MOLECULAR AND CELLULAR BIOLOGY, Mar. 1995, p. 1554–1563 Vol. 15, No. 3
0270-7306/95/$04.00+0

Transcriptional Activators Differ in Their Responses to Overexpression of TATA-Box-Binding Protein

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Received 18 May 1994/Returned for modification 6 September 1994/Accepted 14 December 1994

We investigated how overexpression of human TATA-box-binding protein (TBP) affects the action of estrogen receptor (ER) and compared the response with that of other activators. When ER activates a simple promoter, consisting of a response element and either the collagenase or *tk* TATA box, TBP overexpression potentiates transcription. TBP potentiates only estrogen-induced and not basal transcription and does so independent of spacing between response element and TATA box. TBP overexpression also reduces autoinhibition by overexpressed ER, suggesting that one target of the autoinhibition may be TBP itself. Both AF-1 and AF-2 domains of ER are potentiated by TBP, and each domain binds TBP in vitro. Like ER, chimeric GAL4/VP16 and GAL4/Tat activators are also potentiated by TBP, as is the synergistic activation by ER and GAL4/VP16 on a complex promoter. Unlike ER, GAL4/Sp1 and GAL4/NF-I become less potent when TBP is overexpressed. Furthermore, synergy between ER and Sp1 or between ER and NF-I, whether these are supplied by transfected GAL4 fusions or by the endogenous genes, is inhibited by TBP overexpression. Thus, ER resembles VP16 in response to TBP overexpression and is different from Sp1 and NF-I, which predominate over ER in setting the response on complex promoters.

The estrogen receptor (ER) is an upstream activator protein that binds an estrogen response element (ERE) on DNA and enhances transcription from nearby promoters. Two ER activation domains contribute to this process: AF-1 in the amino terminus, which is constitutive, and AF-2 in the C-terminal ligand-binding domain (LBD), which is active only when hormone is bound (13, 57). Neither of the ER domains is marked by an abundance of glutamine, proline, or acidic amino acids as has been noted for many other transcriptional activation domains.

The mechanism whereby the ER domains contribute to transcriptional activation is unknown, but by analogy with better-understood viral activators, it is thought that interactions with target proteins within the transcriptional apparatus are important (for a review, see reference 55; see also references 11 and 27 and references therein). The TATA-box-binding protein (TBP) is one candidate target. TBP binding to the promoter is a pivotal event leading to unwinding of the DNA at the TATA box and widening of the minor groove (31, 32, 47). TBP binding is needed for the subsequent recruitment of TFIIB and RNA polymerase II (reviewed in reference 60). Several activators bind TBP in vitro. These include the acidic activator VP16 (26, 52), E1a (3, 24, 37), c-Rel (30, 59), and Tax 1 (7). For several of these activator mutations that decrease binding to TBP decrease transcriptional activation, suggesting that the binding is of functional significance. In higher eukaryotes, TBP is tightly associated with a class of coactivators called TBP-associated factors (TAFs). This complex, but not isolated TBP, mediates transcriptional enhancement by activator proteins in vitro (10, 14, 53, 61; reviewed in reference 55). TAFs and activators also appear to directly interact. VP16 binds TAF40. Sp1 binds TAF110 (10, 19, 23), and mutations in Sp1 that decrease binding decrease transcriptional activation (17). A human $TAF_{II}30$ that binds the ER AF-2 domain and is required for transcriptional activation by ER in vitro has recently been described (27).

These observations suggest that upstream activators may regulate some aspect of TBP function, either directly or through an adapter. Further support for the notion that TBP is a target of upstream activators comes from studies of the effects of TBP overexpression. TBP supplied by transfection of mammalian cells potentiates the activity of retinoic acid beta receptor working in concert with E1a (2, 29), of c-Rel (30, 59), of Tax 1 (7), and of bovine papillomavirus E2 (22). The ability of TBP to potentiate bovine papillomavirus E2 was strong on minimal promoters with a TATA box and was limited by the presence of an initiator element, an effect that had earlier been seen in Sp1-TBP interactions in *Drosophila* cells (12).

The foregoing observations raise the question of whether the ER or other steroid receptor activation domains, which have not been examined, interact with TBP. More generally, they raise the question of whether all activators are potentiated by TBP overexpression. In this study, we investigated whether overexpression of human TBP potentiates transcriptional activation by ER on a minimal promoter consisting of an ERE and either a collagenase or *tk* TATA box. We tested the AF-1 and AF-2 domains together and separately, both for TBP potentiation and for TBP binding in vitro. We also compared the response of the ER activation domains with that of representative acidic (herpes simplex virus VP16), glutamine-rich (Sp1), and proline-rich (NF-I/CTF) activation domains, each tested on the same reporter gene. We also tested a domain from the human immunodeficiency virus activator Tat, which

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has recently been reported to bind TBP (28). We found that TBP overexpression enhances activation by ER, its individual AF-1 and AF-2 domains, and the activation domains of Tat and VP16. TBP overexpression also enhances activation by combinations of ER and VP16 or ER and Tat. However, TBP does not potentiate all activators when tested on these reporters. TBP overexpression inhibits activation by Sp1 and NF-I activation domains and becomes even more inhibitory when these activators synergize with ER on complex promoters.

MATERIALS AND METHODS

Expression vectors and promoter constructs. ERE-tkTATA was prepared by ligation of an oligonucleotide corresponding to a minimal ERE (5′-AGGTCA CAGTGACCT-3′) into the *Hin*dIII site of tkTATA, which was previously described and spans *tk* sequences −32 to +45 with respect to the *tk* transcriptional initiation site (38, 39). This plasmid has two β-globin transcription termination signals upstream of the promoter to eliminate the influence of plasmid transcripts. A single binding site for GAL4 (5′-GCGGAGTACTGTCCTCCGA-3′) was placed at the *Sal* site between the ERE and the core promoter. It should be noted that this and other reporter plasmids used in this work have the pUC AP-1 site (35) removed. Spacing mutations were constructed by digesting ERE-tkTATA with *Xba*I (43 space), *Sph*I (39 space), or *Bam*HI and *Sph*I (15 space) followed by limited mung bean nuclease treatment. Vectors were self-ligated, and ligation products were sequenced to determine the relative spacing of the ERE and TATA box. ERE-tk109 was prepared by ligation of the minimal ERE into the *Hin*dIII site of pBLCAT3, which had been modified to remove the pUC AP-1 site (40) by digestion with *Nco*I and *Eco* 109. CollTATA was prepared by ligation of an oligonucleotide, spanning interstitial collagenases sequences from −32 to +8 relative to the start site of transcription, into tkTATA from which *tk* upstream sequences had been removed by digestion with *Xba*I and *Pst*I. ERE-CollTATA was prepared by ligation of the minimal ERE into the *Hin*dIII site of CollTATA. ERE-Coll60 was prepared by ligation of the minimal ERE upstream of interstitial collagenase sequences −60 to +63 (40) in a vector without the pUC AP-1 site.

LEN-TBP was prepared by subcloning the *Bgl*II-*Bam*HI fragment, spanning the TBP cDNA, from vector pGPP26 (48) into the *Bam*HI site of pLEN (36). Human ER expression vectors HE0, HE19, HE15, and HE11 have been previously described (34), as have mouse ER expression vectors MOR121-599 and its mutant derivatives (13). The GAL4, GAL4/VP16 (50), GAL4/NF-I (53), and GAL4/Tat (16) expression vectors have been described. An expression vector for GAL4 (amino acids [aa] 1 to 147) fused to Sp1 (aa 83 to 778) and driven by the Rous sarcoma virus promoter was a kind gift of Brian West and Dale Leitman (37a). The reporter gene for transfection efficiency, in which the β-actin promoter drives expression of human choriogonadotropin (hCG), has been described elsewhere (58).

To express glutathione *S*-transferase (GST) fusions to the amino-terminal domain (aa 1 to 185) of the human ER, an *Eco*RI–blunt-ended *Kpn*I fragment spanning this domain from EGE (34) was cloned into *Sma*I- and *Eco*RI-digested pGEX-5X-1, one of the vectors of the pGEX series (Pharmacia Biotech Inc., Piscataway, N.J.). A similar fusion of the wild-type (Gly-400) LBD (aa 282 to 595) was constructed in two steps. An *Xba* fragment from HE19G (56) was inserted into the equivalent position of *Xba*I-digested SG5-HE14, which spans the ER LBD (34) to correct the mutation at aa 400 from Val to Gly (56). Then, an *Eco*RI fragment spanning the wild-type ER LBD was cloned into pGEX-3X. A fusion of the wild-type full-length receptor to GST was prepared by inserting an *Eco*RI fragment from HEG0 spanning the ER cDNA into pGEX5X-1. Vectors to express GST fusions to the mouse ER LBD (aa 313 to 599), either wild type or mutant, were constructed by inserting a *Sal*I fragment with repaired ends spanning the domain into pGEX-2X (51).

For the GST-TBP fusion pull-down assays, pGexTBP, a plasmid that encodes sequences corresponding to the full-length human TBP protein (aa 1 to 339) cloned into pGEX2TK (43, 44), was used for expression. For in vitro expression of the human full-length, N-terminal, and C-terminal ER proteins, HE0, HE15, and HE19 plasmids (34), respectively, were used. Plasmid pBX49, which encodes the carboxy-terminal region of the UL80 protein from human cytomegalovirus (1), was also used for in vitro expression as a negative control protein for these studies.

Cell lines. Both HeLa and Chinese hamster ovary (CHO) cells were grown in Coon's F-12–Dulbecco's modified Eagle's (1:1) medium supplemented with 10% calf serum (Sigma Serumax4, batch 19F-0156, low in E_2) and 1% penicillin-streptomycin in a 5% CO_2 atmosphere. CHO cells transfected with ER cDNA (clone D20) were grown in the same medium and supplemented by 40 mM $CdSO_4$ and 50 mM $ZnSO_4$ after transfection (58). For cell counts, the cells were diluted in trypan blue-containing buffer, and viable, dye-excluding cells were counted in a hemocytometer. Cell viability was greater than 95%.

Transient transfections. HeLa and CHO cells were transiently transfected in triplicate with different combinations of plasmids as described for each experiment. Expression vector DNA was held constant in each experiment by the addition of an empty expression vector, and the total amount of transfected DNA was kept below 20 μg/1 million cells. Gene transfer was by electroporation as described previously (58). The cells were then electroporated at 960 μF and plated in growth medium in six-well plates (Corning). Chloramphicol acetyltransferase (CAT) activity, corrected to background from mock-transfected cells and normalized for transfection efficiency with a cotransfected reporter gene in which β-hCG was driven by the human β-actin promoter (58), was determined after 48 to 72 h, using chloramphenicol and 8 mCi of [^{3}H]acetyl coenzyme A (Du Pont, Wilmington, Del.) per ml as described previously (46). The β-hCG reporter was assayed with a standard kit using iodinated antibody (Hybri-Tech Tandem Assay). Results were expressed as mean ± standard error of the mean. CAT activity is calculated as the increase in counts per minute per hour at room temperature (corrected for background) for 10 μl of cell extract and normalized to production of 100 standard units of β-hCG.

Receptor measurement. The ER concentration in transfected cells was measured in triplicate by a whole-cell binding assay (54). The growth medium was replaced by serum-free medium containing 0.1% bovine serum albumin (BSA) and 1 nM [^{3}H]17β-estradiol (specific activity, 92.5 Ci/mmol; Du Pont, New England Nuclear, Boston, Mass.) in the presence or absence of 100 nM unlabeled 17β-estradiol to determine nonspecific binding. After 90 min, the cells were washed with phosphate buffered saline (PBS) containing 0.5% BSA once at room temperature for 30 min and twice in 4°C and then lysed with ethanol and counted in a beta counter. Results were calculated as receptor sites per cell and expressed as mean ± standard error of the mean.

Gel electrophoresis and TBP immunoblots. Cells transfected with either TBP (10 μg) or ER (0.5 μg) or both were scraped from culture plates directly into sample buffer containing 0.4% sodium dodecyl sulfate (SDS), and each sample was sonicated at 30% intensity for 15 s (Dismembrator model 300; Fisher). The samples, which contained 100 μg of protein, were subjected to electrophoresis in 4°C for 4 h at 50 V (Miniprotean; Bio-Rad, Richmond, Calif.) in 10% polyacrylamide gel slabs. Samples were transferred to nitrocellulose at 100 mA overnight, using a Bio-Rad Transblot system. The blots were then incubated for 1 h with mouse anti-human TBP monoclonal antibody 58 at a 1:1,000 dilution. After washing, the blot was incubated with a rabbit anti-mouse polyclonal antibody (Cappel, Durham, N.C.) as the secondary antibody and then with horseradish peroxidase-linked donkey anti-rabbit antibodies (Amersham). The reaction was developed by using a Renaissance kit (Du Pont, New England Nuclear), and the film was exposed for 1 min.

GST fusion proteins. Fusions of GST to various domains of the human and mouse ER were prepared as described previously (51). Briefly, bacteria expressing the fusion proteins were resuspended in buffer IPAB-80 (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 80 mM KCl, 6 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, 1 mM ATP, 0.2 mM phenylmethylsulfonyl fluoride, protease inhibitors [pH 7.9]) and sonicated mildly, and the debris was pelleted at 12,000 rpm for 1 h in an SS34 rotor. The supernatant was incubated for 2 h with 500 μl of glutathione-Sepharose 4B beads that were previously washed with 5 volumes of PBS–0.2% Triton X-100 and equilibrated with 5 volumes of IPAB-80. GST fusion protein beads were then washed with 5 volumes of PBS–0.05% Nonidet P-40 and resuspended in 1 ml of IPAB-80 for storage at 4°C until use. All the foregoing procedures were done in a cold room at 4°C.

Assays of GST-ER fusions were carried out in a 100-μl volume that contained 40 μl of bead suspension (equivalent to 10 μl of compact bead volume) and 1 to 2 μl of ^{35}S-labeled in vitro-translated TBP (prepared with plasmid GPP 26 [48]) in IPAB-80–2.5% nonfat milk and incubated for 1.5 h at 4°C. Beads were washed five to six times with IPAB-80 containing 0.05% Nonidet P-40. Input labeled proteins, proteins bound to GST, GST-human ER, GST-mouse LBD, or GST-mouse LBD-L547A/M548A beads were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) in 10% acrylamide and then to autoradiography.

For assays of GST-TBP fusions, appropriate ER and UL80 cDNAs were transcribed in vitro, translated, and labeled with ^{35}S, using the TNT7-coupled rabbit reticulocyte lysate system as described by the manufacturer (Promega Corp., Madison, Wis.). The TBP-GST fusion protein was prepared and purified as previously described (20). Interaction studies involving the TBP-GST fusion and the in vitro-translated products were carried out as follows. Five hundred nanograms of GST-TBP fusion protein coupled to glutathione-Sepharose beads was added to 2 to 5 μl of radiolabeled, in vitro-translated ER proteins or UL80 protein in 0.2 ml of Z′ buffer (25 mM HEPES [pH 7.5], 12.5 mM $MgCl_2$, 20% glycerol, 0.1% Nonidet P-40, 150 mM KCl, 20 μM $ZnSO_4$) and rocked for 1 h at room temperature. The beads were then washed three times in 1 ml of NETN buffer (100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 20 mM Tris-HCl [pH 8.0]), pelleted by centrifugation, and boiled in 1× SDS sample buffer. Protein bound to the beads were resolved by SDS-PAGE.

RESULTS

TBP overexpression potentiates transcriptional activation by ER. To determine whether TBP is limiting for transcriptional activation by ER, we examined the effect of overexpress-

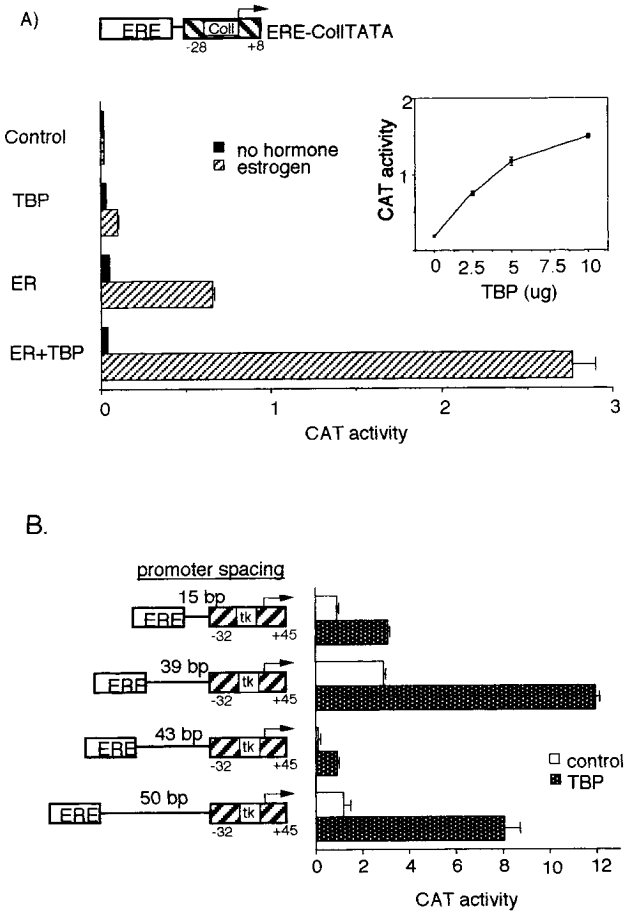

FIG. 1. Potentiation of ER action by overexpressed TBP. (A) TBP stimulation of a CAT reporter (ERE-CollTATA, illustrated) driven by a palindromic ERE and the collagenase promoter TATA box. The CAT activity of the reporter in HeLa cells transfected with expression vectors for ER (0.5 μg), TBP (10 μg), or both and in the presence (striped bars) or absence (black bars) of estrogen (10^{-7} M estradiol) is shown. The inset shows the response of the reporter to various amounts of cotransfected TBP when cotransfected with ER and exposed to estrogen. (B) TBP stimulation of reporters driven by an ERE and herpes simplex virus *tk* TATA box. Spacing of the ERE and TATA box is indicated. CAT activities of each reporter in the absence (open bars) and presence (shaded bars) of expression vector for TBP are shown. All samples were cotransfected with ER as described above, and all were exposed to estrogen.

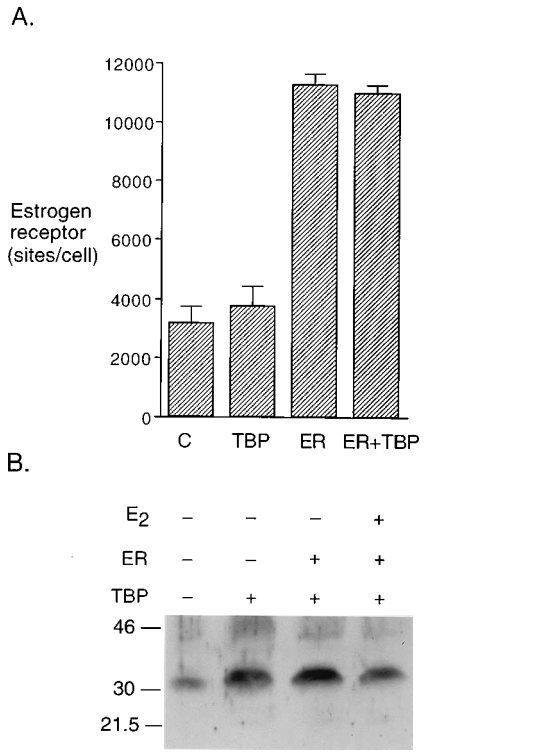

FIG. 2. Overexpression of TBP does not effect ER concentration. HeLa cells were transiently transfected with expression vectors for either ER (0.5 μg) or TBP (10 μg) or both. The cells were grown for 48 h before the assay. (A) Whole-cell binding assay for ER, expressed as sites per cell, was performed as described in Materials and Methods. Note that ER was increased in the ER-transfected cells, and the concentration was not dependent on the presence of coexpressed TBP. (B) Western blot (immunoblot) analysis for TBP. E_2 indicates the presence of estrogen. Note that the TBP concentration was increased in the pool of TBP-transfected cells and that the concentration was not dependent on the presence of unliganded or liganded ER.

ing TBP on the response of estrogen-regulated reporter genes in transiently transfected HeLa cells. For these experiments, we used an ER variant (G400V) that, unlike wild-type-transfected receptor, absolutely requires ligand for transcriptional activation (56). The CAT reporter contained a collagenase gene TATA box downstream of a single palindromic consensus ERE. As expected, transfection of ER alone caused three- to sixfold activation in response to estradiol (Fig. 1A). Cotransfection of TBP along with ER potentiated the activation by an additional 3- to 8-fold, resulting in a 40-fold total activation by estrogen. Transfection of TBP without ER caused a minimal increase in estrogen-induced CAT activity from the reporter, which may reflect potentiation of a low concentration of endogenous ER in HeLa cells by TBP (49a). The potentiation of ER by TBP was dose dependent and was not saturated even in the presence of 10 μg of TBP expression vector (Fig. 1A, inset). Basal activity showed no response to TBP in HeLa cells (Fig. 1A), nor did the activity of a promoter that lacks the ERE (not shown). Similar effects were found in CHO cells (not shown). These observations suggest that TBP is specifically limiting for ER-activated transcription of the collagenase minimal promoter in HeLa and CHO cells.

To test whether TBP potentiated ER action on core promoters other than the collagenase promoter, we tested several herpes simplex virus *tk* promoter constructions. We also varied the ERE-TATA box distance in these constructions. The action of the ER at these promoters varied with the relative rotational position of the ERE, consistent with previous observations (15, 49) (Fig. 1B). Nonetheless, TBP potentiated the estrogen-induced activity of all of the constructions to similar degrees. These observations indicate that the effects of overexpressed TBP are not limited to particular core promoters or particular arrangements of promoter and response element. They also reinforce the conclusion that TBP is limiting for ER action at minimal promoters.

To rule out the possibility that the expression of TBP changed the concentration of ER in the transfected cells, or vice versa, we measured the concentration of each of the two proteins. The mean concentration of ER in the pool of transfected HeLa cells increased approximately three- to fourfold with transient transfection, and this increase was not significantly influenced by the presence or absence of coexpressed TBP (Fig. 2A). Similarly, transfection of TBP led to an increase in the immunoblotted TBP, and this increase was not influenced by the presence or absence of cotransfected ER (Fig. 2B).

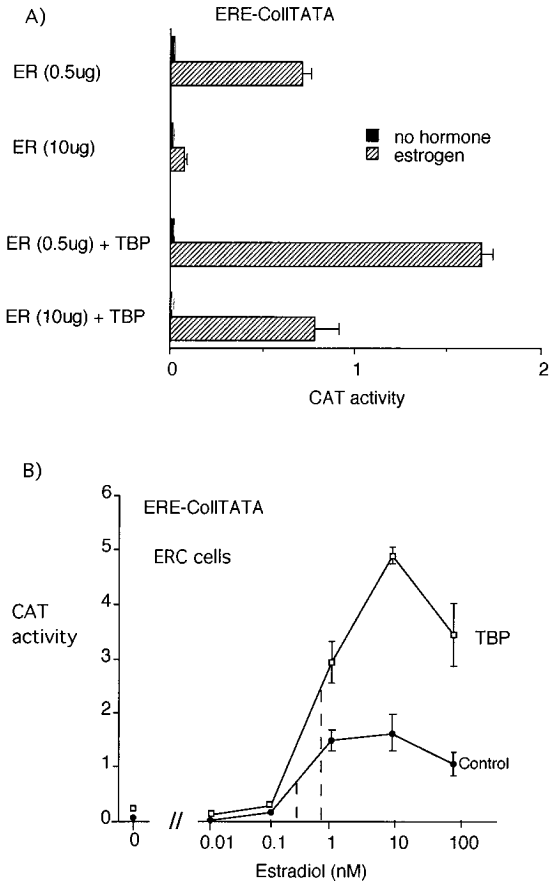

FIG. 3. TBP relief of autoinhibition by ER. (A) Autoinhibition of transcriptional activation by excess ER, supplied by 10 μg of expression vector, in the presence or absence of TBP. CAT activity generated by the ERE-CollTATA reporter is shown. Note that excess ER represses promoter activity 90% in the absence of TBP but only 50% fold in the presence of TBP. (B) Autoinhibition in a cell line (ERC-1 [58]) that stably expresses excess levels (1 million ER molecules per cell) of ER, in the presence or absence of TBP. CAT activity generated by the ERE-CollTATA reporter in response to increasing levels of estrogen is shown. Note the shift of the half-maximal response (dashed line) to the right in the presence of TBP.

TBP expression reduces autoinhibition observed at high ER concentration. Overexpression of ER causes autoinhibition of estrogen-induced transcriptional activation (45, 58). Autoinhibition may occur when excess ER interacts with a limiting component of the basal transcription machinery, thereby decreasing its availability. Since TBP is limiting for estrogen responses, we tested whether overexpression of TBP would relieve autoinhibition by ER. As shown in Fig. 3A, when the amount of transfected expression vector for ER was increased from 0.5 to 10 μg, the estrogen response of the ERE-collTATA promoter to estrogen was diminished by almost 90%. This autoinhibition was reduced to only 50% by TBP overexpression. As a separate test of the effect of TBP overexpression on ER autoinhibition, we used CHO derivatives that express more than 1 million ER molecules per cell (58) and in which inhibition can be demonstrated by increasing the amount of estrogen in the medium. As shown in Fig. 3B, the overexpression of TBP potentiated the expression of the ERE-collTATA promoter. In addition, there was a shift in the half-maximal dose of estrogen to a fourfold-higher concentration (indicated). This shift, which was observed in three independent repetitions of this experiment, is consistent with a relief of autoinhibition. Taken together, these results indicate that autoinhibition induced by a high concentration of ER is partly due to limiting amounts of TBP. Nonetheless, the persistence of some autoinhibition in the presence of overexpressed TBP suggests that TBP is not the sole target.

TBP potentiates AF-1 and AF-2. The ability of overexpressed TBP to potentiate transcriptional activation by ER is likely to reflect an interaction with the ER transcriptional activation functions. To examine this, we tested whether overexpressed TBP potentiates transcription by each of the individual activation domains of ER. The action of AF-1 was tested in two ways. The first used a truncated receptor in which the LBD and its AF-2 function had been removed (HE15; Fig. 4A and B) and which shows constitutive activity of AF-1. The second method was to test the full-length receptor (HE0; Fig. 4A and B) liganded to tamoxifen and hence inactivated for AF-2 (57). When TBP was not present, the full-length receptor liganded to tamoxifen (HE0) and the receptor missing the LBD (HE15) barely activated transcription of the reporter genes (ERE-collTATA [Fig. 4A] and ERE-tkTATA [Fig. 4B]) over the basal level (one- to threefold activation). This absence of response is consistent with the weak activity of AF-1 when tested in isolation in HeLa cells (57). In the presence of TBP, however, both the HE15 receptor and the full-length receptor liganded to tamoxifen showed greatly increased ability to stimulate transcription (up to 20-fold). This pattern was seen with both reporters. We conclude that AF-1 acting in isolation from AF-2 is potentiated by overexpressed TBP. The action of the ligand-dependent AF-2 function was tested with a truncated ER lacking the amino terminus and its AF-1 function (HE19; Fig. 4). Transcription supported only by HE19 was weak (no more than 1.5-fold) on the simple promoters used for these studies. This finding is consistent with previous reports that AF-2 is weak on promoters consisting of an ERE and a minimal TATA box (57). Nonetheless, in the presence of overexpressed TBP, the isolated AF-2 domain in HE19 activated transcription to a readily measured degree, especially with the ERE-tkTATA promoter (25-fold induction). Thus, each of the transcriptional activation domains of ER acting in isolation is potentiated by overexpressed TBP.

The cooperation between overexpressed TBP and the HE19 truncated receptor appears to require the AF-2 function itself. This is indicated by the failure of the tamoxifen-liganded HE19 receptor derivative to stimulate transcription when TBP is present (Fig. 4A and B). To confirm that AF-2 is needed for the cooperative interaction, we tested some derivatives of the mouse ER that bear mutations in the LBD that abolish the ability of this domain to activate transcription when tested with either its own or a heterologous DNA-binding domain. These mutations thus abolish AF-2 function. They are known to leave intact the ability of the receptor to bind hormone and to promote dimerization and DNA binding (13). These mutations cluster in a small region of interdigitated hydrophobic and acidic amino acids that is highly conserved among steroid receptors. We tested mutations of both the hydrophobic and acidic amino acids in the context of a truncated mouse receptor missing the amino-terminal AF-1 domain. The truncated derivative of the mouse ER (MOR121-599; Fig. 4C) cooperated with TBP to stimulate transcription from the test promoter (Fig. 4C), but derivatives bearing mutations in a pair of adjacent hydrophobic amino acids (L543A/L544A) or in three closely spaced acidic residues (D542N/E546Q/D549N) failed to stimulate transcription either with or without overexpressed TBP. Thus, TBP potentiation of HE19 action requires AF-2 function and hence reflects cooperation between TBP and AF-2 itself.

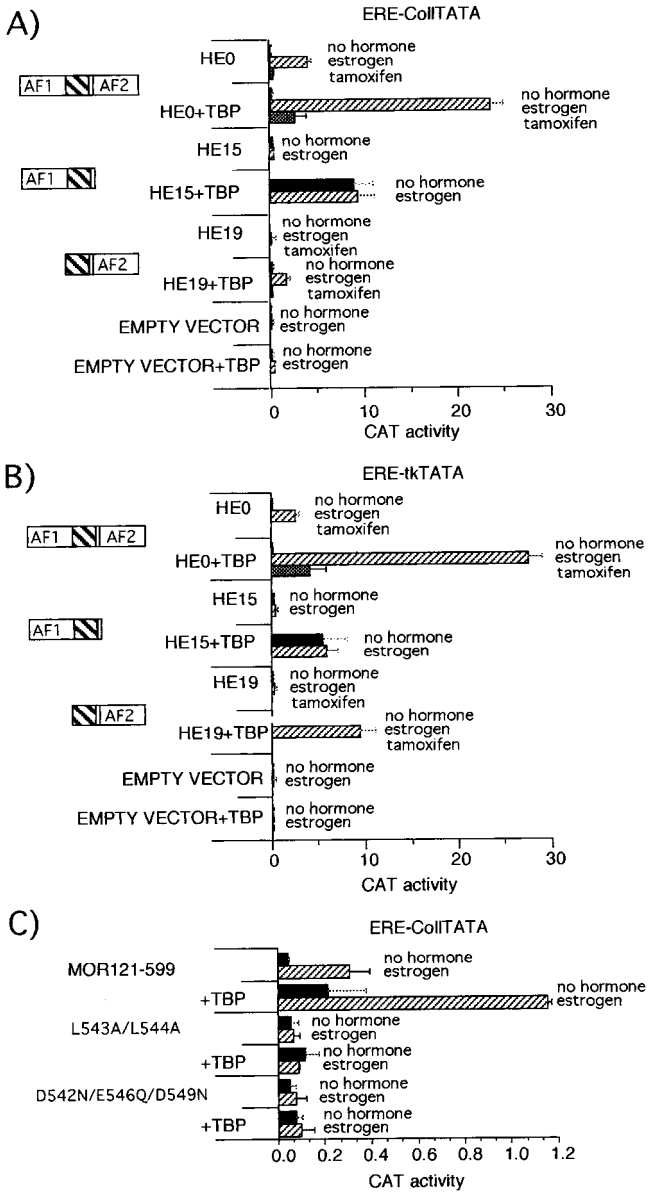

FIG. 4. Potentiation of AF-1 and AF-2 by TBP. The CAT activity of the ERE-CollTATA reporter gene (A) or the ERE-tkTATA reporter (B) in HeLa cells transfected with the indicated human ER expression vectors (0.5 μg) in the presence or absence of overexpressed TBP (10 μg) is shown. The ligand is either no hormone, estrogen, or tamoxifen (not done with HE15). Note that TBP potentiates under conditions in which AF-1 is active (HE15 and HE0 with tamoxifen) and also in which AF-2 is active (HE19). (C) Similar experiment with the AF-2 domain of the mouse ER (MOR121-599) and with two mutant derivatives (L543A/L544A and D542N/E546Q/D549N). Note that the two mutations, which disable AF-2 function, block cooperation with TBP.

Each of the ER activation domains binds TBP. To test whether the functional interaction of ER and TBP might reflect biochemical interactions, we examined the abilities of various ER derivatives fused to GST and attached to glutathione beads to pellet in vitro-translated, ^{35}S-radiolabeled TBP. We tested full-length human ER and the isolated AF-1 and AF-2 domains. We also tested three mutants, mentioned above, in the mouse ER AF-2 domain that are known to eliminate AF-2 function but maintain estrogen binding (13). Either full-length human ER (data not shown), the AF-1 region (Fig. 5A, lane 4), or the AF-2 region (Fig. 5A, lane 3) bound radiolabeled TBP. Control GST beads did not bind (Fig. 5A, lane 2). The isolated LBD of the mouse ER also bound TBP (Fig. 5B, lane 3). Mutant versions of the mouse LBD with changes in a small region around aa 545 that abolish AF-2 activity (L547A/M548A [not shown], L543A/544A [lane 4], and D542N/E546Q/D549N [lane 5]), however, also bound. Moreover, estrogen had no effect on the ER binding to TBP (data not shown). We therefore retested the binding between ER and TBP in a reverse assay in which TBP was fused to the GST beads and ER was translated and labeled in vitro. Once again, full-length human ER (HE0 [Fig. 5C, lane 5]) and truncations missing the LBD (HE15 [Fig. 5C, lane 6]) or N terminus (HE19 [Fig. 5C, lane 7]) bound to GST-TBP but not to control GST beads. A control protein from the cytomegalovirus UL80 protein (lane 8) did not bind the GST-TBP beads. None of these proteins bound control beads with GST. Since these assays are carried out in the presence of a vast excess of unlabeled control proteins, the TBP binding to ER is likely to be specific and not merely a reflection of a general tendency of TBP or ER to bind other proteins.

Thus, both full-length ER and the isolated AF-1 and AF-2 domains can interact with TBP. Because the interaction with TBP is independent of hormone and occurs with the mutant LBD derivatives that are unable to stimulate transcription, it appears that AF-2 function must involve additional aspects separate from TBP interactions. The possible nature of these separate steps is discussed below.

Activators respond differently to TBP. To investigate whether other types of transcriptional activator become more potent when TBP is overexpressed, we used expression vectors in which various activation domains were fused to the DNA-binding domain of the yeast protein GAL4. We tested these activators in HeLa cells, using a reporter with a single GAL4-binding site (and also an ERE) next to the collagenase TATA box. ER, included for comparison, gave a very strong activation in this series of experiments (Table 1), whereas the isolated GAL4 DNA-binding domain had no activity. The chimeric GAL4 activators varied in potency. TBP coexpression potentiated transcription mediated by the acidic activator GAL4/VP16, and by the HIV activator GAL4/Tat (Table 1). In contrast, TBP cotransfection did not potentiate and slightly diminished activation mediated by the glutamine-rich activator GAL4/Sp1 or the proline rich activator GAL4/NF-I. Thus, when tested with the same core promoter, TBP potentiates some activators and inhibits others.

We further investigated the pattern of interaction with TBP when transcription was stimulated by ER and a second activator in concert. We first tested ER with GAL4/Tat or GAL4/VP16, since TBP had potentiated each of these when tested individually. GAL4/Tat and ER did not synergize when both were active on the test promoter containing a cognate site for each (Fig. 6), but the combined effect of ER and GAL4/Tat was potentiated threefold by TBP. GAL4/VP16 and ER synergized strongly with each other (Fig. 6, ER and GAL4/VP16, no TBP). The combined effect of the two was further potentiated by TBP (approximately twofold; Fig. 6). Thus, even when these two potent activators synergize strongly, TBP potentiates their concerted activity.

We next tested the combination of ER with either GAL4/Sp1 or GAL4/NF-I. These activators had been slightly inhibited by overexpressed TBP. GAL4/Sp1 or GAL4/NF-I and ER synergized strongly with each other when cotransfected into HeLa cells (Fig. 7). When TBP was overexpressed, however, the cooperative stimulation of transcription by ER and GAL4/Sp1 or by ER and GAL4/NF1 was diminished (Fig. 7). The

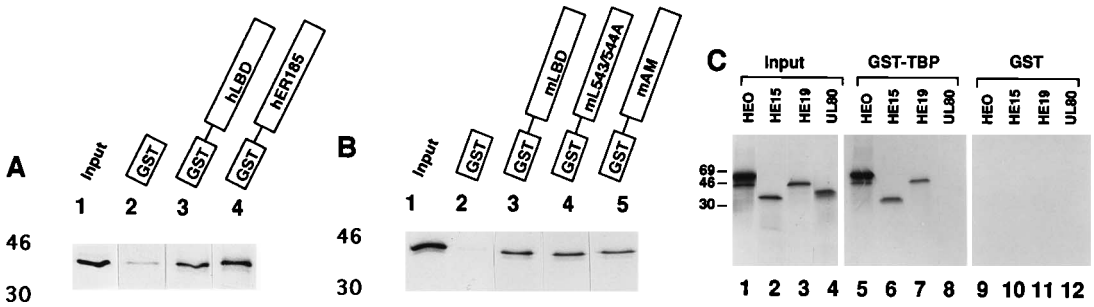

FIG. 5. Binding of ER and TBP in vitro. Shown are autoradiograms of PAGE analysis of in vitro-translated proteins after binding to the indicated fusion proteins. (A) Binding of in vitro-translated TBP to fusions of human ER domains with GST. TBP is either unreacted (lane 1) or bound to control GST (lane 2), to a GST fusion with the human ER LBD (hLB) that spans AF-2 (lane 3), or to a GST fusion with the human ER amino-terminal domain (aa 1 to 185; hER185) that spans AF-1 (lane 4). (B) Binding of in vitro-translated TBP to fusions of the mouse ER LBD and mutants thereof. TBP is unreacted (lane 1) or bound to control GST (lane 2), to GST fused with the wild-type mouse ER LBD (mLBD; lane 3), or to derivatives with the L543A/L544A mutation (mL543A/L544A; lane 4) or the acidic mutations (mAM) D542N/E546Q/D549N (lane 5). (C) Binding of in vitro-translated human ER derivatives to GST-TBP. Shown are full-length ER (HE0), ER missing the LBD (HE15), ER missing the N terminus (HE19), or a control protein, the C terminus of cytomegalovirus protein UL80, either unreacted (lanes 1 to 4), bound to GST-TBP (lanes 5 to 8), or bound to control GST beads (lanes 9 to 12). Sizes are indicated in kilodaltons.

inhibitory effect of TBP on transcription stimulated by ER and GAL4/NF-I was modest (about 30%) and similar to that seen with GAL4/NF-I acting alone (20%). The inhibitory effect of TBP on transcription stimulated by ER and GAL4/Sp1, however, was strong (70%) and more marked than that with GAL4/Sp1 alone (about 10%). Indeed, the contribution of GAL4/Sp1 to the transcriptional activation was nearly eliminated by overexpressed TBP. Thus, GAL4/NF1 and GAL4/Sp1 predominate over ER in determining the reaction to TBP.

Endogenous Sp1 and NF-I binding to native sites change potentiation of ER action by overexpressed TBP into repression. The studies described above show that when ER and GAL4/Sp1 or GAL4/NF-I supplied by transfection activate a reporter gene with synthetic GAL4 binding sites, the overexpression of TBP inhibits transcription. We wondered, therefore, whether reporter genes that contain an ERE upstream of a native promoter with sites for endogenous Sp1 and NF-I would show a similar inhibition with overexpressed TBP. We therefore examined the response to overexpression of TBP of a reporter gene (ERE-tk109) with an ERE upstream of a segment of the herpes simplex virus *tk* promoter that includes two binding sites for Sp1 and a single binding site for NF-I (42). The ERE-tk109 reporter was strongly activated by ER and estrogen, an indication of synergy between the various activators (Fig. 8). Nonetheless, overexpression of TBP diminished activation of ERE-tk109. TBP had little effect on the tk109 promoter in the absence of activation by ER, indicating that the inhibitory effects of TBP overexpression are exerted on the synergy between ER and the other activators. In contrast to the negative influence of TBP on ERE-tk109, transcription of a control reporter in which the Sp1 and NF-I sites are deleted (ERE-tkTATA [Fig. 8]; see also Fig. 1B) was potentiated by TBP. Hence, endogenous Sp1 and NF-I binding to their native sites in the *tk* promoter act similarly to the fusion proteins GAL4/Sp1 and GAL4/NF-I acting at GAL4 sites. In each case, the activators synergize with ER but confer a sensitivity to inhibition by TBP.

Because we had previously seen that TBP can potentiate ER action with a reporter gene containing an ERE and the collagenase gene TATA box, we also tested a reporter in which an ERE was placed upstream of the proximal collagenase promoter (ERE-Coll60 [Fig. 8]). This promoter includes a binding site for an activator protein (box 1 [33]) that increases basal activity (Fig. 8). Overexpression of TBP did not increase the activity of the collagenase promoter activated by endogenous

TABLE 1. Potentiation or repression of different activators by TBP[a]

Activator	Fold activation		TBP effect (fold)
	Without TBP	With TBP	
ER	27	103	3.8
	1.0	1.0	1.0
$GAL4_{1-147}/VP16_{413-490}$	36	277	7.7
$GAL4_{1-147}/Tat_{1-82}$	0.9	2.1	2.3
$GAL4_{1-147}/Sp1_{83-778}$	6.9	6.2	0.9
$GAL4_{1-147}/NF\text{-}I_{399-499}$	1.9	1.5	0.8

[a] Each value is a mean for three to seven independent determinations, each in triplicate, normalized to the level for the GAL4 DNA-binding domain (aa 1 to 147) ($GAL4_{1-147}$), which was set at 1. TBP effect on either ER, GAL4/VP16, or GAL4/Tat was significantly different from TBP effect on GAL4/Sp1 or GAL4/NF-I ($P < 0.035$, two-tailed t test).

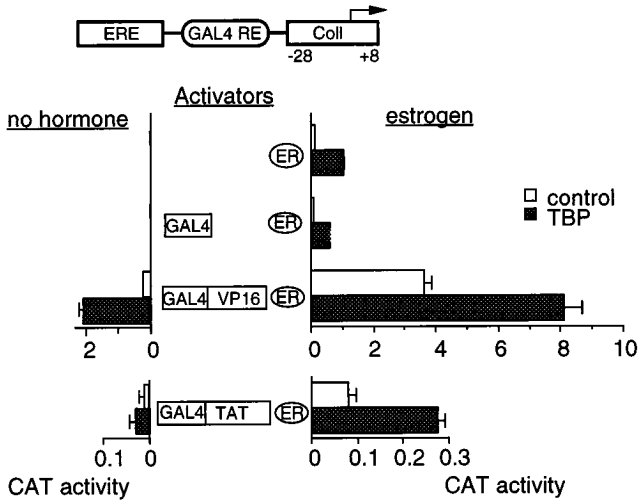

FIG. 6. TBP potentiation of transcription activated by ER and GAL4/VP16 or GAL4/Tat. Shown is the CAT activity of the ERE-GAL4RE-CollTATA reporter in HeLa cells transfected with expression vectors (0.5 μg) for ER and either GAL4 (DNA-binding domain only), GAL4/VP16, or GAL4/Tat in the absence (open bars) or presence (shaded bars) of TBP. Activities without (left) and with (right) estrogen are shown. Note that when both the second activator (VP16 or Tat) and ER are active (estrogen is present), TBP increases the promoter activity.

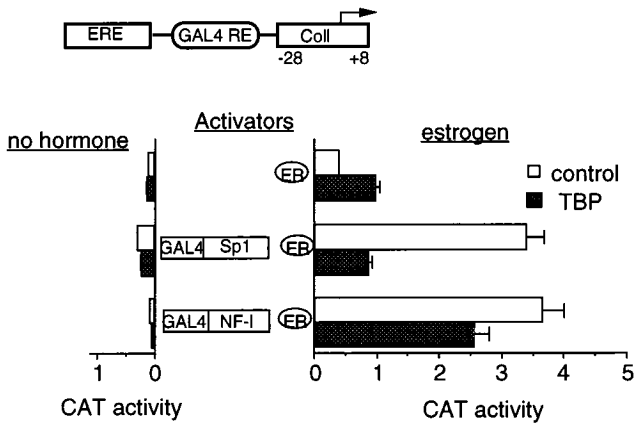

FIG. 7. TBP inhibition of transcription activated by both ER and GAL4/NF-I or GAL4/Sp1. Shown is the CAT activity of the ERE-G4RE-CollTATA reporter in HeLa cells transfected with expression vectors (0.5 μg) for ER and either no second activator, GAL4/Sp1, or GAL4/NF-I in the absence (open bars) or presence (shaded bars) of TBP. Activities without (left) and with (right) estrogen are shown. Note that when both the second activator (GAL4/Sp1 or GAL4/NF-I) and ER are active (estrogen is present), although the activators synergize strongly, TBP decreases the promoter activity.

box 1 factor (Fig. 8, Coll60). In the presence of estrogen, ER and box 1 factor cooperated and activated strongly (Fig. 8, ERE-Coll60). TBP, however, diminished their cooperative activation. ER action on a control reporter without the box 1 sequences (Fig. 8, ERE-CollTATA), in contrast, was potentiated by TBP. Thus, endogenous box 1 activator also synergizes with ER but confers a sensitivity to inhibition by TBP.

These observations of TBP effects on ER-activated transcription from complex promoters indicate that the presence of binding sites for other activators may abolish the potentiation of ER action by TBP.

DISCUSSION

Interactions between the ER activation domains and TBP. The studies described above indicate that overexpression of TBP in HeLa and CHO cells potentiates transcription activation by ER in a dose-dependent manner. The potentiation, which varies between 3- and 10-fold, occurs on promoters that contain an ERE and a minimal TATA box region from either the human collagenase promoter or the herpes simplex virus *tk* promoter. Potentiation persists despite varying distances between the ERE and the TATA box. Control experiments show that overexpression of TBP does not change the concentration of ER, nor does ER change the accumulation of TBP. Importantly, TBP overexpression in these cells has no effect on basal activation. These observations indicate that TBP is specifically limiting for ER-activated transcription of these minimal promoters. Previous studies of several activators in mammalian cells have reported a similar pattern of interaction with overexpressed TBP (2, 7, 59). TBP potentiated activated but not basal transcription from core promoters with a TATA box. On some more complex promoters, TBP is not limiting for ER action because of inhibitory interactions between TBP and other transcription factors that predominate over the interaction with ER. These inhibitory interactions are discussed in detail below.

ER has two separate activation domains, AF-1 and AF-2, and each of these is potentiated by TBP when tested in isolation. Mutations that abolish AF-2 activity abolish the activity of this domain seen in the presence of overexpressed TBP. This

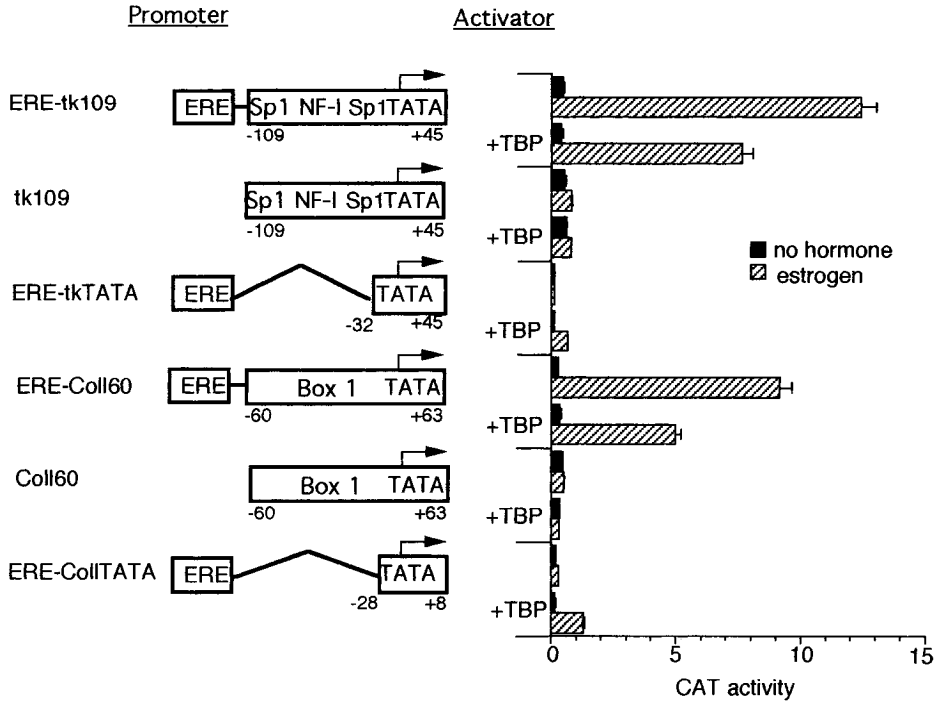

FIG. 8. TBP effects on ER action on promoters that contain an ERE and binding sites for endogenous activators. Shown is CAT activity generated by promoters containing an ERE and the *tk* flanking region including its binding sites for Sp1 and NF-I (ERE-tk109), by the collagenase promoter flanking region including the box 1 site, or by control promoters containing an ERE and lacking the other sites (ERE-tkTATA and ERE-CollTATA). Each promoter is tested with transfected ER (0.5 μg) or both ER and TBP (10 μg) and in the presence (striped bars) or absence (black bars) of estrogen. Note that whereas TBP overexpression increases ER action at ERE-tkTATA and at ERE-CollTATA, TBP decreases ER action at ERE-tk109 and ERE-Coll60.

finding indicates that TBP does not potentiate the activity of this domain by uncovering a cryptic activation function not related to the activity in the absence of TBP. Using the GST fusion protein system, we have seen that in vitro-translated TBP directly binds full-length ER and each of the activation domains. However, variants of the AF-2 domain bearing mutations in a small region around aa 545 that eliminate all AF-2 function are still able to bind TBP. This indicates that mutations of the aa 545 region disrupt a step in ER action in addition to TBP binding. This step does not appear to be binding to TFIIB, because although the ER LBD binds TFIIB (reference 25 and unpublished data), the mutations do not affect such binding (27a). It has recently been shown that these mutations disrupt the binding of ER to a candidate coactivator, RIP/ERAP (6, 8, 9, 21). Our preliminary mapping studies (not shown) place the region of interaction between ER and TBP outside of the region that mediates interaction with RIP/ERAP. It may thus be possible to identify mutations that disrupt specifically the ER-TBP interaction. Our binding studies are consistent with the previous studies of activation by retinoic acid receptor and E1A, by c-Rel, and by Tax-1 (2, 7, 29, 30, 59). Each of these proteins binds TBP in vitro; and the action of each is potentiated by overexpressed TBP. However, the connection between the ability of TBP to bind ER in vitro and the ability to potentiate ER action in vivo is currently speculative, as is true for TBP effects on the other activators noted above. An adequate test will require the in vivo testing of TBP derivatives that disrupt binding in vitro.

The ER shows potent autoinhibition when overexpressed, and we find that coexpression of TBP partly relieves this autoinhibition. The behavior of ER is similar to the previously reported behavior of overexpressed viral activator Tax-1 (7). The autoinhibition of transcription produced by overexpression of activator proteins is commonly explained by the formation of nonfunctional complexes between the excess activator and a limiting target (18, 41, 45). Thus, our observations suggest that TBP may be among the targets of autoinhibition by transcriptional activators. However, some autoinhibition by ER continues when TBP is overexpressed, and TBP fails to relieve autoinhibition at complex promoters (reference 58 and data not shown). This finding suggests that there are other targets involved. Recent in vitro studies suggest that autoinhibition by ER, E1a, or VP16 can be fully relieved by TFIID complexes and not by TBP alone (3–5). Thus, a second likely candidate for a target of autoinhibition by ER is a TFIID-associated coactivator molecule needed for ER-activated transcription. A human TAF present in only a subfraction of TFIID that preferentially stimulates transcription by the ER activation domains (4, 5) has recently been cloned (27). This protein, $TAF_{II}30$, may be among the targets. In addition, a pair of proteins, RIP and ERAP, that bind the ER LBD only in the presence of agonist ligands and do not bind the LBD of the aa 545 region mutants defective in transcriptional activity have been identified (9, 21) and cloned (6, 8). These proteins are also candidates for the target of autoinhibition.

Different activators respond differently to overexpressed TBP. We have compared the response of the ER activation domains to overexpressed TBP with that of several other activation domains. Like ER, transcription activated by GAL4/VP16 or GAL4/Tat is further potentiated by overexpressed TBP. Although ER activation domains are not similar in composition to VP16, some similarities in function have been noted (4, 5). Unlike the case for ER, activation by GAL4/Sp1, GAL4/NF-I, or endogenous Sp1 and NF-I either is not increased or is diminished by TBP. The reporter gene was held constant in these studies. Thus, different activators show different responses to overexpressed TBP, at least when tested with the collagenase or herpes simplex virus *tk* core promoter.

Our finding that Sp1 does not respond to TBP contrasts with previous observations that Sp1 activation of a promoter consisting of multiple response elements and the adenovirus major late promoter TATA box is potentiated by TBP overexpression in *Drosophila* Schneider cells (12). We do not know the reason for these differences in the Sp1 response to TBP. The differences may reflect the potent increase in basal transcription that was seen in the Schneider cells after overexpression with TBP (12). In the mammalian cells and promoters used in our studies, no increase in basal transcription occurred with TBP. In the Schneider cells, TBP stimulated basal transcription more than 100-fold and Sp1 activated transcription only 2- to 3-fold. These discrepancies may also reflect differences in the core promoters used in the different studies. In this latter regard, it should be noted that in Schneider cells, TBP failed to activate core promoters that contained an initiator element and lacked a TATA box.

When the ER is tested in concert with GAL4/VP16 on the collagenase core promoter, the paired activators synergize and are further potentiated by TBP. Thus, TBP can superactivate promoters that are already very strongly activated. When, on the other hand, ER is paired with GAL4/NF-I or GAL4/Sp1, although the activators synergize, TBP overexpression inhibits transcription. The inhibition by TBP is especially strong with the ER-GAL4/Sp1 combination. Thus, GAL4/Sp1 appears to predominate over ER and sensitizes the promoter for inhibition by TBP. Endogenous Sp1 and NF-I acting on the *tk* promoter, or the box 1 factor acting on the collagenase promoter, also synergize strongly with ER acting on an upstream ERE and confer a sensitivity to inhibition by TBP. Thus, whereas TBP is limiting for ER action on the minimal collagenase or herpes simplex virus *tk* promoter, on complex promoters, the binding of other upstream activators along with ER can make TBP no longer limiting.

Our observations of inhibitory effects of TBP overexpression on synergy of ER and Sp1 are consistent with a recent report of TBP interactions with the bovine papillomavirus E2 activator (22). TBP inhibits E2-Sp1-synergized transcription but potentiates E2 in the absence of Sp1 (22). Again the effects of promoter context must be considered. Our studies were done with the collagenase or *tk* core promoter containing the TATA box and immediate downstream sequences. Although the immediate downstream sequences are not known to have initiator function in these promoters, we did not test the isolated TATA boxes or potential initiator elements. However, in the study of E2-Sp1-activated transcription recently reported, TBP inhibited synergy between these two activators, whether from the isolated adenovirus major late promoter TATA box or an isolated initiator element. Hence, the ability of Sp1 to both synergize with other activators and confer sensitivity to inhibition by overexpressed TBP may be a general property.

The mechanisms underlying the different responses to TBP overexpression of different activators are obscure. One possibility is that they reflect differences in activator function. For example, an activator that was able to recruit TBP efficiently to the TATA box in a cell with normal concentrations of TBP might not have improved function when TBP concentrations are elevated. An activator that was poor at recruiting TBP, in contrast, might have improved function when TBP concentrations are elevated. A second possibility is that overexpression of TBP alters the composition of the different types of TBP-TAF (TFIID) complexes present in the cell. ER has recently been shown to activate transcription with a distinct TFIID complex that is not active with some other activators (27). It is

possible that TBP overexpression favors the overproduction of this complex.

In summary, ER differs from other transcriptional activators, such as Sp1 and NF-I, with respect to its ability to be potentiated by overexpressed TBP. This may point to differences in the mechanisms of action of these transcriptional activators.

ACKNOWLEDGMENTS

We thank Brian West, Greg Peterson, Grace Gill, Robert Tjian, Boris Peterlin, Danny Reinberg, Richard Metz, Rodrigo Bravo, Ellen Baum, and Pierre Chambon for plasmids. We thank Geoffrey Greene and Robert Weinzierl for antibody. We also thank Marrieta Dunaway, David Gardner, and Fred Schaufele for their critical comments on the manuscript.

This work was supported by grant BE61E from the American Cancer Society to P.J.K.

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