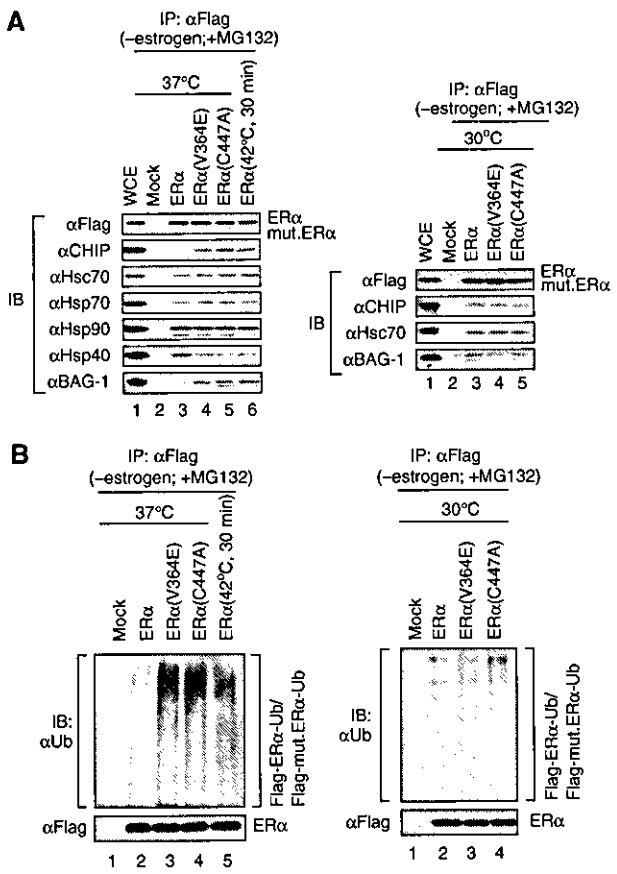

Figure 7 The misfolding of ERα induced the recruitment of CHIP and BAG-1 to the complex. (**A**) CHIP and BAG-1 preferentially recognized and bound misfolded ERα. Flag-tagged ERα, ERα(V364E) or ERα(C447A) (100 ng) was transfected into 293 cells. These cells were cultured with MG132 (10^{-6} M) at 30°C (permissive temperature; right panel), 37°C (normal/nonpermissive temperature; left panel) or under thermally stressed conditions (42°C for 30 min; left panel). Extracts prepared from these cells (lanes 3–6) or untransfected cells (Mock) were subjected to immunoprecipitation using anti-Flag M2 antibody and then Western blotted using antibodies as indicated. The whole-cell extract is shown in lane 1 (WCE). (**B**) The ubiquitination status of the temperature-sensitive mutants or heat-shocked ERα was enhanced. Flag-tagged ERα, ERα(V364E) or ERα(C447A) (500 ng) was transfected into 293 cells. These cells were cultured with MG132 (10^{-6} M) at 30°C (right panel), 37°C (left panel) or under thermally stressed conditions (42°C for 30 min; left panel). Extracts prepared from these cells (lanes 2–5) or untransfected cells (Mock) were subjected to immunoprecipitation using anti-Flag M2 antibody. The ubiquitination status of ERα and mutants was analyzed by Western blotting using anti-ubiquitin antibody.

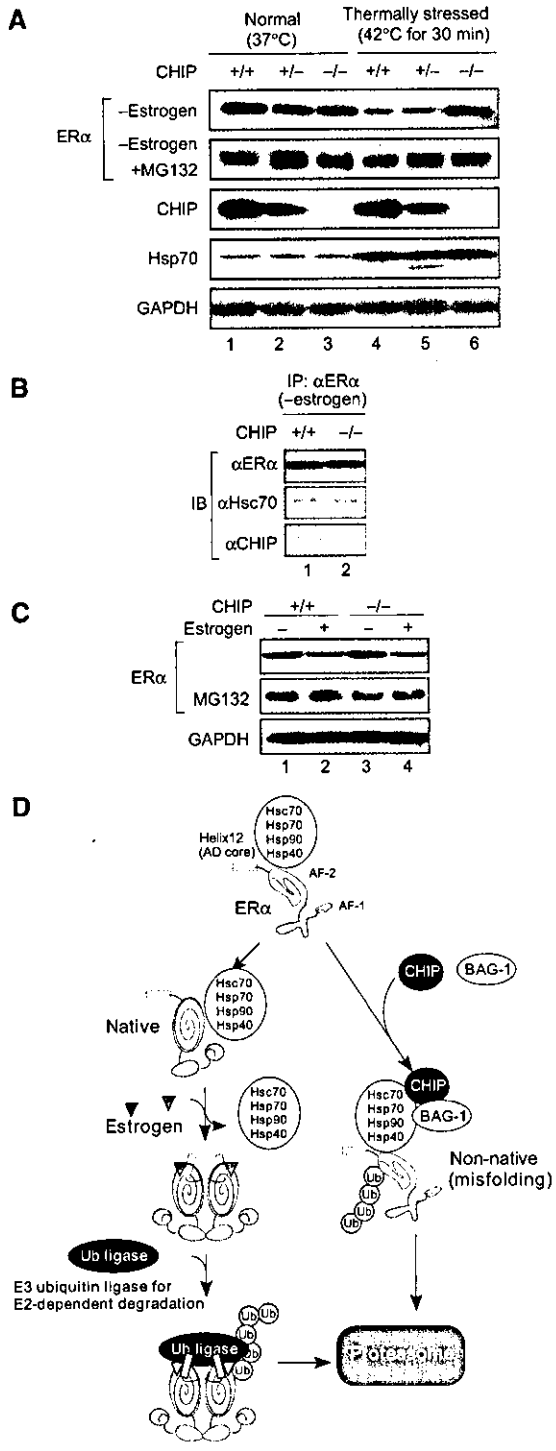

Figure 8 Liganded but not unliganded ERα degradation was observed in CHIP–/– MEF cells. (**A**) Thermally induced degradation of ERα was not observed in CHIP–/– cells. MEF cells were isolated from CHIP–/–, CHIP+/– mice and wild-type littermates (CHIP+/+). MEF cells were cultured under normal conditions (37°C) or thermally stressed conditions (42°C for 30 min) without estrogen. Extracts prepared from the MEF cells were subjected to Western blotting using the indicated antibody. (**B**) CHIP+/+ or CHIP–/– cells were lysed and subjected to immunoprecipitation using anti-ERα antibody in the absence of estrogen. Precipitates were Western blotted with antibodies for ERα, Hsc70 and CHIP. (**C**) Estrogen induced degradation of ERα in CHIP–/– cells. MEF cells were cultured in the presence or absence of estrogen (10^{-8} M), and cell extracts prepared from these cells were subjected to Western blotting using anti-ERα antibody. (**D**) ERα degradation may be regulated by two independent ubiquitin-proteasome pathways.

degradation machinery for the unliganded receptor but not for the liganded. Otherwise, there may be a change in the conformation of the receptor, which would protect the receptor from degradation. Reid *et al* (2003) also demonstrated that unliganded ERα is subject to proteasome-mediated turnover, which is mechanistically different from the turnover of liganded ERα.

Several lines of evidence indicate that estrogen, progesterone and glucocorticoid receptors (GRs) are degraded in the presence of their cognate ligands (Nawaz *et al*, 1999a; Wallace and Cidlowski, 2001). However, this is contrasted with observations of androgen and vitamin D receptors, which are accumulated in the presence of their agonist

ligands (Li *et al*, 1999). From our results, these inconsistent observations might be explained by the balance between the two degradation pathways in the cells. When the degradation pathway for unliganded receptors is more active than that for liganded receptors, these receptors would stabilize in the presence of ligands. In contrast, when the liganded receptor degradation pathway is stronger than the unliganded receptor degradation pathway, the protein level of receptors is down-regulated by ligand treatment.

CHIP containing a protein complex specifically binds and ubiquitinates unliganded estrogen receptor

To address the mechanism of the ubiquitination and degradation of unliganded ERα, we purified proteins using GST-fused ERαLBD, and identified CHIP, which specifically bound to unliganded ERαLBD. Our findings indicate that CHIP binds unliganded ERα as a protein complex containing Hsp90, Hsc70, Hsp70, Hsp40 and BAG-1, all of which are known to possess or assist chaperoning functions, and a Dna J-like protein, KIAA0678. Dna J is a member of the Hsp40 family of molecular chaperones, which regulate the activity of Hsp70s. Dna J-like proteins that contain regions closely resembling a Dna J domain are suggested to regulate the activity of Dna J proteins during protein translocation, assembly and disassembly (Cheetham and Caplan, 1998).

CHIP expression with ERα enhanced the conjugation of ubiquitin to the receptors and stimulated degradation. Receptor ubiquitination and degradation was abrogated when cells were treated with estrogen. These results are in good agreement with the results obtained from binding experiments. Furthermore, OHT and ICI, both of which inhibited the interaction between CHIP and ERα, reduced the CHIP-mediated degradation of ERα. These findings confirmed the idea that unliganded ERα ubiquitination is mediated by CHIP. In immunostaining, CHIP was largely detected in the cytoplasm (Figure 6C). The localization of CHIP was not changed when cells were cultured under heat-stressed conditions (data not shown). According to these results, CHIP-dependent ERα ubiquitination may occur mainly in the cytoplasm. However, we cannot exclude the possibility that a small amount of CHIP is involved in the ubiquitination of ERα in the nucleus.

Recently, CHIP was reported to induce ubiquitination of the GR bound to Hsp90 for proteasomal degradation (Connell *et al*, 2001). While our findings indicate that CHIP selectively binds to unliganded ERα and ubiquitinates it, CHIP-mediated GR degradation is observed in the presence of ligands. Recent reports indicate that in the presence of ligands, nuclear receptors do not remain permanently bound at a promoter, but rather undergo cycles of binding and unbinding (Shang *et al*, 2000; Stenoien *et al*, 2001; Galigniana *et al*, 2004). The cycling of ligand-bound ERα requires proteasomal activity (Reid *et al*, 2003). Together with these reports and our observations, it is possible that the binding of estrogen to ERα induces the dissociation of CHIP and the association of other ubiquitin ligases, which are involved in receptor cycling at a promoter. The ligand-dependent cycling of GR is known to be much faster than that of ERα and both chaperones and proteasomes are thought to be important for GR cycling since the disruption of either leads to alterations in the exchange rate (Galigniana *et al*, 2004). According to these results, it is possible that, while the chaperone complex containing CHIP mainly resides in the cytoplasm, it may translocate into the nucleus and regulate the cycling of liganded GR.

CHIP is involved in the quality control of estrogen receptor

Since CHIP selectively bound to and ubiquitinated unliganded ERα, CHIP seemed not to be directly involved in transcriptional regulation. Recently, it was shown that CHIP is involved in the ubiquitination of the immature cystic fibrosis transmembrane conductance regulator (CFTR) in the endoplasmic reticulum-associated degradation (ERAD) pathway (Wickner *et al*, 1999; Meacham *et al*, 2001). Based on these findings, it is speculated that CHIP may be a new category of E3 enzyme responsible for the quality control of cellular proteins linked to the function of molecular chaperones. However, there is no experimental evidence to show that CHIP indeed acts as E3 ubiquitin ligase capable of distinguishing the non-native states from native states of target proteins *in vivo*.

In this study, we have shown that temperature-sensitive mutants of ERα preferentially recruited CHIP to ubiquitinate and degrade these receptors under nonpermissive temperatures. In addition, the ubiquitination and degradation of unliganded ERα was enhanced when cells were cultured under thermally stressed conditions. These observations suggest that CHIP preferentially induces the hydrolysis of abnormal or mutant forms. Using MEF cells derived from CHIP−/− or wild-type littermates, we confirmed the importance of the observation of CHIP-mediated unfolded ERα degradation. These observations provide direct *in vivo* evidence that CHIP selectively ubiquitinated thermally denatured ERα. Our observations provide the first *in vivo* evidence that CHIP functions as 'quality-control E3' involved in the selective ubiquitination of target proteins by recognizing the non-native state in a molecular chaperone-assisted manner. Furthermore, estrogen treatment induced the degradation of ERα in CHIP−/− cells to the same extent as in CHIP+/+ cells, suggesting that CHIP is not involved in estrogen-dependent degradation, and supporting the idea that there are two independent ubiquitin–proteasome pathways for ERα. Considering that nuclear receptors have conserved LBDs and that some are known to associate with a chaperone complex, our findings raise the possibility that other members of the nuclear receptor family may also be regulated by two independent ubiquitin–proteasome pathways.

Materials and methods

Expression vectors, antibodies, cell culture and transfection

These are available as Supplementary data at *The EMBO Journal* Online.

Co-immunoprecipitation and Western blotting

293 cells were transfected with the indicated plasmids, lysed in TNE (10 mM Tris–HCl (pH 7.8), 1% NP-40, 0.15 M NaCl, 1 mM EDTA, 1 μM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin) buffer. Extracted proteins were immunoprecipitated with the antibody-coated protein A/G Sepharose (Amersham) or anti-Flag M2 agarose (Sigma). The bound proteins were separated by SDS–PAGE, transferred onto polyvinylidine difluoride membranes (Millipore) and detected with indicated antibodies, and secondary antibodies conjugated with horseradish peroxidase. Specific proteins were detected using enhanced chemiluminescence (ECL) Western blot detection system (Amersham).

Ubiquitination assay

MCF7 and 293 cells, which were transfected with or without Flag-tagged ERα and HA-tagged CHIP, were lysed with radioimmunoprecipitation (RIPA) buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with COMPLETE protease inhibitor mixture (Roche) and kept for 20 min on ice. The extracts clarified by centrifugation were immunoprecipitated with anti-Flag agarose for 1 h at 4°C. After washing the resin with RIPA buffer, the bound proteins were eluted by incubation for 1 h at 4°C with Flag peptide in RIPA buffer (0.4 mg/ml). Immunoprecipitates were immunoblotted with the indicated antibody.

Protein purification

Immobilized GST-ERαLBD fusion proteins were preincubated for 1 h at 4°C in GST-binding buffer (20 mM Tris–HCl (pH 7.9), 180 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 1 mM DTT) containing BSA (1 mg/ml) with or without estrogen (10^{-6} M). Bead-immobilized proteins were then incubated at 4°C for 6–10 h with HeLa cell extracts in the presence or absence of 10^{-6} M estrogen. After washing with GST buffer (GST-binding buffer with 0.1% NP-40) three times, the beads were further washed with GST buffer containing 0.2% *N*-lauroyl sarkosine. Proteins bound to ERα were eluted with 15 mM reduced glutathione in elution buffer (50 mM Tris–HCl (pH 8.3), 150 mM KCl, 0.5 mM EDTA, 0.5 mM PMSF, 5 mM NaF, 0.08% NP-40, 0.5 mg/ml BSA, 10% glycerol). For purification of the Flag/HA-CHIP complex, HeLa cells stably expressing Flag/HA-CHIP were extracted with TNE buffer and extracted proteins were incubated with anti-Flag M2 agarose for 2 h at 4°C. After washing the resin with TNE buffer, the bound proteins were eluted by incubation for 1 h at 4°C with Flag peptide in TNE buffer (0.4 mg/ml). For further purification, eluted fractions were incubated with anti-HA agarose for 2 h at 4°C. After washing with TNE buffer, the bound proteins were eluted with a small aliquot of HA peptide in TNE buffer (0.05 mg/ml).

Pulse chase

MCF7 and 293 cells were transfected with or without ERα and CHIP, and 48 h post-transfection, the cells were labeled for 30 min at 37°C with 50 μCi [^{35}S]methionine per ml in methionine-free Dulbecco's modified Eagle's medium (DMEM). The cells were then washed twice and incubated in DMEM containing 10% FBS for the indicated time periods (chase). At each time point of the chase, cell lysates were immunoprecipitated with anti-ERα antibody. The immunoprecipitates were resolved by SDS–PAGE and visualized by autoradiography. Phosphoimager was used to quantify the metabolically labeled ERα present at each time point.

Immunofluorescence

The 293 cells were grown on poly-L-lysine-coated eight-well chamber culture slides, and transfected with plasmids. At 24 h post-transfection, the cells were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with Triton buffer (50 mM Tris–HCl (pH 7.5), 0.5% Triton X-100, 150 mM NaCl, 2 mM EDTA) for 15 min. The cells in each well were blocked with PBS containing 1% BSA and 0.5% goat serum for 3 h at 37°C. The cells were incubated with anti-HA and ERα antibody in PBS containing 1% BSA for 2 h at 37°C. After washing with PBS, the cells were incubated with Alexa fluor 488 goat anti-rat IgG and Alexa fluor 594 goat anti-mouse IgG (Molecular Probes) for 1 h at 37°C and washed with PBS. The sample was mounted in VECTASHIELD mounting medium (Vecter Labs) and analyzed with Leica TCS SP2 spectral confocal scanning system.

RNAi

MCF7 cells maintained in the DMEM medium containing charcoal-stripped FBS were cotransfected with CHIP siRNA vector or luciferase siRNA vector (control) and pUC19 vector carrying puromycin-resistant gene. At 24 h post-transfection, the transfected cells were changed to the medium containing 1 μg/ml of puromycin. At 48 h after puromycin selection, the puromycin-resistant cells were harvested and lysed with TNE buffer. The equal amounts of extracted protein were subjected to Western blotting.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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BRCA1 function mediates a TRAP/DRIP complex through direct interaction with TRAP220

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Breast cancer susceptibility gene 1 (*BRCA1*) is a tumor suppressor gene mutated in a high percentage of hereditary breast and ovarian cancers. The multifunctional BRCA1 protein acts on cell cycle control, exerting several highly specialized DNA repair processes through diverse domains. Gene regulation through its C-terminal domain (BRCT) is indispensable for BRCA1-mediated tumor suppression, suggesting the possibility that the BRCT domain interacts with co-regulator complexes. Using a biochemical approach with HeLa S3 nuclear extracts, we isolated BRCT-associated complexes and identified one of the purified components as TRAP220. We then performed interaction studies *in vivo* (co-immunoprecipitation) and *in vitro* (glutathione S-transferase pull-down assays) and showed that BRCT directly interacted with TRAP220. This *in vitro* interaction was completely abolished by BRCT point mutations typical of those found in patients with BRCA1 that lack transactivation function. BRCA1 transactivation function was dependent on TRAP220 expression level in a transient expression assay. Moreover, a cell survival assay showed that antisense TRAP220 expression to disrupt endogenous TRAP220 expression significantly reduced the survival rate potentiated by BRCA1 after DNA damage. These results suggested that a TRAP220 complex play an important role as putative co-activator complexes in BRCA1-mediated tumor suppression.
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Germ-line mutation of *BRCA1* is well known to predispose women to early onset of breast and ovarian cancer (Venkitaraman, 2002). The *BRCA1* gene encodes a relatively large protein of 1863 amino acids, and apart from an N-terminal zinc-binding RING domain and two C-terminal tandem copies of a BRCT motif, displays little similarity to other known proteins (Futreal *et al.*, 1994; Miki *et al.*, 1994). The C-terminal BRCT domain has been shown to be involved in double-stranded DNA repair and homologous recombination (Callebaut and Mornon, 1997; Moynahan *et al.*, 1999; Scully *et al.*, 1999; Zhong *et al.*, 1999). However, the major function of BRCT is thought to be as a gene regulator, mediating BRCA1 function as a tumor suppressor. This hypothesis is based on several lines of evidence, including that the autonomous transactivation function of BRCA1 was preserved in a recombinant protein consisting of the BRCT domain fused to a GAL4 DNA-binding domain (Miyake *et al.*, 2000). In addition, missense and point mutations in the BRCT domain derived from patients with inherited breast cancer result in the loss of transcriptional activity, and BRCA1 can also act as a negative regulator on some gene promoters (Chapman and Verma, 1996; Monteiro *et al.*, 1996; Zheng *et al.*, 2001; Kawai *et al.*, 2002). Reflecting the complex nature of BRCT transactivation function, this domain has already been shown to physically interact with a number of transcription factors and co-regulators (presumably in complexes), and also associate with chromatin remodeling complexes (Anderson *et al.*, 1998; Yu *et al.*, 1998; Zhang *et al.*, 1998; Yarden and Brody, 1999; Bochar *et al.*, 2000). Moreover, transcriptional squelching between BRCA1 and estrogen receptor (ER) has recently been reported (Fan *et al.*, 1999; Zheng *et al.*, 2001). As ER is a member of the nuclear receptor (NR) gene superfamily and acts as a ligand-induced transcription factor (Mangelsdorf *et al.*, 1995; Watanabe *et al.*, 2001; Yanagisawa *et al.*, 2002), limited cellular amounts of a common co-activator complex for both BRCA1 and ER could explain the transcriptional squelching phenomenon.

To better understand the BRCT transactivation function, we screened for putative transcription co-activator complexes that directly interacted with the BRCT domain using a biochemical approach. We established an affinity column whereby the BRCT domain (amino acids 1528–1863) was immobilized as a glutathione S-transferase (GST)-fusion protein. Fractions of HeLa S3 nuclear extract, presumably containing multiprotein complexes, were applied to the affinity

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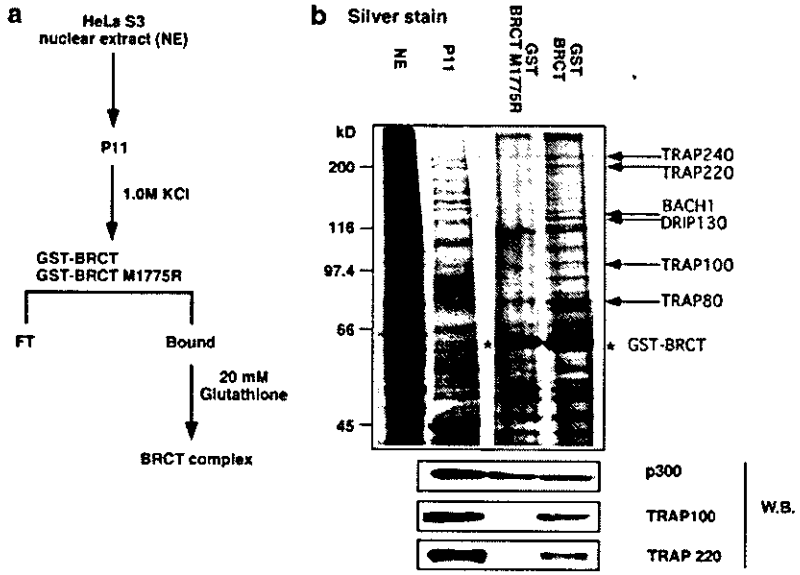

Figure 1 Affinity purification of BRCT-containing complexes (a) Purification scheme. HeLa S3 nuclear extract was fractionated using a phosphocellulose (P11) column and a GST-BRCT (amino acids 1528–1863) affinity column. The 1 M KCl P11 eluate was concentrated onto the GST-BRCT affinity column at 4°C for 10–12 h. Bound proteins were washed extensively with buffer, and subsequently eluted by buffer containing 20 mM reduced glutathione. As a negative control, purification from P11 eluates was performed using another affinity column (immobilized GST-BRCT M1775R). (b) Purified fractions were boiled, separated by electrophoresis and analysed by SDS–PAGE followed by silver staining or Western blot analysis using antibody, as shown on the right of the figure. Molecular weight standards are shown to the left of the figure. The asterisk denotes the molecular bait (GST-BRCT, GST-BRCT M1775R) after elution. Arrows show proteins identified by mass spectrometry (Yanagisawa *et al*., 2002)

column (Yanagisawa *et al*., 2002; Kitagawa *et al*., 2003), and several of the transcription co-regulators subsequently verified in the BRCT-interacting complexes by Western blotting (Figure 1b, lower panel). Western blotting of the BRCT-interacting complexes identified TRAP220 and TRAP100 (Figure 1b), which were confirmed as factors that associate with BRCT by time-of-flight mass spectrometry (TOF-MS). TRAP220 contains two LXXLL motifs, which are consensus interacting motifs and part of the core activation domain in NR ligand-binding domains (Fondell *et al*., 1999; Rachez *et al*., 1999). Thus, TRAP220 is thought to act as a major and direct interactant with ER (Yanagisawa *et al*., 2002), as well as with other NRs, as part of the common TRAP/DRIP co-activator complex (Rachez *et al*., 1999). It is therefore possible that the functional role of TRAP220 in the TRAP/DRIP complex may account for the reported transcriptional squelching between ER and BRCA1. Indeed, several other BRCT-associated proteins we identified were also TRAP/DRIP complex components, such as TRAP220, TRAP240, DRIP130, and TRAP80 (Ito *et al*., 1999). BACH1 (Cantor *et al*., 2001) and p300 (Pao *et al*., 2000) were also detected by TOF-MAS and Western blotting (Figure 1b), respectively, and have been previously shown to interact with BRCT, which confirmed the efficacy of our purification method.

To address the functional importance of the BRCT-TRAP220 interaction, a BRCT point-mutant, derived from a breast cancer patient and deficient in transcriptional activity, was used to isolate interacting complexes. As clearly shown in Figure 3b, the GST-fused BRCT point-mutant (M1775R) protein lacked the ability to retain TRAP220 on the column. This indicated that TRAP220 may function as a co-activator in the BRCA1 complex. To determine whether full-length BRCA1 protein interacted with TRAP220 in human cells, we expressed full-length BRCA1 (FLAG epitope-tagged) and/or TRAP220 (His/Myc epitope-tagged) in 293T cells. Significant expression of the tagged proteins was confirmed by Western blotting. After immunoprecipitation with anti-FLAG M2 to obtain full-length BRCA1, the immunoprecipitants were blotted with anti-Myc to identify TRAP220-containing complexes (Figure 2a). Both TRAP220 and BRCA1 were detected in cell lysate immunoprecipitates (Figure 2a), which supported the hypothesis that BRCA1 physically associates with TRAP220 in living cells. This hypothesis was further confirmed by *in vivo* association between endogenous BRCA1 and TRAP220 in MCF-7 cells expressing both proteins (Figure 2b). No such association was observed when the BRCA1 point-mutant (M1775R) was used instead of wild-type BRCA1 in the immunoprecipitation experiment (Figure 2a), as expected from the results of the column purification experiments.

To map the region of TRAP220 that interacted with BRCT, a GST pull-down assay was performed using TRAP220 deletion mutants (Figure 3a). FLAG-tagged TRAP220 fragments, abbreviated TR1, TR2, TR3, TR4 and TR5 (as described in Figure 3a), were *in vitro* translated in the presence of [^{35}S]methionine and incubated with GST-fused BRCT protein-bound resin. Only TR1 was trapped, which suggested that only the TR1 region interacted with BRCT. Interestingly, the

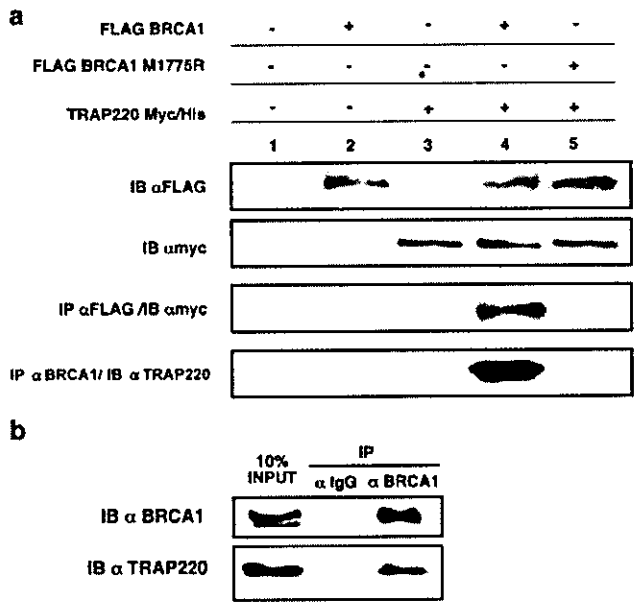

Figure 2 *In vivo* association between TRAP220 and BRCA1. (a) Formation of BRCA1 and TRAP220 complexes in 293T cells was analysed by co-immunoprecipitation (IP) using the anti-FLAG monoclonal M2 antibody (Sigma Aldrich) followed by immunoblotting (IB) using anti-Myc. 293T cells were transiently transfected with combinations of expression vectors as indicated. The expression of proteins in transfected cell extracts was determined by Western blot analysis using FLAG or Myc tags. (b) Detection of endogenous BRCA1-TRAP220 interaction by Western blotting. MCF-7 nuclear extracts were applied for immunoprecipitation with 5 μg of anti-BRCA1 (MS-BRC14-UP50, GeneTex, Inc.) and IgG, respectively. Then bound proteins in 30 μl of protein G sepharose™ 4 Fast Flow (Amersham Biosciences, NJ, USA) were detected by Western blotting. The 10% amount of the tested nuclear extracts is shown as positive control as input (Kitagawa *et al*., 2003)

BRCT column did not retain either TR2 or TR3, which contain LXXLL motifs thought to interact with liganded NRs. Reflecting the associations between TRAP220 and wild-type or point-mutant BRCA1 as observed in our *in vivo* experiments, the point-mutations A1708E, P1749R, and M1775R that exhibit no BRCA1 transactivation function caused the loss of TRAP220 interaction *in vitro* (Figure 3b).

To examine the co-activator activity of TRAP220 toward BRCT transactivation function, a transient transfection assay was performed using a luciferase reporter plasmid driven by the adenovirus major late promoter (AdMLP) containing GAL4DBD-binding sites. A BRCT-GAL4DBD-fusion protein (GAL-BRCT) alone potently stimulated transcription (Figure 4b). TRAP220 expression in human 293T cells led to an approximately twofold increase in luciferase activity compared to GAL-BRCT alone, while such co-activation was not detected in a TRAP220 deletion mutant lacking the BRCT interacting TR1 region (amino acids 1–326, see Figure 3a) (Figure 4b). This enhancement of transactivation by TRAP220 was not observed when either GAL4 DNA-binding domain alone or GAL-BRCT mutants (A1708E, P1749R, or M1775R) were used, as expected from the *in vivo* and *in vitro* TRAP220 experiments. Consistent with these findings, antisense TRAP220 expression, that disrupted endogenous TRAP220 expression (Figure 4a), reduced the transcriptional activity of BRCT (Figure 4b). This again suggested a significant role for TRAP220 in BRCA1 transactivation function.

Finally, we then tested the significance of TRAP220 activity in the DNA damage response mediated by BRCA1. BRCA1 was transfected into HCC1937 cells (a

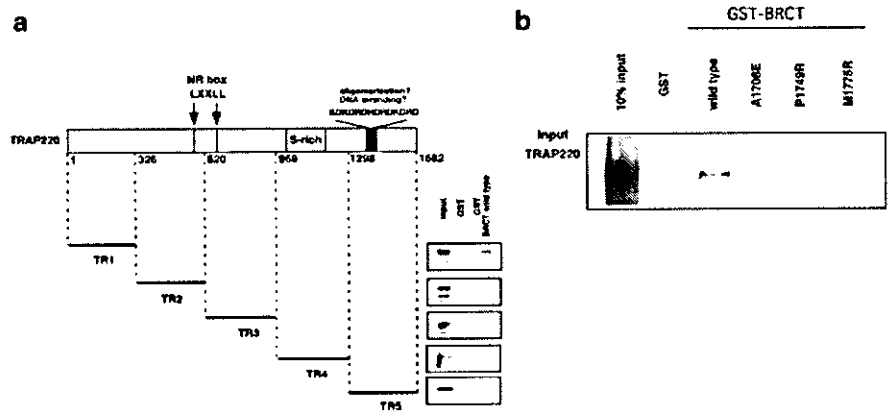

Figure 3 *In vitro* association between TRAP220 and BRCT, and mapping of the BRCT-interacting region of TRAP220. (a) Mapping of the BRCT-interacting region of TRAP220 using GST-BRCT and TRAP220 fragments. Bacterially expressed GST-fusion proteins immobilized on beads were used in *in vitro* pull-down assays. A schematic diagram of the structure of TRAP220 is shown. TRAP220 'TR1' (amino acids 1–326), 'TR2' (326–620), 'TR3' (620–969), 'TR4' (969–1298), and 'TR5' (1298–1582) were *in vitro* translated in the presence of [^{35}S]methionine (Amersham Pharmacia Biotech) using a TNT coupled *in vitro* translation system (Promega). Each labelled TRAP220 fragment was then incubated with either GST alone or GST-BRCT. The mixtures were washed and subjected to SDS-PAGE and analysed. Polyacrylamide gels were stained briefly with Coomassie Brilliant Blue to verify the loading of equal amounts of fusion proteins prior to drying and autoradiography (Ohtake *et al*., 2003). (b) *In vitro* association of TRAP220 with BRCT or BRCT point mutants that lack transcriptional activity were performed by incubating GST, GST-BRCT, GST-BRCT A1708E, P1749R, or M1775R with *in vitro* translated TRAP220

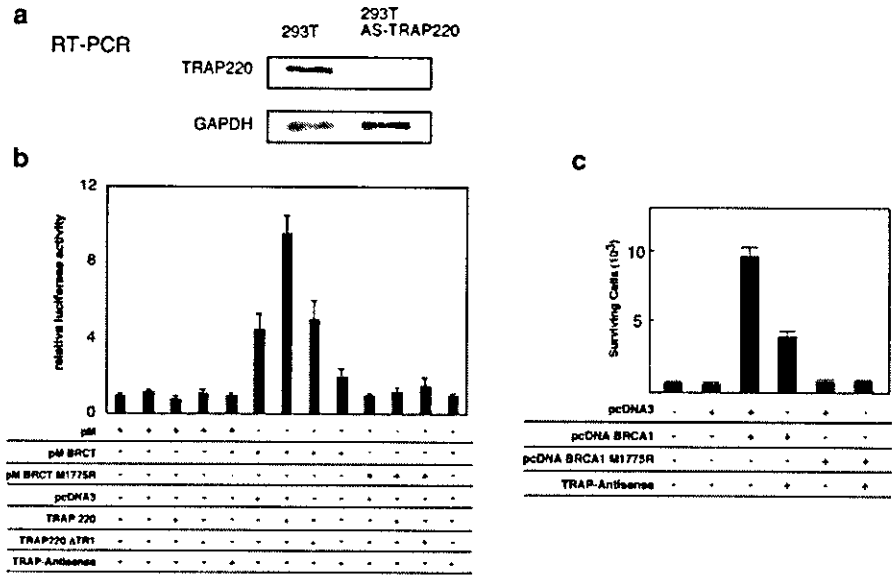

Figure 4 TRAP220 activates transcription by GAL-BRCT, while antisense TRAP220 disrupts the DNA damage response of BRCA1. (a) Transfection of antisense TRAP220 in 293T cells reduced TRAP220 expression as shown by RT-PCR analysis. (b) Transient transfection assays of GAL-BRCT and FLAG-TRAP220 using a luciferase reporter (Promega) containing the GAL4 DNA-binding site (17M8) showed specific enhancement of transcription. 293T cells were transfected with each luciferase reporter (E1b-Luc and AdMLP-Luc), pM or pM-BRCT, pRL CMV-Luc as a control of transfection efficiency, and either pcDNA3 (Invitrogen) empty expression vector, pcDNA FLAG-TRAP220, pcDNA TRAP220ΔTR1 or antisense TRAP220. Measurements of luciferase (Promega) activity were performed according to the manufacturer's instructions. Error bars indicate the standard deviation. Each experiment was repeated at least three times in triplicate (Watanabe *et al.*, 2001). (c) HCC1937 cells were transfected with constructs based on the pcDNA3 plasmid. Cultures were treated with 0.1% MMS for 50 min, and surviving cells were counted after 8 days, as previously described by Zhong *et al.* (1999)

mutated BRCA1 cell line) that are hypersensitive to DNA damaging agents such as methylmethane sulfonate (MMS). By counting the number of surviving HCC1937 cells, we observed a protective effect of BRCA1 expression in response to DNA damage. Antisense TRAP220 expression resulted in specific deterioration in the DNA damage response potentiated by BRCA1 (Figure 4c), which verified the importance of TRAP220 in BRCA1 function.

BRCA1 is a multifunctional protein that acts as a tumor suppressor controlling gene expression, as well as a sequence-specific regulator and a co-regulator controlling DNA damage (Venkitaraman, 2002). Therefore, it can be speculated that BRCA1 acts as a platform protein that associates with a number of factors, regulators, and complexes to accomplish the diverse functions attributed to BRCA1. Indeed, discrete classes of factors and complexes involved in gene regulation and DNA repair associated with BRCA1 have been identified, and indirect associations with further related factors and complexes are supposed. Nevertheless, clear relationships between *BRCA1* gene mutation and consequent malfunctions of the identified factors and complexes remain to be established. To this end, we searched for co-activator complexes that recognized the BRCT domain, as mutations in this domain modulate BRCA1 transactivation function and are highly related with breast and ovarian cancer incidence (Humphrey *et al.*, 1997; Greenman *et al.*, 1998). Also, a previous report found that BRCA1 competed with ER in terms of transcriptional control via the BRCT domain, which suggested the possibility of common co-activator complexes between ER and BRCA1, presumably including the TRAP/DRIP complex already identified as an ER co-activator complex (Ito *et al.*, 1999; Rachez *et al.*, 1999; Yanagisawa *et al.*, 2002).

In this study based on biochemical approaches (Yanagisawa *et al.*, 2002; Kitagawa *et al.*, 2003), we showed that a TRAP220-containing complex associated with wild-type BRCT through physical interaction with TRAP220. As TRAP220 binding of BRCA1 was abrogated *in vivo* and *in vitro* when clinically relevant BRCA1 mutants that lacked transactivation function (Chapman and Verma, 1996) were used as bait, the association between BRCT and TRAP220 complexes appears to be critical for normal BRCA1 function. This is supported by the findings that TRAP220 alone potently enhanced BRCA1 transactivation function, and that the disruption of endogenous TRAP220 by antisense TRAP220 led to a clear reduction in BRCA1-mediated DNA damage repair. This last result suggested that TRAP220-containing complexes may also play a role in the DNA damage repair function of BRCA1.

The TRAP/DRIP mediator complex was originally isolated as a co-activator complex for different classes of activators, including NRs, by several independent groups (Fondell *et al.*, 1996; Rachez *et al.*, 1999). Further study of the isolated complex components

revealed that the complexes formed a class of co-activator complexes that shared common major components along with limited numbers of specific factors (Gu *et al*., 1999). Combinations of these specific components generate a number of TRAP/DRIP complex subclasses (Freedman, 1999). The co-activator function of TRAP/DRIP complexes in *in vitro* transcription systems illustrates the direct link of the activator complex to the basal transcription machinery. The 220-kDa component of the complex, referred to as TRAP220/DRIP230, was identified as a subunit with unique properties in that it directly binds to NRs in a ligand-dependent manner through a region containing NR recognition motifs (LXXLL, NR box) (Ito *et al*., 1999; Rachez *et al*., 1999). Given that BRCA1 may compete with ER with respect to gene regulation, and that mutations in the BRCT region lead altered BRCA1-dependent gene regulation and enhanced tumorigenesis in estrogen-dependent cancers (presumably through modulation of ER-mediated estrogen signaling), interaction between TRAP220 and BRCA1 may account for the transcriptional squelching observed between BRCA1 and ER (Fan *et al*., 1999; Zheng *et al*., 2001).

The results of our study showed that TRAP220 bound directly to wild-type BRCA1, but not to BRCA1 mutants. Thus, breast cancer predisposition caused by genetic mutations in the BRCT domain may be due, at least in part, to insufficient interaction with TRAP complexes. In this respect, it is perhaps surprising that gene amplification or overexpression of TRAP220 is observed in some cancer cell lines (Zhu *et al*., 1999). Our transient transfection assay showed that TRAP220 enhanced BRCT-mediated transactivation function, such that the TRAP complex clearly served as a co-activator complex in the promoters of BRCA1 target genes. In conclusion, we propose that the failure of binding between BRCT and TRAP220 is a key event in cancer predisposition. Further investigations should be performed to elucidate the molecular mechanisms that underlie the formation BRCA1-TRAP complexes in normal cell growth, thereby revealing its role as a tumor suppressor in estrogen-responsive organs, especially the breast and ovary.

Abbreviations

BRCA1, breast cancer susceptibility gene 1; GST, glutathione S-transferase; CMV, cytomegalovirus; AdMLP, adenovirus major late promoter; NR, nuclear receptor.

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Alleviation of PC4-mediated Transcriptional Repression by the ERCC3 Helicase Activity of General Transcription Factor TFIIH*

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Positive cofactor 4 (PC4), originally identified as a transcriptional coactivator, possesses the ability to suppress promoter-driven as well as nonspecific transcription via its DNA binding activity. Previous studies showed that the repressive activity of PC4 on promoter-driven transcription is alleviated by transcription factor TFIIH, possibly through one of its enzymatic activities. Using recombinant TFIIH, we have analyzed the role of TFIIH for alleviating PC4-mediated transcriptional repression and determined that the excision repair cross complementing (ERCC3) helicase activity of TFIIH is the enzymatic activity that alleviates PC4-mediated repression via β-γ bond hydrolysis of ATP. In addition, the alleviation does not require either ERCC2 helicase or cyclin-dependent kinase 7 kinase activity. We also show that, as complexed within TFIIH, the cyclin-dependent kinase 7 kinase does not possess the activity to phosphorylate PC4. Thus, TFIIH appears to protect promoters from PC4-mediated repression by relieving the topological constraint imposed by PC4 through the ERCC3 helicase activity rather than by reducing the repressive activity of PC4 via its phosphorylation.

Positive cofactor 4 (PC4)[1] was originally identified in the upstream-factor stimulatory activity that augments activator-dependent transcription *in vitro* (1, 2). PC4 stimulates transcription *in vitro* with diverse kinds of activators, including VP16 (3, 4), thyroid hormone receptor (5), octamer transcription factor-1 (6), and BRCA-1 (7), presumably by facilitating assembly of the preinitiation complex through bridging between activators and the general transcriptional machinery (4, 8). Studies on the interaction of PC4 with activators and TFIIA, as well as *in vitro* functional analyses, suggest that interaction between TFIIA and PC4 plays a pivotal role for facilitating the preinitiation complex (PIC) assembly (3, 4). Further studies also demonstrated the importance of PC4 for transcriptional activation by AP-2 (9) and HIV transactivator Tat (10) *in vivo*. In addition, a yeast homologue of PC4, SUB1/TSP1 (11, 12), which is essential for viability in the presence of TFIIB mutations (12), was shown to function as a coactivator for GCN4 and HAP proteins. The N-terminal region of PC4 contains a serine-rich portion termed the SEAC domain, which exhibits similarity to viral immediate-early proteins (3). Phosphorylation of the serine residues in the SEAC domain negatively regulates the coactivator activity of PC4 (3, 13) possibly by a conformational change.

In addition to the role as coactivator, PC4 was subsequently shown to repress promoter-driven transcription as well as nonspecific transcription *in vitro* (14, 15). The analyses of PC4 mutants demonstrated that the repressive activity is a separate function from the coactivator activity (14); therefore, the repressive activity of PC4 may play an as yet unknown function in regulating transcription *in vivo*. In fact, the primary function of PC4 *in vivo* could possibly be to repress transcription rather than to enhance transcription because phosphorylated PC4, which is inactive as a coactivator but retains repressive activity, is the predominant form (~95%) within the cells (13). Transcriptional repression by PC4 correlates with the single-stranded (ss) DNA binding activity present in its C-terminal region, which shows preferential binding to melted double-stranded (ds) DNA and to heteroduplex DNA (14). The structural studies show that PC4 forms a homodimer via its C-terminal region that contains four-stranded β-sheets rich in positively charged and aromatic residues involved directly in binding to ssDNA (16, 17). Interestingly, in contrast to its coactivator activity, the ssDNA binding activity of PC4 is augmented by phosphorylation of its N-terminal region (8). Further studies indicate that PC4-mediated repression of specific transcription from promoters is alleviated by TFIIH, possibly through its enzymatic activities that require β-γ hydrolysis of ATP (14, 15). However, the identity of the enzymatic activity responsible for the alleviation as well as the mechanism by which TFIIH alleviates PC4-mediated repression remains unknown.

Here we used the recombinant TFIIH mutants that lack one of the enzymatic activities (cdk7 kinase, ERCC2 helicase, or ERCC3 helicase) (18) and examined the mechanism by which TFIIH counteracts the repressive effect of PC4. We have found that TFIIH counteracts PC4-mediated repression via ERCC3 helicase activity and that neither ERCC2 helicase nor cdk7 kinase activity is required for alleviating the repression, an observation further supported by the fact that TFIIH does not phosphorylate PC4. Our results suggest that PC4 and the ERCC3 helicase activity of TFIIH may act together to increase the specificity of transcription and also to provide more intricate regulation of transcription.

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[1] The abbreviations used are: PC4, positive cofactor 4; ERCC, excision repair cross-complementing; cdk, cyclin-dependent kinase; PIC, preinitiation complex; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; RNAPII, RNA polymerase II; TBP, TATA box-binding protein; CTD, carboxyl-terminal domain; Ni-NTA, nickel-nitrilotriacetic acid; TF, transcription factor; nt, nucleotide; HIV, human immunodeficiency virus.

EXPERIMENTAL PROCEDURES

Purification of Transcription Factors—PC4 was expressed in *Escherichia coli*, BL21(DE3)pLysS, harboring the plasmid pET11c-PC4, and the extract was prepared by sonication in buffer A (20 mM Hepes-KOH, pH 7.9, 10% glycerol, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol containing 100 mM KCl). The extract was applied onto a HiTrap SP column, and the bound proteins were eluted with a 5-column volume of a linear gradient of 0.1–0.6 M KCl. The eluted fractions were diluted to adjust the conductivity to that of 0.1 M KCl and then loaded onto a HiTrap heparin column. The bound proteins were eluted with a 5-column volume of linear gradient of 0.1–0.6 M KCl. RNA polymerase II (RNAPII), TFIIB, TFIIE, TFIIF, and FLAG-tagged TBP (f:TBP) were prepared essentially as described (19).

Preparation of Recombinant TFIIH—Recombinant TFIIH and its mutants were reconstituted in High Five cells using three baculoviruses, each of which expresses three subunits of TFIIH (18, 19). The purification of TFIIH was done essentially as described (19) except that TALON™ metal affinity resin (Clontech) was used in place of Ni-nitrilotriacetic acid (NTA) superflow (Qiagen). The amount of each TFIIH, whose cdk7 subunit is C-terminal-tagged with a FLAG epitope, was adjusted by using silver-stained gels as well as quantitative immunoblots with anti-FLAG M2 antibody.

In Vitro Transcription—*In vitro* transcription reactions were carried out in a 25-μl reaction containing 12 mM Hepes-KOH, pH 7.9, 6% glycerol, 60 mM KCl, 0.6 mM EDTA, 8 mM $MgCl_2$, 5 mM dithiothreitol, 20 units of RNase inhibitor (TaKaRa), 0.2 mM ATP, 0.2 mM UTP, 0.1 mM 3′-*O*-methyl GTP, 12.5 μM CTP, 10 μCi of [α-^{32}P]CTP, 20 ng TFIIA, 10 ng TFIIB, 4 ng f:TBP, 10 ng TFIIE, 20 ng TFIIF, 20 ng recombinant TFIIH, 100 ng RNAPII, and the indicated amount of PC4. All the transcription reactions contained negatively supercoiled pMLΔ53 (100 ng) as a template. The reactions were performed at 30 °C for 1 h, stopped by the addition of 20 mM EDTA, 0.2% SDS, and 5 μg of proteinase K, and further incubated at 37 °C for 1 h. After phenol/chloroform extraction and ethanol precipitation, the transcripts were analyzed by electrophoresis on a 5% denaturing polyacrylamide gel, followed by autoradiography.

Kinase Assays—Phosphorylation of GST-CTD (carboxyl-terminal domain) and PC4 by TFIIH was performed essentially as described (19). Where indicated, casein kinase II (New England Biolabs) was used in place of TFIIH as indicated.

RESULTS

Requirement of β-γ Bond Hydrolysis of ATP for the Alleviation of PC4-mediated Repression by TFIIH—To investigate the functional relationship between TFIIH and PC4, we prepared recombinant TFIIH reconstituted in the insect cells infected with three baculoviruses that expressed TFIIH subunits (18, 19). *In vitro* transcription assays were performed with recombinant TBP, TFIIB, TFIIE, TFIIF, and TFIIH together with RNAPII purified from HeLa cells (Fig. 1*A*), using a linearized pMLΔ53C2AT template that contained the adenovirus major late promoter fused with a 380-bp G-less cassette (19). The specific 390-nt transcript was observed only in the presence of all factors. No transcription was observed when one of the factors was omitted from the reaction, indicating that there was no cross-contamination among the factors (Fig. 1*B*).

We next tested whether recombinant TFIIH could alleviate transcriptional repression by PC4. As shown in Fig. 2*A*, even in the absence of TFIIH, the negatively supercoiled template allowed production of the specific 390-nt transcript (*lane 1*), which was suppressed to less than 5% by the addition of PC4 (*lane 2*). Adding the increasing amounts of TFIIH, however, gradually restored the levels of transcription (*lanes 3–6*) to 40–60% of those seen in the absence of both TFIIH and PC4 (*lane 1*), indicating that recombinant TFIIH can reverse the repressive effect of PC4 in a dose-dependent manner as does natural TFIIH (14, 15).

Using the highly purified reconstituted system, we then tested the requirement for β-γ bond hydrolysis by substituting ATP with adenylyl-imidodiphosphate (AMP-PNP) and adenosine-5′-*O*-(thiotriphosphate) (ATP-γS), both of which can be incorporated into growing RNA chains during transcription but

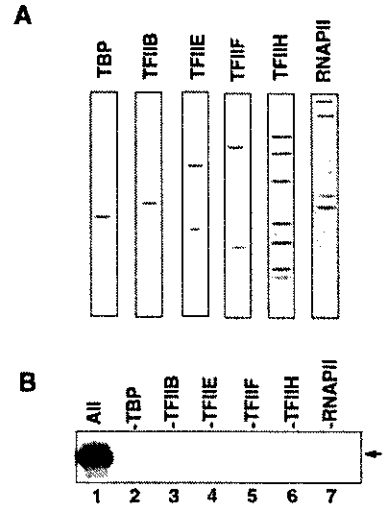

FIG. 1. **Transcription factors used for *in vitro* transcription.** *A*, purified proteins were separated by 10% SDS-PAGE and silver stained. *B*, reconstituted transcription analysis *in vitro*. Transcription reactions were performed with a linearized pMLΔC2AT that requires TFIIH for the production of the 390-nt-specific transcript. Transcription assays were performed in the presence of all factors (*lane 1*) or in the absence of TBP (*lane 2*), TFIIB (*lane 3*), TFIIE (*lane 4*), TFIIF (*lane 5*), TFIIH (*lane 6*), or RNAPII (*lane 7*). The *arrow* indicates the position of the 390-nt transcript.

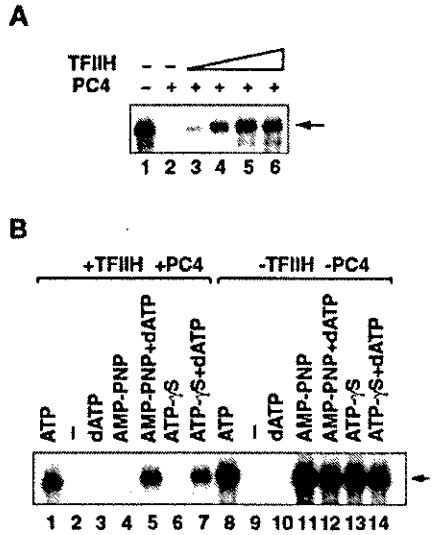

FIG. 2. **Requirement of β-γ bond hydrolysis of ATP for alleviating PC4-mediated repression by TFIIH.** *A*, recombinant TFIIH was used for alleviating PC4-mediated transcriptional repression. Transcription reactions consisting of TBP, TFIIB, TFIIE, TFIIF, and RNAPII contained 200 ng of PC4 (*lanes 2–6*) together with 5 (*lane 3*), 10 (*lane 4*), 20 (*lane 5*), or 40 ng (*lane 6*) of TFIIH. The *arrow* indicates the position of the 390-nt transcript from pMLΔC2AT. *B*, transcription reactions contained TBP, TFIIB, TFIIE, TFIIF, and RNAPII, with (*lanes 1–7*) or without TFIIH and PC4 (*lanes 8–14*). The reactions also contained 100 μM ATP (*lanes 1* and *8*), no ATP (*lanes 2* and *9*), 100 μM dATP (*lanes 3* and *10*), 100 μM AMP-PNP (*lanes 4* and *11*), 100 μM AMP-PNP and 100 μM dATP (*lanes 5* and *12*), 100 μM ATP-γS (*lanes 6* and *13*), or 100 μM ATP-γS and 100 μM dATP (*lanes 7* and *14*).

cannot be hydrolyzed at the β-γ bond. When ATP was replaced by non-hydrolyzable AMP-PNP or ATP-γS in the transcription reactions containing both PC4 and TFIIH, virtually no transcription was observed (Fig. 2*B*, *lanes 4* and *6*), indicating that β-γ bond hydrolysis of ATP was absolutely required for counteracting PC4-mediated repression. Transcription was restored, however, when AMP-PNP and ATP-γS were further supplemented with dATP (Fig. 2*B*, *lanes 5* and *7*), which could provide β-γ bond hydrolysis. These results show the require-

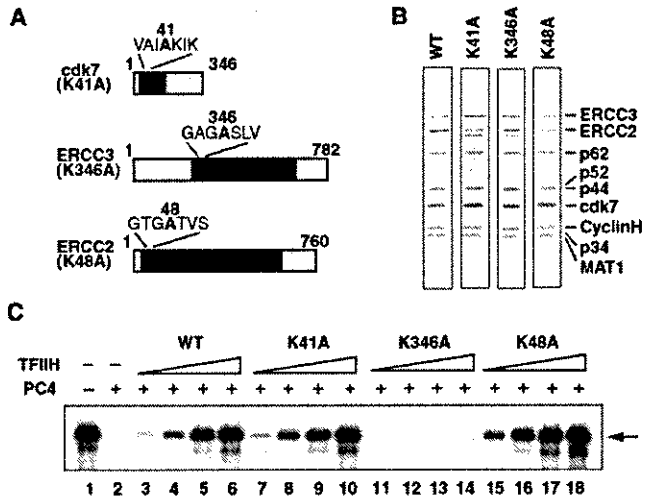

FIG. 3. **Requirement of ERCC3 helicase activity for alleviating PC4-mediated repression.** *A*, diagram of cdk7, ERCC3, and ERCC2, indicating portions of amino acid sequences including alanine residues (*boldfaced*) that were introduced in place of lysine. The lysine residues (residues 41, 346, and 48 of cdk7, ERCC3, and ERCC2, respectively) in the conserved ATP binding domains, as shown in *filled boxes*, were mutated to alanine using oligonucleotide-directed mutagenesis. *B*, purified recombinant wild-type TFIIH (*WT*) and TFIIH mutants that have a mutation in cdk7 (*K41A*), ERCC3 (*K346A*), or in ERCC2 (*K48A*). *C*, alleviation of PC4-mediated repression requires ERCC3 helicase activity. The transcription reactions contained TBP, TFIIB, TFIIE, TFIIF, PC4, and RNAPII, together with 5, 10, 20, and 40 ng of wild-type TFIIH (*lanes 3–6*), K41A (*lanes 7–10*), K346A (*lanes 11–14*), or K48A (*lanes 15–18*) as indicated.

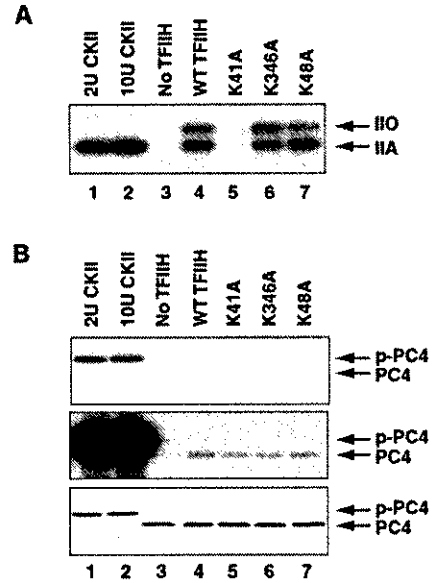

FIG. 4. **Phosphorylation of PC4.** *A*, phosphorylation of GST-CTD by casein kinase II (*CKII*) and TFIIH were tested. Phosphorylation reactions contained GST-CTD as a substrate together with 2 (*lane 1*) and 10 (*lane 2*) units of casein kinase II or with 100 ng of wild-type TFIIH (*lane 4*), K41A (*lane 5*), K346A (*lane 6*), and K48A (*lane 7*). The *arrows* indicate the position of hyperphosphorylated (*IIO*) and hypophosphorylated (*IIA*) forms of GST-CTD. *B*, phosphorylation reactions contained casein kinase II and TFIIH mutants as shown in *panel A*, with 200 ng of PC4 as a substrate in place of GST-CTD. The *top* and *middle panels* show the short (1.5 h) and long exposures (20 h) of the autoradiogram; the *bottom panel* shows Coomassie Blue staining of the same gel. The *arrows* indicate the positions of the phosphorylated (*p-PC4*) and non-phosphorylated (*PC4*) forms of PC4, which migrated as ~20 and ~15 kDa, respectively.

ment for β-γ bond hydrolysis of ATP (or dATP) for alleviating PC4-mediated repression even in the highly pure transcription system. Because TFIIH is the only known factor that utilizes β-γ bond hydrolysis of ATP in this well defined transcription system, the results clearly demonstrate the involvement of the enzymatic activities of TFIIH in the alleviation.

The ERCC3 Helicase Activity of TFIIH Is Essential for Alleviating PC4-mediated Repression—The requirement of β-γ bond hydrolysis suggested that one of the enzymatic activities of TFIIH was required for alleviating the repression by PC4. To determine which enzymatic activity of TFIIH was responsible for the alleviation, we utilized three recombinant TFIIH mutants, each of which is defective in either cdk7 kinase, ERCC3 helicase, or ERCC2 helicase activities (Fig. 3, *A* and *B*). These mutants have alanine instead of the conserved lysine within the ATP binding site of Walker type A motifs, at the 41st residue of cdk7, 346th residue of ERCC3, and 48th residue of ERCC2, respectively (Fig. 3*A*) (18, 20). Substitution of the lysine with either arginine or alanine in these motifs is known to eliminate the ability to hydrolyze ATP, resulting in the inactivation of each enzymatic activity. As shown in Fig. 3*C*, TFIIH with the mutated cdk7 kinase (K41A) and with the mutated ERCC2 helicase (K48A) alleviated PC4-mediated repression as well as wild-type TFIIH, whereas TFIIH with the mutated ERCC3 helicase (K346A) could not alleviate the repression at all. These results demonstrate that ERCC3 helicase activity is the sole enzymatic activity required for alleviating PC4-mediated repression, and neither the cdk7 kinase nor the ERCC2 helicase plays any role in alleviating PC4-mediated repression through ATP hydrolysis.

TFIIH Does Not Phosphorylate PC4—Because the previous result showed that PC4 is released from the template upon phosphorylation by TFIIH (21), the dispensability of the cdk7 kinase for PC4-mediated repression was somewhat unexpected. Furthermore, lack of any consensus phosphorylation site for cdks (*S/TPXR/K*) in PC4 prompted us to re-address whether TFIIH is indeed able to phosphorylate PC4 *in vitro* as previously reported (15, 21). As shown in Fig. 4*A*, wild-type TFIIH, K48A, and K346A phosphorylated CTD efficiently but K41A did not phosphorylate CTD, indicating that the substitution of lysine with alanine at the 41st residue of cdk7 eliminated the kinase activity to an undetectable level. Phosphorylation of CTD by TFIIH produced the hypophosphorylated form as well as the hyperphosphorylated form that showed a slower migration on the SDS gel (Fig. 4*A*). Casein kinase II also phosphorylated CTD, although phosphorylation did not shift the migration of GST-CTD (Fig. 4*A*, *lanes 1* and *2*).

We next tested whether casein kinase II and the same set of TFIIH mutants could phosphorylate PC4. Casein kinase II efficiently phosphorylated PC4 as previously reported (3, 13) and altered PC4 from the faster migrating form (~15 kDa) to the slower migrating form (~20 kDa) (Fig. 4*B*, *bottom panel*, *lanes 1* and *2*). In contrast, wild-type TFIIH, K346A, and K48A, all of which retain cdk7 kinase activity (Fig. 4*A*, *lanes 4*, *6*, and *7*), did not phosphorylate PC4 (Fig. 4*B*, *lanes 4*, *6*, and *7*). The low levels of PC4 labeling observed on a longer exposure of the gel (Fig. 4*B*, *middle panel*, *lanes 3–7*) is not because of the TFIIH kinase activity because the TFIIH mutant K41A, which lacks the kinase activity (Fig. 4*A*, *lane 5*), showed the same degree of labeling as wild-type TFIIH. Our results demonstrate that TFIIH does not phosphorylate PC4 and argue against the involvement of PC4 phosphorylation by TFIIH for alleviating PC4-mediated repression of transcription.

Quantitative Analysis of PC4-mediated Repression in the Absence of the ERCC3 Helicase Activity—The *in vivo* concentration of PC4 is estimated to be ~1 μM in HeLa cells, and ~95% of PC4 is phosphorylated *in vivo*, presumably by casein kinase II (3, 13, 14). Therefore, we tested whether phosphorylated and non-phosphorylated PC4 can distinguish the presence and ab-

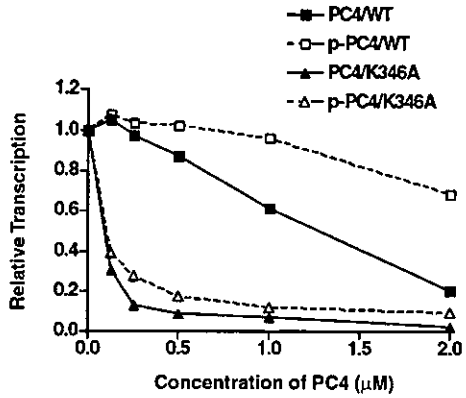

FIG. 5. **Repression of transcription by phosphorylated and non-phosphorylated PC4 in the absence of the ERCC3 helicase activity.** The relative levels of transcription are shown. The level of transcription in the absence of PC4 was arbitrarily defined as 1.0. Transcription reactions contained TBP, TFIIB, TFIIE, TFIIF, and RNAPII in the presence of either wild-type TFIIH (*WT*) or the ERCC3-deficient TFIIH mutant (*K346A*), together with indicated amounts of either non-phosphorylated or phosphorylated PC4. The values indicate the level of transcription in the presence of wild-type TFIIH and non-phosphorylated PC4 (*filled squares, solid line*), wild-type TFIIH and phosphorylated PC4 (*open squares, dotted line*), K346A and non-phosphorylated PC4 (*filled triangles, solid line*), and K346A and phosphorylated PC4 (*open triangles, dotted line*).

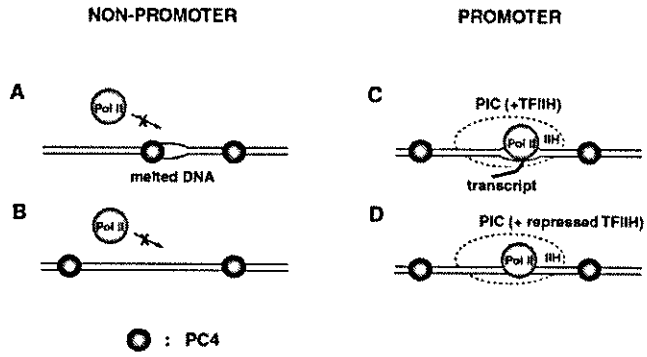

FIG. 6. **Model for PC4 in the regulation of transcription from non-promoter and promoter regions.** *A*, PC4 binds to dsDNA and ssDNA regions and prevents the binding of RNAPII to ssDNA regions by a direct competition. PC4 bound to dsDNA regions may also serve as a reservoir for PC4 recruited to ssDNA regions. *B*, PC4 binds dsDNA regions and prevents the binding of RNAPII to DNA by restricting the formation of transient ssDNA regions. *C*, the PIC (*dotted line*) containing TFIIH can initiation transcription from the promoter DNA that is bound by PC4. *D*, the PIC whose TFIIH activity is repressed (such as by FBP interacting repressor) fails to initiate transcription from the promoter DNA that is bound by PC4.

sence of ERCC3 helicase activity within the general transcriptional machinery at the physiological PC4 concentration. PC4 was first phosphorylated by CKII as shown in Fig. 4*B*, and then increasing amounts of both phosphorylated and non-phosphorylated PC4 were added to the transcriptional reactions containing either wild-type TFIIH or K346A. As shown in Fig. 5, the levels of transcription were reduced to less than 40% at 0.125 μM of PC4 and to ~10% at 1 μM of PC4 in the absence of ERCC3 helicase activity. Non-phosphorylated PC4 repressed transcription slightly better than phosphorylated PC4 in the absence of ERCC3 helicase activity (Fig. 5). By contrast, in the presence of wild-type TFIIH, transcription remained markedly more resistant to repression by PC4 (Fig. 5) (14). These results indicate that PC4 represses transcription regardless of its phosphorylation status in the absence of ERCC3 helicase activity. In addition, because repression by PC4 occurs similarly in the presence of K346A (Fig. 5) as in the absence of TFIIH (data not shown) (14), mutual exclusion of PC4 and TFIIH on the promoter is an unlikely mechanism for the antagonistic effect of PC4 and TFIIH.

DISCUSSION

Our results show that the ERCC3 helicase activity of TFIIH counteracts PC4-mediated transcriptional repression and that neither the ERCC2 helicase nor the cdk7 kinase has any role in this process. The fact that the ERCC3 helicase, but not the cdk7 kinase, of TFIIH relieves PC4-mediated repression provides a clue as to the mechanism by which TFIIH and PC4 act antagonistically to regulate transcription. Negatively supercoiled templates allow specific transcription by RNAPII in the absence of TFIIH and ATP *in vitro* (22, 23), presumably by the transfer of free energy stored on the negatively supercoiled templates (24–26). This transfer of free energy appears to be constrained by PC4, because the property of negatively supercoiled DNA templates bound by PC4 is similar to that of linear DNA templates with regard to the absolute requirement of TFIIH and ATP for specific promoter-driven transcription (22, 23). This effect of PC4 transmitted indirectly through DNA to the general transcriptional machinery is consistent with the functional antagonism between TFIIH and PC4 that does not involve the mutual exclusion of TFIIH and PC4 on the promoter (Fig. 5). Thus, the role for ERCC3 helicase activity may be to overcome the topological constraint conferred by PC4 on negatively supercoiled templates, a process that could potentially prompt the release of PC4 from the promoter region (21). Our results, however, rule out the possibility that the cdk7 kinase of TFIIH phosphorylates PC4 (15, 21) and facilitates its release from the promoter (21).

In light of our study as well as a previous study (14), we propose two possible mechanisms by which PC4 represses promoter-independent transcription: *i.e.* "direct" and "indirect" mechanisms. In the direct mechanism, PC4 binds to ssDNA regions via its ssDNA binding ability, competing directly with RNAPII, and thus physically displaces RNAPII from ssDNA regions (Fig. 6*A*). By contrast, in the indirect mechanism PC4 binds dsDNA regions via its dsDNA binding ability and renders DNA more "rigid" so that the free energy stored in negative superhelicity (24–26) will not generate transiently melted ssDNA regions that permit RNAPII to initiate random transcription (Fig. 6*B*). It is conceivable that the indirect mechanism provides the primary protection against spurious transcription and the direct mechanism provides a backup. In this scenario, PC4 bound to dsDNA regions may also serve as a reservoir that can be recruited quickly to ssDNA regions where the possibility of spurious transcription is greater. In agreement with the recruitment of PC4 from dsDNA to ssDNA, PC4 binds to ssDNA more strongly than to dsDNA (14).

PC4-mediated repression of transcription from non-promoter regions as described above may facilitate the efficient allocation of the limiting amount of RNAPII *in vivo* (27, 28), which could be otherwise sequestered onto transiently melted ssDNA regions. In the living cells, DNA is predominantly negatively supercoiled and is also undergoing dynamic topological changes during DNA replication, transcription, and repair, possibly exposing melted ssDNA regions frequently. Spurious transcription from these melted ssDNA regions is likely to be suppressed mainly by phosphorylated PC4, which constitutes ~95% of PC4 *in vivo* (13), because phosphorylated PC4 can strongly suppress promoter-independent (and thus, general transcription factor-independent) transcription from the melted DNA region *in vitro* (14).

PC4 may also play a role in preventing spurious transcription from promoters, which *in vivo* is likely to be negatively supercoiled and from which transcription could be potentially initiated in the absence of TFIIH. When the ERCC3 helicase of

TFIIH is active within the general transcriptional machinery, transcription is probably not repressed by PC4 *in vivo* (Fig. 6*C*) because the TFIIH ERCC3 helicase activity counteracts the repressive activity of phosphorylated PC4 at the physiological concentration (~1 μM) (Fig. 5). Indeed, when PC4 is overexpressed in cells in the absence of the HIV transactivator, transcription from the HIV promoter is only marginally reduced or not reduced at all, depending upon the assay conditions (10). However, if the ERCC3 helicase activity of TFIIH is inhibited (Fig. 5), such as by negative regulator of activated transcription and by FBP interacting repressor (29, 30), phosphorylated PC4 may further reduce the low background transcription from promoters even at the physiological PC4 concentration (Fig. 6*D*). Because TFIIH appears to be sub-stoichiometric (20–30%) to other general transcription factors *in vivo* (27), a fraction of PIC might even lack TFIIH and could be repressed by PC4, though this possibility must be rigorously examined *in vivo*. In any event, regulation of promoter-dependent transcription with a high level of dynamic range *in vivo* is likely to be contingent upon the presence of PC4, because negatively supercoiled DNA *in vivo* may permit inadvertent transcription from promoters and could potentially reduce the dynamic range of transcriptional regulation.

Several lines of evidence suggest the importance of PC4 in regulating transcription *in vivo*. First, a yeast homolog of human PC4, SUB4, enhances transcriptional activation by the activators GCN5 and HAP4 (12), and though PC4 is not essential for viability, its deletion results in inositol auxotrophy, a phenotype observed in the mutations of transcriptional regulators such as SNF/SWI, SRB, and the CTD of RNA polymerase II (31–34). Second, PC4 enhances TAT-dependent transcription from the HIV promoter (10) and restores the reduced AP-2 activity in the *ras*-transformed cell lines by relieving AP-2 self-interference (9). Finally, PC4 may play a role as a tumor suppressor in lung and bladder cancers, because the loss of heterogeneity of the PC4 gene is often observed in these cancer cells (35, 36). These results demonstrate the importance of PC4 as a regulator of transcription and possibly as a tumor suppressor *in vivo*. Though the importance of PC4 *in vivo* has been mainly interpreted in the context of its coactivator activity, the predominance of the repressive form of PC4 *in vivo* (13) suggests that some of the observed effects may well be attributed to the reduced precision of transcriptional regulation caused by the loss of the repressive activity of PC4.

In conclusion, the repressive activity of PC4 may be essential for the intricate regulation of transcription in conjunction with the ERCC3 helicase of TFIIH. The repressive activity of PC4, and possibly of other ssDNA-binding proteins, may play an important but yet under-appreciated role for more elaborate and fine-tuned regulation of reactions involving DNA molecules.

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Transcriptional Coactivator PC4 Stimulates Promoter Escape and Facilitates Transcriptional Synergy by GAL4-VP16

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Positive cofactor 4 (PC4) is a coactivator that strongly augments transcription by various activators, presumably by facilitating the assembly of the preinitiation complex (PIC). However, our previous observation of stimulation of promoter escape in GAL4-VP16-dependent transcription in the presence of PC4 suggested a possible role for PC4 in this step. Here, we performed quantitative analyses of the stimulatory effects of PC4 on initiation, promoter escape, and elongation in GAL4-VP16-dependent transcription and found that PC4 possesses the ability to stimulate promoter escape in response to GAL4-VP16 in addition to its previously demonstrated effect on PIC assembly. This stimulatory effect of PC4 on promoter escape required TFIIA and the TATA box binding protein-associated factor subunits of TFIID. Furthermore, PC4 displayed physical interactions with both TFIIH and GAL4-VP16 through its coactivator domain, and these interactions were regulated distinctly by PC4 phosphorylation. Finally, GAL4-VP16 and PC4 stimulated both initiation and promoter escape to similar extents on the promoters with three and five GAL4 sites; however, they stimulated promoter escape preferentially on the promoter with a single GAL4 site. These results provide insight into the mechanism by which PC4 permits multiply bound GAL4-VP16 to attain synergy to achieve robust transcriptional activation.

Transcription of mRNA-coding genes involves RNA polymerase II and six general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) which comprise the basal transcription machinery that recognizes the core promoter elements and elicits the basal level of transcription (50). Activated transcription requires the binding of activators to the regulatory DNA sequences typically present upstream of the core promoter and their interactions with the general transcription machinery (32, 49). Despite the well-documented direct interactions of activators with the general transcription factors and RNA polymerase II, activated transcription requires yet another group of transcription factors, termed mediators or coactivators, that confer on the general transcription machinery a markedly enhanced responsiveness to activators (2, 18, 20, 36, 41).

A wide array of coactivators may be grouped into two broad categories according to the requirement of chromatin for their action in biochemical assays. The coactivators which function on the templates without chromatin include the TATA box binding protein-associated factors (TAFs) present in TFIID (58), positive cofactors (PCs) (PC1, PC2, PC3, and PC4) derived from the upstream factor stimulatory activity (USA) cofactor fraction (20), and metazoan multiprotein complexes that are structurally related to the yeast mediator (40) (TRAP/SMCC, ARC, DRIP, NAT, murine mediator, human mediator, CRSP, and PC2) (36, 41). The coactivators which require chromatin templates for their functions include CBP/p300, PCAF and its related GCN5 proteins, and p160 family proteins that display histone acetyltransferase activities (4, 65). Given their structural complexity and diversity, these coactivators are expected to show not only redundancy and cooperativity but activator and promoter selectivity as well, posing significant challenges for complete understanding of the various mechanisms by which coactivators facilitate transcription.

One way to approach the mechanisms of coactivator functions is to employ a well-defined transcription system that supports activated transcription in response to the smallest possible numbers of activators and coactivators and to identify the steps of transcription that are targeted physically and functionally by the activators and coactivators. A system well suited for this minimalist approach would be the transcription system that allows activated transcription in response to GAL4-VP16 or other GAL4-derivatives in the presence of coactivator PC4. PC4 is a coactivator that was initially identified in the USA fraction that enhances transcription by various transcriptional activators in vitro (13, 27, 38) and turned out to be identical to the 15-kDa single-stranded DNA (ssDNA)-binding protein. Although PC4 possesses both ssDNA- and double-stranded DNA (dsDNA)-binding activities, which are important for transcriptional repression, only its dsDNA-binding activity appears to correlate with the coactivator activity (62, 63). The coactivator activity of PC4 and its interaction with activators, but not the ssDNA-binding activity, are lost upon phosphorylation of the serine residues within its N-terminal region by casein kinase II (14, 27).

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Since PC4 interacts with both transcriptional activators and TFIIA (13, 16) and also with TFIIB in the case of its yeast homolog, SUB1/TSP1 (16, 23), PC4 is proposed to promote the assembly of the preinitiation complex (PIC) (13, 21, 27) in activated transcription. However, given that transcription is a multistep process consisting of PIC assembly, promoter opening, initiation, promoter escape, elongation, and reinitiation, steps other than PIC assembly are potential targets for regulation as well. Indeed, despite predominant effects of activators—presumably in conjunction with coactivators—on PIC assembly, the effects on the subsequent steps (1, 28, 31, 37) have also been demonstrated in various systems, including promoter opening (60), promoter escape (10, 29), elongation (66), and reinitiation (26, 67). However, the relative contributions of activators and coactivators in stimulating individual steps of transcription remain to be more clearly defined.

In this study, we have systematically analyzed the stimulatory effects of GAL4-VP16 and PC4 and assigned their quantitative contributions to the stimulation of individual steps of transcription. Our results show that PC4 contributed to the stimulation of promoter escape as well as initiation in the presence of GAL4-VP16 and that these effects were contingent upon the presence of TFIIA and TAFs in TFIID. Consistent with the previously demonstrated requirement of the ERCC3 helicase activity of TFIIH in stimulating promoter escape (10), PC4 was found to interact specifically with TFIIH through its coactivator domain, a region that also interacted with GAL4-VP16. Furthermore, the number of GAL4 sites (and thus the number of bound GAL4-VP16 dimers) on the promoter influenced the degree of stimulation of each step by PC4, revealing possible links between the physical interactions involving PC4 and their functional consequences on the steps of transcription. Together, these results provide important clues as to the mechanism by which PC4 assists GAL4-VP16 in transcriptional activation.

MATERIALS AND METHODS

DNA templates for in vitro transcription. DNA templates pG1HMC2AT and pG3HMC2AT were created by replacement of the five GAL4-binding sites of pG5HMC2AT with the annealed oligonucleotides. The following synthetic oligonucleotides were used for one and three GAL4-binding sites: 5′-AATTCGA GCTCGGTACCAGGGGACTAGAGTCTCCGCTCGGAGGACAGTACTCC GACCTGCA-3′ and 5′-GGTCGGAGTACTGTCCTCCGAGCGGAGACTCT AGTCCCCTGGTACCGAGCTCG-3′, respectively, for pG1HMC2AT and 5′-AATTCGAGCTCGGTACCAGGGGACTAGAGTCTCCGCTCGGAGGACA GTACTCCGCTCGGAGGACAGTACTCCGCTCGGAGGACAGTACTC CGACCTGCA-3′ and 5′-GGTCGGAGTACTGTCCTCCGAGCGGAGTACTG TCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGACTCTAGTCCCCTGG TACCGAGCTCG-3′, respectively, for pG3HMC2AT. The mixtures of the complementary oligonucleotides were denatured at 90°C for 5min and were then cooled slowly to room temperature in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA–50 mM NaCl. Then, the annealed oligonucleotides were used to replace the EcoRI-PstI fragment encompassing the five GAL4-binding sites of pG5HMC2AT. The obtained DNA templates were sequenced completely on both strands to rule out the possibility of spurious mutations.

Purification of transcription factors and in vitro transcription assays. Purification of recombinant factors (TFIIA, TFIIB, TFIIE, TFIIF, TFIIH, and GAL4-VP16), epitope-tagged TFIID, and RNA polymerase II were performed as described previously (10, 12). Recombinant PC4 was purified as described previously (11). For 390- and 20-nucleotide (nt) transcripts, in vitro transcription reaction mixtures (25 μl) contained 50 ng of negatively supercoiled pG5HMC2AT or its derivative, 12 mM HEPES-KOH (pH 7.9), 6% glycerol, 60 mM KCl, 0.6 mM EDTA, 8 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 20 U of RNase inhibitor (TaKaRa), 0.2 mM ATP, 0.2 mM UTP, 0.1 mM 3′-*o*-methyl GTP, 12.5 μM CTP, 10 μCi of [α-^{32}P]CTP, 20 ng of TFIIA, 10 ng of TFIIB, 1 μl of FLAG-tagged TFIID (which corresponds to ~0.1 ng of TATA binding protein [TBP]), 10 ng of TFIIE, 20 ng of TFIIF, 20 ng of recombinant TFIIH, and 100 ng of RNAPII. Where indicated, the reaction mixtures contained 25 ng of GAL4-VP16 and 200 ng of PC4 as well. Transcription reactions for the initiation product were performed as described previously (10), and the derived pppApC was treated with calf intestinal phosphatase to form ApC before electrophoresis (29). After electrophoresis, the transcripts were quantified by using a Fujix Bas2000 bioimaging analyzer. Stimulation of promoter escape (*n*-fold) was calculated by dividing the stimulation of the 20G transcript (*n*-fold) by that of ApC. Similarly, stimulation of elongation (*n*-fold) was calculated by dividing the stimulation of the 390-nt transcript (*n*-fold) by that of the 20G transcript.

Preparation of PC4-GST. The coding region of PC4 was amplified by PCR with the primers 5′-GGCCTCTAGACATATGCCTAAATCAAAGG-3′ and 5′-GGCCGGATCCCAGCTTTCTTACTGCGTC-3′, which both incorporated XbaI and NdeI sites at the 5′ end and a BamHI site in place of the stop codon at the 3′ end. After digestion with XbaI and BamHI, the XbaI-BamHI fragment was subcloned into pBluescript II KS(−) (Stratagene) that was digested with XbaI and BamHI to create pBKS(−)-PC4. Then, the coding region of *Schistosoma japonicum* glutathione *S*-transferase (GST) was amplified by PCR from expression vector pGEX2TL(+) by using the primers 5′-GGCCGGATCCCCT ATACTAGGTTATTG-3′ and 5′-GGCCGGATCCAGATCTCAGTCAGTCA TTTTGGAGGATGGTCGCC-3′, which both incorporated a BamHI site at the 5′ end and BglII and BamHI sites at the 3′ end. The amplified PCR product was digested with BamHI, and the derived BamHI-BamHI fragment was inserted into the BamHI site of pBKS(−)-PC4 to create pBKS(−)-PC4-GST. After confirmation of its DNA sequence, the entire PC4-GST coding region was cut out with NdeI and BglII digestion and inserted into the NdeI and BamHI sites of pET3a (TaKaRa). PC4 deletion mutants, either as GST-PC4 or as PC4-GST, were created by using the same strategy and were inserted into the same expression vectors in place of wild-type PC4.

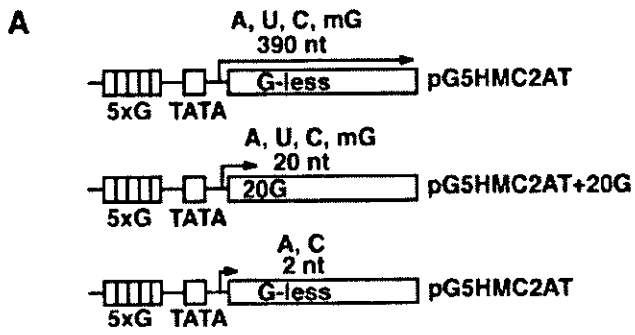
Recombinant GST-PC4 and PC4-GST and their derivatives were expressed in *Escherichia coli* BL21(DE3) p*Lys*S at 30°C for 3 h, and the extract was prepared by sonication in buffer C (20 mM HEPES-KOH [pH 7.9], 10% glycerol, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM DTT) containing 100 mM KCl. After removal of insoluble material by centrifugation, the soluble fraction was used for GST pull-down assays.

GST pull-down assays. Ten microliters of glutathione-Sepharose 4B (Amersham Pharmacia Biotech) was equilibrated with buffer C containing 100 mM KCl and 0.1% Triton X-100 and was incubated with *E. coli* extract containing a GST-fusion protein at 4°C for 1 h. The quantity of each *E. coli* extract was adjusted so that an equal amount of GST fusion proteins could be retained on the resin. In all assays, each GST fusion protein immobilized on glutathione-Sepharose 4B was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to ensure that essentially the same amount of GST fusion proteins was immobilized on each resin. After extensive washing of the resin with the same buffer, either 200 ng of recombinant TFIIH or 240 ng of FLAG-tagged GAL4-VP16 was added to the resin suspended in 100 μl of buffer C, and the mixture was incubated at 4°C for 1 h with constant rotation. After the resin had been washed extensively with the equilibration buffer, the bound proteins were eluted with 10 μl of buffer C containing 1 M KCl and 0.1% Triton X-100. Two microliters of each eluate was separated by SDS–12% PAGE and detected by Western blotting with anti-FLAG M2 antibody (Sigma).

Phosphorylation of PC4-GST. Fifty micrograms of PC4-GST immobilized on 10 μl of glutathione-Sepharose 4B was phosphorylated in reaction mixtures (200 μl) containing 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.2 mM ATP, and 50 U of casein kinase II (New England Biolabs)/μl at 30°C for 1 h. Phosphorylation of PC4-GST was confirmed by both radiolabeling with [γ-^{32}P]ATP and the mobility change on a SDS–15% polyacrylamide gel.

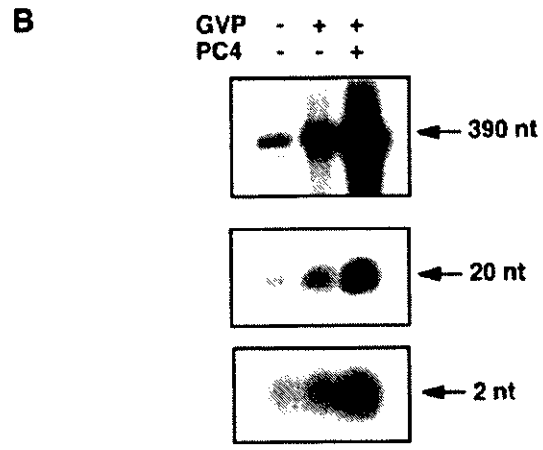
DNase I footprint assays. The promoter regions that contained GAL4 sites were PCR amplified from pG1HMC2AT, pG3HMC2AT, and pG5HMC2AT by using the primers 5′-GTAAAACGACGGCCAGT-3′ and 5′-CCGGGGATCC GGGGATGAGAGTGAATGATGATAGATTTG-3′. After digestion with EcoRI and BamHI, the PCR fragments were subcloned between the EcoRI and BamHI sites of pUC19 to create pUC19-G1, pUC19-G3, and pUC19-G5, which were then entirely sequenced to rule out the possibility of spurious mutations. The plasmids were digested with PvuI and XbaI, and the DNA fragments containing GAL4 sites were isolated after separation on a 4% polyacrylamide gel. Four picomoles of the DNA fragments were labeled by Klenow fragment (New England Biolabs) by using 50 μCi [α-^{32}P]dCTP. Each labeled DNA fragment was then diluted with the same unlabeled fragment to obtain ~2 × 10^4 cpm per 24 femtomoles of each fragment.

DNA binding reaction mixtures (25 μl) contained 12 mM HEPES-KOH (pH



C

Transcript	Fold Stimulation	
	+GVP	+GVP, +PC4
2 nt	2.1	3.2
20 nt	3.0	13.0
390 nt	3.5	16.6



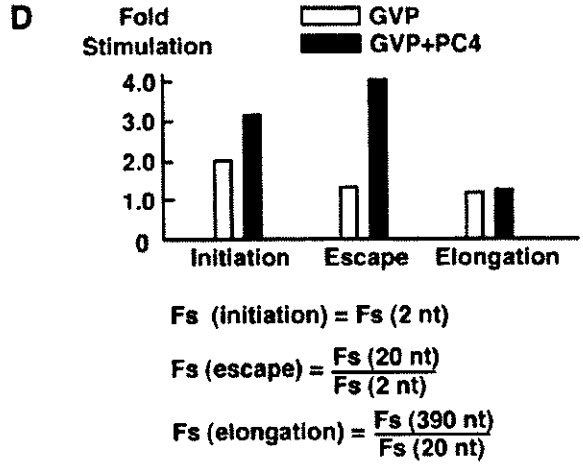


FIG. 1. Effect of PC4 on promoter escape. (A) DNA templates used for in vitro transcription analyses. The template pG5HMC2AT contains five GAL4-binding sites upstream of the human immunodeficiency virus TATA box and the initiator from the Ad2 ML promoter fused to a 380-bp G-less cassette. This template produces the 390-nt transcript in the presence of ATP (A), CTP (C), UTP (U), and 3′-*o*-methyl GTP (mG) and the 2-nt transcript (initiation product) in the presence of ATP and CTP. The template pG5HMC2AT+20G, which is identical to pG5HMC2AT except that it contains a guanine residue at the +20 position, produces the 20-nt transcript in the presence of ATP, CTP, UTP, and 3′-*o*-methyl GTP. (B) Effect of GAL4-VP16 and PC4 upon the 390-, 20-, and 2-nt transcripts. All transcription reactions contained general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) and RNAPII in the presence or absence of GAL4-VP16 and PC4. The transcripts were separated on a denaturing polyacrylamide gel and autographed. (C) The levels of stimulation (*n*-fold) for the 2-, 20-, and 390-nt transcripts. The transcripts were quantified by using Fujix Bas 2000, and stimulation (*n*-fold) was determined for transcripts in the presence of GAL4-VP16 or GAL4-VP16 and PC4 by using the level for the transcript in the absence of GAL4-VP16 and PC4 as the basal level of transcription. (D) Fold stimulation (Fs) for each step of transcription was determined as indicated by using the values of Fs for the 2-, 20-, and 390-nt transcripts shown in panel C. Open bars indicate Fs in the presence of GAL4-VP16, whereas closed bars indicate Fs in the presence of GAL4-VP16 and PC4. For example, a value of 4.1 for Fs (escape) in the presence of GAL4-VP16 and PC4, indicated by a closed bar above "Escape" in the bar graph, was obtained by dividing of Fs (20 nt) (13.0) by Fs (2 nt) (3.2) in the presence of GAL4-VP16 and PC4.

7.9), 6% glycerol, 60 mM KCl, 0.6 mM EDTA, 8 mM $MgCl_2$, 5 mM DTT, the indicated amount of GAL4-VP16, and 24 fmol of the labeled fragment and pUC19, which corresponds to approximately 50 ng of pG5HMC2AT used for in vitro transcription reactions. The reaction mixtures were incubated at 30°C for 60 min, and the DNA fragment was digested with DNase I for 2 min at room temperature by adding 25 μl of 5 mM $CaCl_2$–10 mM $MgCl_2$ containing 0.002 U of DNase I (TaKaRa). The DNase I digestion was stopped by adding 150 μl of stop solution (0.2% SDS, 20 mM EDTA), 20 μg of glycogen, and 5 μg of proteinase K, and the reaction mixtures were further incubated at 37°C for 60 min. After extraction with phenol and chloroform, the DNA fragment was precipitated with ethanol and rinsed twice with 70% ethanol. The dried pellet was redissolved in 2 μl of 90% formamide–0.025% (wt/vol) xylene cyanol and separated on a 4% denaturing polyacrylamide gel.

RESULTS

Promoter escape is a target for the coactivator activity of PC4. To gain a mechanistic insight into the coactivator function in transcriptional activation, we utilized a model in vitro transcription system that included GAL4-VP16 as an activator and PC4 as a coactivator. This in vitro transcription system contained well-defined components, including recombinant factors (TFIIA, TFIIB, TFIIE, TFIIF, TFIIH, PC4, and GAL4-VP16) as well as highly purified HeLa cell-derived FLAG-tagged TFIID and RNA polymerase II (10–12), and exhibited marked transcriptional activation in response to GAL4-VP16 in a highly PC4-dependent manner. These features of this system provided an excellent opportunity to analyze mechanistic aspects of the coactivator function of PC4 in a quantitative manner.

To accurately quantify the effects of PC4, we focused exclusively on measuring the amounts of the 2-, 20-, and 390-nt transcripts (Fig. 1A). We took this approach because measuring the amount of PIC (either by immunoblotting or by gel-mobility shift) and the degree of promoter opening (by potas-

sium permanganate footprinting) did not give sufficiently accurate values compared to measuring the amounts of transcripts and therefore did not provide useful information for detailed quantitative analyses. For this reason, the effects of PC4 on PIC assembly and promoter opening are subsumed in the effects on the 2-nt ApC formation, which corresponds to the initiation step on the templates used in this study (Fig. 1A). Accordingly, unless otherwise stated, the term "initiation," used hereafter for brevity, includes all three steps: PIC assembly, promoter opening, and ApC formation.

Using this reconstituted in vitro transcription system, we measured the levels of the 2-, 20-, and 390-nt transcripts produced from the template pG5HMC2AT or its derivatives (Fig. 1A). The 2-nt initiation transcript, which is ApC on the template pG5HMC2AT, was produced in the presence of ATP and CTP. The 20-nt transcript was produced in the presence of ATP, CTP, UTP, and 3′-*o*-methyl GTP from template pG5HMC2AT+20G, which contained a G residue at the +20 position, at which transcription terminates through incorporation of 3′-*o*-methyl GTP (Fig. 1A). The 390-nt transcript was produced from template pG5HMC2AT in the presence of ATP, CTP, UTP, and 3′-*o*-methyl GTP. After the relative amounts of these transcripts had been determined, the effects of GAL4-VP16 or GAL4-VP16 and PC4 on initiation, promoter escape, and elongation were estimated (Fig. 1B and C). The effect on initiation was estimated directly from the stimulation (*n*-fold) of the 2-nt transcript. Also, the effect on promoter escape was estimated by dividing the stimulation (*n*-fold) of the 20-nt transcript by that of the 2-nt transcript, and likewise, the effect on elongation was estimated by dividing the stimulation (*n*-fold) of the 390-nt transcript by that of the 20-nt transcript.

As shown in Fig. 1B and C, GAL4-VP16 alone stimulated the production of the 2-, 20-, and 390-nt transcripts 2.1-, 3.0-, and 3.5-fold, respectively. Thus, the effects of GAL4-VP16 on initiation, promoter escape, and elongation were calculated as 2.1-, 1.4-, and 1.2-fold, respectively, indicating that GAL4-VP16 stimulated mostly initiation and had lesser effects on promoter escape and elongation (Fig. 1D). Further inclusion of PC4 in these reactions stimulated the production of the 2-, 20-, and 390-nt transcripts 3.2-, 13.0-, and 16.6-fold, respectively (Fig. 1C). Thus, the combined stimulatory effects of GAL4-VP16 and PC4 on initiation, promoter escape, and elongation were 3.2-, 4.1-, and 1.3-fold, respectively, indicating that PC4 augments the ability of GAL4-VP16 to stimulate initiation, promoter escape, and, to a lesser degree, elongation (Fig. 1D). It is notable that the effects of PC4 on the coactivation of GAL4-VP16 are more pronounced at the promoter escape step than at the other steps (Fig. 1D). Taken together, these results not only corroborate a previous demonstration that PC4 acts through the facilitated PIC assembly (13, 21) but also highlight promoter escape as yet another step facilitated by the coactivator activity of PC4.

PC4 requires TFIIA and TAFs for stimulating promoter escape in response to GAL4-VP16. Previous biochemical studies demonstrated that TFIIA and the TAF subunits of TFIID greatly enhance transcriptional activation in vitro. Despite the well-documented roles of TFIIA and TFIID during the assembly of PIC (7, 8, 24, 33), their effects on promoter escape in activated transcription remain undefined. Moreover, observations that TBP alone supports transcriptional activation by various activators, including GAL4-VP16 (39, 43, 59, 64), suggest that some steps may be stimulated without TAFs.

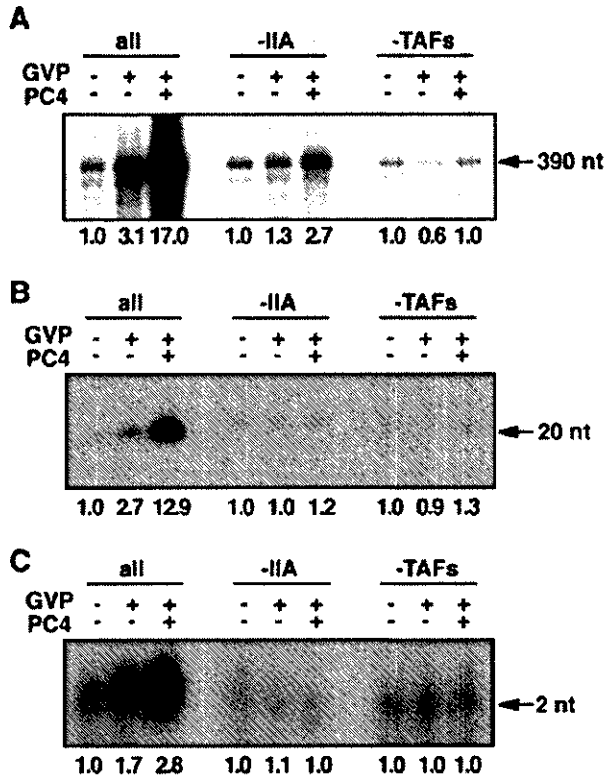


FIG. 2. Requirement of TFIIA and TAFs for stimulation of promoter escape by PC4. Stimulation of the 390-nt (A), 20-nt (B), and 2-nt (C) transcripts in the absence of TFIIA or TAFs. Transcription reactions were done in the absence of TFIIA (−IIA) or the TAF subunits of TFIID by using pG5HMC2AT or pG5HMC2AT+20G. The levels of stimulation of each transcript (*n*-fold) are indicated below the autoradiograms of the gels. In the absence of TFIIA or TAF subunits of TFIID, little stimulatory effect was observed on initiation, promoter escape, and elongation in the presence of GAL4-VP16 or in the presence of both GAL4-VP16 and PC4 except for a small stimulatory effect (i.e., ~2.3-fold) on elongation by PC4 in the absence of TFIIA, an effect that was dependent upon the presence of TFIID.

To determine whether TFIIA and TAFs are required for mediating the stimulation of promoter escape by GAL4-VP16 and PC4, we performed in vitro transcription assays in the presence or absence of TFIIA and TAFs. When TFIIA was removed from the reactions, the 2-, 20-, and 390-nt transcripts were stimulated 1.1-, 1.0-, and 1.3-fold, respectively, in the presence of GAL4-VP16 alone and 1.0-, 1.2-, and 2.7-fold, respectively, in the presence of GAL4-VP16 and PC4 (Fig. 2). Thus, in the absence of TFIIA, there was little stimulation of initiation and promoter escape by PC4 in response to GAL4-VP16. Interestingly, an approximately twofold stimulatory effect on elongation by GAL4-VP16 and PC4 remained intact even in the absence of TFIIA, albeit in a TAF-dependent manner (Fig. 2A and B), although this effect was not pursued further in this study. When TFIID was replaced by TBP, the 2-, 20-, and 390-nt transcripts were stimulated 0.6-, 0.9-, and 1.0-fold, respectively, in the presence of GAL4-VP16 and 1.0-,

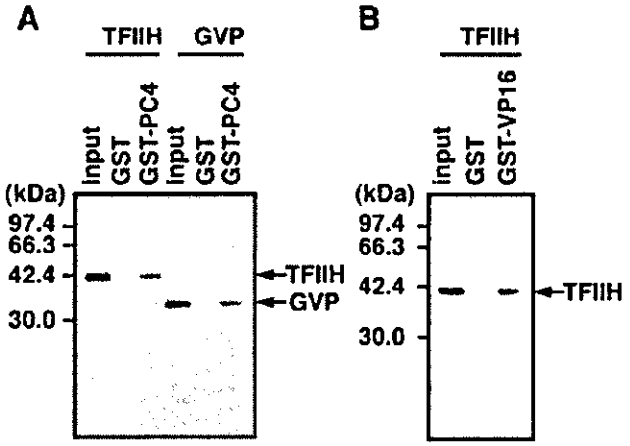


FIG. 3. PC4 interacts directly with TFIIH. (A) Interaction of PC4 with TFIIH. PC4 was fused to the C terminus of GST and expressed in *E. coli* as GST-PC4. TFIIH and GAL4-VP16 were allowed to interact with GST or GST-PC4 prebound to glutathione-Sepharose, and, after extensive washing, bound proteins were eluted, separated by SDS-PAGE along with ~20% of the amount of the input protein, and detected by immunoblotting. TFIIH and GAL4-VP16 were detected with anti-FLAG M2 antibody, since the MO15 subunit of TFIIH and GAL4-VP16 were tagged with a FLAG epitope. The positions of molecular mass markers are indicated on the left. The positions of MO15 (TFIIH) and GAL4-VP16 (GVP) are also indicated on the right. (B) Interaction of TFIIH and GAL4-VP16. GST pull-down assays were done under the same conditions as used for the tests presented in panel A, with GST-VP16 being used in place of GST-PC4.

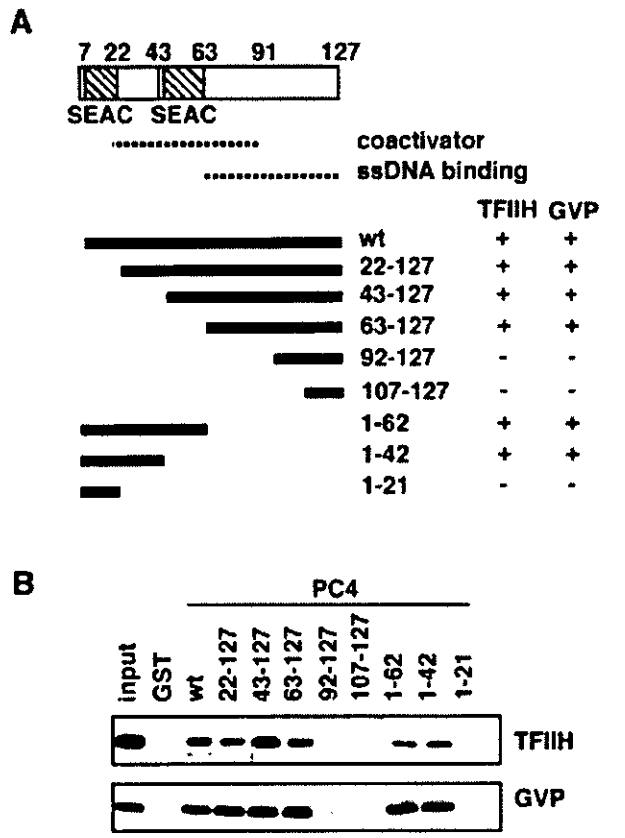


FIG. 4. The interaction of TFIIH and GAL4-VP16 with the coactivator domain of PC4. (A) Schematic representation of the domain structure of PC4. Two serine-rich domains, termed SEAC, are present between amino acid residues 7 and 22 and between residues 43 and 63. The domain for binding single-stranded DNA (dotted line) is localized between residues 63 and 127, and the 89th tryptophan residue is critical for its activity. The coactivator domain (dotted line) is localized to the region between residues 63 and 91, partially overlapping the ssDNA-binding domain. The lower panel shows the tested deletion mutants and the results of the GST pull-down assays for their interactions with PC4 or GAL4-VP16. The 127-amino-acid full-length PC4 is indicated by "wt." Binding and nonbinding are indicated by "+" and "−," respectively, on the right side of the lower panel. (B) GST pull-down assays for PC4 deletion mutants, as detected with Western blots. Note the variation in the amounts of bound TFIIH, which was reproducible, in marked contrast to the constant level of GAL4-VP16 binding.

1.3-, and 1.0-fold, respectively, in the presence of GAL4-VP16 and PC4 (Fig. 2), demonstrating that TAFs are essential for stimulating all of the steps, including initiation, promoter escape, and elongation, at least under the present assay conditions. Together, these observations suggest that both TFIIA and TAFs are indispensable for PC4 to effect noticeable stimulation of promoter escape as well as initiation (probably via facilitated PIC assembly) in response to GAL4-VP16.

PC4 interacts with TFIIH and GAL4-VP16 via its coactivator domain. A previous study showed that TFIIH is required for the stimulation of promoter escape because of its ERCC3 helicase activity (10) in activated transcription by GAL4-VP16 and PC4. The fact that both PC4 and TFIIH are required simultaneously to facilitate promoter escape prompted us to examine a possible physical interaction between PC4 and TFIIH. To do this, we performed GST pull-down assays by using PC4 fused to the C terminus of GST, which was expressed in *E. coli* and retained on the glutathione-Sepharose resin. Since recombinant TFIIH has a FLAG tag at the C terminus of its MO15 subunit and GAL4-VP16 has an N-terminal FLAG tag, both proteins were detected by Western blotting with anti-FLAG M2 antibody after separation by SDS-PAGE. As shown in Fig. 3A, TFIIH was found to bind to GST-PC4 but not to GST alone, indicating that PC4 interacts with TFIIH specifically. The interaction between PC4 and TFIIH seemed as strong as the well-characterized interaction between PC4 and VP16 (Fig. 3A) and that between TFIIH and the VP16 activation domain (Fig. 3B), since similar proportions (~10%) of input TFIIH and GAL4-VP16 were bound to GST-PC4 under the same conditions.

To explore the relevance of the interactions of PC4 with TFIIH and with GAL4-VP16 for the coactivator activity of PC4, we localized the region of PC4 that interacted with TFIIH and GAL4-VP16. We created N-terminal and C-terminal deletion mutants of PC4, as shown in Fig. 4A, and tested their interactions with TFIIH and GAL4-VP16. As shown in Fig. 4B and also summarized in Fig. 4A, the mutants PC4(22-127), PC4(43-127), PC4(63-127), PC4(1-62), and PC4(1-42) interacted with both TFIIH and GAL4-VP16, whereas PC4(92-127), PC4(107-127), and PC4(1-21) did not interact with either TFIIH or GAL4-VP16 (Fig. 4A and B), showing that PC4 interacts with TFIIH and GAL4-VP16 through the region from residue 22 to residue 91, a domain necessary and sufficient for the coactivator activity of PC4 (21, 27). Furthermore, the PC4 mutants PC4(1-62) and PC4(63-127), which do