Different TBP-associated factors are required for mediating the stimulation of transcription *in vitro* by the acidic transactivator GAL-VP16 and the two nonacidic activation functions of the estrogen receptor

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ABSTRACT

The estrogen receptor (ER) contains two nonacidic transcriptional activation functions, AF-1 and AF-2 (formerly TAF-1 and TAF-2). In this study we show that AF-1 and AF-2 are able to stimulate transcription *in vitro* in a HeLa cell system when fused to the DNA binding domain of the yeast activator GAL4. We also demonstrate that a factor(s) required for the function of the ER AFs is chromatographically separable from a factor(s) necessary for the activity of the acidic activation domain of VP16. Moreover, immunoprecipitation experiments using a monoclonal antibody directed against the TATA box binding protein (TBP) indicate, that these different factors are associated with TBP in distinct TFIID complexes.

INTRODUCTION

The estrogen receptor (ER) is a ligand-dependent transcription factor, belonging to the nuclear receptor superfamily which includes receptors for steroid, thyroid hormones, vitamin D3, retinoic acid and ecdysone as well as orphan receptors which have no known ligands (1,2,3,4,5,6,7; and references therein). Upon binding estradiol the ER activates transcription from promoters containing estrogen response elements (EREs). Antiestrogens, such as hydroxytamoxifen and ICI164,384, also bind to the ER and modify or inhibit its action by still unclear mechanisms (8; and references therein). Several different approaches have shown that the ER can be dissected into physical and functional domains required for site-specific DNA binding, hormone binding, dimerization, nuclear localization and transcriptional activation (1,8,9,10,11,12,13,14,). The ER has two well characterised nonacidic transcriptional activation functions, AF-1 (formerly TAF-1) which is located in the N-

terminal A/B region, and AF-2 (formerly TAF-2) which is located in the hormone binding domain (E) and whose activity is hormone-dependent *in vivo* (11,15,16,17).

Initiation of transcription *in vitro* by RNA polymerase II (B) requires the ordered assembly, on sequences flanking the initiation site, of the basic transcription machinery which, in addition to RNA polymerase II, includes the general transcription factors TFIIA, IIB, IID, IIE, IIF, IIH or BTF2, and IIJ (18,19,20,21,22,23,24; and refs. therein). The first step in preinitiation complex formation is template commitment due to the binding of the TATA-binding protein (TBP), present in the TFIID fraction, to its cognate TATA box element (19,20,25,26). The activity of the basic transcription machinery can be modulated *in vitro* by transactivators bound to sites located either upstream or downstream of the initiation site (for reviews see 27,28,29).

The results of *in vivo* (17,30,31,32,33,34,35,36) transcriptional 'interference' or 'squenching' experiments have suggested the existence of transcriptional intermediary factors (TIFs), possibly equivalent to coactivators or mediators, which are required for the activity of transactivators, but not for basal level transcription. Studies *in vivo* of homo- and hetero-synergism, and transcriptional interference/squelching, with the two ER activation functions and the acidic activation domain (AAD) of VP16 (15,17) have led to the proposal that distinct TIFs may mediate the effect of different classes of activation functions.

Two models have been proposed to account for the function of intermediary factors. In the first model, TIFs serve as bridging molecules between the activator and one of the general transcription factors (17,32,38,39). In the second model, transactivators directly contact certain components of the general transcription machinery, and the TIFs may stabilize these interactions (43,45,46,47,48). Purification and characterization of the putative TIFs is a necessary first step in testing the validity of these two models.

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Recent *in vitro* transcription experiments using cloned and/or purified general transcription factors have confirmed the existence of factors whose presence is required for stimulation of transcription *in vitro* by purified sequence-specific transactivators. Comparison of the functional properties of purified recombinant TBP with those of partially purified TFIID have revealed striking differences. In contrast to recombinant TBP, the transcriptionally active component of the TFIID fraction migrates as a high molecular weight complex (18,21,37). Moreover, purified recombinant TBP is able to interact with the other general factors to reconstitute basal transcription, but in contrast to the native TFIID fraction, will not support activation of transcription by several distinct activators (35,38,39,40,41,42,43). Immunoprecipitation experiments using anti-TBP antibodies have indicated the existence of several TBP-associated factors (TAFs) (39,42). The function of at least one of these TAFs appears to be necessary for the stimulation of transcription by transactivators. Furthermore, a distinct coactivator called 'USA', which is chromatographically separable from the general transcription factors including TFIID, and is required for the *in vitro* function of activators such as SP1 and NF κ B, has been described (41,42).

Recently, using a partially reconstituted HeLa cell *in vitro* transcription system and immunoprecipitation with a monoclonal antibody against TBP, we have shown that stimulation of transcription by the chimeric activators GAL-VP16, GAL-TEF-1 and GAL-ER(EF) requires factors which are associated with TFIID complexes (60). Moreover, the activity of GAL-TEF-1 was mediated by two chromatographically distinct populations of TFIID, one of which functioned selectively with this activator, but not with GAL-ER(EF) or GAL-VP16 (60). These results directly indicated the existence of factors specifically required for the function of activators with distinct classes of activation domains.

In this report, we demonstrate that both activation functions of the ER, AF-1 and AF-2, can function individually *in vitro* when fused to the DNA binding domain of the yeast activator GAL4. Furthermore, we extend the results of our previous study by providing the first direct biochemical evidence indicating that the activities of ER AF-1 and AF-2 are mediated *in vitro* by a factor(s) which is different from that required for stimulation of transcription by the acidic transactivator GAL-VP16. Our results also indicate that these different factors are associated with TBP in chromatographically separable TFIID complexes.

MATERIALS AND METHODS

Reporter plasmids

Reporter plasmids pAL7, 17M5/pAL7 (35) and pG1 (49) have been previously described.

Overexpression and purification of the chimeric activator proteins

GAL-VP16 and GAL(1–147) were overexpressed and purified as described in (35). ER(AB)-GAL was constructed as follows: the EcoRI-Kpn I fragment, containing the sequences for amino acids 1–184 of hER was excised from HE28 (50) and inserted 5' of the GAL4 DNA binding domain (DBD) in the EcoRI-Hind III sites of pG4 poly(I) (12) using a KpnI-Hind III adaptor, the stop codon between the Hind III site and the ATG start site of the GAL4 DBD was then removed by site directed mutagenesis creating the following amino acids Gly-Thr-Gly-Thr-Ser-Leu.

Next, the ATG coding for the first amino acid of ER(AB)-GAL was mutated to an NdeI site by site-directed mutagenesis. The NdeI/BamHI fragment, now containing the entire ER(AB)-GAL coding region was inserted into the NdeI/BamHI digested pET10b vector [created by deleting the small EcoRV fragment of pET3b (51; a gift of D.Metzger)]. The ER(AB)-GAL protein was overexpressed using the T7 expression system in *E. coli* (51) and purified from cells which were sonicated in buffer A (20mM Tris-HCl pH7.9, 20% glycerol, 0.5 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF) containing 400mM KCl together with 0.1 mM PMSF. Following centrifugation to remove cellular debris and insoluble material, the supernatant was loaded on a DEAE-BioGel A column, the flow-through fraction was diluted to 100 mM KCl and loaded on a heparin-Ultrogel column, the bound protein was eluted with a linear gradient from 100–600 mM KCl in buffer A. The ER(AB)-GAL containing fractions (tested by gel shift and Western blot) were then further purified on a DNA affinity column containing oligomerized 17-mer GAL4 DNA binding sites (52).

The pSG5 based GAL-ER(EF) expression vector has been described previously [called GAL-ER (147–282) in (12)]. Prior to transfer to the baculovirus vector the mutation in the hormone binding domain at amino acid 400 was changed to a glycine (53), the GAL-ER(EF) sequences were then excised with EcoRI and inserted into the corresponding site of the baculovirus vector pVL1392 (54). Generation of recombinant virus and infection of *Spodoptera frugiperda* (Sf 9) cells were performed according to (54). To purify GAL-ER(EF), Sf9 cells were lysed in buffer A, containing 400 mM KCl, using a Dounce homogenizer. Extracts were spun for 30 min at 40,000g and the supernatant was further purified as described for ER(AB)-GAL (see above). The DNA-affinity purified ER(AB)-GAL and GAL-ER(EF) were run on SDS-polyacrylamide gels and either silver stained or assayed by Western blotting with the monoclonal B10 or F3 antibody respectively (Metzger et al., in preparation; see Fig. 1A).

Gel shift assay

DNA binding assays with the purified chimeras was carried out as described in (55) except that the binding buffer contained either no or only 50 ng of poly d(I-C) and 20 μ g of BSA for the GAL-ER(EF), and 0.1 % NP40 for the ER(AB)-GAL reactions. The different monoclonal antibodies used are described in (43) and in Metzger et al. (in preparation).

Fractionation of HeLa whole cell extract (WCE)

All operations were performed at 4°C. HeLa WCE (500 mg) was prepared according to (56), dialysed against buffer A (see above) containing 50 mM KCl and loaded on the different columns as shown in Fig. 3A. Bound proteins were eluted stepwise with buffer A for the low pressure columns and with buffer B (25mM Tris-HCl, pH7.9; 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol) for the HPLC columns containing the indicated KCl concentrations (see Fig. 3A). The PC0.5 fraction was prepared as previously described (60). Briefly, HeLa cell nuclear extract was loaded on a heparin ultrogel column. The column was sequentially washed with buffer A containing 0.24M KCl and 0.6M KCl. The 0.6M KCl fraction was then diluted 1.5 times and adjusted to 2.26 M ammonium sulphate. After stirring for 1 hour at 4°C the precipitated protein was collected by centrifugation and resuspended in buffer C [50 mM Tris-HCl

pH 7.3 (at 25°C), 20% (vol/vol) glycerol, 0.5 mM DTT, 0.5 mM EDTA]. The protein was then loaded on a phosphocellulose P11 column. The bound protein was then eluted with sequential washes with buffer C containing 0.1, 0.3, 0.5, and 1.0M KCl.

In vitro transcription

In vitro transcription reactions (20–25 μ l) contained 25 ng of pAL7 or 17M5/pAL7 and 25 ng of pG1 internal control plasmid (as indicated) along with 50 μ g of WCE or 10 μ g of Hep0.45 and 0.5 μ g of partially purified STF [TFIIA, (56)], 10 μ l aliquots of chromatography fractions (where indicated), together with 20–80 ng of purified recombinant activator proteins (as indicated). After 30 min preincubation at 25°C the reactions were initiated by addition of nucleotide triphosphates (0.5 mM) and $MgCl_2$ (5 mM) and incubated at 25°C for 45 min. Reactions were stopped by adding 300 μ l of 0.1% SDS and 5 μ g tRNA, extracted with phenol and then with chloroform and ethanol precipitated. Correctly initiated transcripts were analysed by quantitative S1 nuclease analysis (15).

Western blot analysis

Purified GAL4 chimeras (~100 ng) or extracts containing 10 μ g protein were resolved on 10% SDS-polyacrylamide gels, electrotransferred to a nitrocellulose filter and probed either with specific ER antibodies (see Fig.1A) or with a monoclonal antibody against the N-terminus of TBP [3G3, (60); see Fig.3D] as described in (34).

Immunoprecipitation

Monoclonal antibody 3G3 was prepared from ascites fluid by caprylic acid precipitation followed by precipitation with 50% ammonium sulphate. The chromatography fractions, derived either from the Hep0.45 or the SP0.3 fractions, were dialysed against IP buffer [25 mM Tris-HCl pH 7.9, 5 mM $MgCl_2$, 10% (vol:vol), 0.1% NP40, 0.5 mM DTT, 0.1 M KCl]. To pre-clear the purifie fractions they were incubated with protein G-sepharose (Pharmacia). The purified antibody was also incubated with protein G-sepharose for 2hours and the antibody-protein G complexes were pelleted by centrifugation at 2000 g and washed five times with IP buffer. The antibody-protein G-sepharose and the pre-cleared fractions were then mixed and incubated with rotation for 2 hours at 4°C. The antibody-protein G-TFIID complexes were then pelleted and washed 5 times with 10 volumes of IP buffer. The immunoprecipitated TFIID complexes were eluted by addition of an 1000 fold excess of an N-terminal peptide (PA81) corresponding to the first 17 amino-acids of TBP, which was found to react with the 3G3 antibody (60).

RESULTS

The two independent activation functions of the ER stimulate transcription *in vitro* when fused to the GAL4 DNA binding domain

To study the activation properties of the two AFs of the ER independently, they were fused to a heterologous DNA binding domain (see Materials and Methods, and Fig. 1A). The DNA-binding domain of the yeast activator GAL4 was chosen, as this domain has previously been used to create chimeras which stimulate transcription from promoters containing 17-mer GAL4 binding sites in HeLa cell extracts (34,35,39,57). The two GAL4-ER chimeras were overexpressed in either *E.coli*

Figure 1. Overexpression, purification and DNA-binding activities of the ER(AB)-GAL and GAL-ER(EF) fusion proteins. (A) The schematic organization of the ER(AB)-GAL and the GAL-ER(EF) chimeras is shown at the bottom of the panel. The numbering of the amino acids is as in the original ER and GAL4 proteins (see also Materials and Methods). The regions containing the AFs of the ER are indicated. ER(AB)-GAL and GAL-ER(EF) were overexpressed in *E.coli* or in baculovirus systems, respectively and purified as described in Materials and Methods. After purification the proteins were resolved on SDS-polyacrylamide gels and detected by either silver staining (lanes 1 and 3) or by Western blot with the B10 or F3 antibodies, respectively (lanes 2 and 4). To stabilize the purified proteins either BSA (lane 1) or insulin (lane 3) were added to 200ng/ μ l final concentrations. The positions of standard proteins with the relative molecular masses (in kDa) indicated are shown to the left of the figure. (B) and (C) Gel shift assays using the purified fusion proteins. A 5'-[32 P]-labeled oligonucleotide containing the GAL4 DNA binding site was incubated with the purified ER(AB)-GAL (B) and GAL-ER(EF) (C) proteins either alone or in combination with different antibodies (as indicated) for 15 min at 25°C and the gel shift assay was carried out as described in Materials and Methods. The positions of the specific complexes are shown with arrows. The lower arrow in (C) points to the GAL-ER(EF) complex that migrates faster in the presence of estradiol (E2), lane 6. The specific anti-B domain antibody (B10, panel B lane 3) and the anti-F domain antibody (F3, panel C lane 2) gave supershifted complexes with ER(AB)-GAL and GAL-ER(EF) respectively. The anti-GAL4 DNA binding domain antibody [2GV10, (43)] inhibited the binding of the chimeras to the labelled GAL4 binding site (panel B lane 2, panel C lane 3). The control antibodies [5GV2 and SP2, (43)] did not influence the binding of these chimeras (panel C lanes 4 and 5 and data not shown).

[ER(AB)-GAL] or baculovirus [GAL-ER(EF)] systems, and purified to homogeneity using DNA affinity chromatography (see Materials and Methods, and Fig. 1A). The affinity-purified fusion proteins were estimated to be 90–95% pure from silver stained gels (Fig. 1A, lanes 1 and 3). In Western blot analysis both, the

Figure 2. (A) Transcriptional activation *in vitro* by the ER(AB)-GAL and the GAL-ER(EF) chimeras. *In vitro* transcription reactions were carried out in HeLa WCE with either the pAL7 or 17M5/pAL7 templates (schematically represented in the bottom panel) in the absence (–) or in the presence (+) of ER(AB)-GAL (A, lanes 1–4), GAL-ER(EF) (A, lanes 5–8 and B lanes 1–5), GAL(1–147) (A, lanes 9,10) and GAL-VP16 (A lane 11) activator proteins. Quantitative S1 nuclease analysis was used to determine the amount of correctly initiated RNAs from the AdMLP promoter (AdMLP+1) in pAL7 and 17M5/pAL7 and from the reference rabbit β -globin promoter (Glob+1). ER(AB)-GAL stimulated transcription ~5 fold while stimulation by GAL-ER(EF) was ~7 fold as determined from several independent experiments. (B) Ligand-independent stimulation of *in vitro* transcription by GAL-ER(EF). Purified GAL-ER(EF) was incubated with estradiol (E2), hydroxytamoxifen (OHT) or ICI (164, 384) (ICI) as described in Results and assayed in *in vitro* transcription reactions as described above. [The binding of these ligands to the GAL-ER(EF) chimera was verified in gel shift assays (see Fig. 1C and data not shown)].

ER(AB)-GAL and the GAL-ER(EF) chimeras were appropriately recognized by specific antibodies raised against the B (antibody B10) or the F (antibody F3) regions of the ER (Metzger et al., in preparation), respectively (Fig. 1A, lanes 2 and 4). In gel shift assays both fusion proteins bound to the GAL4 DNA binding site, and addition of the B10 and F3 antibodies resulted in supershifted complexes with ER(AB)-GAL and GAL-ER(EF), respectively, confirming the nature of the complexes (Fig. 1B lanes 1 and 3, and Fig. 1C lanes 1 and 2). Moreover, an anti-GAL4 DNA binding domain antibody [2GV10 (43)] inhibited the binding of these chimeras to the GAL4 binding site (Fig. 1B lane 2, and Fig. 1C lane 3). The addition (1h at 4°C) of estradiol (50nM) or anti-estrogens [hydroxytamoxifen (1 μ M) or ICI164,384 (1 μ M)] to the GAL-ER(EF) did not modify its DNA binding, but resulted in a modification of the electrophoretic mobility of the complexes (Fig. 1C, compare lanes 1 and 6; and data not shown) indicating that the hormone binding domain in the GAL-ER(EF) chimera is able to bind these ligands. Similar differences in electrophoretic mobility have been observed for the full-length ER in the presence of different ligands (16,58; Metzger et al., in preparation). Taken together, these results indicate that both chimeric activator proteins bind DNA efficiently and specifically, and that GAL-ER(EF) can bind both estrogens and anti-estrogens.

The chimeric activator proteins were tested for their ability to stimulate transcription *in vitro* in a HeLa whole cell extract system (WCE). To this end, transcription from reporter plasmids

which contained no (pAL7) or five GAL4 binding sites (17M5/pAL7) in front of the adenovirus-2 major late promoter (AdMLP) TATA box was compared in the presence or absence of ER(AB)-GAL and GAL-ER(EF) activators (Fig. 2). The plasmid pG1 which contains the rabbit β -globin promoter (49) was included in the transcription reactions as an internal control to correct for variations in transcription efficiencies (Glob +1 signal in figures). Both ER(AB)-GAL and GAL-ER(EF) stimulated transcription 5–7 fold in HeLa WCE (Fig. 2A). This stimulation was strictly dependent on the presence of the 17-mer GAL4 DNA binding sites (Fig. 2A, compare lanes 2 with 4 and lanes 6 with 8), and on the presence of the ER activation domains since the E.coli overexpressed and purified GAL4 DNA binding domain alone (GAL1–147) gave only a weak stimulation (1.5 fold) (Fig. 2A, lanes 9 and 10). Thus, both ER AFs can independently activate transcription *in vitro*.

Interestingly, no changes in the extent of stimulation were observed when estradiol (E2) or the anti-estrogens hydroxytamoxifen (OHT) and ICI 164, 384 (ICI) were added to the transcription reaction (Fig. 2C, compare lanes 1 and 2 with lanes 3, 4 and 5). From these and above results, we conclude that *in vitro* the hormone is not required for DNA binding and transactivation by GAL-ER(EF), and that the constitutive ER AF-2 activity *in vitro* is not inhibited by ligands which abrogate AF-2 activity *in vivo* (11).

Distinct factors are required for the stimulatory activity of the ER AFs and VP16 AAD *in vitro*

HeLa WCE was fractionated by chromatography on DEAE and heparin-agarose (Fig. 3A) to identify factors necessary for transactivation by ER AF-1 and AF-2. The factors required for accurately initiated transcription from the two promoters used in this study (AdMLP and rabbit β -globin) elute between 0.05 and 0.25 M KCl from a DEAE-BioGelA column, and subsequently between 0.24 and 0.45 M KCl from a heparin-Ultrogel column (see Figs. 2A and B, and data not shown), with the exception of STF (TFIIA) which was prepared as described previously (56). The heparin 0.45 M KCl fraction (Hep0.45) supplemented with STF (Hep0.45 + STF system) was then tested for its ability to support stimulation by the two chimeric GAL-ER activators and the chimeric acidic activator GAL-VP16 (35,59). In the presence of affinity-purified GAL-VP16 (0.5 pmol), transcription was strongly stimulated (~15 fold; Fig. 3B compare lanes 5 and 6). This result indicates that the Hep0.45+STF system is not limiting in general transcription factors, as in the presence of GAL-VP16 a high level of transcription was obtained, and that the factor(s) required for stimulation by GAL-VP16 is present in the Hep0.45 fraction.

In contrast, transcription in the Hep0.45+STF system was not stimulated by either ER(AB)-GAL or GAL-ER(EF) (Fig. 3B, lanes 1 to 4). However, the combination of the Hep0.45+STF system with the fraction eluted from the heparin column between 0.45 and 0.7 M KCl (Hep0.7) resulted in a stimulation of transcription by both GAL-ER(EF) and ER(AB)-GAL (Fig. 3C, lane 1 and 2, and 11 and 12). Thus, the Hep0.7 fraction contains a factor(s) which is not absolutely required for basal transcription or for mediating the activity of the VP16 AAD, but is necessary for stimulation by ER AF-1 and AF-2. Note that the Hep0.7 contains no detectable basal transcription activity on its own (data not shown), although its addition to the Hep0.45+STF system stimulates basal transcription from the AdMLP and the globin promoter used as internal control (Fig. 3C lanes 1 and 11). Taken

Figure 3. Identification of a factor(s) required for transcriptional stimulation by AF-1 and AF-2 of ER which is distinct from the factor(s) that is necessary for stimulation by GAL-VP16. **(A)** HeLa WCE fractionation scheme. Abbreviations: WCE, whole cell extract; AS, ammonium sulfate. Proteins were eluted from the different columns stepwise as indicated. **(B)** The protein fraction eluting in a step between 0.24 and 0.45 M KCl from a heparin-Ultrogel column (Hep0.45) supplemented with STF (TFIIA) supports basal level transcription and contains a factor that is selectively required for stimulation by GAL-VP16. Reaction mixtures contained 25 ng of 17M5/pAL7 and pG1 templates (see legend to Fig. 2) and 6 μ l (10 μ g) of Hep0.45 fraction supplemented with 1 μ l (0.5 μ g) STF (TFIIA) in the absence (-) or in the presence (+) of the different purified activator proteins as indicated. **(C)** The proteins which elute from the heparin-Ultrogel column between 0.45 and 0.7 M KCl (Hep0.7) were further chromatographed on a sulfopropyl (SP) column. The Hep0.7 and the subsequent SP column fractions (as indicated) were assayed in the Hep0.45 + STF system as described above (see panel B lanes 1 to 4) in the absence or in the presence of ER(AB)-GAL (lanes 1–10) or GAL-ER(EF) (lanes 11–20). The stimulatory activity for the two ER AFs elutes in the SP0.3 fraction. **(D)** Western blot analysis of the Hep0.45, Hep0.7 and the SP column fractions. 10 ng of partially purified human recombinant TBP (rTBP) together with 10 μ g protein from the indicated fractions (see also panel A) were analysed with a monoclonal anti-human TBP antibody (3G3). The position of TBP is indicated.

together these results indicate that a factor(s) necessary for transcriptional stimulation by ER AF-1 and AF-2 is chromatographically separable from that required for activation by GAL-VP16.

The factors necessary for the activity of the VP16 or ER activation functions copurify with different TFIID populations

To further characterize the factor(s) required for the activity of ER AF-1 and AF-2, the Hep0.7 fraction was chromatographed on a sulfopropyl 5PW HPLC column. Bound protein was eluted with steps of 0.13M (SP0.13), 0.3M (SP0.3) and 1M KCl (SP1M) and analyzed by addition to the Hep0.45 + STF system (Fig. 3A and C). The factors required for the stimulatory activity of both ER(AB) and ER(EF) was found in fraction SP0.3 (Fig. 3C lanes 7 and 8, and 17 and 18, respectively). We have previously shown using an alternative reconstituted transcription system that factors required for the activity of GAL-ER(EF) and GAL-VP16 were associated with TBP. Therefore, to determine whether the factors detected in the present study were TBP-associated, the Hep0.45, Hep0.7, and the subsequent sulfopropyl column fractions were tested for the presence of TBP by Western blot analysis, using the monoclonal anti-TBP antibody 3G3 (60)

(Fig. 3D). TBP was present in several distinct chromatographic fractions [Fig 3D, see also (60)]. Interestingly, the Hep0.45 fraction, which supports activation by GAL-VP16, and the Hep0.7 or the subsequent SP0.3 fractions which allow stimulation by the ER(AB) and ER(EF) domains, all contained TBP. Note also that the SP1M fraction which inhibits basal transcription when added to the Hep0.45 + STF system, and which does not contain any detectable stimulatory activity, also contains TBP (Fig. 3C lanes 9 and 10, and 19 and 20, and 3D).

The SP0.3 fraction, containing the stimulatory activity for the ER(AB) and ER(EF) domains, was further purified on a hydrophobic phenyl 5PW HPLC column. Proteins were eluted with a linear 0.9 to 0 M ammonium sulphate (AS) gradient and again the stimulatory activity for both ER domains coeluted with TBP between 0.3 and 0.05 M AS (data not shown).

The factors required for the activity of the VP16 or ER activation functions are associated with TBP

The above results suggest that the different factors necessary for stimulation by the GAL-VP16 AAD and the ER AFs may be associated with TBP in chromatographically separable TFIID complexes. To test this hypothesis, the TBP present in the

Figure 4. The different factors required for transcriptional stimulation by the two AFs of ER and by the GAL-VP16 AAD are associated with TBP. (A–C) *In vitro* transcription reactions with the three different activators were carried out, using 25ng of 17M5/pAL7 and pG1 templates, in the PC0.5+STF(TFIIA) basal transcription system (60). In this system none of the activators stimulate transcription efficiently (lanes 7 and 8 in A, B, and C.). Lanes 3 and 4 show the *in vitro* transcription in the PC0.5+STF(TFIIA) system combined with the SP0.3-derived phenyl 5PW fraction (SP0.3/P5PW). The TBP-containing complexes from the Hep0.45 and the SP0.3/P5PW fractions were immunoprecipitated with the 3G3 monoclonal antibody and eluted with an excess of peptide (see Materials and Methods). The peptide-eluted TFIID complexes from the different fractions (as indicated) were combined with the PC0.5+STF(TFIIA) system and tested for their ability to support stimulation in the absence (–) or in the presence (+) of the three different activators (A, B and C lanes 1, 2 and 5, 6) as described in Materials and Methods. In the presence of Hep0.45 eluted TFIID GAL-VP16 stimulated the transcription 7 to 10 fold while ER(AB)-GAL and GAL-ER(EF) gave no or 1.5 fold stimulation, respectively, in several independent experiments (compare lanes 1 and 2 in A, B, and C and data not shown). In the presence of the SP0.3/P5PW eluted TFIID ER(AB)-GAL and GAL-ER(EF) stimulated the transcription ≈ 3.5 fold, while with GAL-VP16 there was no more stimulation than in the PC0.5+STF system itself (compare lanes 5 and 6 in A, B, and C).

Hep0.45 and the SP0.3-derived phenyl 5PW fractions (designated as SP0.3/P5PW in Fig.4) was immunoprecipitated with the monoclonal anti-TBP antibody 3G3. The immunoprecipitated complexes were then eluted (called eluted TFIID in Fig.4) with an excess of the synthetic peptide (PA81), corresponding to the first 17 amino acids of human TBP, which were shown to react with the 3G3 antibody (60). Since GAL-VP16 stimulates transcription in the Hep0.45+STF system (Fig. 3B), the different eluted complexes were tested for their ability to support stimulation by all three chimeric activators in another HeLa cell basal transcription system composed of a phosphocellulose 0.5M KCl fraction (PC0.5) supplemented with partially purified STF(TFIIA) (as described in 60, and see also Materials and Methods). Transcription in this system is low due to limiting quantities of TFIID and is only weakly stimulated by the addition of GAL-VP16, ER(AB)-GAL and GAL-ER(EF) (compare lanes 7 and 8 in Fig. 4A–C). Moreover, the addition of purified recombinant TBP to this system raises basal transcription but does not support activation by GAL-VP16, GAL-ER(EF) or ER(AB)-GAL(60, and data not shown).

Consistent with the results obtained above with the Hep0.45+STF system, the addition of the peptide-eluted TFIID complexes derived from the Hep0.45 fraction to the PC0.5+STF system allowed an efficient stimulation of transcription by GAL-VP16 (Fig. 4C lanes 1 and 2). However, stimulation by either ER(AB)-GAL or GAL-ER(EF), in the presence of the Hep0.45 eluted TFIID, was not greater than in the PC0.5+STF system itself (Fig. 4A and B compare lanes 1 and 2 with 7 and 8). In contrast, the combination of the PC0.5+STF system with the phenyl 5PW fractions derived from the SP0.3 fraction (SP0.3/P5PW) resulted in activation by ER(AB)-GAL and GAL-ER(EF) (Fig. 4A and B, lanes 3 and 4), whereas much lower levels of GAL-VP16 activated transcription were observed (Fig. 4C, lanes 3 and 4). Moreover, in the presence of the peptide-eluted TFIID complexes derived from these SP0.3/P5PW fractions, both ER(AB)-GAL or GAL-ER(EF) activated transcription, while in contrast stimulation by GAL-VP16 was much less efficient than with the eluted TFIID derived from the Hep0.45 fraction (Fig. 4A–C, compare lanes 5 and 6 with 1 and 2).

Taken together all of these data strongly suggest that the distinct factors which are required to mediate activation of transcription by GAL-VP16 and ER AF-1 and AF-2, are tightly associated with chromatographically separable TBP-containing complexes.

DISCUSSION

The two independent ER activation functions, AF-1 and AF-2, activate transcription *in vitro*

In the present study we show that ER AF-1 and AF-2 independently activate transcription 5–7 fold in a HeLa WCE system *in vitro*, when added as fusion proteins with the DNA-binding domain of GAL4. This activation is specific to the ER activating domains as the GAL4 DNA binding domain alone (GAL1–147) did not significantly stimulate transcription (Fig.2).

In vitro AF-2, which is contained within the hormone binding domain(ER region E), stimulates transcription irrespective of the presence or absence of hormone or anti-hormones (Fig. 2C). Similar results were obtained with the full-length ER (61), although in this case it was not determined whether the observed transcriptional activation was due to AF-1 or AF-2, or both. These *in vitro* results are in apparent contradiction with *in vivo*

observations, as *in vivo* transcriptional activation by ER AF-2 [either in the full ER or GAL-ER(EF)], is a hormone-dependent event (9,11). Several possible explanations can be proposed to account for the constitutive activity of AF-2 *in vitro*, while it is estrogen-dependent and inhibited by anti-estrogens *in vivo*. Firstly, estrogens may be required *in vivo* to 'unmask' an otherwise constitutive AF-2, whose activity could be blocked in a protein complex which may possibly include the 90kDa heat-shock protein [HSP90, for review and refs. see (8)]. This complex may not be formed in the insect *S.frugiperda* cells used to produce GAL-ER(EF), or be dissociated during the purification. Secondly, the manipulation of GAL-ER(EF) *in vitro* may result in a conformational transition in the ER region E which would occur *in vivo* only in the presence of estrogens. In addition, one would have to assume that both anti-estrogens, OHT and ICI, are unable to promote unmasking or conformational change of the ER *in vivo*, and furthermore that they cannot block the activity of AF-2 once it is established *in vitro*, even though they are both bound by GAL-ER(EF) (Fig.1C and data not shown). Thirdly, one should also consider the possibility that the effect of estrogens and anti-estrogens involve events which occur on chromatin-assembled templates, and are not seen with *in vitro* transcription systems under conditions where a chromatin structure is not reconstituted (43, and refs. therein). Further studies are clearly required to elucidate this puzzling constitutivity of ER AF-2 activity *in vitro*.

The factors which mediate the activity of the ER AFs and VP16 AAD are associated with TBP in distinct TFIID complexes

The results presented in this study indicate that HeLa WCE can be resolved by chromatography into a fraction, Hep0.45, which contains all the factors required for basal level transcription, and supports activation by the VP16 AAD, but not by the ER AF-1 and AF-2. The combination of the Hep0.45 and Hep0.7 fractions, however, does restore the stimulatory activity of both ER AFs. Thus, at least one of the components necessary for the function of the ER AFs is distinct from those required for the function of the VP16 AAD. While previous reports have indicated the existence of HeLa cell factors, required for the *in vitro* activity of several transcriptional activators (35,39,41,62), no differential effect of these factors on the activity of different classes of activating domains (ADs) was reported.

It has been shown that TBP-associated factors (TAFs) are involved in mediating the activity of the SP1 activator or chimeric activators containing proline or glutamine-rich activating domains (39,62). Using the chromatography protocols employed in this study or that described in (60), it is possible to resolve several populations of TBP-containing complexes, which can be defined functionally as TFIID. We have recently shown that the activity of chimeric GAL-TEF-1, GAL-VP16, and GAL-ER(EF) activators was mediated by factors present in such chromatographically separable TFIID complexes (60). These results also indicated that the activity of the GAL-TEF-1 activator was mediated by TFIID complexes derived from the PC0.3 fraction, whereas these TFIID complexes could not support transactivation by GAL-VP16 or GAL-ER(EF) (60). However, using the chromatography protocol employed in our previous study we could not separate the TFIID complexes mediating the activity of GAL-VP16 and GAL-ER(EF). The results of the present immunoprecipitation experiments show that the factors mediating the activity of both the ER AFs are also associated

with TBP. More importantly however, the results of the present study indicate that the TBP present in the Hep0.45 fraction in this study is associated with a factor(s) which is specifically required for the activity of the VP16 AAD, while that present in the Hep0.7 fraction is associated with factors which selectively mediate the activity of the ER AFs. Note also, that the chimeric GAL-TEF-1 activator (34,60) does not stimulate transcription in the Hep 0.45+STF system further suggesting that different factors are required for the activity of the TEF-1 and VP16 ADs (our unpublished data). Thus, taken together with those of our previous study, the results presented here indicate that distinct factors appear to be required for stimulation by the activation functions of TEF-1, VP16, and ER. In the present study, however, we were unable to identify chromatographically separable activities required for the function of the two ER AFs (see also below). These results suggest that either these activities are associated with TBP in distinct complexes which cannot be separated using the protocol described here, or that they are associated with TBP in the same complex.

Although the final peptide eluted fractions are highly enriched in the TBP-containing complexes, compared to the Hep0.45 or the SP0.3 fractions (data not shown), the stimulation by each activator is no more efficient than that observed with the starting fractions. There are several possible explanations for these results. The starting fractions may contain 'coactivators' which are not associated with TBP and which are eliminated during purification and/or by immunoprecipitation. Such non-TBP associated coactivator like activities have been characterised in several studies (63,64). Furthermore, it is possible that proteins which selectively inhibit basal but not activated transcription, allowing high levels of stimulation (41,65,66, and our unpublished results), are also eliminated by immunoprecipitation. Note also that, the addition of increasing quantities of the eluted TFIID fractions to the PC0.5+STF system results in a marked increase of basal transcription but not in transactivation. Thus, it seems that, in addition to the TAFs, other negatively and/or positively acting non-TBP associated factors are necessary to obtain high levels of activated transcription.

The existence of distinct factors required for the function of activators with different classes of ADs was inferred from previous *in vivo* transcriptional interference/squelching experiments (17,67), using activators with both acidic and non-acidic ADs (15). In particular it was shown that each activator can squelch its own activity (self-squelching) and that both ER AFs could interfere with activation by the VP16 AAD, while the latter AAD could not interfere with the action of the ER AFs (17). The validity of this prediction is supported by the present and previous (60) results which suggest that besides a set of core TAFs, which may be common to several or all TFIID complexes, activator specific TAFs may be associated with specific TFIID populations. The presence of a set of heterogeneous TFIID complexes which contain factors selectively required for the function of one or several activators facilitates the understanding of the self-squelching previously observed *in vitro* with several activators (32,33,34,35,36,). TFIID complexes containing the TAFs required for the function of a given activator could be titrated by an excess of this activator leading to an abrogation of stimulation, but basal transcription could still be obtained due to the presence of other TFIID complexes, which do not contain these factors and thus, are not titrated out. However, the present results do not yet allow us to formulate a simple model to explain the hetero-squelching between the ER AFs and the VP16 AAD

described above, or the homo- and heterosynergism observed between different activators (15,17). A full understanding of the molecular mechanisms by which transcriptional activators function will require the cloning of all of the actors involved in activation of initiation of transcription.

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