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Androgen Receptor Interacts with the Positive Elongation Factor P-TEFb and Enhances the Efficiency of Transcriptional Elongation*

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Androgen receptor (AR) may communicate with the general transcription machinery on the core promoter to exert its function as a transcriptional modulator. Our previous report demonstrated that the AR interacted with transcription factor IIH (TFIIH) under physiological conditions and that overexpression of Cdk-activating kinase, the kinase moiety of TFIIH, enhanced AR-mediated transcription in prostate cancer cells. In an effort to further dissect the mechanisms implicated in AR transactivation, we report here that AR interacts with PITALRE, a kinase subunit of positive elongation factor b (P-TEFb). Cotransfection of the plasmid encoding the mutant PITALRE (mtPITALRE), defective in its RNA polymerase II COOH-terminal domain (CTD)-kinase activity, resulted in preferential inhibition of AR-mediated transactivation. Indeed, AR transactivation in PC-3 cells was preferentially inhibited at the low concentration of 5,6-dicloro-1-β-D-ribofuranosylbenzimidazole (DRB), a CTD kinase inhibitor. These results suggest that CTD phosphorylation may play an important role in AR-mediated transcription. Furthermore, a nuclear run-on transcription assay of the prostate-specific antigen gene, an androgen-inducible gene, showed that transcription efficiency of the distal region of the gene was enhanced upon androgen induction. Taken together, our reports suggest that AR interacts with TFIIH and P-TEFb and enhances the elongation stage of transcription.

Molecular studies of eukaryotic transcription suggest that the process of transcription can be divided into the following steps: preinitiation complex assembly on the core promoter, initiation, promoter clearance, elongation, and termination (1). To initiate transcription, general transcription factors need to be recruited to the promoter either in a stepwise fashion or in a form of holoenzyme (1). The promoter clearance is defined as a point when RNA polymerase II leaves the initiation complex to start elongation of transcripts (2). Phosphorylation of the COOH-terminal domain (CTD)[1] of the largest subunit of RNA polymerase II is required to establish and maintain the elongation complex (3, 4).

Activators have been demonstrated to stimulate one or more steps of the transcription cycle by direct or indirect communication with the general transcription factors (1, 5). In addition, activators may also interact with auxiliary factors, called coregulators, to enhance recruitment of the general transcription machinery on the promoter (6, 7). Direct interactions of activators with coregulators and/or general transcription factors have been suggested to be mechanisms for transcriptional activation (5–7). Nuclear run-on transcription and RNase protection analyses revealed three classes of activation domains (8). Type 1 activators, such as Sp1 and CTF, stimulate an initiation stage of transcription. Type 2A activators, such as Tat encoded by human immunodeficiency virus type 1, stimulate an elongation stage, thus type 2A activators may prevent abortive elongation by arrest of RNA polymerase II at poorly defined sites. Type 2B activators, such as VP16 and p53, stimulate both an initiation and an elongation stage.

The AR is a member of the steroid receptor superfamily that is composed of a variable amino-terminal domain, a highly conserved DNA-binding domain, and a ligand-binding domain (9). Ligand-dependent transcriptional activation of steroid receptors is mediated by the COOH-terminal domain that includes a ligand-binding domain and activation function-2 (10). Crystallographic studies show that ligand-bound steroid receptors undergo a conformational change in the activation function-2 core motif (11, 12). The ligand-induced conformational change presumably recruits coregulators as well as the basal transcriptional machinery for the target gene expression (6, 7). The coregulators of nuclear receptors (ARA24, ARA54, ARA55, ARA70, ARA160, CBP/p300, p/CIP/ACTR/AIB1, Rb, RIP140, SRC-1/NCoA-1, TIF-2/GRIP1, and TRAPs/DRIPs) have recently been cloned and characterized (6, 7, 13–17). It has been proposed that coregulators function as a bridge between activators and the basal transcription machinery (5–7). They may potentiate transactivation of nuclear receptors in transient transfection or in *in vitro* transcription assays through the modification of nucleosomal structure or the efficient recruitment of basal transcription machinery (6, 7). A growing number of coregulators, such as SRC-1, ACTR, and PCAF, of steroid receptors have been reported to possess and/or recruit histone acetyltransferase activity to induce modification of nucleosomal structures leading to activation of transcription (6). In contrast to coactivators, corepressors, such as NCoR-1 and SMRT, bind to the

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[1] The abbreviations used are: CTD, COOH-terminal domain of RNA polymerase II largest subunit; AR, androgen receptor; DHT, dihydrotestosterone; DRB, 5,6-dicloro-1-β-D-ribofuranosylbenzimidazole; NELF, negative elongation factor; PSA, prostate-specific antigen; P-TEFb, positive-transcription elongation factor b; TFIIF and TFIIH, transcription factor IIF and IIH, respectively; FBS, fetal bovine serum; MMTV, murine mammary tumor virus; AR-NDBD, AR amino-terminal and DNA-binding domain.

nuclear receptors in the absence of ligands, recruit histone deacetyltransferase, and lead to condensation of nucleosomal structures for repression of transcription (6, 18).

The amino-terminal domain of steroid receptors contains a ligand-independent activation function-1, which is under the control of activation function-2 (10). The amino-terminal domain of steroid receptors has been reported to interact with general transcription factors, as exemplified by AR interaction with transcription factor IIF (TFIIF) (19) and transcription factor IIH (TFIIH) (20). Transcription factor IIB has been reported to interact with thyroid receptor (21), vitamin D receptor (22), and hepatocyte nuclear factor 4 (23). However, the molecular mechanism by which activation function-1 synergistically activates transcription remains unclear.

We reported previously that the AR interacted with TFIIH under physiological conditions and that overexpression of Cdk-activating kinase, the kinase moiety of TFIIH, enhanced AR-mediated transactivation in prostate cancer cells (20). In an effort to further dissect the mechanisms implicated in AR transactivation, we found that AR interacts with PITALRE, a kinase subunit of positive elongation factor b (P-TEFb) (24), and that cotransfection of the plasmid encoding the mutant PITALRE (mtPITALRE), which is defective in its CTD kinase activity (25), results in preferential inhibition of AR-mediated transactivation. AR transactivation is also preferentially inhibited at the low concentration of DRB, a CTD kinase inhibitor. In addition, a nuclear run-on transcription assay of the PSA gene, an androgen-inducible gene, using LNCaP nuclei showed that the transcription efficiency of the distal region of the gene was enhanced upon androgen induction. These results suggest that AR interacts with TFIIH and P-TEFb and enhances the elongation stage of transcription.

MATERIALS AND METHODS

Plasmids—The complementary DNA fragments for PITALRE and mtPITALRE were generous gifts (25) and subcloned into the eukaryotic expression vector pSG5 (Stratagene). The complementary DNA fragment for negative elongation factor-D (NELF-D) (26) was generated by polymerase chain reaction and subcloned into pSG5.

Cell Culture and Transfection Assay—DU145 and PC-3 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% heat inactivated fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium/F-12 supplemented with 7% FBS, respectively. Non-prostate cancer H1299 and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS. All media contain 50 units/ml penicillin and 50 μg/ml streptomycin. Cells were seeded to be a density of 50–60% confluence for transfection. Cells in 35-mm dishes were refed with fresh medium 2 h before transfection and transfected with 2 μg of DNA according to the "SuperFect transfection" instructions (Qiagen). After 2–3-h incubation, cells were treated with medium supplemented with charcoal-dextran-treated FBS containing either ethanol or ligands. Cells were further incubated at 37 °C for 24 h, washed with PBS, and harvested. Cell lysates were prepared and used for luciferase assay according to the manufacturer's instructions (Promega). Relative luciferase activities were plotted using the activity of AR in the absence of ligand and coactivator as 1. The results were obtained from at least three sets of transfection and presented as mean ± S.D.

Coimmunoprecipitation—LNCaP whole cell extracts were prepared as described previously (27) and aliquots stored at −70 °C. For immunoprecipitation, protein A-Sepharose (Amersham Pharmacia Biotech) resins were incubated with bovine serum albumin (1 mg/ml) overnight, washed with PBS, and coupled to anti-human PITALRE polyclonal antibody (Santa Cruz) as follows. Swollen protein A-Sepharose (100 μl) was incubated with 50 μl of anti-PITALRE antibody (Santa Cruz). The conjugated resins (30 μl bed volume) were incubated with 1 mg of whole cell extract for 4 h at 4 °C, then extensively washed with 20 mM potassium phosphate (pH 8.0) and 100 mM KCl. The resins were incubated with 0.2 M glycine HCl (pH 2.5) to elute proteins. The resins were extensively washed with 20 mM potassium phosphate (pH 8.0) and were incubated with 0.2 M ethanolamine (pH 11.5) to elute proteins. The eluted proteins were combined and analyzed on SDS-polyacrylamide gels. Western blotting was performed using the ECL system (Amersham Pharmacia Biotech).

Biochemical Binding Assay—A recombinant protein, 6 histidine-tagged AR amino-terminal and DNA-binding domain (AR-NDBD), amino acids from 38 to 643, was expressed in *Escherichia coli*. Bacterial cells were lysed in 5 ml of binding buffer (20 mM HEPES (pH 7.5), 500 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol, and 10% glycerol). Recombinant proteins were purified using Ni^{2+} resin (Novagen) affinity chromatography according to the manufacturer's instructions. Purity of the proteins was over 90%, judged by Commassie Blue staining of the gel.

Proteins obtained from 50 ml of culture were incubated with 100 μl bed volume of Ni^{2+} resins. The resins were incubated with ^{35}S-labeled TNT-expressed PITALRE for 4 h and washed extensively with 20 mM HEPES (pH 7.5), 0.5 mM EDTA, 20% glycerol, and 400 mM NaCl. The bound proteins were eluted by 2× SDS loading buffer (100 mM Tris·HCl (pH 6.8), 4% SDS, 20% glycerol, and 200 mM β-mercaptoethanol) separated on 12% SDS-polyacrylamide gels, and analyzed using a Molecular Dynamics PhosphorImager.

Nuclear Run-on Transcription—Nuclear run-on transcription assay was performed as described elsewhere (28). Briefly, LNCaP cells were maintained in RPMI 1640 supplemented with 10% FBS. Cells at ~60–70% confluence were treated with medium supplemented with charcoal-dextran-treated FBS containing either ethanol or 1 nM DHT. Cells were further incubated at 37 °C for 16 h, washed with PBS, and harvested. Cells were resuspended in 10 mM Tris·HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40 and incubated on ice for 10 min. The nuclei pellets were spun down at 500 × *g* and resuspended in 10 mM Tris·HCl (pH 8.0), 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA. Nuclei were frozen and stored in liquid nitrogen in portions of 100 μl corresponding to 2×10^7 nuclei. The nuclei were mixed with 100 μl of 10 mM Tris·HCl (pH 8.0), 5 mM $MgCl_2$, 300 mM KCl, 0.5 mM each ATP, GTP, UTP, and 100 μCi of [α-^{32}P]CTP (800 Ci/mmol) and incubated for 30 min at 30 °C. RNase free DNase I was added, and the incubation was continued for 10 min at 37 °C. Protease K was added to a final concentration of 300 μg/ml in 0.1% SDS, and the reaction mixture was incubated 30 min at 37 °C. The labeled RNA transcripts were isolated by phenol extraction, phenol/chloroform extraction, and ethanol precipitation. Nuclear transcripts were separated from unincorporated nucleotides using Sephadex G-50 columns equilibrated with 10 mM Tris·HCl (pH 7.8), 0.5 mM EDTA, and 0.3% SDS.

The DNA fragments containing the PSA exons 1 and 2 were obtained by polymerase chain reaction of the plasmid containing the genomic PSA. The DNA fragments were gel-purified and digested with *Eco*RI. The distal and proximal PSA DNA fragments (Fig. 6*A*) were gel-purified. The plasmid-containing 7SK gene (29) was digested with *Pst*I. Each DNA fragment was denatured and immobilized onto the nylon membrane using a slot blot (Schleicher & Schuell) as described elsewhere (28). The membrane was prehybridized for 2 h at 60 °C in 6× SSC, 10× Denhardt's reagent, 1% SDS, and 100 μg/ml denatured salmon sperm DNA. Hybridization was carried out at 60 °C for 24 h in 6× SSC, 1.0% SDS, 100 μg/ml denatured salmon sperm DNA, and 1×10^6 cpm of labeled RNA transcripts. The filters were washed twice in 2× SSC for 30 min at 60 °C and then treated in 2× SSC containing RNase A (5 μg/ml) for 20 min at 30 °C to remove unhybridized regions of RNA. The filters were washed twice in 2× SSC for 10 min at 37 °C. Signals were detected using a Molecular Dynamics PhosphorImager.

RESULTS

AR Interaction with TFIIH—Biochemical studies of protein-protein interactions between AR and the general transcription factors indicated that AR may interact with TFIIH and TFIIF under physiological conditions (19, 20). Recruitment of TFIIH completes the assembly of the preinitiation complex on the promoter and results in promoter opening and/or the early elongation/promoter clearance steps (1, 3). The kinase activity in TFIIH has been reported to phosphorylate the CTD of the largest subunit of RNA polymerase II (3) and to stimulate the elongation stage of transcription by several activators (8). TFIIH-mediated CTD phosphorylation could lead to promoter clearance by dissociation of proteins recruited for the initiation steps of the preinitiation complex assembly, resulting in establishing an elongation-competent transcription complex (30). The significance of CTD phospho-

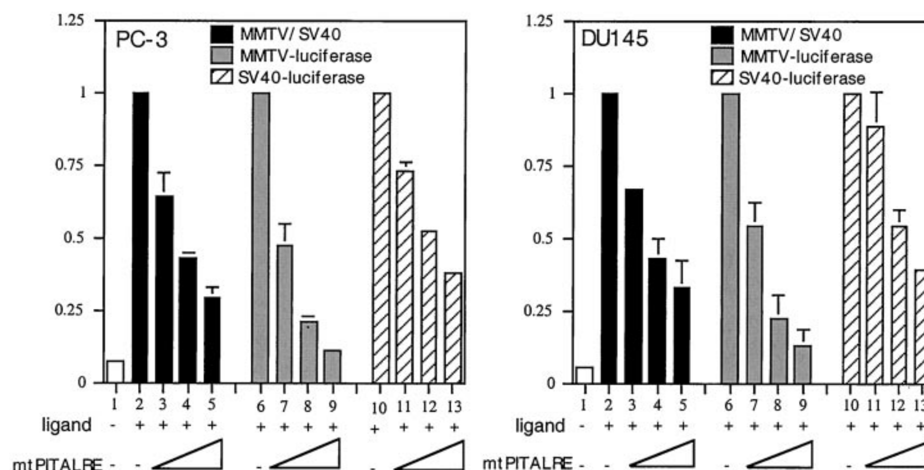

FIG. 1. **Preferential inhibition of AR-mediated transactivation by the mutant PITALRE in prostate cancer PC-3 and DU145 cells.** *A*, AR-negative PC-3 cells were transiently transfected using SuperFect transfection reagent (Qiagen) with 600 ng of MMTV-luciferase reporter plasmid, 10 ng of pRLSV40-luciferase as an internal control, 30 ng of AR expression plasmid, and without or with increasing amounts of the mutant PITALRE plasmid as indicated. The total amounts of plasmids were adjusted to 2 μg with vector plasmid pSG5. The luciferase activities with the MMTV-luciferase reporter plasmid and pRLSV40-luciferase plasmid are shown as *shaded* and *hatched bars*, respectively. The ratios of luciferase activity with pMMTV-luciferase over that with pRLSV40-luciferase are shown as *closed bars*. The result obtained in the absence of 1 nM DHT is shown as an *open bar*. Relative luciferase activities were plotted using the activity without mutant PITALRE as 1. *B*, experiments were performed and analyzed as described above using prostate cancer DU145 cells.

rylation for the elongation stage of transcription was demonstrated by Yankulov *et al.* (31). The elongation stage of transcription in *Xenopus* oocytes was inhibited by microinjection of antibodies against TFIIH subunits, but not by microinjection of antibodies against TFIIB, a general transcription factor specific for transcription initiation. In summary, phosphorylation of the CTD of RNA polymerase II may be associated with the transition from the initiation to the elongation stage of transcription (30). Based on intensive molecular and biochemical studies of transcription mechanisms, interaction of AR with TFIIH reported in our previous study led us to analyze whether AR enhances transcription mainly at the elongation stage of transcription.

The Mutant PITALRE (mtPITALRE) Inhibits AR Transactivation in Prostate Cancer Cells—Given the fact that general elongation factors, such as P-TEFb, transcription factor IIS, and Elongins, also regulate the elongation stage of transcription (4), we analyzed the effects of positive and negative elongation factors on AR-mediated transcription. P-TEFb is composed of 124- and 43-kDa polypeptides and a key regulator controlling RNA polymerase II in the elongation stage of transcription (24). The small subunit of P-TEFb, PITALRE, possesses protein kinase activity capable of phosphorylating the CTD of the largest subunit of RNA polymerase II, which has been known to be a key step required to enter an elongation mode from the preinitiation complex formation on the promoter (4). Recent studies show that the Tat protein encoded by the human type 1 immunodeficiency virus (HIV-1) genome, a notable transcriptional modulator, which activates the elongation stage of transcription, requires P-TEFb kinase activity for the efficient transactivation both *in vivo* and *in vitro* (25, 32).

Since P-TEFb is an abundant general elongation factor, cotransfection of the PITALRE wild type expression plasmid showed little, if any, effect on transcription of the reporter gene (data not shown). Thus, we used mtPITALRE, defective in its kinase activity (25), to analyze the effect of P-TEFb on AR-mediated transcription. It is necessary to compare the inhibitory effect of mtPITALRE on transcription with the promoter containing AR-responsible elements to the effect on transcription with the other promoters. We took advantage of a dual luciferase assay (Promega) using pMMTV-luciferase as a reporter gene and pRLSV40-luciferase as an internal control. Cells were cotransfected with pMMTV-luciferase, pRLSV40-luciferase, the AR expression plasmid, and variable amounts of the mtPITALRE expression plasmid. Overexpression of mtPITALRE inhibited AR-mediated transcription from the MMTV-reporter DNA ~10-fold (*lane* 6 *versus lane 9* in Fig. 1) and SV40 enhancer-mediated transcription from the pRLSV40-luciferase less than 3-fold (*lane 10 versus lane 13* in Fig. 1) in both prostate cancer PC-3 and DU145 cells. These results indicate that mtPITALRE preferentially inhibited AR-mediated transcription over SV40 enhancer-mediated transcription.

However, when we used non-prostate cancer H1299 (Fig. 2) and HeLa cells (data not shown), cotransfection of the mtPITALRE expression plasmid inhibited AR-mediated transcription and SV40 enhancer-mediated transcription to the same degree, resulting in little preferential inhibition of AR-mediated transcription by mtPITALRE. These results suggest that there might be another factor(s), which modulates activity of P-TEFb in prostate cancer PC-3 and DU145 cells and enhances AR-mediated transcription more efficiently. Since our preliminary data indicate that AR activates androgen-responsive genes mainly at the elongation stage of transcription, we propose that the specific factor(s) modulating activity of PITALRE may play a role in prostate cancer progression. To analyze whether AR utilizes a specific set of general elongation factors for regulation of AR transactivation, a cotransfection assay of the expression plasmid encoding NELF-D, a subunit of a recently identified negative elongation factor (26), was performed. Cotransfection of the NELF expression plasmid inhibited both AR-mediated transcription and SV40 enhancer-mediated transcription to about the same degree in PC-3 (Fig. 3) and DU145 (data not shown) cells, resulting in no preferential inhibition of AR transactivation by NELF. Since cotransfection of the NELF expression plasmid did not show preferential inhibition of AR-mediated transcription in prostate cancer cells, we expect that AR does not utilize NELF to regulate AR transactivation and that the specific factor(s) modulating activity of PITALRE may not interact with NELF.

AR Interaction with PITALRE—Results demonstrating preferential inhibition of AR-mediated transcription by mtPI-

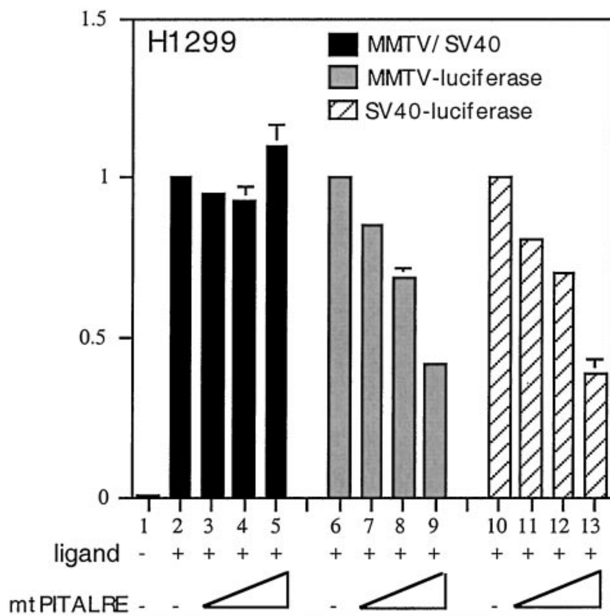

FIG. 2. **No preferential inhibition of AR-mediated transactivation by mutant PITALRE in non-prostate cancer cell line H1299 cells.** Experiments were performed and analyzed as described in the legend to Fig. 1 using non-prostate cancer H1299 cells.

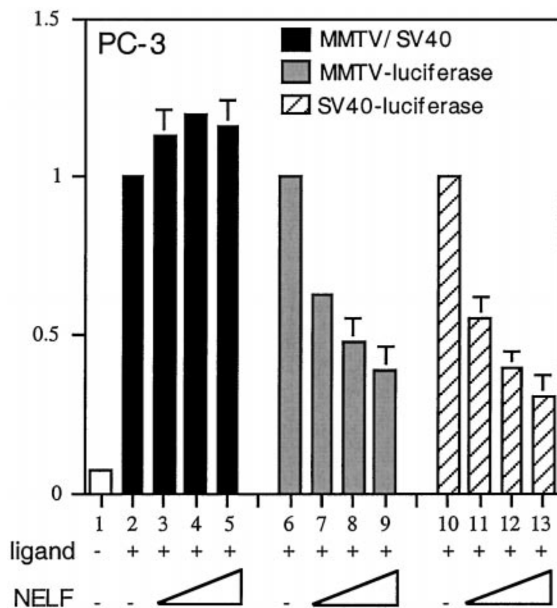

FIG. 3. **No preferential inhibition of AR-mediated transactivation by the negative elongation factor NELF in PC-3 cells.** PC-3 cells were transiently transfected with 600 ng of pMMTV-luciferase, 10 ng of pRLSV40-luciferase, 30 ng of AR expression plasmid, and without or with increasing amounts of NELF expression plasmids as indicated. Total amounts of plasmids were adjusted to 2 μg using pSG5. Experiments were analyzed as described in the legend to Fig. 1.

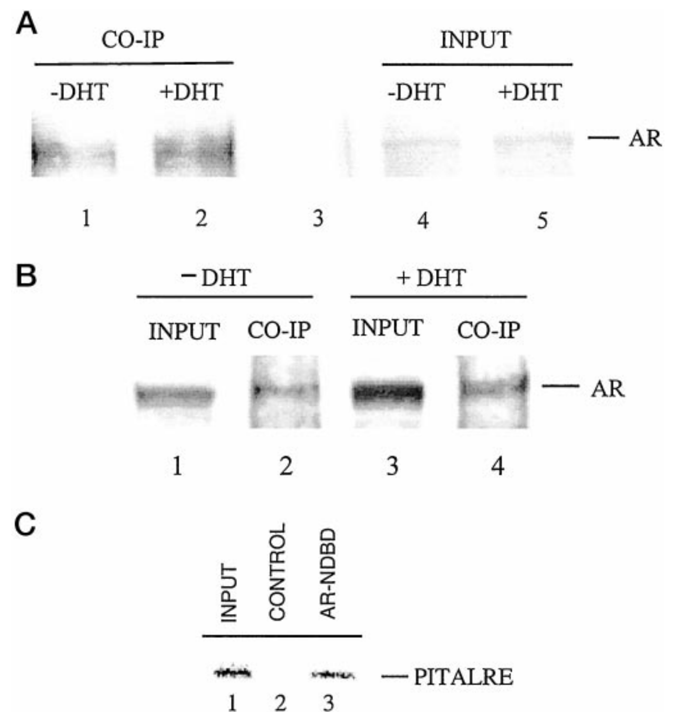

FIG. 4. **AR interaction with PITALRE.** *A*, whole cell extracts of LNCaP (1 mg each) were prepared as described elsewhere (27) and used for coimmunoprecipitation. *Lanes 1* and *4* were obtained with whole cell extract from ethanol-treated cells. *Lanes 2* and *5* were obtained with whole cell extracts from 1 nM DHT-treated cells. About 0.5% of input was loaded in *lanes 4* and *5*. The immunoprecipitated samples with anti-PITALRE antibody-bound protein A-Sepharose were loaded in *lanes 1* and *2*. The immunoprecipitated sample with protein A-Sepharose was loaded in *lane 3* as a control. *B*, PC-3 cells were transiently transfected with the plasmids encoding AR and wild type PITALRE as described in the legend to Fig. 1. Whole cell extracts were prepared as described elsewhere (27) and used for coimmunoprecipitation. *Lanes 1* and *2* were obtained with whole cell extract from ethanol-treated cells. *Lanes 3* and *4* were obtained with whole cell extracts from 1 nM DHT-treated cells. About 7.5% of input was loaded in *lanes 1* and *3* as a control. Immunoprecipitated samples were loaded in *lanes 2* and *4*. *C*, bacterial lysate containing histidine-tagged AR amino-terminal plus DNA-binding domain (AR-NDBD) was incubated with Ni^{2+} resins. The resins were incubated with ^{35}S-labeled TNT-expressed PITALRE and extensively washed with 20 mM HEPES (pH 7.8), 20% glycerol, 0.5 mM EDTA, and 400 mM NaCl. Proteins were eluted and analyzed on SDS-polyacrylamide gels, followed by a PhosphorImager. For the control, bacterial lysate without histidine-tagged AR-NDBD was used in parallel (*lane 2*). About 5% of TNT-expressed samples was loaded on *lane 1*.

TALRE led us to analyze whether PITALRE interacts with AR in prostate cancer cells. The whole cell extract of AR-positive LNCaP prostate cancer cells was prepared and used for a coimmunoprecipitation assay with protein A-Sepharose beads coupled with anti-PITALRE antibody. The immunoprecipitated samples were analyzed by a Western blot assay using anti-hAR antibody (NH27). As shown in Fig. 4*A*, AR was detected in the immunoprecipitated samples obtained using protein A-Sepharose beads coupled with anti-PITALRE antibody, but not in the samples obtained using protein A-Sepharose beads alone, indicating AR interaction with PITALRE in a ligand-independent manner under physiological conditions. This interaction was also analyzed using AR-negative prostate cancer PC-3 cells with cotransfection of plasmids encoding AR and PITALRE (Fig. 4*B*). AR interaction with P-TEFb in a ligand-independent manner was further confirmed by a biochemical binding assay (Fig. 4*C*). Since glutathione *S*-transferase fused with the AR NH_2-terminal plus DNA-binding domain (AR-NDBD) gave low purity due to protein degradation, histidine-tagged AR-NDBD was used. Consistent with the results of coimmunoprecipitation assay, ^{35}S-labeled PITALRE was retained by the resins containing histidine-tagged AR-NDBD, but not by the resins containing *E. coli* proteins prepared by the same method used for the histidine-tagged AR-NDBD.

Effect of DRB, a CTD Kinase Inhibitor, on AR Transactivation—The purine nucleotide analog, DRB, has been known to preferentially reduce the synthesis of promoter-distal transcripts and has a minimal effect on the synthesis of promoter-proximal transcripts both *in vitro* and *in vivo*, thus it is an inhibitor for the elongation stage of transcription by RNA

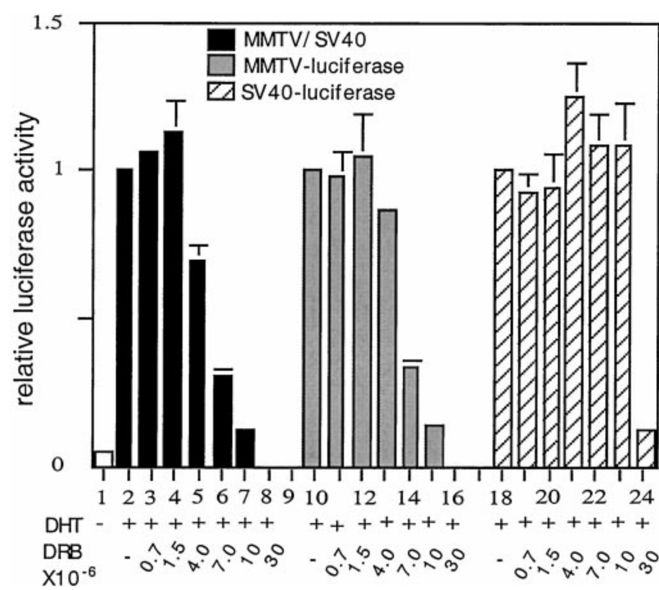

FIG. 5. **Preferential inhibition of AR-mediated transcription by DRB, a CTD kinase inhibitor.** DU145 cells were transiently transfected with pMMTV-luciferase, pRLSV40-luciferase, and pSG5-AR as described in the legend to Fig. 1. The result obtained in the absence of DHT is shown as an *open bar*. Various concentrations of DRB were treated as shown in the figure. Relative luciferase activities were plotted using the activity without DRB treatment as 1.

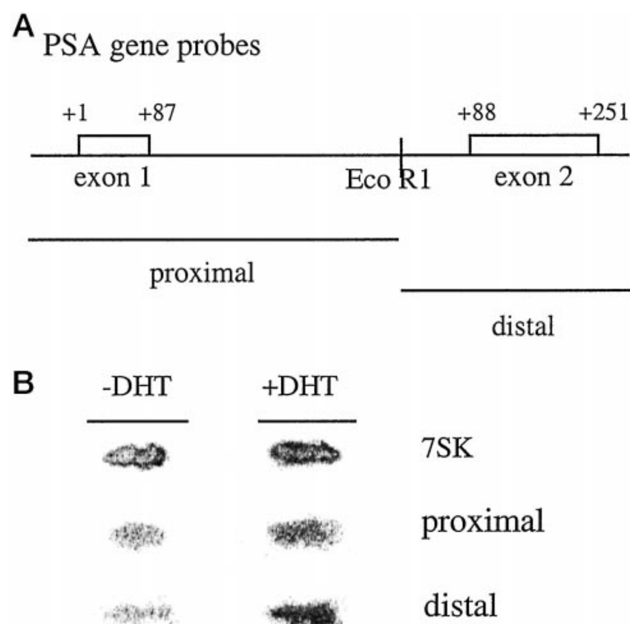

FIG. 6. **Nuclear run-on transcription assay of the PSA gene.** The schematic diagram of proximal and distal probes for PSA RNA transcripts is shown in *A*. The introns and exons are shown as *lines* and *open boxes*, respectively. The numbers for the nucleotide positions of the PSA transcript are shown. The results of the nuclear run-on transcription assay are shown in *B*. LNCaP nuclei were prepared from cells incubated in the presence of 1 nM DHT or EtOH. Radioactively labeled RNA transcripts were prepared and hybridized with the denatured 7SK, proximal, and distal probes. The signals were analyzed using a Molecular Dynamics PhosphorImager.

polymerase II (33, 34). The mechanism of DRB inhibition at the transcriptional elongation stage was well characterized by the finding that DRB is an inhibitor for CTD kinases (35). P-TEFb has been reported to possess a DRB-sensitive CTD kinase activity. A cotransfection assay was performed in the presence of various concentrations of DRB to analyze the significance of AR modulation during transcriptional elongation. As shown in Fig. 5, transcription from both the reporter pMMTV-luciferase and the control pRL-luciferase was slightly enhanced at the concentration of DRB lower than 10^{-6} M, presumably due to the inhibition of nonspecific random initiation of RNA polymerase II resulting in an increase in specific initiation. However, AR-mediated transcription was markedly inhibited from 4×10^{-6} M DRB, while SV40 enhancer-mediated transcription was not inhibited. These results indicate that efficient AR-mediated transcription is highly dependent on the CTD phosphorylation of RNA polymerase II, which is a key step required to enter the elongation stage (3). Together with cotransfection results obtained with the mtPITALRE, preferential inhibition of AR-mediated transcription by DRB indicates that AR may enhance androgen responsible genes mainly at the elongation stage of transcription by communicating with P-TEFb and TFIIH.

Nuclear Run-on Transcription Assay of PSA Gene—Since the specific sequences of certain genes play a role in pausing or premature termination of RNA polymerase II (36), a nuclear run-on transcription assay of PSA gene, an androgen-inducible gene, was performed to exclude a possibility that preferential inhibition of AR-mediated transcription, either by mtPITALRE or DRB in a reporter gene assay, was due to the structure of the reporter gene. Radioactively labeled RNAs were prepared using LNCaP nuclei and hybridized with the single-stranded 7SK gene probe as a control, the proximal probe of PSA gene containing an exon 1 and the distal probe containing an exon 2 (Fig. 6*A*). Although eukaryotic RNA polymerase II pausing or arrest signals are poorly characterized, RNA polymerase II pausing or arrest are frequently caused within a few hundred nucleotides from the initiation. The exons 1 and 2 of the PSA gene are separated by 1200 nucleotides, thus the exon 1 and 2 were chosen as a proximal and distal probe, respectively. As shown in Fig. 6*B*, the ratio of the signal detected by the distal probe to that detected by the proximal probe increased ~2–3-fold upon androgen induction. This result clearly indicates that preferential inhibition of AR-mediated transcription was due to *bona fide* characteristics of AR-mediated transcription.

DISCUSSION

AR is required to communicate with the general transcription machinery on the core promoter to exert its function as a transcriptional modulator. AR interaction with TFIIH and P-TEFb reported in our previous (20) and current studies indicates that AR may utilize TFIIH and P-TEFb to regulate the general transcription machinery on the core promoter. The functional significance of protein-protein interaction between AR and P-TEFb was analyzed using a transient transfection assay. Cotransfection of the expression plasmid encoding mtPITALRE, defective in its kinase activity (25), showed preferential inhibition of AR-mediated transcription in prostate cancer PC-3 and DU145 cells, when we compared the effect of mtPITALRE on AR-mediated transcription with that on SV40 enhancer-mediated transcription. SV40 enhancer-mediated transcription is a good control for analyzing the effect of mtPITALRE on AR-mediated transcription, because SV40 enhancer-mediated transcription is stimulated by several activators, such as AP-2, PU.1, Sp-1, and TEF-1 (37). However, preferential inhibition of AR-mediated transcription by mtPITALRE was not observed when we used non-prostate cancer cells. These results suggest that specific factor(s) may interact with P-TEFb in prostate cancer cells and that cotransfection of the plasmid encoding mtPITALRE may squelch out the specific factor(s) that may regulate AR-mediated transcription by modulating the activity of P-TEFb. In addition, this preferential

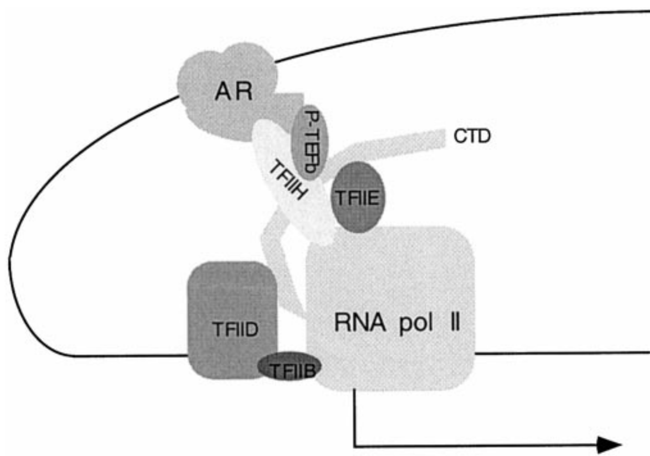

FIG. 7. **A working model for AR-mediated transcription.** Nuclear-specific coregulators and general coregulators are omitted in the figure to simplify the relative positions of TFIIH and P-TEFb with AR.

inhibition of AR-mediated transcription was not observed with cotransfection of the expression plasmid encoding NELF, a recently identified negative elongation factor (26). These results indicate that AR utilizes a specific set of elongation factors for efficient AR-mediated transcription. The working model for AR-mediated transcription based on our results is shown in Fig. 7.

Both TFIIH and P-TEFb possess subunits that can phosphorylate the CTD domain of RNA polymerase II (3, 4). However, TFIIH and P-TEFb function at different stages of transcription. TFIIH is required for promoter clearance, which is defined as a point when RNA polymerase II leaves the initiation complex to start formation of transcripts (30), while P-TEFb is required to prevent arrest of RNA polymerase II within a few hundred nucleotides of the promoter (4). P-TEFb has been reported to be required for the efficient transcription of many, but not all, genes, which explains inhibition of both SV40 enhancer-mediated transcription and AR-mediated transcription. However, AR-mediated transcription appears to suffer more severely from the frequent arrest of RNA polymerase II than SV40 enhancer-mediated transcription. This difference may reflect the possibility that the preinitiation complexes on the pRLSV40 promoter for SV40 enhancer-mediated transcription differ from those on the pMMTV promoter for AR-mediated transcription. Development of a well defined transcription system may be necessary to characterize the mechanisms by which AR enhances transcription.

Since AR interacts with both TFIIH and P-TEFb, it is plausible to speculate that AR activates transcription mainly at the elongation stage. Given the fact that PITALRE is a DRB-sensitive CTD kinase (4), the effect of DRB on AR-mediated transcription was analyzed to demonstrate that CTD phosphorylation is a rate-limiting step for efficient AR transactivation. DRB dramatically increases the frequency of RNA polymerase II arrest within a few hundred nucleotides from the transcription initiation site by inhibiting phosphorylation of the CTD (32, 33). SV40 enhancer-mediated transcription was not inhibited in the presence of 10^{-6} M DRB, while AR-mediated transcription was severely inhibited. Transcriptional activators, such as AP-2, PU.1, Sp1, and TEF-1, modulate SV40 enhancer-mediated transcription (37), thus phosphorylation of the CTD by P-TEFb may not be a rate-limiting step for efficient transcription by these activators. This result is consistent with the observation that most transcriptional activators enhance the rate of transcriptional initiation (8). SV40 enhancer-mediated transcription was inhibited only at the high concentration (5×10^{-5} M) of DRB (data not shown). This result indicates that AR-mediated transcription requires efficient CTD phosphorylation. Consistent with the results obtained by a reporter gene assay using mtPITALRE and DRB, a nuclear run-on transcription assay of the PSA gene, an androgen-inducible gene, using LNCaP nuclei indicated that transcription efficiency of the distal region of the PSA gene was enhanced upon androgen induction (Fig. 6). This result clearly suggests that preferential inhibition of AR-mediated transcription by mtPITALRE or DRB was not due to the artifact of a reporter gene assay (*e.g.* RNA polymerase II pausing or arrest signal in a reporter gene). The preinitiation transcription complex activated by AR may require a high level of CTD phosphorylation for efficient transcription. All together, AR may increase the processivity of RNA polymerase II upon androgen induction. A reporter gene assay with a reporter gene containing only the multicopy of AR-responsible elements did not give detectable induction by androgens (data not shown). This phenomenon may result from the possibility that AR enhances transcriptional elongation. AR may need other activators to enhance transcriptional initiation. A recent study of androgen regulation of the p21 gene indicated that binding sites for AR and Sp1 on the p21 promoter showed synergistic activation (38). Given the fact that Sp1 enhances the rate of transcriptional initiation (8), this study suggests a cooperation between an activator for elongation and an activator for initiation for efficient transcription of p21 gene. Characterization of mechanisms implicated in AR transactivation may facilitate identification of additional coregulators required for efficient AR transactivation as well as development of potential therapeutic drugs for effective prevention of prostate cancer.

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