mutation in this region was found. Mutant 5 (P448S) bound T_3 about 40% as well as wild-type TR yet could not activate the TRE_{pal} reporter in *S. cerevisiae* either on its homologous DNA-binding domain or as a GAL4 fusion. Thus, this mutant would appear to have a minor impairment in T_3 binding but a major defect in its ability to transactivate. This proline is mutated to three different amino acids in separate alleles of GRTH (44).

The preponderance of superactivator mutations in a region where we might have expected to find inactivating mutations was initially puzzling. However, in retrospect, this phenotype is consistent with expected properties of mutations in the transactivation domain of a mammalian transcription factor selected in a yeast transcriptional system. We postulate that although the TR transactivation domain has been conserved in evolution to a degree sufficient to allow it to interact with the yeast basal transcriptional machinery, this interaction would not in fact be expected to be optimal. The consequence would be that TR serves as a relatively weak activator in the context of *S. cerevisiae*. Mutations that give rise to the superactivating phenotype would presumably create a stronger interaction between the transactivation domain of TR and yeast transcriptional components. This hypothesis is supported by the observation that virtually all of the superactivator mutants retain

hormone dependence for transcriptional activation. If this is true, then we might expect these same mutations to weaken the interaction with the mammalian transcriptional machinery for which TR has been evolutionarily optimized. Indeed, this is exactly the result we obtained; the mutant TR that superactivated in *S. cerevisiae* were transcriptionally crippled in mammalian cells. Thus, these data support a critical role of residues in the C-terminal region in the transactivation process.

The importance of this C-terminal region in transactivation has been supported by several studies that have appeared during the course of this work. Danielian et al. (16) noted that several C-terminal residues of the E domain were conserved across many family members. Mutation of two of these conserved positions, E-546 and D-549, of the mouse estrogen receptor seriously blunted its ligand-dependent transcriptional activation. The hormone-binding and DNA-binding capacities of this mutant were unaffected. These mutations were also introduced into the glucocorticoid receptor with similar results. On the basis of these and other data, Danielian et al. (16) propose that this region is a component of the ligand-dependent activation domain (AF-2) of estrogen and glucocorticoid receptors and probably other members of the receptor family as well. One of the dominant negative mutations of the estrogen receptor isolated by Ince et al. (26) also maps to this region. These authors suggest that the dominant negative phenotype results from AF-2 inactivation.

These results have been extended to include members of the thyroid/retinoid subfamily of receptors. Saatcioglu et al. (52) introduced simultaneous changes at glutamate residues 401 and 404 in chicken TRα1 (corresponding to E-452 and E-455 of TRβ). This mutated receptor was inactive. Other mutations in the C-terminal nine amino acids also produced receptors defective in transcriptional activation. Thus, these investigators also argued that this region must be important for transcriptional activity. However, all of the mutant TR were significantly reduced in T3 binding, as well as in transcriptional activation. Thus, this study cannot unambiguously distinguish between effects on hormone binding and on transactivation. More recently, Barettino et al. (7) extended these results for the chicken TRα by mutating several additional C-terminal residues. They found a single mutant, carrying mutation E401Q, which retained hormone-binding capacity but lost transactivation. Furthermore, they demonstrated that a fusion of the GAL4 DNA-binding domain with the 35 C-terminal residues of TRα resulted in a protein capable of activating transcription in mammalian cells. Although the level of activation was reduced significantly relative to that of a GAL4 fusion of the entire D and E domains and lacked hormone dependence, these data suggest that this region of TRα behaves as an autonomous activation domain.

As mentioned previously, mutations that affect transactivation could do so by altering intramolecular signal transduction triggered by hormone binding or by affecting intermolecular interactions between TR and the transcriptional machinery. Upon hormone binding, TR is known to undergo a conformational change (37, 62). This change presumably allows the transactivation domain of TR to productively interact with the transcriptional machinery. The characteristics of these superactivators strongly argue that the C-terminal domain of TR is directly involved in the transactivation function. If these mutations caused their phenotype by mimicking the conformational change that occurs upon hormone binding, the expected phenotype would be a hormone-independent activator. Instead, we find that virtually all of the superactivators retain a dependence on hormone binding. Thus, a hormone-dependent conformational change is necessary to expose the activation domain of the superactivator mutants. This concept is consistent with the data of Barettino et al. (7) demonstrating that this region of TRα can impart transactivation to a heterologous DNA-binding domain. However, the retention of hormone dependence for activation of the superactivator TRs more strongly supports the physiological role of this domain.

What is the active TR species in *S. cerevisiae?* Recent studies have suggested that TR function in mammalian cells is intimately linked with that of the RXRs. Using gel retardation assays, we have not been able to detect an RXR-like activity in yeast extracts. Similar efforts to detect a factor that enhances DNA binding of TR by using the less stringent avidin-biotin complex DNA binding assay were also unfruitful (data not shown). While we cannot completely exclude the existence of such a factor, we strongly suspect that TR functions as either a monomer or a homodimer in *S. cerevisiae*. Recently, two laboratories have shown that coexpression of RXR and TR in *S. cerevisiae* leads to enhanced TR activation, as expected from results in mammalian cells (24, 34). Consequently, we will be testing the mutant TR isolated in this study in the context of RXR in yeast cells.

An assessment of homodimer action in mammalian cells is complicated by the background of RXR found in virtually all cells. However, there is growing evidence that RXR-independent pathways of transcriptional activation do exist for members of the retinoid, thyroid, and vitamin D family of receptors. For the vitamin D receptor, RXR-independent and -dependent pathways have been defined (60). Recent work of Saatcioglu et al. (53) has shown that TRα can function as a hormone-independent activator from a unique Rous sarcoma virus DNA element. This action is not dependent on RXR. Kurokawa et al. (31) presented evidence that TR were able to form stable homodimers in solution. The RXR-TR interaction was approximately threefold higher than the TR-TR interaction in this assay. Katz and Koenig (29) used a PCR-based binding site selection strategy to identify an 8-bp element (5′-TAAGGTCA) that binds strongly to TRα monomers. This element also confers TR-dependent activation to a linked reporter gene in cotransfection assays. Recent work of Schrader et al. (59) confirms these findings. All of these observations suggest that in addition to heterodimers, homodimers and monomers of TR expand the repertoire of gene regulation by the thyroid hormones.

In conclusion, we have identified specific amino acid residues in TR which play a critical role in the process of hormone-dependent activation in yeast and mammalian cells. In particular, two regions of TRβ contained multiple mutations: the D region and a carboxy-terminal segment of the E region. Our data suggest that mutations in these residues affect the process of transcriptional activation, independent from hormone binding. These mutants should prove useful in further delineating the pathway of action of TR in mammalian cells.

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