Oestrogen receptor facilitates the formation of preinitiation complex assembly: involvement of the general transcription factor TFIIB

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The action of oestrogen hormones is mediated through the oestrogen receptor (ER), a member of a large superfamily of nuclear receptors that function as ligand-activated transcription factors. Sequence-specific transcription factors, including the nuclear receptor superfamily, are thought to interact either directly or indirectly with general transcription factors to regulate transcription. Although numerous studies have focused on the identification of potential co-activators interacting with isolated trans-activation domains of ER, few have investigated the mechanisms by which ER transmits its signal to the basal transcription machinery. We show that ER does not stabilize the binding of the TATA-box binding protein (TBP) of the TFIID complex, or of TFIIB to the promoter, although a stable ER–TBP–TFIIB–promoter complex was detected, suggesting that ER, TBP and TFIIB might interact with each other to form a complex to the promoter. We also demonstrate that ER binds specifically to TFIIB, a key component of the preinitiation complex. Affinity chromatography with immobilized deletion

mutants of ER maps a TFIIB interaction region that encompasses the DNA-binding domain. The addition of excess TFIIB to transcription reactions *in vitro* did not, however, affect the magnitude of transcriptional activation by ER. These results indicate that, in contrast with current models, ER does not activate transcription by increasing the rate of assembly of TFIIB into the transcription complex. An increased concentration of TFIIB was unable, by itself, to overcome the requirement for ER. By using an immobilized promoter-template assay employing nuclear extract from HeLa cells, recombinant human ER increased the stable association of subsequent components of the transcription machinery (TFIIE and TFIIF), in correlation with ER-induced transcription. Our results suggest that ER acts, in an early step, during or immediately after the formation of template-committed complexes containing TFIIB, favouring the recruitment of one or more components of the basic transcription machinery as well as co-activators.

INTRODUCTION

The oestrogen receptor (ER) is a ligand-dependent transcription regulatory factor that belongs to the nuclear receptor (NR) superfamily (reviewed in [1]). Like other members of the superfamily, ER exhibits a modular structure composed of three regions, with the N-terminal region containing a constitutive trans-activation function (AF-1), the DNA-binding central domain (DBD), and the C-terminal region that encompasses the ligand-binding domain (LBD), a dimerization surface and a ligand-dependent trans-activation function (AF-2) [2]. On activation by hormone binding, the receptor interacts specifically with a *cis*-acting DNA sequence called the oestrogen-responsive element (ERE) and enhances transcription from the nearby promoter [2]. The molecular mechanisms by which ER contributes to transcriptional activation have not been completely elucidated. Promoter-specific initiation of mRNA synthesis in eukaryotic cells requires the assembly of RNA polymerase II and basal transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIF at core promoter sequences (reviewed in [3,4]). Biochemical dissection of TFIID has revealed that this transcription factor consists of TATA-box binding protein (TBP) and as many as 13 bound subunits, the TBP-associated factors (TAFs), ranging in size from approx. 18 kDa to 250 kDa [5,6]. For regulating gene expression, transcription factors, including NRs, have to interact with one or more components of the transcription initiation complex either directly or indirectly

through intermediary factors, the so-called co-activators/mediators [7]. A new TBP-associated factor, TAF_{II}30, has been shown to interact specifically *in vitro* in a ligand-independent manner with the ER LBD [8]. It has been recently proposed that TAFs are critical targets for transcription activators and act as co-activators that mediate transcriptional activation of upstream enhancer-binding factors [9,10]. Several putative co-activators that interact with AF-2 of ER in a hormone-dependent fashion have been identified, including ERAP-160 and ERAP-140 [11], RIP-140 and RIP-160 [12], RING finger proteins TIF1 [13], TRIP1 [14] (homologous with the yeast SUG1 protein characterized as 3′–5′ DNA helicases [15]) and NR transcription mediators SRC-1 [16] and TIF2 [17], which function in transcriptional activation through their histone acetyltransferase activities. However, their targets within the preinitiation complex (PIC) remain unknown at present with the exception of TRIP1, which can interact *in vitro* with TBP and TAF_{II}30 [14]. So far only SRC-1 and TIF2 have been demonstrated to stimulate AF-2 activity significantly in transfection experiments [16,17]. In addition, it has been demonstrated that the co-activator of the cAMP response element-binding protein (CREB) transcription factor, CREB-binding protein (CBP) and the related E1A-associated p300 protein, enhance steroid receptor-dependent transcription [18–21]. However, the CBP/p300 co-activators have a more general role and are known to stimulate the activity of an increasing number of transcription factors [22].

Abbreviations used: AdML promoter, adenovirus major late promoter; DBD, DNA-binding domain; DTT, dithiothreitol; ER, oestrogen receptor; ERE, oestrogen-responsive element; EMSA, electrophoretic mobility-shift assay; GST, glutathione S-transferase; h, human; HNF-4, hepatocyte nuclear factor 4; LBD, ligand-binding domain; NR, nuclear receptor; PIC, preinitiation complex; PR, progesterone receptor; TAF, TBP-associated factor; TBP, TATA-box binding protein.

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In contrast with the identification of these potential co-activators, little is known about the mechanisms by which ER propagates its signals to the basal transcription machinery. Interestingly, ER has been reported to interact *in vitro* with TBP [12] and TFIIB [23]. By using glutathione S-transferase (GST) fusion protein, it has been shown that TBP directly binds full-length ER and each of the activation domains AF-1 and AF-2. An interaction between TFIIB and a truncated ER mutant lacking AF-1 has also been reported but the domain within the receptor required for this interaction has not yet been identified. However, the significance of these interactions has not been determined. In the present study we investigated whether TBP and TFIIB might have a role in transcriptional activation by ER. We report here that ER does not stabilize the binding of TBP to DNA in the absence of TFIIB, suggesting that ER, TBP and TFIIB might interact with each other to form a stable complex with the promoter. Using an immobilized-template assay we show that ER increases the stable association of downstream basal transcription factors (TFIIE and TFIIIF) and that this stable association is correlated with ER-induced transcription.

EXPERIMENTAL

DNA constructs

Oligonucleotide primers containing *EcoRI* and *SstI* sites at the ends were used to isolate adenovirus major late (AdML) promoter fragments of different lengths. The -54 to -17 fragment of the AdML promoter was subcloned upstream of the G-free cassette into plasmid pC₂AT19 [24], and two copies of the ERE-binding site derived from the *Xenopus* vitellogenin A₂ gene (5'-AGGTCACAGTGACCT-3') were inserted at the unique *EcoRI* site, giving rise to the plasmid pERE₂Ad G-free. For electrophoretic mobility-shift assay (EMSA) and immobilized-template assays the -54 to $+53$ fragment of the AdML promoter was subcloned into Bluescript vector (Stratagene) and one or two copies of the ERE-binding site were inserted at the *EcoRI* site, giving rise to the plasmids pERE-AdTATA and pERE₂-AdTATA.

Protein production and purification

Calf uterus ER was purified as described previously [25]. Human ER (hER) was expressed in Sf9 cells with the pBlueBac His baculovirus expression system (Invitrogen Corp.). Sf9 cells in spinner culture were infected with 6His-hER baculovirus and cultured for 72 h. Cells were then harvested by centrifugation and lysed by freeze-thawing in a buffer containing 40 mM Tris/HCl, pH 7.4, 1 mM EDTA, 10% (v/v) glycerol, 1 mM dithiothreitol (DTT), 0.2 mM PMSF and 5 µg/ml leupeptin. The homogenate was centrifuged at 105 000 g for 1 h. The clear supernatant was loaded on a DEAE-Sephacel column (Pharmacia). After washing, elution was performed by applying a linear 0–0.4 M KCl gradient. Receptor concentration was determined by hormone-binding analysis. The hER-containing samples were stored at -70°C . Further purification over Ni²⁺/NTA-agarose (Qiagen) was unsuccessful.

Recombinant human TBP and TFIIB were tagged with six histidine residues at the N-terminus, expressed in *Escherichia coli* from the T7 promoter of the pET28a vector (Novagen), and purified over a Ni²⁺/NTA-agarose as described previously [26,27]. Recombinant purified untagged hTBP and hTFIIB were from Promega. The expression and purification of GST-ER mutants were performed as described previously by Jacq et al. [8].

EMSA

The 142 bp DNA fragment obtained after digestion of the pERE-AdTATA by *EcoRI* and *SstI* was end-labelled with [γ -³²P]ATP and T4 polynucleotide kinase (Boehringer). Purified calf ER (70 pmol/ml), purified recombinant untagged TBP (150 pmol/ml) and TFIIB (20 nmol/ml) were incubated alone or together with 0.2–0.4 ng of end-labelled probe [$(2-4) \times 10^4$ c.p.m.] in a binding buffer containing 20 mM Hepes, pH 7.9, 1 mM DTT, 5 mM MgCl₂, 100 ng of poly(dG-dC) (Boehringer), 5% (v/v) glycerol and 2 µg of BSA in a final volume of 20 µl. Binding reactions were left for 30 min at 30 °C. Protein–DNA complexes and free DNA were resolved on a non-denaturing 4% (w/v) polyacrylamide gel with running buffer containing 0.25 × TBE (1 × TBE is 89 mM Tris/89 mM boric acid/2 mM EDTA) and 2 mM MgCl₂. The gel was cast in running buffer supplemented with 0.5 mM DTT, 2 mM MgCl₂ and 10% (v/v) glycerol. After suitable separation, gels were dried and autoradiographed at -70°C .

Transcription assays *in vitro*

Transcription assays contained 25 mM Hepes, pH 7.9, 50 mM KCl, 5 mM MgCl₂, 10% (v/v) glycerol, 2 mM DTT, 2.5 mM creatine phosphate (Sigma), 0.4 mM ATP, 0.4 mM CTP, 16 µM UTP, 10 µCi of [α -³²P]UTP (400 Ci/mmol; Amersham), 10 units of RNase T1, 100 µM 3'-O-methyl-GTP (Pharmacia), 30 units of RNasine and 1 µg of sonicated herring sperm DNA in a final volume of 30 µl. Partly purified recombinant hER (300 fmol) and HeLa nuclear extract (40–60 µg of proteins) were incubated with 100 ng of pERE₂Ad G-free for 20 min at 30 °C. For the transcription assays *in vitro* with TFIIB-depleted nuclear extract, anti-TFIIB monoclonal antibody (Promega) was coupled to Protein A–Sephacel (Pharmacia) and the nuclear extract was incubated for 1 h at 4 °C with the resin. After centrifugation the supernatant was saved and analysed by transcription *in vitro* in the absence or presence of ER and with various concentrations (0.4–400 ng) of purified His-TFIIB. Transcriptions were initiated by the addition of nucleotide triphosphates and continued for 1 h at 30 °C. Reactions were terminated by the addition of 270 µl of stop mix [0.5% SDS/0.3 M Tris/HCl (pH 7.4)/0.3 M sodium acetate/2 mM EDTA/3 µg/ml tRNA] followed by extraction with phenol/chloroform/3-methylbutan-1-ol and precipitation with ethanol. The transcripts were analysed on a 6% (v/v) polyacrylamide sequencing gel and dried, then autoradiographed for 24–48 h at -70°C with an intensifying screen. All transcription assays *in vitro* were quantified by densitometric scanning of the autoradiographs.

Protein–protein interaction assays

Far-Western blotting

The pGEM7Zf plasmid (Promega) harbouring the hER cDNA was transcribed and translated in a rabbit reticulocyte lysate in the presence of [³⁵S]methionine with a commercial translation kit (TNT Promega). Partly purified recombinant His-TFIIB was subjected to SDS/PAGE [12.5% (v/v) gel] and electroblotted to nitrocellulose. After denaturation/renaturation in 6 to 0.187 M guanidinium chloride in buffer TGKE [20 mM Tris/HCl (pH 7.9)/0.25 M KCl/0.2 mM EDTA/10 mM 2-mercaptoethanol/0.5 mM PMSF/10% (v/v) glycerol], filters were saturated at 4 °C in buffer TGKE containing 5% (w/v) skimmed milk powder and then incubated overnight at 4 °C in buffer TGKE containing 1% skimmed milk in the presence of ³⁵S-labelled ER. The filters were then washed three times with buffer TGKE, three times with buffer TGKE without glycerol, once

with 15 % (w/v) trichloroacetic acid and finally with 50 % (v/v) ethanol. Filters were dried and exposed for autoradiography.

GST pull-down

Recombinant TFIIB cDNA in pSG5 was transcribed and translated in rabbit reticulocyte lysates in the presence of [³⁵S]-methionine. ³⁵S-labelled TFIIB was then incubated with GST-ER mutants immobilized on glutathione-agarose beads containing 5 % (w/v) skimmed milk. The beads were washed twice with NTEN buffer [20 mM Tris/HCl (pH 8)/1 mM EDTA/0.5 % Nonidet P40 (Sigma)/100 mM NaCl] and with NTEN buffer containing 250–350 mM NaCl. Samples were analysed by SDS/PAGE [12.5 % (w/v) gel] after elution of the bound proteins by SDS-sample loading buffer, and the amounts of ³⁵S-labelled TFIIB were measured by autoradiography.

Sequence-specific DNA affinity resin

The ER was immobilized on the ERE DNA-affinity resin as described [28]. Thereafter the procedure followed was similar to that in GST pull-down experiments except that the bound ³⁵S-labelled TFIIB translated *in vitro* was eluted with 0.5 M KCl. Samples were analysed by SDS/PAGE [12.5 % (w/v) gel], and the amounts of ³⁵S-labelled TFIIB were measured by autoradiography.

Immobilized DNA-template assay

We used the original procedure [29] with some modifications. The *Eco*RI/*Sma*I fragment of pERE₂-Ad G-free was end-labelled with biotin-14-dATP (BRL) with the Klenow fragment of DNA polymerase I. Dynabeads M-280 streptavidin (Dyna) were prewashed three times in TEN buffer [10 mM Tris/HCl (pH 7.5)/1 mM EDTA/1 M NaCl]. Dynabeads (400 µg/µg of DNA) were incubated overnight at 25 °C in TEN buffer. The Dynabeads coupled with DNA were then purified with a magnetic particle concentrator (Dyna). The interaction of partly recombinant purified hER (1 nmol) with general transcription factors supplied by HeLa cell nuclear extracts (50 µg of proteins) (Promega) took place on biotinylated immobilized DNA template by using 20 µl of beads (10 pmol of DNA) per 250 µl (final volume) of transcription buffer [20 mM Hepes (pH 7.9)/100 mM KCl/0.2 mM EDTA/0.5 mM DTT/20 % (v/v) glycerol]. Reaction mixtures were incubated for 1 h at 25 °C and for 30 min on ice. Complexes were purified magnetically; the beads were then washed three times in transcription buffer containing 0.003 % Nonidet P40. Transcription assays *in vitro* were performed and analysed as described above except that washed beads were incubated for 1 h at 30 °C. For immunoblot detection of bound proteins, the beads were eluted with SDS-sample loading buffer, the proteins were resolved by SDS/PAGE [12 % (w/v) gel] and electroblotted to nitrocellulose. The membranes were incubated with a monoclonal antibody against ER (H222) and with monoclonal antibodies against four basal transcription factors TBP, TFIIB (Promega), TFIIF (RAP30) and TFIIE (TFIIE-α subunit) (Santa Cruz Biotechnology). The immune complexes were detected with an enhanced chemiluminescence detection kit (Amersham).

Cell transfection

Chinese hamster ovary cells were routinely maintained in Phenol Red-free Dulbecco's modified Eagle's medium/Ham's F12 supplemented with 10 % (v/v) charcoal-treated fetal calf serum and were transfected by the CaPO₄ co-precipitation method. The

transfected DNA included a reporter plasmid, ERE₂-37tkCAT (10 µg), 2 µg of pCH110 used as an internal control for transfection efficiency (Pharmacia), 25 or 500 ng of pSG5-hER (pER) in the presence of variable amounts of pSG5-hTFIIB (pTFIIB), and pSG5 vector to a total of 20 µg of DNA per dish. After transfection, cells were maintained in the presence or absence of 10 nM oestradiol. After 24 h, cell extracts were assayed for chloramphenicol acetyltransferase activity as described [30]. β-Galactosidase activity was used to correct for differences in transfection efficiency in all experiments [31].

RESULTS

Characterization of ER-TBP-TFIIB-DNA complex by EMSA

To test whether ER influences an early step in PIC assembly, we used purified recombinant TBP and TFIIB, purified ER [25] and a 140 bp DNA fragment containing an ERE site and the TATA box of the AdML promoter separated by 35 bp. The results of the EMSAs are presented in Figure 1.

ER and TBP were each able to form complexes with the DNA probe; these complexes were distinguishable by their electro-

Figure 1 ER enhances the stability of TBP-TFIIB-DNA complexes

Upper panel: purified ER (210 fmol), TBP (450 fmol) and TFIIB (10 pmol) were incubated with the ³²P-labelled DNA probe (approx. 25 000 c.p.m.) as indicated above each lane for 30 min at 30 °C in 20 µl before electrophoresis in a 4 % (w/v) polyacrylamide gel. The migrations of specific complexes are indicated at the right. Lower panel: preformed ER-TBP-TFIIB-DNA complexes were incubated with 1.5 µl of anti-ER (H222) (90 µg/ml), 1.5 µl of anti-TBP (180 µg/ml) or 6 µl of anti-TFIIB (220 µg/ml) for 1 h at room temperature and 30 min at 4 °C before electrophoresis in a 4 % (w/v) polyacrylamide gel. The run was performed at 4 °C.

Figure 2 ER increases the formation of TBP-TFIIB-DNA complexes by recruitment into complexes of lower mobility

TBP-DNA and ER-TBP-DNA complexes were formed with the 32 P-labelled DNA probe (approx. 15 000 c.p.m.) as described in the legend to Figure 1, in the absence (lanes 2 and 6) or presence of increasing amounts of TFIIB (lanes 3 and 7, 2 pmol; lanes 4 and 8, 4 pmol; lanes 5 and 9, 8 pmol).

phoretic mobilities (Figure 1, upper panel). Both ER and TBP, although present in excess over the DNA, retarded only a portion of the probe. Simultaneous incubation of the probe with ER and TBP did not lead to the formation of a stable complex: three retarded bands (ER-TBP-DNA, TBP-DNA and ER-DNA) could be distinguished, as well as a smear through the gel, suggesting that the ER-TBP-DNA complex was unstable and underwent dissociation during electrophoresis. Thus ER failed to stabilize the TBP-promoter interaction. TFIIB alone did not bind to the probe and did not affect the mobility of the ER-DNA complex (results not shown) but, as expected, TFIIB stabilized the TBP-DNA interaction [32,33] resulting in a higher proportion of the probe in the retarded complex. When the probe was incubated with TBP, TFIIB and ER, all of the free probe was shifted into a single complex of low electrophoretic mobility. Under the same conditions, ER did not bind to a control probe lacking an ERE-binding site (results not shown). The presence of TBP, TFIIB and ER bound to the same probe was demonstrated by the supershifted complexes observed after incubation with monoclonal anti-ER, anti-TBP and anti-TFIIB antibodies (Figure 1, lower panel). Similar results were obtained with recombinant purified His-tagged hTBP and hTFIIB (results not shown). The TBP-TFIIB-DNA complex gave a weak signal at low TFIIB concentrations (Figure 2). This complex became more abundant with increasing amounts of TFIIB. When TFIIB was added to a reaction mixture containing ER, TBP and DNA, formation of the slowly migrating ER-TBP-TFIIB-DNA complex was stimulated appreciably, concomitantly with a decrease in the ER-DNA complex, even at the lowest TFIIB concentration tested. The mobility of the protein-DNA complexes decreased slightly but reproducibly with increasing TFIIB concentrations and thus did not depend on the protein preparations used. These results show that TFIIB stabilized the binding of TBP and ER to DNA. Taken together, these results suggest that ER, TBP and TFIIB interact with each other to form a stable complex with the promoter.

Figure 3 TFIIB binds to the ER-DNA complex

35 S-labelled TFIIB was prepared by translation *in vitro* (as described in the Experimental section) and loaded on an agarose ERE affinity column either with (lane 3) or without (lane 2) DNA-bound ER. After extensive washing, the bound proteins were eluted with 0.5 M KCl, separated on 12.5% (w/v) polyacrylamide gels, and detected by fluorography. The input (lane 1) contained 10% of the labelled TFIIB.

Direct interaction of ER with the basal transcription factor TFIIB

To determine whether ER bound to ERE might contact TFIIB, a sequence-specific DNA-affinity method was used [28]. Oligonucleotides containing ERE-binding sites were coupled to Sepharose resin as described previously [28]. Partly purified recombinant ER was loaded on the column and the unbound material was removed. A control DNA affinity resin that lacked ER protein was also prepared and run in parallel. The [35 S]methionine-labelled TFIIB translated *in vitro* was incubated with each resin and, after being washed, the bound proteins were eluted with a high-salt buffer (0.5 M KCl). The input and eluted material were subsequently analysed by SDS/PAGE followed by fluorography (Figure 3). These experiments showed that TFIIB could be retained on the ER-containing resin. As expected, no TFIIB was retained on the control matrix. These results suggest a direct contact between ER and TFIIB.

In the next series of experiments, direct DNA-independent physical interactions between ER and TFIIB were investigated. First, far-Western blotting experiments (Figure 4) showed an interaction between ER and TFIIB, independently of the presence or absence of hormone or anti-hormone, suggesting that it is an intrinsic property of ER. Secondly, to determine further which domains of ER interact with TFIIB, affinity chromatography was performed with immobilized deletion mutants expressed as GST fusions in *E. coli*, bound on glutathione-agarose beads and incubated with [35 S]methionine-labelled TFIIB. Specific complexes were subjected to several stringent washes and analysed by SDS/PAGE. Fusions between GST and the ABC (GST-HE 15) or the CDEF (GST-HE 19) regions of ER specifically retained TFIIB (Figure 5). To map the interaction domain further, we included a series of C-terminal and N-terminal deletion mutants in our analysis. In addition, region D (residues 259-302) and the N-terminal part of the E region (residues 283-330) of the ER were also expressed as GST fusions. The binding of TFIIB to HE19Δ429 indicates that the integrity of LBD is not necessary for this interaction. From the results obtained with GST-HE 72, GST-HE 259-302 and GST-HE 283-330 we can conclude that the region of the ER that interacts with TFIIB encompasses the DBD.

Figure 4 Far-Western blotting experiments

(A) TFIIB was purified from *E. coli*, subjected to SDS/PAGE, transferred to a membrane and incubated with anti-TFIIB; the immune complexes were detected with antibodies tagged with peroxidase. (B) Far-Western blotting. After denaturation/renaturation as described in the Experimental section, the membrane was probed with ^{35}S -labelled ER incubated without hormone (lane 2), with 100 nM oestradiol (lane 1) or with 100 nM 4-hydroxytamoxifen (lanes 3 and 4). Lane 3 was a control extract containing no TFIIB. Labelled proteins were detected by fluorography. (C) After denaturation/renaturation as described in the Experimental section, the membrane was incubated with ER translated with non-radioactive methionine and, after washing, was incubated with monoclonal H222 anti-ER antibody. The immune complexes were detected with peroxidase-tagged antibodies. Lane 1, control extract containing no TFIIB; lanes 2 and 3, 2 μg and 6 μg of TFIIB-containing extract respectively.

Figure 5 Mapping of sites in ER that interact with TFIIB by GST pull-down experiments

Upper panel: schematic representation of full-length ER and GST-ER mutants. ^{35}S -labelled TFIIB was prepared by translation *in vitro*. Labelled protein probe was incubated with immobilized GST or GST-ER mutants. Equal amounts of ^{35}S -labelled TFIIB were incubated with each resin. After extensive washing of the GST beads, the proteins were eluted, separated on 12.5% (w/v) polyacrylamide gels and detected by fluorography. The input lane contained 5% of the labelled TFIIB.

TFIIB does not enhance the transcriptional activation by ER

To determine whether TFIIB is limiting for transcriptional activation by ER, we examined the effect of TFIIB on ligand-

Figure 6 Effect of TFIIB on oestrogen-dependent transcription in intact cells

pER (25 ng or 500 ng) was co-transfected with different amounts of pTFIIB and the reporter plasmids ERE₂-37tkCAT and pCH110 into Chinese hamster ovary cells. Transcriptional activation is expressed as total induction over the reporter alone in the absence or presence of 10 nM oestradiol. The bars indicate S.D. and were obtained from three independent experiments.

dependent activation of transcription by ER. We have performed transient transfection assays in Chinese hamster ovary cells by using a CAT reporter that contained the herpes simplex virus tk promoter TATA box 37 bp downstream of two palindromic consensus ERE sites (ERE₂-37tkCAT). This reporter does not possess any other *cis* elements. Transfection of pTFIIB without ER did not induce the CAT activity from the reporter (results not shown). Cells co-transfected with pER displayed only a minimal increase in CAT activity in the absence of hormone even on co-expression of TFIIB (results not shown). In the subsequent experiments (Figure 6), cells were co-transfected with an amount of pER (25 ng per dish) sufficient for a maximal oestrogen-dependent CAT activity (7–8-fold induction) as determined by

Figure 7 ER does not recruit TFIIB to the promoter

TFIIB-depleted nuclear extract was incubated with 100 ng of pERE₂Ad G-free in the absence (lanes 1–6) or presence (lanes 7–12) of 4 μ l of partly purified recombinant hER (87 pmol/ml) and 0.4 ng (lanes 2 and 8), 2 ng (lanes 3 and 9), 4 ng (lanes 4 and 10), 20 ng (lanes 5 and 11) or 400 ng (lanes 6 and 12) of purified TFIIB. Lanes 1 and 7, no added TFIIB. The transcripts were analysed on a 6% (w/v) sequencing gel.

dose-response experiments. No significant effect could be detected on co-transfection of an equal amount of pTFIIB. Increasing the amount of expression vectors pTFIIB up to an excess of 20-fold decreased the oestrogen-dependent reporter activation mediated by ER. The control reporter lacking ERE sites or pCH110 used as control were not affected by co-transfection of ER and/or TFIIB (results not shown). Similar results were observed in cell line BPA 31 (Balb/c mouse fibroblasts; data not shown).

At larger amounts of pER (500 ng per dish), a marked inhibition of transcription was observed probably owing to 'auto-inhibition' by an interference/squelching phenomenon as previously reported [34]. The decreased transcriptional activity of an activator under squelching conditions is explained by the fact that an excess of activator competes with activator-bound promoter for one or more limiting factor(s) necessary for its trans-activation. Under squelching conditions, the co-expression of TFIIB failed to relieve auto-inhibition, even when the amount of the TFIIB expression vector was varied over a large range of concentrations (Figure 6). These results indicate that TFIIB is not present in limiting concentration in the cells and that ER requires additional 'limiting' co-activators to mediate its trans-activating effects.

To determine whether the interaction between ER and TFIIB is functional, we analysed the effect of TFIIB on ER-dependent transcription *in vitro*. A template containing two ERE sites and the TATA box of AdML promoter upstream of a G-free cassette of 380 nt was constructed for the transcription assay *in vitro* in a HeLa nuclear extract. The addition of partly purified recombinant hER expressed in *Spodoptera frugiperda* cells (Sf9) to the HeLa nuclear extract resulted in a 3–4-fold stimulation of correctly initiated transcription from ERE₂Ad G-free(380) and was independent of the presence as well as the absence of hormone or anti-hormone (results not shown; see Figure 8, test template). As previously reported [35], in EMSAs, all ER-antagonist complexes bound to the ERE migrated more slowly than ER-oestradiol-ERE complexes; in the absence of ligand, complexes of intermediate mobility were observed. These modifications of electrophoretic mobilities have been attributed to a ligand-induced conformational change in the complexes that take place within the LBD of the ER [35]. These results suggest that although the ligand-dependent change in conformation occurs within the ER LBD, only the ligand-independent trans-activation function is active when the receptor is expressed in Sf9

cells. Similar behaviour has been reported for ER, GAL-ER(EF) and progesterone receptor (PR) produced by recombinant baculovirus [36–38].

Next, transcription was performed with a HeLa nuclear extract depleted of TFIIB by immunoadsorption. The TFIIB-depleted extract showed a weak basal transcriptional activity owing to a small residual amount of TFIIB (Figure 7, lanes 1 and 7). The addition of recombinant hTFIIB restored both basal transcription and the ER-dependent transcription (Figure 7). However, in the presence of different concentrations of TFIIB, the magnitude of transcriptional activation by ER remained relatively constant at approx. 3–4-fold. These results strongly suggest that ER does not recruit TFIIB to the promoter. Consistently with previous findings obtained with other activators, an increased concentration of TFIIB was unable by itself to overcome the requirement for ER.

ER acts in the PIC assembly process

We have addressed the question of whether ER can facilitate PIC assembly. To answer this question we employed an immobilized-template assay [29] to separate the factors stably associated with the promoter from unbound factors. We have used crude HeLa nuclear extract as source of basal transcription factors. A fragment containing two ERE sites and the TATA box of the AdML promoter upstream of a G-free cassette of 380 nucleotides was biotinylated and immobilized on streptavidin magnetic beads. The DNA beads were incubated with the HeLa nuclear extract to allow PIC formation in the absence or presence of partly purified recombinant hER labelled with oestradiol. Complexes were extensively washed to remove factors not stably associated and then analysed for bound factors or for transcriptional potential. The proteins were eluted with SDS-loading buffer and the presence of ER and several basal transcription factors in the eluate was quantified by immunoblot analysis with monoclonal antibodies against ER, TBP, TFIIB, TFIIF (RAP30) and TFIIE (TFIIE- α). As shown in a representative assay (Figure 8), the presence of ER during the incubation slightly increased the amounts of TFIIB and TFIIF (1.5-fold), whereas it substantially increased the amount of TFIIE (3-fold) associated with the immobilized template although ER did not directly bind the subunits of TFIIE in GST pull-down experiments (results not shown). In contrast, the amount of template-bound TBP was approximately the same in the absence and in the presence of ER. Moreover, in the presence of nuclear extract, the amount of ER bound to the promoter was substantially higher. This result suggests that PIC formation stabilized ER binding to the target promoter. Transcription experiments initiated from these purified initiation complexes by the addition of nucleoside triphosphates showed that the addition of ER results in a 3–4-fold stimulation of transcription (Figure 8).

These immunoblot and transcription experiments point to the conclusion that ER increases the stable association of basal transcription factors to form a functional PIC and that this stable association is correlated with ER-induced transcription. These results suggest that ER does not increase the recruitment or the stability of TFIIB binding to the promoter but rather that it induces qualitative changes in the structure of the TBP/TFIID-TFIIB-DNA complex that enhance subsequent basal transcription factor interactions.

DISCUSSION

We show that ER acts, in an early step of the hormone-induced transcriptional activation, during or immediately after the formation of template-committed complexes containing TFIIB,

Figure 8 ER enhances the PIC assembly process

HeLa cell nuclear extracts were incubated in the presence or absence of partly purified recombinant hER with immobilized DNA template or with control beads as described in the Experimental section. The complexes were purified magnetically and washed extensively. For immunoblot analysis, the proteins were eluted with SDS-sample loading buffer, resolved by SDS/PAGE [12% (w/v) gel] and analysed with the H222 monoclonal anti-ER antibody, and monoclonal antibodies against TBP, TFIIB, RAP30 and TFIIE- α . Transcription assays were performed as described in the Experimental section. The test template indicated the correctly initiated transcripts.

leading to a more efficient recruitment of subsequent components of the assembly pathway.

Although ER has been shown to interact with TBP [12] and TFIIB ([23], and the present study), the following observations suggest, in contrast with current models, that ER does not activate the transcription by favouring the assembly of TFIIB into the transcription complex. First, ER did not stabilize TBP or TFIIB on the promoter. However, when ER, TBP and TFIIB were incubated together with the template containing ERE and one TATA box, a stable complex was detected in EMSA. This observation suggests that ER, TBP and TFIIB might interact with each other in the ER-TBP-TFIIB-promoter complex. Secondly, the addition of increasing amounts of TFIIB did not affect the magnitude of transcriptional activation by ER. Lastly, with an immobilized promoter-template assay, ER slightly increased the level of TFIIB associated stably with DNA, whereas the level of TBP remained constant. However, we show that ER enhanced the stable association of the rest of the transcription machinery as determined by the presence of TFIIF, which seems to have a critical role in the recruitment of RNA polymerase II [39], and TFIIE, which requires the presence of RNA polymerase II to associate with the PIC [39]. The level of bound TFIIE is correlated with ER-induced transcription. However, ER did not associate directly with the subunits of TFIIE. To interpret these observations, we propose that the formation of a stable ER-TBP-TFIIB-TFIIE complex favours the stable assembly of other basal transcription factors to form the functional PIC. In addition, ER might induce a conformational change in the TBP-TFIIB-TFIIE complex, thereby enhancing the binding of subsequent components of the transcription complex. Although in the presence of ER no change in the DNase I digestion

protection by TBP-TFIIB over the TATA box is observed (M. Sabbah and G. Redeuilh, unpublished work), the possible change in the conformation of TFIIB might be TAFs-dependent [29]. It is also possible that ER interacts directly or indirectly with other downstream basal transcription factors, i.e. TFIIF, TFIIE or RNA polymerase II itself.

In our assay conditions, ER increased the transcription rate to a maximum of nearly 4-fold over basal levels, independently of the presence of hormone or anti-hormone. It is possible that putative co-activator(s)/mediator(s) essential for ER activity were absent or in low concentration in HeLa nuclear extract used or were non-functional under our assay conditions.

The ER region between residues 160 and 315, which we have shown to interact specifically with TFIIB, encompasses the DBD of ER. Ing et al. [23] reported that ER and a truncated ER (143-C) containing the entire LBD and DBD interact specifically with TFIIB. Interestingly, the homologous DBD of *Xenopus* ER had been shown to induce the constitutive expression of hormone response genes, demonstrating that the receptor DBD retains some ability to activate transcription [40]. The ability of several members of the NR family, such as PR, thyroid hormone receptor α and β , calcitriol receptor and chicken ovalbumin upstream promoter transcription factor, to interact with TFIIB [23,41–43] suggests that they can act at least in part by a similar mechanism of trans-activation. Moreover, transfection experiments have also revealed the presence of a trans-activation function within the DBD of glucocorticosteroid receptor and PR [44,45]. Thus the presence of an activation function within the DBD might be a general feature of the steroid hormone receptor family. Malik and Karathanasis [46] have reported that the orphan receptor hepatocyte nuclear factor 4 (HNF-4) activates transcription by facilitating the assembly of a PIC intermediate consisting of TBP-TFIIB via the direct interaction with TFIIB. The region of HNF-4 that interacted specifically with TFIIB was mapped in the N-terminal 118-residue fragment that includes the DBD. In a second step, HNF-4, in an AF-2-dependent manner, enables the entry of PIC components acting downstream of TFIIB. HNF-4 belongs to a different NR subfamily from ER, on the basis of both amino acid sequence and functional properties (regulation by post-translational modification compared with ligand binding) [46]. Thus it seems that these transcription factors rely on similar initial steps during the formation of an active PIC. However, the specificity of this interaction seems to be limited because TFIIB has also been shown to interact with the acid activation domain of VP16 [47], the glutamine-rich activation domain of fushi tarazu (a *Drosophila* transcription factor) [48] and the proline-rich activation domain of CTF-1 [49].

Transfection experiments show that the overexpression of TFIIB did not augment ligand-dependent activation of ER *in vivo*, suggesting that TFIIB is not limiting in the cell. Moreover, the magnitude of transcriptional activation by ER decreases as the concentration of TFIIB is increased. This phenomenon can be explained by squelching effects due to TFIIB [50]. In addition, the expression of TFIIB failed to relieve the squelching caused by overexpression of ER. These findings are consistent with the existence of several potential co-activator(s) known to bind the ER AF-2 domain such as RIP 140, ERAP 160, TIF1, TRIP1/SUG1, SRC-1 and TIF2, which do not interact with TFIIB.

Trans-activation by ER, *in vivo*, is completely dependent on ligand. It is likely that ER contacts additional components through AF-1 and AF-2 domains. These considerations raise the possibility that ER acts in an early step during the initiation of transcription by interacting with both general transcription factors and TAF_{II} to facilitate PIC assembly. These associations are an intrinsic property of ER, independent of ligand, and can

account for the constitutive enhancement of transcription *in vitro*. In addition, through the ligand-modulated activation domain AF-2, ER might interact with specific co-activators/mediators to produce full receptor transcriptional effectiveness. Ultimately, a reconstitution system *in vitro*, using chromatin-assembly templates, will be critical for understanding the regulatory mechanisms by which ER modulates transcription.

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