We have constructed novel chimeric receptors consisting of the activation region of the herpes simplex virus transcription factor VP16 inserted into the amino-terminal region of the human estrogen receptor containing or lacking the hormone-binding region. By gene transfer into mammalian cells, these chimeric receptors behave in a hormone-dependent or hormone-independent manner, respectively, and are more efficient activators of gene expression than wild-type estrogen receptor. These studies indicate that a potent activation region from a viral transcription factor can be placed under hormonal control when introduced into a steroid receptor molecule and can enhance the receptor’s potency (~10-fold) in activating specific gene expression. It is likely that such chimeric molecules could be designed to increase selected target gene responses to any intracellular receptor in the course of cellular transfection, transformation, or transgenic animal experiments.

Molecular cloning of various steroid receptor cDNAs has revealed that they are a family of hormone-dependent transcription factors having a common structural organization (Hollenberg et al., 1985; Miesfeld et al., 1986; Conneely et al., 1986, 1987; Jeltsch et al., 1986; Green and Chambron, 1986; Weinberger et al., 1986; Sap et al., 1986; Arriza et al., 1987; Giguere et al., 1987; McDonnell et al., 1987; Evans, 1988). In general, most transcription factors consist of two functional domains, a DNA-binding and an activation domain (Brent and Ptashne, 1985; Struhl, 1987; Ptashne, 1988). The DNA-binding domain recognizes a cis-acting element(s) on the control region of target genes (Geise et al., 1982; Renkawitz et al., 1984; Karin et al., 1984; Slater et al., 1985; Klein-Hitpass et al., 1986; Janzen et al., 1987; Jones et al., 1988), and the activation domain recognizes the transcriptional machinery to regulate transcription. Some activation domains have been found to consist of negatively charged regions (Ma and Ptashne, 1987; Hope and Struhl, 1986; Hope et al., 1988). In addition to an excess of acidic residues, some aspect of structure, such as an α helix, is essential for formation of an activating region. Mutations in these regions which result in a net increase of negative charge usually increase the activity of the transcription factor (Gill and Ptashne, 1987); reduction of negative charge results in loss of activity. Thus, higher negative charge usually equates with greater potential for that particular transcription factor to stimulate its target genes.

Consistent with this notion, virus-associated transcription factors that have the greatest transcription activation potential are often highly negatively charged.

The herpes simplex virus transcription factor VP16 contains the strongest activation region identified to date and has a net negative charge of ~22 within its activation domain of ~80 amino acids (Treizemberg et al., 1988a, 1988b). Recent studies have shown that a hybrid protein consisting of the activation domain of VP16 and the DNA-binding domain of the Gal4 transcription factor (GAL4-VP16) created a super-activation factor that has a markedly higher level of activity in yeast than the wild-type Gal4 (Sadowski et al., 1988; Cousens et al., 1989).

Although activation properties have been found in the amino- and carboxyl-terminal regions of steroid receptors, these properties remain poorly characterized (Giguere et al., 1986; Godowski et al., 1987; Webster et al., 1988; Dobson et al., 1989). Recently, two trans-activation domains have been identified in the human glucocorticoid receptor (Hollenberg and Evans, 1988). These two sequences, although structurally unrelated, are both acidic in character. In the present study, we describe chimeric steroid receptors formed by fusing the VP16 activation region to the amino-terminal region of the human estrogen receptor, with or without its hormone-binding domain. We show that these chimeric receptors are better activators of target gene expression than wild-type receptors by 1 order of magnitude. These superactive receptors retain the capacity to function in either a constitutive or a hormone-dependent manner.

**EXPERIMENTAL PROCEDURES**

| Plasmid Constructions—All DNA constructions were performed by using standard procedures (Maniatis et al., 1982). The ER· expression vector was constructed by inserting the 1923-base pair SacI fragment (positions 102–2025) of the human ER cDNA into the SacI site of the SacI site of eukaryotic expression vector PSTC containing the cytomegalovirus promoter/enhancer (~522/73) (Severne et al., 1988). The ER VP16 expression vector was constructed by ligating NotI linkers onto the 231-base pair SalI (1521) to Rsal (1752) fragment of VP16. The correct orientation of the insert was determined by restriction enzyme analysis. The ER(1–422) VP16 expression vector was constructed by removing the hormone-binding domain from the BglII site (1504) to the SacI site (2025) of the ER VP16 expression plasmid. The ER(1–281) expression vector was constructed by inserting the 974-base pair SacI (102) to Hpal (1076) fragment of ER into the SacI site of PSTC. The expression vector was constructed by inserting the 974-base pair SacI (102) to Hpal (1076) fragment of ER into the SacI site of PSTC. | ERE-TKCAT plasmids used in previous studies (Klein-Hitpass et al., 1989) were constructed by insertion of synthetic oligonucleotides (21-mer) containing vifgogen A sequences from ~352 to ~318 (Walker et al., 1984) and BglII overhangs into pBLCAT2 (Lucsek and Schutz, 1987). pBLCAT2 contains the promoter of the herpes simplex virus thymidine kinase gene coupled to the CAT gene of *Escherichia coli* and RNA processing signals of simian virus 40. The **This work was supported by Grant HD-08188 from the National Institutes of Health and Postdoctoral Fellowship PF-3300 (to J. F. E.) from the American Cancer Society. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. |
ERE oligonucleotide contains the sequence 5'-GATCTAGTCTACAGTGACCTA-3'.

CV-1 Cell Transfections—CV-1 cells were maintained in 10-cm Petri dishes at 37 °C in phenol red-free Dulbecco's modified Eagle's medium supplemented with DR-JTS* (Collaborative Research Inc.). Cells were transfected at 30-50% confluence in serum-free media using Polybrene (5 μg/ml) (Kawai and Nishizawa, 1984) followed 4-6 h later by glycerol shock (Lopata et al., 1984). At 48 h post-transfection, cells were harvested, lysed, and 1-100 μg of soluble protein was assayed for CAT activity by the method of Gorman et al. (1982).

Labeling Analysis—At 48 h post-transfection, cells were washed twice with phosphate buffer, harvested, and homogenized in a 2-ml Dounce homogenizer using the B-pestle. 10 mM Tris-HCl (pH 8.5 at 4°C), 1.5 mM EDTA, 10 mM thiglycerol, 10% glycerol, 1.0 μM KCl was added to homogenates in equal volumes. The homogenates were incubated at 4°C for 1 h with resuspension every 15 min. The suspensions were centrifuged at 180,000 × g for 30 min. Protein levels were determined. The extracts were normalized to equivalent protein concentrations (4 μg/μl).

The total receptor content of the fractions was determined by incubating 50 μl of extract with either 20 nM or 0.1 μM [3H]estradiol with and without a 200-fold excess of radiolabeled estradiol overnight at 4°C. After incubation, 50 μl of hydroxyapatite slurry (Bio-Rad, 3 parts of packed gel in 5 parts of 50 mM Tris-HCl (pH 7.2 at 4°C), 1 mM KCl, 1°C) was added to each tube. The tubes were incubated on ice for 30 min with shaking every 10 min and centrifuged at 800 g for 2 min. The hydroxyapatite pellets were washed three additional times in buffer (0.5 ml) and resuspended in 1.0 ml of absolute ethanol. The hydroxyapatite/ethanol solution was vortexed and poured into scintillation vials, and any remaining hydroxyapatite was washed into the vials with two additional washes of ethanol (1 ml). Samples were counted at 25% efficiency.

RESULTS

Strategies for Constructing Superactive Estrogen Receptors—Shown in Fig. 1 are the estrogen receptor constructs generated. Two types of receptors were constructed: those lacking and those containing the hormone-binding domain. The receptors lacking the hormone-binding domain, ER(1-281) and ER(1-422) VP16, contained amino acids 1-281 and 1-422 of the human estrogen receptor (ER VP16), respectively. The VP16 activation domain was inserted into the amino-terminal region of the native estrogen receptor (ER VP16) as well as the chimeric receptor lacking the hormone-binding region (ER(1-422) VP16). We would expect those chimeric estrogen receptors containing the VP16 activation domain, ER(1-422) VP16 and ER VP16, to have a higher level of transcriptional activity than wild-type receptor (ER). Furthermore, those receptors lacking the hormone-binding domain, ER(1-281) and ER(1-422) VP16, should behave in a constitutive or hormone-independent manner; those containing the hormone-binding region, ER and ER VP16, would be expected to behave in a hormone-dependent fashion.

![Fig. 1. Strategies for constructing superactive estrogen receptors.](https://example.com)

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**Strategies for Constructing Superactive Estrogen Receptors**—Shown in Fig. 1 are the estrogen receptor constructs generated. Two types of receptors were constructed: those lacking and those containing the hormone-binding domain. The receptors lacking the hormone-binding domain, ER(1-281) and ER(1-422) VP16, contained amino acids 1-281 and 1-422 of the human estrogen receptor (ER VP16), respectively. The VP16 activation domain was inserted into the amino-terminal region of the native estrogen receptor (ER VP16) as well as the chimeric receptor lacking the hormone-binding region (ER(1-422) VP16).

**Estrogen Response Element Mediates Estrogen Receptor-dependent Activation of CAT Activity**—To determine whether chimeric estrogen receptors are better activators of gene expression than wild-type receptors, monkey kidney (CV-1) cells, which contain nondetectable levels of steroid receptors, were cotransfected with an "effector" plasmid encoding either human estrogen receptor, one of its derivatives, or vector control, and a CAT "reporter" plasmid either containing or lacking the estrogen-responsive element (ERE) of the vitellogenin A2 sequences from -332 to -318. This synthetic oligonucleotide (21-mer) was inserted as a monomer in opposite orientation immediately 5' to the thymidine kinase gene.

All the estrogen receptor mutants (ER VP16, ER(1-281), and ER(1-422) VP16) activated gene expression (Fig. 2, A and B). Although the level of activity of the chimeras containing the VP16 activation domain (ER VP16 and ER(1-422) VP16) was markedly higher than wild-type activity, the truncated ER(1-281), lacking both the hormone-binding and VP16 activation regions, had a reduced level of activity. All forms of the estrogen receptor mediated their level of activity of gene expression through the ERE.

**Estrogen Receptor Activation of CAT Activity**—To quantify the level of activity of the mutant estrogen receptors with regard to wild-type receptor, ER, ER VP16, ER(1-422) VP16, ER(1-281), and vector control plasmids (2 μg) were cotransfected with ERE-TKCAT (2 μg), 1, 2.5, 5, and 10 μg of protein extracts from transfected cells were assayed for CAT activity. Background activity (vector control) was subtracted from total activity, and this specific activity was plotted as percent conversion versus protein assayed (Fig. 3, A and B).

The level of induction was notably greater with the two chimeric receptor forms containing the VP16 activation domain (ER VP16 and ER(1-422) VP16). ER VP16 had a 10-fold greater level of activity than wild-type receptor (Fig. 3A) whereas ER(1-422) VP16 had a nearly 13-fold greater level of activity than wild-type receptor (Fig. 3B). The truncated receptor, ER(1-281), lacking both the hormone-binding and VP16 activation domains, had a reduced level of activity (Fig. 3B). Thus, the presence of the potent VP16 activation domain markedly enhanced the receptors' ability to activate gene expression.

**Estrogen-dependent and -independent Induction of CAT Activity**—To determine whether the mutant estrogen receptors mediate gene expression in a hormone-dependent manner, ER, ER VP16, ER(1-281), and KH(1-422) VP16 plasmids were cotransfected with the ERE-TKCAT reporter plasmid at equivalent DNA concentrations (2 μg) in the presence and absence of 5 nM estrogen. Only in the presence of hormone were ER and ER VP16 able to activate gene expression (Fig. 4A). In contrast, the level of gene expression for the two receptors lacking the hormone-binding region, ER(1-281) and ER(1-422) VP16, was the same whether or not hormone was present (Fig. 4B).

To determine whether the hormone-dependent chimeric receptor (ER VP16) activates gene expression in a dose-dependent manner similar to wild-type receptor, CV-1 cells were cotransfected with expression plasmids (2 μg) for ER or ER VP16 with the ERE-TKCAT reporter plasmid at equivalent DNA concentrations (2 μg) and exposed to 10^{-12} - 10^{-7} M concentrations of hormone or anti-hormone. 20- and 100-μg amounts of cellular protein extracts were incubated for 2 and 18 h, respectively, and assayed for CAT activity. Results were plotted as percent conversion versus concentration (Fig. 5).

ER and ER VP16 activated gene expression in a dose-dependent manner (Fig. 5, A and B). Stimulation of expres-
FIG. 2. Estrogen response element mediates estrogen receptor-dependent activation of CAT activity. To determine whether chimeric estrogen receptors are better activators of gene expression than wild-type receptors, monkey kidney (CV-1) cells were cotransfected with an effector plasmid (2 μg) encoding either human estrogen receptor, one of its derivatives, or vector control, and a CAT reporter plasmid (2 μg) either containing (+) or lacking (−) the estrogen-responsive element of the vitellogenin A2 sequences from −332 to −318.

FIG. 3. Estrogen receptor activation of CAT activity. To quantify the level of activity of the chimeric estrogen receptors with regard to wild-type receptor, ER (○), ER VP16 (■), ER(1-281) VP16 (▲), and vector control plasmids (2 μg) were cotransfected with ERE-TKCAT (2 μg). 1, 2.5, 5, and 10 μg of protein extracts were assayed for CAT activity. Background activity from vector-transfected control was subtracted from total activity, and specific activity was plotted as percent conversion versus protein assayed.

FIG. 4. Estrogen-dependent and -independent induction of CAT activity. To determine whether the chimeric estrogen (Eα) receptors mediate gene expression in a hormone-dependent manner, ER, ER VP16, ER(1-281), and ER(1-422) VP16 were cotransfected with ERE-TKCAT reporter plasmid at equivalent DNA concentrations (2 μg) in the presence and absence of 5 nM estrogen for 48 h.

Expression by wild-type receptor was nearly saturated by 10^{-9} M concentration of hormone. Similarly, expression was stimulated by ER VP16 at concentrations greater than 10^{-10} M estradiol and was nearly saturated by 10^{-8} M estradiol. The anti-estrogen Nafoxidine alone had no effect on gene expression by ER at 10^{-12}–10^{-7} M concentrations but did activate gene expression by ER VP16 at concentrations greater than 10^{-8} M. Although ER and ER VP16 activate gene expression in a dose-dependent manner, their sensitivities to hormone appear to be different.
Quantitation of Receptors in Transfected Cells—To determine whether equivalent levels of wild-type and chimeric receptors were produced, CV-1 cells were transfected with 2 μg of expression plasmids for ER (open symbols) or ER VP16 (solid symbols) with ER-E2CAT at equivalent DNA concentrations (2 μg) and exposed to 10^{-12}-10^{-7} M concentrations of 17β-estradiol (circles) or Nafoxidine (Naf) (triangles). 20 μg (panel A) and 100 μg (panel B) of protein extracts were incubated for 2 and 18 h, respectively, and assayed for CAT activity. Results were plotted as percent conversion versus concentration E2, estrogen.

**DISCUSSION**

We have shown that specific gene expression is efficiently activated by chimeric steroid receptors, which consist of the activation region of the herpes simplex virus transcription factor VP16 inserted into the amino-terminal region of the human estrogen receptor containing or lacking the hormone-binding region. These studies indicate that potent activation regions from viral transcription factors enhance the steroid receptor's potency in activating target gene expression. Our results agree with observations made previously showing that a hybrid protein (GAL4-VP16) activates transcription unusually efficiently in mammalian cells (Sadowski et al., 1988; Cousens et al., 1989).

Gene expression by chimeric estrogen receptor containing the hormone-binding region is dependent upon the presence of hormone. These hormone-dependent forms activate gene expression in a dose-dependent manner comparable to wild-type receptor. This suggests that the mechanism by which hormone activates receptor-induced transcription is conserved in these chimeric receptors. Although dose dependence is conserved, it appears that sensitivity to hormone might be altered slightly. Perhaps the interaction of ligand with receptor is changed slightly in the presence of a potent activation region. This could explain why we see agonistic activity of ER VP16 in the presence of the anti-estrogen Nafoxidine.

Removal of the hormone-binding region uncoupled the receptor from its hormone dependence and resulted in a constitutively active transcription factor. These results agree with previous work showing that removal of the hormone-binding region can render the receptor a less effective but constitutively active molecule (Carson et al., 1987; Godowski et al., 1987). Studies have suggested that the hormone-inducible activation function for both estrogen and glucocorticoid receptors is present within the region that contains the hormone-binding domain (Webster et al., 1988; Hollenbert et al., 1987; Godowski et al., 1987). However, the exact location and nature of the hormone-inducible activation region have yet to be defined. Deletion studies suggest that the hormone-inducible activating domain, which is present in the carboxy-terminal region (amino acids 282-595), is located either very...
close to (amino acids 314–330) or within (amino acids 330–553) the hormone-binding domain of the ER (Kumar et al., 1986, 1987). Since deletion of the carboxy-terminal region resulted in relatively low but constitutive activation of transcription, there must be an additional transcriptional activation region(s) contained within these receptors.

Addition of the VP16 activation domain to the constitutively active receptor or to the hormone-dependent receptor form produced chimeric estrogen receptors that have markedly higher levels of activity than that of wild-type receptor. The enhanced level of transcriptional activity was not due to differences in receptor levels since hormone-binding analysis of native and hormone-dependent chimeric receptors revealed that both were produced at equivalent levels in CV 1 cells. Although the levels of hormone-independent receptors could not be detected in this manner, our results suggest that the greater than 10-fold difference in level of activity by these hormone-independent forms is unlikely to be due to a difference in receptor levels.

The precise mechanism by which the VP16 activation region enhances the estrogen receptor's potential for activating specific gene expression is unknown. It has been suggested that the acidic region of VP16 interacts with unusual avidity with some component of the transcriptional apparatus (Lin et al., 1988). What is of great interest is that this strong activating region can be placed under hormonal control. How a potent activation region inserted into the amino-terminal region of the receptor can be placed under hormonal control is conjectural. Perhaps hormone binding to the receptor triggers a conformational change in the protein which unmask (derepresses) activation domains inherent to its functional configuration. This model, proposed by Godowski et al. (1987), predicts that constitutively active receptors function in the absence of hormone because the region that masks or represses the DNA-binding and activation domains has been deleted. The deleted portion of the receptor may mask these functional domains either directly or by contacting proteins that interact with the intact receptor. Even though the VP16 activation domain is in the amino-terminal region, the hormone-binding domain appears still to allow direct or indirect masking of receptor functional domains. Alternatively, this potent activation domain may not be masked, but hormone is still necessary to derepress functional DNA binding.

In vitro transcription studies will allow us to examine this important hormone-mediated response system under more defined biochemical conditions and to determine whether direct interactions of estrogen receptors with auxiliary factors of the transcription machinery exist. In addition, the ability to enhance the steroid receptor's potency in activating selected gene expression and to place potent viral activation domains under hormonal control offers interesting possibilities for studying the molecular mechanisms involved in growth, development, and oncogenesis both in vitro and in vivo.