

Identification of a negative regulatory function for steroid receptors

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ABSTRACT This report describes the identification of a negative regulator of estrogen and progesterone receptor function. Using a reconstituted estrogen-responsive transcription system in *Saccharomyces cerevisiae*, we have identified a "repressor function," which when mutated, increases the transcriptional activity of the estrogen and progesterone receptors. In the case of the estrogen receptor this mutation increases the sensitivity of estrogen-mediated activation by at least four orders of magnitude. Analysis of derivatives of the estrogen receptor indicated that this repressor specifically affects the transcription activity of the TAF1 activation domain of the estrogen receptor. The repressor was cloned by complementation and identified as SSN6, a previously described mediator of glucose repression in yeast. Our results indicate that SSN6 is likely to be involved also in the repression of other cellular activators. Interestingly, deletion of the SSN6 protein allows the antiestrogens ICI 164384 and nafoxidine to behave as more potent agonists of estrogen receptor function, while RU486 also becomes a more potent activator of progesterone receptor function. These data suggest that in wild-type cells the role of hormone is twofold: it promotes DNA binding of the receptor and it also induces a conformational change in the receptor which overcomes the effects of this repressor function.

Our understanding of the mechanism of action of steroid hormones has been enhanced by the cloning of the cDNAs for their intracellular receptors (1). Reconstitution of hormone-responsive transcription units in heterologous cells and ensuing mutagenesis studies have defined discrete domains that are responsible for DNA binding, hormone binding, nuclear localization, interaction with heat-shock proteins, and transactivation (2–7).

Our present interest is to study the biochemical mechanism by which activation domains of receptors interact with the transcription apparatus to modulate the rate of transcription of target genes. For this purpose, we chose the estrogen receptor as our model. This receptor has two well-characterized activation domains: TAF1, which is located at the amino terminus of the receptor, and TAF2, which is tightly regulated by hormone and located in the carboxyl terminus (8–10). This study concentrates on the TAF1 activator. This activator is of particular interest in that it is hormone independent and, once delivered to DNA, can activate transcription (11, 12). A molecule having undergone a deletion of the hormone-binding domain and containing only a single TAF1 activator was shown to have different activities in various cell types. In HeLa cells the transactivation activity is about 5% that of the wild-type receptor, in chicken embryo fibroblasts it is 58% (10), and in GC cells (13) it activates transcription indistinguishably from the wild-type receptor. This pattern suggests that the differences in asso-

ciated transcription factor pools in these cells may determine the efficacy of steroid receptor function.

Fortuitously, we had noticed that a similar estrogen receptor containing only amino acids 1–282 had minimal transcriptional activity in *Saccharomyces cerevisiae* (12). This result was in contrast to the results of White *et al.* (14), who showed that an identical construct had 100% of the activity of the wild-type receptor in yeast. Our observation suggested that differences in the transcription factor pools between the two yeast strains may exist, analogous to what has been proposed for mammalian cells.

The present study was designed to identify factors which modulate the activity of the TAF1 activator of the estrogen receptor. A genetic approach was developed in yeast. Our premise was that factors identified in yeast may be functionally related to mammalian counterparts and would enhance our understanding of TAF1 activator function.

MATERIALS AND METHODS

Biochemicals. Restriction enzymes were purchased from Promega, Boehringer Mannheim, and Amersham. T4 polynucleotide kinase and T4 DNA ligase were purchased from Bethesda Research Laboratories. Chemicals were purchased from Sigma.

Steroids. 17 β -Estradiol and nafoxidine were purchased from Sigma. ICI 164384 was a gift from ICI. RU486 was a gift from Roussel-Uclaf.

Yeast Strains. The following two strains were used throughout this study; BJ5409 (*Mata, ura3-52, trp1, leu2 Δ 1, his3 Δ 200*) and BJ3505 (*Mata, ura3-52, trp1 Δ 101, lys2-208*).

DNA Constructions. All steroid receptor plasmids and the reporter plasmids have been described previously (15, 16).

Assay of Promoter Activity. Samples of cytosol were assayed for β -galactosidase activity as described previously; all activities shown in the figures were weighted with respect to protein concentration (17).

Mutagenesis. Cells containing the effector plasmid ERN282G and the reporter plasmid YRPE2 were grown overnight in selective media. The cells were washed twice in 100 mM sodium phosphate buffer, pH 7.0, and resuspended twice in the same buffer. The cells were diluted 100-fold and cell number was estimated by direct counting. To 1 ml of diluted cells 30 μ l of 100% ethyl methanesulfonate was added. These cells and a control sample were then incubated for 50 min at 30°C. The reaction was stopped by addition of 9 ml of 5% Na₂S₂O₃. The cells were then washed with water and spread on selective plates containing 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-Gal) at a density of 2000–3000 cells per 150-mm Petri plate. These plates were incubated at room temperature for 5 days. Those surviving cells that were blue were selected for further analysis.

Plasmid Curing. Plasmids were evicted from yeast strains by successive passages in nonselective media. For plasmids

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Abbreviation: X-Gal, 5-bromo-4-chloro-3-indolyl β -D-galactoside.

containing a *URA3* marker, cells were grown on selective plates containing 5-fluoroorotic acid (Sigma). The resulting strains were analyzed by replica plating.

Complementation Analysis. Samples of the mutant strain containing the yeast reporter YRpE2-Leu and the effector plasmid were transformed with a *S. cerevisiae* YCp50 genomic library (obtained from Mark Rose, Princeton, NJ). Several different fractions of this library were used. The transformants were plated on selective plates containing X-Gal and incubated at 37°C for several days.

Gene Disruption. The *SSN6* locus was disrupted by using the plasmid PJS22 and a previously described protocol (18).

RESULTS

Activation of Transcription by Estrogen Receptor Derivatives in Yeast. Our study focuses on the identification of proteins that interact with the TAF1 activator. The rationale was to generate cells by mutagenesis in which the inactive TAF1 activator would now activate transcription. To develop a suitable screen we used the construct ERN282G, containing only the TAF1 activator and the DNA-binding domain of the estrogen receptor, to transform the yeast strain BJ5409 (Fig. 1A). For comparative purposes the wild-type estrogen receptor and a derivative containing the DNA-binding region and the hormone-binding region (ER179C) were also expressed. Western immunoblot analysis and quantitative immuno-dot-blot analysis demonstrated that these receptor derivatives were produced intact and in similar amounts in yeast (data not shown).

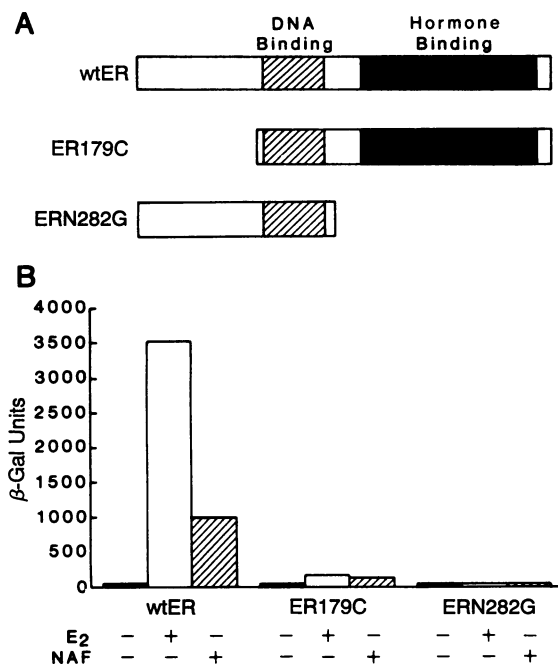


FIG. 1. Activity of estrogen receptor derivatives in the *S. cerevisiae* strain BJ5409. (A) Diagram of the three estrogen receptor derivatives used in this study. wtER, wild-type estrogen receptor. ER179C has undergone a deletion of the first 179 amino acids of the receptor. ERN282G contains only the first 282 amino acids of the receptor (15). (B) Yeast cells were transformed with an expression plasmid (as indicated) carrying the estrogen receptor fragment and a second plasmid containing the estrogen-responsive *CYC1-lacZ* fusion reporter plasmids. The cells were then grown overnight in minimal medium containing 2% glucose in the absence of hormones or in the presence of 1 μ M estradiol (E₂) or nafoxidine (NAF). The β -galactosidase activities were measured and weighted with respect to protein concentration (17).

To test the transcriptional activities of these receptor derivatives we cointroduced the reporter YRPE2 into strains producing the effectors. The reporter vector contains two copies of the palindromic estrogen response element (ERE) inserted into the promoter of a *CYC1-lacZ* fusion vector (19). Cells containing both the expression plasmid and the reporter were grown overnight in selective media containing glucose either in the absence of hormone or in the presence of estrogen or nafoxidine. Cells were harvested and β -galactosidase activities were measured. The results of this assay shown in Fig. 1B illustrate that in this yeast strain the wild-type estrogen receptor is a ligand-dependent activator and responds appropriately to hormones. The construct containing the TAF1 activator alone (ERN282G) was minimally active and displayed less than 5% of the wild-type activity. A construct containing only the TAF2 activator (ER179C) was also weakly active (<10%). The fact that the sum of the activities of each individual activator is less than that of the wild-type receptor suggests that considerable synergism must exist between these motifs in the wild-type molecule. Interestingly, the activity of ER179C was similar in the presence of both estrogen and the antiestrogen nafoxidine and suggests that the antiestrogenic activity of nafoxidine in the full-length receptor could be influenced by the inability of the TAF1 activator to operate. In wild-type receptor, antiestrogens may not induce the conformational change required to allow the TAF1 and TAF2 activators to synergize efficiently.

Isolation of Mutants. Cells containing ERN282G and the reporter were mutagenized with ethyl methanesulfonate, spread on X-Gal indicator plates, and grown at room temperature for 5 days. From 100,000 surviving clones, we identified several clones that grew with a significant blue halo. One isolate, E5UA, demonstrated considerable transcriptional activity. Initial comparisons indicated that ERN282G was 50-fold more active in the mutagenized E5UA strain than it was in the parent yeast strain; under optimized conditions this clone was \approx 100-fold more active than was observed in the parental wild-type strain (data not shown).

In addition, when we replaced the *CYC1* promoter with the proximal promoter from the *GAL1* gene we got identical results (data not shown). These data ruled out the possibility that the mutant phenotype was promoter specific. Further characterization of the mutant indicated that the phenotype resulted from a genomic mutation and was unrelated to the expression vector or the reporter plasmids (data not shown).

Mutant Locus Specifically Affects TAF1. We next wished to compare the relative activities of full-length estrogen receptor and the other receptor derivatives in this mutant cell (E5UA) background (Fig. 2). For comparative purposes we also reassayed the activity of these molecules in the unmutagenized parent strain. As seen before, the ERN282G molecule was constitutively active in the mutant strain and was inactive in the wild-type BJ5409 background. In the absence of hormone or in the presence of estradiol the intact receptor behaved in a similar manner in the wild-type and mutant cell strains. The slightly lower activity of the estrogen receptor in the mutant was observed reproducibly and may result from the fact that the mutant strain grows slower (see below). We observed that the antihormone nafoxidine, normally a weak agonist, behaves as a potent agonist in the mutant strain (Fig. 2A and B). Also, the antiestrogen ICI 164384 acted as only a weak agonist of the estrogen receptor in wild-type cells but was a very efficient agonist in the E5UA strain. This suggests, as in the case of nafoxidine, that this compound delivers receptor to DNA. This is in contrast with the *in vitro* data of Fawell *et al.* (20), which suggest that this compound prevents the association of the receptor with DNA by interfering with receptor dimerization. Importantly, the E5UA mutant strain reveals little difference from wild-type in func-

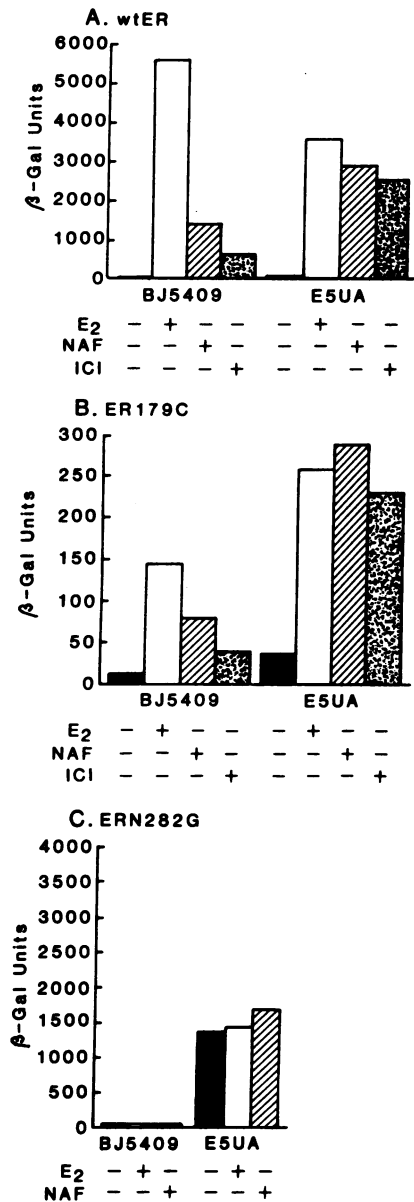


FIG. 2. Activity of estrogen receptor derivatives in the wild-type BJ5409 strain and the mutant E5UA strain. Both strains were transformed with wild-type receptor (A), ER179C (B), or ERN282G (C) expression vector and an estrogen-responsive reporter plasmid. Cotransformants were identified by uracil and tryptophan prototrophy. The cells were grown in selective media containing 2% glucose to an optical density at 600 nm of 1. The medium was supplemented with 1 μ M estradiol (E₂), 1 μ M nafoxidine (NAF), or 10 μ M ICI 164384 (ICI) as indicated. Note the β -galactosidase activity scales on the panels are different.

tion of the TAF2 activator (ER179C). In comparison, the function of the TAF1 activator was increased 50- to 100-fold, while the activity of TAF2 is increased only 2- to 3-fold. Specificity was further demonstrated by showing that the yeast *CUP1* locus was unaffected in this strain (data not shown). Finally, when the TAF1 activator in the estrogen receptor was replaced by the VP16 acidic activator, we were unable to detect any differences in activity (data not shown).

We were interested next in testing the activity of another steroid receptor in this mutated strain. For this purpose we transformed yeast with an expression vector that contained human progesterone receptor and a reporter vector that contained progesterone-responsive elements (16). When assayed in the wild-type strain the receptor functioned as a

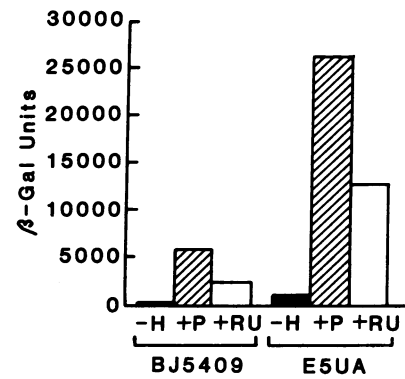


FIG. 3. Comparison of the activity of the human progesterone receptor in mutant (E5UA) and wild-type (BJ5409) backgrounds. Cells were transformed with an expression plasmid for the human progesterone receptor (YEpHPr1) and a progesterone-responsive receptor (YRpCG3) (16). Cotransformants were selected for by uracil and tryptophan prototrophy. The cells were grown in selective media containing 2% glucose in the absence of hormone (-H) or in the presence of 1 μ M progesterone (+P) or 1 μ M RU486 (+RU). The cells were harvested when they had reached an optical density of 1.0 at 600 nm and transcriptional activity (β -galactosidase) was measured.

ligand-dependent activator of transcription (Fig. 3). The progesterone antagonist RU486 behaved as only a partial agonist. When this reconstituted system was analyzed in the E5UA mutant strain we observed a 5-fold increase in progesterone receptor activity. There was a similar increase in the potency of RU486 as an agonist.

It was puzzling, however, that whereas in the mutant strain we observed a large effect on the progesterone receptor and on the TAF1 activator of the estrogen receptor, we saw only minimal effects on the full-length estrogen receptor. We considered whether the mutation may have compromised the ability of this strain to take up estrogen from the medium or whether the dose-response curve was shifted. To address these possibilities we assayed the activity (accumulation of target gene product) of the full-length estrogen and progesterone receptors in the presence of widely varying concentrations of estradiol or progesterone in both wild-type and mutant cells. The results are shown in Fig. 4. As expected, at 10⁻⁶ M estradiol (the concentration routinely used for assays) the activity of the receptor in the wild-type cells was roughly equivalent to that observed in the mutant strain (Fig. 4A). The normal amount of hormone required for half-maximal induction of the transcriptional response was $\approx 5 \times 10^{-9}$ M in the wild-type strain, but surprisingly, it was less than 10⁻¹³ M in the mutant strain. The increased activity of the estrogen receptor was not related to receptor level or hormone binding affinity; the K_d for estrogen binding to authentic receptor was 0.5×10^{-9} M in wild-type cells and 0.3×10^{-9} M in the E5UA cells. A similar but less pronounced (≈ 100 -fold) effect on the sensitivity of the progesterone receptor to hormone was noted in the mutant cells (Fig. 4B).

Identification of the Mutant Locus. An analysis of the E5UA strain revealed that the mutant phenotype was recessive. In addition the cells grew slowly, aggregated, and were temperature sensitive for growth.

The temperature sensitivity and the growth defects of the E5UA strain gave us a powerful screen with which to identify a complementing clone. The mutant cells containing the truncated receptor and the reporter plasmid were transformed with a low-copy-number yeast genomic library. The transformed cells were plated on X-Gal indicator plates and incubated for 4 days at 37°C to identify temperature-resistant transformants. A single colony was identified (ES-3). When reexamined in liquid culture, this yeast clone allowed little

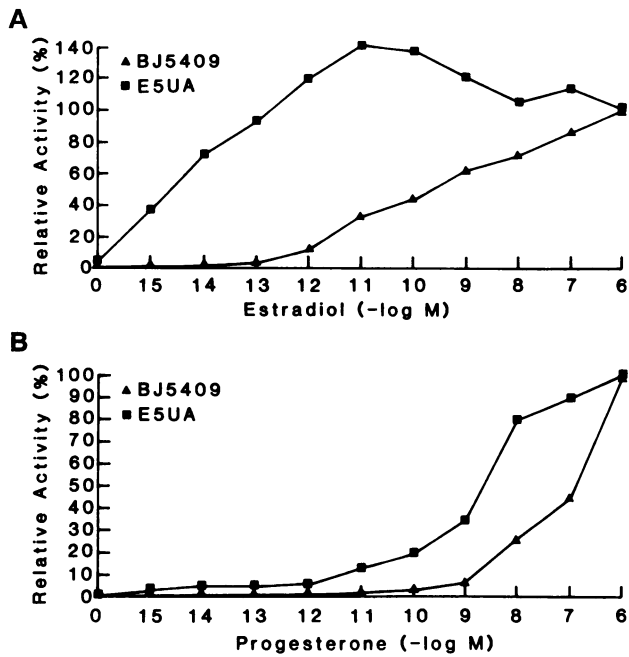


FIG. 4. Activity of the wild-type estrogen receptor and the human progesterone receptor in the mutant and wild-type backgrounds at different hormone concentrations. (A) Cells transformed with the estrogen receptor expression plasmid and the estrogen-responsive promoter were grown in minimal media supplemented with 2% glucose in the absence of hormone or in the presence of estradiol at various concentrations. (B) Cells transformed with the progesterone receptor expression plasmid and a plasmid containing a progesterone-responsive promoter were grown up in the absence of hormone or in the presence of progesterone at various concentrations. The relative activity of the effectors is expressed as the activity at a given concentration relative to the activity of the effector in the presence of 10^{-6} M hormone.

transcriptional activity of ERN282G (data not shown). Upon eviction of the complementing plasmid, the strain reverted to the prior mutant phenotype (E5UA), in which ERN282G efficiently activates transcription.

Characterization of the Mutant Gene. The complementing plasmid (YCp50-3) was isolated from the transformant described above (Fig. 5). The plasmid contained ≈ 10 kilobases (kb) of genomic DNA. This DNA was restriction mapped, and unique restriction sites were used to produce a series of deletions within the clone. These were reintroduced into the mutant strain containing the reporter and ERN282G. The results of the complementation test are shown in Fig. 5. The smallest piece of complementing DNA obtained was ≈ 7 kb and was designated Δ -RCLA. Concurrently, we sequenced the 5' and 3' ends of the YCp50-3 insert. A comparison of this sequence to published sequences in the GenBank data base indicated that the 5' end of the clone contained sequence

identical to the structural gene *LYS2*, whereas the 3' end was identical to that reported for the *SSN6* gene. Further mapping and sequencing confirmed that the *SSN6* gene was wholly contained within the complementing Δ -RCLA clone.

The *SSN6* locus has been studied extensively and has been shown to function as a negative regulator of a variety of genes that are involved in growth, mating, and sporulation (18, 21). We have shown that the transcriptional activity of ERN282G in our mutant strain is independent of the carbon source utilized, dissociating the ability of TAF1 to function from catabolite repression (data not shown).

***SSN6* Is a Repressor of ERN282G Function.** An examination of sequences in GenBank that were related to *SSN6* revealed a striking primary sequence similarity to *GAL11* (data not shown). The similarity of *SSN6* and *GAL11* raised the possibility that the mutant we obtained was not a null mutation in a repressor but rather a structural alteration in an activator that allowed it to interact more efficiently with the transcription apparatus (22). To examine this possibility we created a null allele of *SSN6* in BJ5409 by gene disruption. The activity of the estrogen receptor in the *SSN6* strain was similar to that in the E5UA mutant strain (Fig. 6). These data indicate that the *SSN6* molecule is behaving normally as an inhibitor of estrogen receptor-mediated transcription and in this regard is dissimilar to the more popular concept of *GAL11* function (22, 23).

DISCUSSION

These studies concentrated on the function of the TAF1 activator sequence, which is located in the amino terminus of the estrogen receptor. In our yeast strains, we found TAF1 to be minimally active (12). A search for a suppressor mutation of this phenotype identified the locus *SSN6*, which, when mutated, allowed a high constitutive activity of the truncated receptor containing TAF1.

The involvement of the *SSN6* protein in glucose repression, mating type suppression, and sporulation suggests that it is a transcriptional inhibitor with a low specificity (18, 24). Nevertheless, specificity is suggested by our data showing that the *SSN6* protein primarily affects the function of the TAF1 activator but not the TAF2 activator. It therefore suggests a regulatory preference for the type of activator sequences contained in the TAF1 region.

Although the *SSN6* molecule has not been shown to interact directly with DNA, its nuclear localization and primary sequence would seem to suggest a nuclear function (24, 25). On the basis of our results and those of others, we suggest two dissimilar models of action. In the first model a mutation of the *SSN6* locus leads to a constitutive derepression of transcription due to a global alteration in chromatin. As a result, weak activators would be capable of inducing transcription. Opposing this model is the fact that only a limited subset of genes have been reported to be derepressed in a mutant *SSN6* background (25). We show further, in the

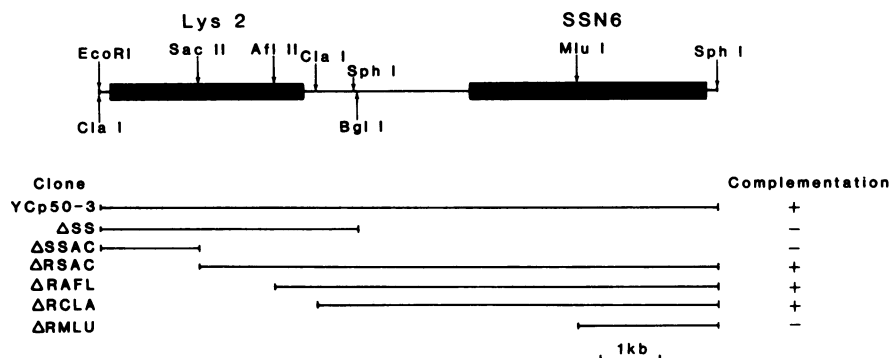


FIG. 5. Identification of the complementing gene within the plasmid YCp50-3. The plasmid from the complementing cells was isolated and mapped. A series of deletion mutants were constructed that when re-assayed for their ability to complement the transcription defect allowed the localization of the complementing locus. Sequencing of this clone and some of the deletions allowed the identification of two major reading frames. The identities of these reading frames were revealed upon searching the GenBank data base. The locations of the *LYS2* and *SSN6* loci within the clone are indicated. kb, Kilobase.

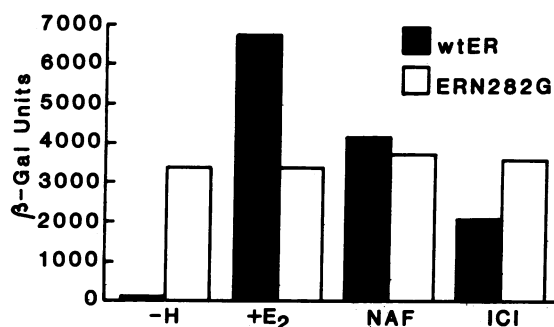


FIG. 6. Assay of the activity of the estrogen receptor in an *SSN6* null background. Wild-type cells (BJ5409) were transformed with an expression vector carrying either the full-length estrogen receptor or the truncated ERN282G receptor and an estrogen-responsive reporter. The *SSN6* locus in this strain was disrupted by a single-step disruption due to the plasmid PJS22 (18). Cells containing these plasmids and the disrupted *SSN6* were grown in selective media containing 2% glucose in the absence of hormone (-H) or in the presence of 1 μ M estradiol (+E₂), 1 μ M nafoxidine (NAF), or 10 μ M ICI 164384 (ICI). The cells were harvested when they had reached an OD₆₀₀ of 1.0 and the β -galactosidase activity was measured.

case of the estrogen receptor, that the effects of the *SSN6* defect are limited to the TAF1. An alternative model, which we favor, is that *SSN6* mediates repression of a specific subset of activator sequences within gene regulatory proteins, of which TAF1 is a member. The *SSN6* molecule may be the repressor itself or it could promote the interaction of another repressor protein with the TAF1 activator.

We have demonstrated previously that the hormone estradiol is responsible for a two-step activation process in yeast (19). The first step included the dissociation of heat-shock proteins from the receptor and the subsequent conversion of the receptor to a DNA-binding form. This is accomplished by both estrogens and also the antiestrogens nafoxidine, ICI 164384, tamoxifen, LY 156, and LY 117 (21). Nevertheless, DNA binding alone is insufficient for activation (19). We demonstrate in the present study that a single mutation can result in a phenotype which permits activation by antiestrogens, following binding to DNA. Thus, we suggest the possibility that the second step required for activation of gene transcription by estrogen receptor in yeast includes also a derepression from the effects of the *SSN6* molecule. Our model suggests that, in wild-type yeast cells, hormone is capable of inducing an allosteric change in the receptor, resulting in the displacement of a repressor. In this conformation, distinct TAF1 and TAF2 activation sequences can interact productively with the transcription apparatus. The model further suggests that antiestrogens, even though they may alter the structure of the receptor, are not effective in overcoming the repressive effect of the *SSN6* inhibitor.

In the mutant yeast strain the sensitivity of the estrogen receptor to hormone increases by four orders of magnitude. It would appear, therefore, that estrogen has a catalytic rather than a stoichiometric effect on transcription. One explanation is that the *SSN6* protein is involved in termination of the transcriptional effect of the estrogen receptor. In this model the net transcriptional effect is a product of the positive effect of estrogen receptor and the opposing effect of the *SSN6* protein. In the mutant strain the *SSN6* protein is absent and so transcription proceeds unopposed. In support of this model we have shown that the apparent increase in

hormone sensitivity results from a difference in the rate of accumulation of the target gene product rather than an increase in efficacy of the transcription factor (data not shown).

The concept of a functional cellular repressor for receptor function has not been reported previously to our knowledge. Identifying a mammalian counterpart would be very interesting.

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