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# Activation of the DNA-dependent Protein Kinase Stimulates Nuclear Export of the Androgen Receptor *in Vitro*[*]

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**The androgen receptor undergoes nuclear import in response to ligand, but the mechanism by which it undergoes nuclear export is poorly understood. We developed a permeabilized cell assay to characterize nuclear export of the androgen receptor in LNCaP prostate cancer cells. We found that nuclear export of endogenous androgen receptor can be stimulated by short double-stranded DNA oligonucleotides. This androgen receptor export pathway is dependent on ATP hydrolysis and is enhanced by phosphatase inhibition with okadaic acid. Fluorescence recovery after photobleaching in permeabilized cells, under the conditions that stimulate androgen receptor export, suggested that double-stranded DNA-dependent export does not simply reflect the relief of a nuclear retention mechanism. A radiolabeled androgen was used to show that the androgen receptor remains ligand-bound during translocation through the nuclear pore complex. A specific inhibitor to the DNA-dependent protein kinase, NU7026, inhibits androgen receptor export and phosphorylation. In living cells, NU7026 treatment increases androgen-dependent transcription from endogenous genes that are regulated by androgen receptor. We suggest that DNA-dependent protein kinase phosphorylation of the androgen receptor, or an interacting component, helps target the androgen receptor for export from the nucleus.**

As a member of the nuclear receptor (NR)[2] superfamily of ligand-responsive transcription factors (1), the androgen receptor (AR) is composed of an N-terminal transcriptional activation domain, a central DNA binding domain (DBD), a hinge region, and a C-terminal ligand binding domain. AR is important for proper development of male secondary sexual characteristics and for normal prostate function (2). AR is also thought to be critical for the tumorigenesis of prostate cancer (PCa) and for the progression of PCa from an androgen-sensitive to an androgen-insensitive state (3–8). Thus, in PCa xenografts in mice, elevation of AR mRNA levels has been correlated with PCa progression to hormone-insensitive status (9).

AR transcriptional regulatory functions are dependent on the proper subcellular localization of the receptor. AR is thought to associate with a heat shock protein 90 (hsp90)-based chaperone complex in the cytoplasm (10) until the binding of cognate ligand induces a conformational change in AR, chaperone dissociation, and subsequent AR nuclear import (discussed in Refs. 11, 12). Once in the nucleus, AR can bind specific androgen-response elements (AREs) to enhance or repress transcription of associated androgen-responsive genes. The type of ligand bound to AR is critical for its regulatory activity, as agonist and antagonist are known to differentially affect AR subnuclear localization (11–14) and intranuclear mobility (12, 15). AR can also undergo export from the nucleus (11). The DBD of AR is sufficient to direct nuclear export of a reporter protein, and point mutations in the DBD of full-length AR reduce nuclear export without affecting import (16). The PCa xenograft LAPC-4 has been used to show that under low androgen conditions the steady state localization of AR is different in androgen-dependent and androgen-independent forms of the tumor (17). Thus, defining the mechanisms responsible for regulating AR protein levels and subcellular localization could provide insight into the molecular events that underlie PCa progression.

Multiple signal transduction pathways operate upstream of the AR and can modulate its transactivation function (18) A member of the phosphatidylinositol 3-kinase family, DNA-dependent protein kinase (DNA-PK), has recently been implicated in AR transcriptional regulation in human prostate cancer (LNCaP) cells (19). Comprised of a large (~470 kDa) catalytic and two Ku regulatory subunits, DNA-PK is involved in the nonhomologous end-joining response to DNA damage, V(D)J recombination, and retroviral DNA integration (20, 21). DNA-PK has been reported to associate with members of the NR superfamily (22–24) and can phosphorylate the glucocorticoid receptor (GR) *in vitro* (25, 26).

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[2] The abbreviations used are: NR, nuclear receptor; AF-1, activation function 1; AMP-PNP, adenylylimidodiphosphate; AR, androgen receptor; ATM, ataxia telangiectasia, mutated (kinase); ATR, ATM- and Rad3-related (kinase); DBD, DNA binding domain; DNA-PK, DNA-dependent protein kinase; ds, double-stranded; ERS, energy regenerating system; FRAP, fluorescence recovery after photobleaching; FKBP51, FK506-binding protein 51; GFP, green fluorescent protein; GMP-PNP, guanylylimidodiphosphate; GR, glucocorticoid receptor; hsp90, heat shock protein 90; NES, nuclear export sequence; NFAT, nuclear factor of activated T cells; NPC, nuclear pore complex; OA, okadaic acid; PCa, prostate cancer; PDF, prostate-derived factor; PR, progesterone receptor; PSA, prostate-specific antigen; RCC1, regulator of chromatin condensation 1; DTT, dithiothreitol; PBS, phosphate-buffered saline; ARE, androgen-response element; JNK, c-Jun N-terminal kinase; RT, reverse transcription.

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Furthermore, the Ku subunits of DNA-PK can be localized to the promoter of the endogenous AR-responsive *PSA* gene by chromatin immunoprecipitation, and they act as AR transcriptional coactivators in reporter assays (19).

In this study we set out to analyze the mechanism of AR nuclear export by developing an *in vitro* assay based on digitonin-permeabilized LNCaP cells. Using this assay, we found that short double-stranded DNA (dsDNA) oligonucleotides stimulated robust AR export in an ATP-dependent reaction. Okadaic acid (OA) modestly enhanced the reaction and promoted the phosphorylation of exported AR. Significantly, both AR export and phosphorylation were prevented by chemical inhibitors of the phosphatidylinositol 3-kinase kinase family, including an inhibitor that is specific for DNA-PK. We also found that an export factor, exportin 5, can stimulate AR export in permeabilized cells independently of DNA-PK. These findings together with our previous work showing that mitogen-activated protein kinases regulate AR export (27) lead us to conclude that AR uses multiple pathways to exit the nucleus. We propose that kinases, including p38, JNK, and DNA-PK, can regulate AR export, possibly by providing AR with a "license" to exit the nucleus.

## EXPERIMENTAL PROCEDURES

*Cell Culture and Transfection*—Human prostatic carcinoma LNCaP cells (28) were cultured in T medium (Invitrogen) containing phenol red and 5% fetal bovine serum. The GFP-AR plasmid used for fluorescence recovery after photobleaching (FRAP) analysis and single-cell export assays encodes an N-terminally GFP-tagged form of human AR in a pEGFP-C1 vector backbone (Clontech). LNCaP cells were transfected using transfectin (Bio-Rad).

*Recombinant Protein Preparation Exportin 5*—A pQE60 plasmid (Qiagen) encoding C-terminally His-tagged human exportin 5 (Addgene plasmid 12553) was transformed into the TG1 bacterial strain. Following growth to log phase in LB medium with 2% ethanol, cultures were induced using 300 $\mu$M isopropyl 1-thio-$\beta$-D-galactopyranoside overnight at 25 °C. Cultures were centrifuged and resuspended in 20 mM HEPES, pH 8, 500 mM NaCl, 10% glycerol, and 14 mM $\beta$-mercaptoethanol. Resuspension and wash buffers contained 1 mM phenylmethylsulfonyl fluoride and 5 $\mu$g/ml aprotinin, leupeptin, and pepstatin. Following three rounds of lysis using a French press, lysates were clarified and incubated with Talon beads (Clontech) at 4 °C for ≥2 h. Talon resin was washed with 20 mM HEPES, pH 8, 500 mM NaCl, 10% glycerol, 14 mM $\beta$-mercaptoethanol, and 10 mM imidazole, and then with 20 mM HEPES, pH 8, 200 mM NaCl, 10% glycerol, 7 mM $\beta$-mercaptoethanol, and 10 mM imidazole. Recombinant exportin 5 was eluted using 20 mM HEPES, pH 8, 200 mM NaCl, 10% glycerol, 14 mM $\beta$-mercaptoethanol, and 250 mM imidazole before dialysis into 1× transport buffer (20 mM HEPES, pH 7.4, 110 mM potassium acetate, 2 mM magnesium acetate, 0.5 mM EGTA) supplemented with 10% glycerol, 2 mM DTT, and 1 $\mu$g/ml aprotinin, leupeptin, and pepstatin. Exportin 5 was snap-frozen in single-use aliquots.

*GST-DBD-Hinge*—A pGEX4T3 vector (GE Healthcare) encoding a fragment of AR that includes the DBD and hinge domains (residues 549–671) was transformed into BL21 cells. Following growth to log phase, protein expression was induced with 1 mM isopropyl 1-thio-$\beta$-D-galactopyranoside in the presence of 2% ethanol for 3 h at room temperature. Pelleted cells were resuspended in PBS supplemented with protease inhibitors. Following French press-mediated lysis, lysates were clarified and incubated with glutathione beads for 1.5 h at 4 °C. The bound fraction was washed with PBS containing 1% Triton X-100, then washed with PBS containing 500 mM NaCl, and then washed with PBS alone. GST-AR-DBD-Hinge protein was eluted with 100 mM Tris, pH 8, 10 mM glutathione, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, aprotinin, leupeptin, and pepstatin. Dialysis into 1× transport buffer (20 mM HEPES, pH 7.4, 110 mM potassium acetate, 2 mM magnesium acetate, 0.5 mM EGTA) containing 1 mM DTT preceded snap freezing in single-use aliquots.

*In Vitro AR Export Assay*—The assay was based on selective permeabilization of the plasma membrane of cultured cells with digitonin, an approach used to study protein import and export mechanisms (29). LNCaP cells expressing endogenous AR were supplied with 1–10 nM synthetic androgen (R1881; PerkinElmer Life Sciences) for 1–3 h prior to a wash with phosphate-buffered saline (Invitrogen). Cells were subjected to a brief trypsinization (0.05% trypsin/EDTA (Invitrogen)) that was quenched with serum-containing medium, then subjected to gentle centrifugation (~200 × *g*) before resuspension in ice-cold 1× transport buffer. Cells were permeabilized on ice for 6–8 min with 50 $\mu$g/ml high purity digitonin (Calbiochem) in ice-cold complete transport buffer (CTB: 1× TB containing 2 mM DTT, 1 $\mu$g/ml aprotinin, and 0.5 $\mu$g/ml leupeptin and pepstatin). Cells were then washed with ice-cold CTB and aliquoted into standard polystyrene tubes commonly used for DNA transfection (Falcon 352058). Ice-cold export reaction mixes were added to aliquots of permeabilized cells on ice. Final reaction volume was ~125 $\mu$l (100-$\mu$l reaction mix and 25-$\mu$l cell suspension); final permeabilized cell concentration in each reaction was ~$5 \times 10^6$ cells/ml. The tubes containing the permeabilized cells and reaction mixes were incubated at 30 °C for 20–30 min. Following the incubation, reaction components were centrifuged (~18,000 × *g* for 12 min) to isolate the reaction supernatant (containing the exported AR fraction) from the cell pellet (containing the nonexported, nuclear AR). Equal volumes of reaction supernatants were then separated on 7.5% acrylamide SDS-polyacrylamide gels. Following SDS-PAGE separation, proteins were transferred onto PVDF membranes (Millipore) and immunoblotted using the polyclonal $\alpha$-AR antibody PG-21 (directed against the N-terminal 21 amino acid residues of AR (Upstate)), providing a semi-quantitative measure of the amount of AR exported during the assay. Comparison of the exported fractions with cell pellets (nuclei) in multiple experiments established that equivalent numbers of permeabilized cells were dispensed and recovered under different conditions and that on average ~40% of LNCaP AR was exported in a 30-min reaction (data not shown).

*In vitro* export assays conducted in the presence of CTB alone served as negative control for AR export. Our energy-regenerating system (ERS) consisted of bovine serum albumin (5 mg/ml final in export reaction), creatine phosphokinase (80 units/ml final), creatine phosphate (1.6 mg/ml final), ATP

(1 mM final), GTP (1 mM final), and aprotinin. Nonhydrolyzable nucleotide analogs AMP-PNP and GMP-PNP were also used at 1 mM final concentration. Okadaic acid (Calbiochem) was used at a final concentration of 200 nM. Casodex (10 $\mu$M; Fisher), wortmannin (20 $\mu$M (MP Biomedicals)), LY294002 (50 $\mu$M (Calbiochem)), and NU7026 (2.5 $\mu$M (Sigma)) were added to LNCaP cells 1–2 h prior to the start of the *in vitro* export assay. Chemical inhibitors were routinely maintained in pre-export wash buffers and in export reactions. For export assays in the absence of agonist, cells were washed with phenol red-free RPMI (Invitrogen) containing 5% charcoal-stripped serum, and then incubated in this medium for 6–24 h prior to permeabilization and *in vitro* export assays.

For phosphatase treatment of exported AR, export reactions were first carried out in the presence of ERS, OA, and ARE (1 $\mu$M). Following centrifugation, the export supernatants (36 $\mu$l) were treated with Antarctic phosphatase (10 units; PerkinElmer Life Sciences) for 2 h at 37 °C in the presence of the manufacturer's supplied buffer (final 1× concentration). Reaction components were subjected to SDS-PAGE, and AR was immunoblotted with the PG-21 antibody.

For *in vitro* export assays using [$^3$H]R1881, LNCaP cells were incubated in the presence of 2 nM [$^3$H]R1881 (PerkinElmer Life Sciences) for 1–3 h prior to washing and cell permeabilization. *In vitro* export reactions ensued at 30 °C for 30 min. Reaction supernatants containing the exported AR fraction were then collected, applied to Whatman paper, dried, and measured by scintillation counting. For immunoprecipitations, reaction supernatants were incubated with control resin or AR-21 anti-AR resin in the presence of 0.1% Triton X-100 for 4 h at 4 °C. Resins were washed five times in complete transport buffer containing 0.1% Triton X-100 prior to release of bound [$^3$H]R1881 with 2% SDS at 90 °C for 10 min. Released fractions were applied to Whatman paper, dried, and measured by scintillation counting.

For *in vitro* export assays followed by detergent extraction, LNCaP cells were subjected to standard export reactions prior to centrifugation and harvest of reaction supernatants containing the exported AR fraction. Cell pellets containing residual, nonexported AR were then subjected to extraction with 0.5% Triton X-100 in complete transport buffer supplemented with bovine serum albumin (5 mg/ml final), glycerol (0.5% final), and aprotinin, for 20 min on ice. Subsequent centrifugation yielded supernatant (detergent-sensitive) and pellet (detergent-resistant) fractions. In Fig. 6*C* (*Triton in the Export Rxn* samples), 0.5% Triton X-100 was included during standard export reactions to disrupt the nuclear envelope and yielded a single released AR fraction.

Recombinant exportin 5 was used at a final concentration of 0.1 and 0.2 mg/ml. Dialysis buffer obtained from the preparation of recombinant exportin 5 served as negative control in the export reactions.

For *in vitro* AR export in adherent cells, LNCaP cells were plated on glass coverslips (Fisher) coated with poly-D-lysine (Sigma) and grown in T medium containing phenol red and supplemented with 5% fetal bovine serum. Following treatment with 10 nM R1881, cells were washed three times (4 min/wash) with ice-cold TB, permeabilized with 50 $\mu$g/ml digitonin in ice-cold CTB for 6–8 min, and then washed again three times with ice-cold CTB. Export reaction mixes were supplied to the cells before incubation at 30 °C for 20–30 min. Cells were washed two more times with ice-cold CTB prior to fixation with 3.7% formaldehyde.

In antibody access experiments to test the integrity of the nuclear envelope during *in vitro* export assays, an antibody to the nuclear protein RCC1 (Santa Cruz Biotechnology) was included in the export reaction mix (7 $\mu$g/ml final). As a positive control for envelope disruption, 0.2% Triton X-100 was also included in the export reaction. Following the export assay, washes, and fixation, cells were then permeabilized with 0.2% Triton X-100 and subjected to standard immunofluorescence processing using an anti-AR primary antibody (AR-21) and secondary antibodies to both the anti-RCC1 and anti-AR primary antibodies. Cells were mounted and viewed using a Nikon Eclipse E800 upright microscope.

*DNA Oligonucleotides*—Oligonucleotides used to stimulate AR export *in vitro* are from Sigma Genosys. The ARE duplex (duplex *a*, see Fig. 7*A*) contains both half-sites of the ARE in the C(3)1 intron of the prostatic binding protein gene (30). The mutant ARE duplex (duplex *b*) contains four point mutations within the 12-residue positions comprising the tandem of ARE half-sites (30). Oligonucleotides *c* and *d* are the sense and antisense strand components, respectively, of the wild-type ARE duplex (duplex *a*). Duplexes *f*–*h* are derivatives of the wild-type ARE duplex. In duplex *i*, the two ARE half-sites are separated by a noncomplementary bubble, 12 residues in length on each duplex strand. In oligonucleotide *j*, the upstream ARE half-site is adjoined to a 13-nucleotide noncomplementary loop (see Fig. 7*A* for schematic representations of these oligonucleotides). The NRE duplex is a derivative of the ARE duplex and harbors an NRE1 sequence (26) instead of an ARE. The ARE-NRE duplex contains an NRE1 sequence and an ARE in *cis*. NS1, NS2, and NS3 are intended as nonspecific duplexes that harbor neither an ARE nor an NRE1 sequence.

*FRAP Analysis and AR Export in Single Cells*—LNCaP cells were grown on Delta T dishes (Fisher) coated with poly-D-lysine. Following expression of GFP-AR, cells were treated with R1881 and subjected to standard permeabilization and washes with complete transport buffer on ice. Cells were then placed on a Bioptechs stage warmer, and GFP-AR-expressing cells were identified using the wide field mode of a Zeiss LSM 510 Meta confocal laser scanning microscope, and export reaction mixes were added to cells. For single-cell export assays, nuclear GFP fluorescence intensity was recorded immediately prior to and immediately following a 30-min export reaction at 37 °C. The ratio of these two values (plotted in Fig. 4*D*) provides a measure of nuclear GFP fluorescence lost, and therefore of GFP-AR nuclear export, during the reaction. For FRAP measurements, recovery of GFP fluorescence into the photobleached area was monitored in increments of 2.5 min. Recovery curves represent the ratio of the fluorescence intensity within the photobleached rectangular area to the nuclear fluorescence intensity outside this photobleached area plotted as a function of time. Following addition of an export reaction mix, up to six recovery curves per dish were generated sequentially, each in a separate cell. All recovery curves for a given export

reaction condition were averaged, and a representative recovery curve is plotted for each condition in Fig. 4*B*. The $t_{1/2}$ value (Table 1) indicates the time at which the half-maximal amount of fluorescence recovery into the photobleached area has occurred, under a given reaction condition.

*DNA Oligonucleotides*—Oligonucleotide sequences are as follows: duplex *a*, sense, GGGAGCTTACATAGTACGTGATGTTCTCAAGGTCGA, and antisense, TCTCGACCTTGAGAACATCACGTACTATGTAAGCT; duplex *b* (residues mutated relative to duplex *a* are in boldface), sense, GGGAGCTTACAT**T**GT**T**C**T**TGA**G**GTTCTCAAGGTCGA, and antisense, TCTCGACCTTGAGAAC**C**TCA**A**G**A**AC**A**ATGTAAGCT; oligonucleotide *c* (identical to the sense strand of duplex *a*), GGGAGCTTACATAGTACGTGATGTTCTCAAGGTCGA; oligonucleotide *d* (identical to the antisense strand of duplex *a*), TCTCGACCTTGAGAACATCACGTACTATGTAAGCT; duplex *e*, sense, AGCTTACATAGTACGTGATGTTCTCAAGGTCGACT, and antisense, TCGACCTTGAGAACATCACGTACTATGTAAGCTGGG; duplex *f*, sense, GGGAGCTTACATAGTACGTGATGTTCTCAAGGTCGACT, and antisense, TCTCGACCTTGAGAACATCACGTACTATGTAAGCTGGG; duplex *g*, sense, GGGAGCTTACATAGTACGTGATGTTCTCAAGGTCGAGA, and antisense, TCTCGACCTTGAGAACATCACGTACTATGTAAGCTCCC; duplex *h*, sense, CATAGTACGTGACCCCCCCCCCCCCTGATGTTCTCAA, and antisense, TTGAGAACATCACCCCCCCCCCCCTCACGTACTATG; oligonucleotide *i*, CATAGTACGTGACCCCCCCCCCCCCTCACGTACTATG; and duplex *j*, sense. CGCGC($I_{26}$)CGCGC, and antisense, GCGCG($C_{26}$)GCGCG; NRE, sense, GGGAGCTAACTGAGAAAGAGAAAGACGACAGGTCGA, and antisense, TCTCGACCTGTCGTCTTTCTCTTTCTCAGTTAGCT; ARE-NRE, sense, AACTGAGAAAGAGAAAGACGAGCTTACATAGTACGTGATGTTCTCAAGGT, and antisense, ACCTTGAGAACATCACGTACTATGTAAGCTCGTCTTTCTCTTTCTCAGTT; NS1, sense, TCGAGATGGATTATAAAGCATTTGATAATCTTTAAT, and antisense, CTAGATTAAAGATTATCAAATGCTTTATAATCCATC; NS2, sense, GTCCATCTTGTCGTCTTGAGAAATGTTATGAAGCAGGG, and antisense, CCCTGCTTCATAACATTTCTCAAGACGACAAGATGGAC; and NS3, sense, AGAAAAAATCCCACATCCTGCTCAGAGCGCTTCTACCAGCTCACC, and antisense, GGTGAGCTGGTAGAAGCGCTCTGAGCAGGATGTGGGATTTTTTCT. The fluorescent ARE duplex used in Fig. 1*C* contains a fluorescein moiety on the 5′ end of the antisense strand of the wild-type ARE duplex (duplex *a*).

*Real Time RT PCR*—Real time RT PCR analysis was done on an iCycler optical system (Bio-Rad) using the IQ SYBR Green PCR master mix. LNCaP cells were pretreated with NU7026 for 16 h and then the cells were untreated or treated with 1 nM R1881 for 24 h. Total RNA was extracted from cells using the RNeasy kit (Qiagen). DNase I treatment was performed directly on the RNeasy mini column with 27 Kunitz units of DNase I (Qiagen) for 15 min at room temperature according to the Qiagen protocol. RNA was quantified using Ribogreen (Molecular Probes). 500 ng of RNA was reverse-transcribed in a 20-$\mu$l reaction volume using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol. The human-specific PCR primers used were as follows: forward PSA, 5′-TGGTGCATTACCGGAAAGTGGATCA-3′, and reverse PSA 5′-GCTTGAGTCTTGGCCTGGTCATTTC-3′; forward S100P, 5′-ATGACGGAACTAGAGACAGCC-3′, and reverse S100P 5′-AGGAAGCCTGGTAGCTCCTT-3′; forward FKBP51, 5′-CATCAAGGCATGGGACATTGG-3′, and reverse FKBP51 5′-TCGAGGGAATTTTAGGGAGACT-3′; forward PDF, 5′-GGGCAAGAATCTCAGGACGG-3′, and reverse PDF 5′-TCTGGAGTCTTCGGAGTGCAA-3′; and forward $\beta$-glucuronidase, 5′-CCGACTTCTCTGACAACCGACG-3′, and reverse $\beta$-glucuronidase, 5′-AGCCGACAAAATGCCGCAGACG-3′. Nonreversed transcribed RNA was subject to PCR as a control; no DNA contamination was observed. Primer annealing temperatures were 66 °C (PSA), 58 °C (S100P), 60 °C (FKBP51), 58 °C (PDF), or 68 °C ($\beta$-glucuronidase). Mean starting quantity of each androgen-induced gene is normalized to mean starting quantity $\beta$-glucuronidase; this ratio (with standard error for the ratio) is shown in Fig. 9, *A–D,* for a representative experiment in which each sample is tested in duplicate.

## RESULTS

*AR Export in Permeabilized LNCaP Cells*—We developed a permeabilized cell assay to investigate the mechanism(s) of AR export. In this assay, cultured LNCaP cells were pretreated with the synthetic androgen R1881 to induce nuclear import of endogenous AR. Cells were harvested, washed, and treated with digitonin to selectively permeabilize the plasma membrane. Permeabilized cells were then supplemented with reaction mixes to stimulate nuclear export of AR into the reaction supernatant, which was examined by immunoblotting with affinity-purified anti-AR antibody. In the course of optimizing reaction conditions for the assay, we tested whether a dsDNA oligonucleotide containing an ARE (Fig. 1*A*) could improve cytosol-dependent nuclear export by relieving presumed nuclear retention of AR. This approach had been used by Kehlenbach *et al.* (31) to improve cytosol-dependent NFAT export in permeabilized cells. Unexpectedly, we found that ARE together with an energy-regenerating system (ERS) stimulated robust AR export without the addition of cytosolic factors (Fig. 1*B*). Fluorescently labeled ARE entered the nuclei of digitonin-permeabilized LNCaP cells during the export assay, which is consistent with ARE stimulating AR export from within the nuclei (Fig. 1*C*). We also verified that the nuclear envelope remains intact under conditions that promote AR export. An antibody to the nuclear protein RCC1 failed to detect its antigen unless the nuclear envelope was disrupted by Triton X-100 treatment (Fig. 1*D*). Together, these data show that ARE stimulates energy-dependent AR translocation through the nuclear pore complex (NPC).

*ARE-dependent AR Export Requires ATP Hydrolysis*—Depending on the export substrate, ATP (31, 32) and/or GTP (33) can stimulate nuclear export in permeabilized cells. As our ERS contains both ATP and GTP, we tested whether one or both nucleotides is required for AR export. ATP, but not GTP, stimulated AR export in the presence of the ARE oligonucleotide (Fig. 2). The nonhydrolyzable ATP analog AMP-PNP did not support AR export, and the nonhydrolyzable GTP analog GMP-PNP did not inhibit export stimula-

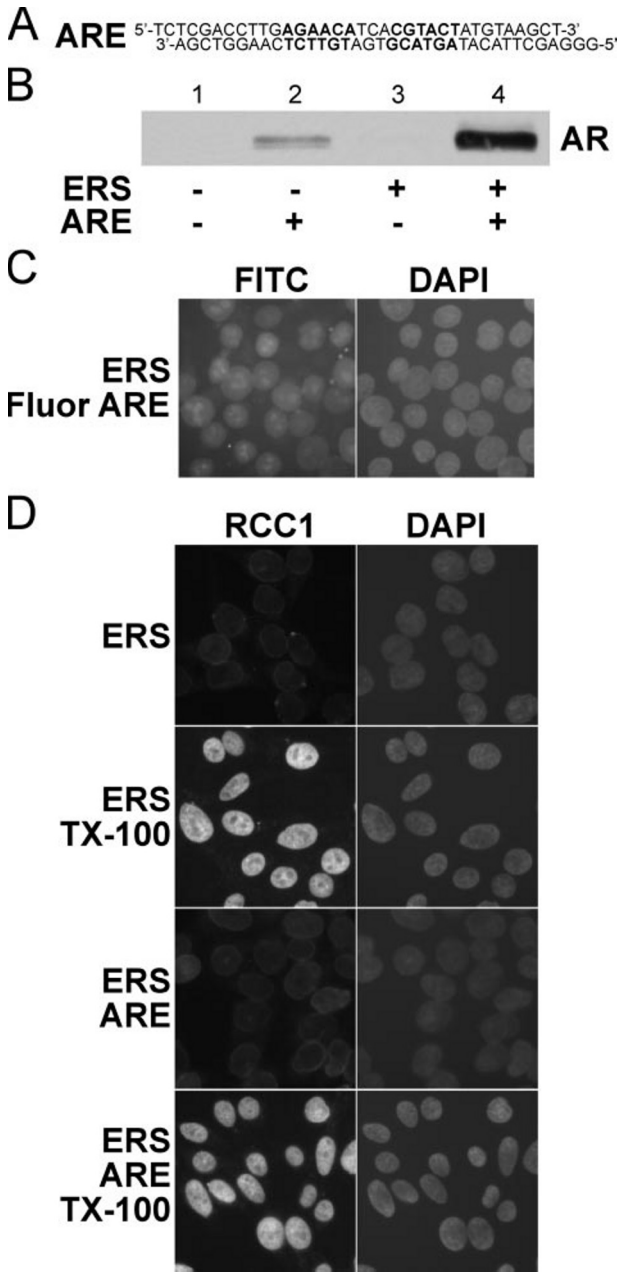


FIGURE 1. **A double-stranded DNA oligonucleotide containing an ARE stimulates nuclear export of endogenous AR in permeabilized LNCaP cells.** *A,* schematic representation of a 33-bp double-stranded oligonucleotide (ARE) that contains the C3(1) ARE (70) and possesses two 5′ overhangs. *B,* ARE (1 $\mu$M) and an ERS (contains ATP, GTP, creatine phosphate, creatine phosphokinase, and bovine serum albumin) were added in the combinations shown to permeabilized LNCaP cells to induce AR nuclear export *in vitro* (as described under "Experimental Procedures"). Reaction supernatants containing the exported AR fraction were subjected to SDS-PAGE (7.5% acrylamide) and immunoblotted using the anti-AR PG-21 antibody. *C,* adherent LNCaP cells were permeabilized with digitonin and subjected to standard *in vitro* export reactions in the presence of a fluorescein-labeled ARE. Fluorescein (*FITC*) and 4′,6-diamidino-2-phenylindole (*DAPI*) channels show fluorescent ARE and DNA localization, respectively. *D,* standard *in vitro* assays using adherent LNCaP cells were conducted in the presence or absence of 0.2% Triton X-100 (*TX-100*). All export reactions contained 7 $\mu$g/ml of anti-RCC1 antibody. Cells were then washed, fixed, exposed to a secondary antibody against the anti-RCC1 primary antibody, and analyzed using immunofluorescence microscopy.

tion by ATP (Fig. 2). Our results indicate that ATP hydrolysis, and not GTP hydrolysis, is rate-limiting for ARE-dependent AR export.

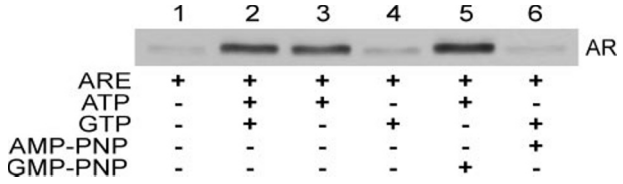


FIGURE 2. **ATP hydrolysis is required for ARE-dependent AR nuclear export.** Permeabilized LNCaP cells were subjected to standard AR *in vitro* export assays in the presence of ARE (1 $\mu$M) and the presence (*lane 2*) or absence (*lane 1*) of a complete energy-regenerating system. ATP, GTP, AMP-PNP, and/or GMP-PNP were added in the combinations shown (each 1 mM final). Reaction supernatants containing the exported AR fraction were immunoblotted using the anti-AR PG-21 antibody.

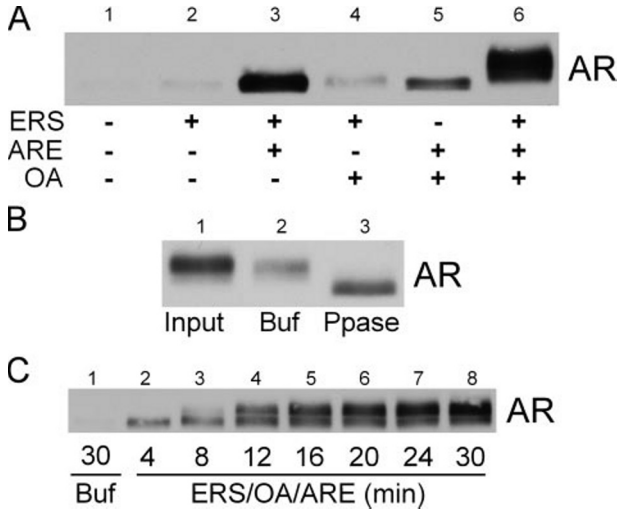


FIGURE 3. **OA enhances ARE-dependent AR nuclear export and stimulates phosphorylation of exported AR.** *A,* standard AR *in vitro* export assays were conducted in the presence of ARE (1 $\mu$M), ERS, and/or OA (200 nM) as shown. Reaction supernatants containing the exported AR fraction were immunoblotted with the anti-AR PG-21 antibody. *B,* permeabilized LNCaP cells were first treated with ERS/OA/ARE to stimulate AR nuclear export. Reaction supernatants containing the exported AR fraction (*lane 1*) were then incubated in the presence of buffer (*Buf, lane 2*) or phosphatase (*Ppase*, *lane 3*) for 2 h at 37 °C. *C,* standard *in vitro* AR export assays were conducted in the presence of ERS/OA/ARE (*lanes 2–8*) and harvested at the times indicated. A control reaction in the presence of buffer alone (*lane 1*) continued for 30 min. Exported AR fractions were immunoblotted using the PG-21 antibody.

This ATP requirement could reflect the involvement of a kinase(s) or protein chaperone(s) in ARE-stimulated AR export. Because phosphatase activity has been implicated in the regulation of GR nucleocytoplasmic trafficking (34), we tested the phosphatase inhibitor okadaic acid in our permeabilized cell assay. We found that OA modestly enhanced AR export and caused an upward shift in AR electrophoretic mobility (Fig. 3*A*, compare *lanes 3* and *6*). This AR mobility shift is strongly dependent upon ERS and ARE (Fig. 3*A*) and can be reversed by alkaline phosphatase treatment of exported AR (Fig. 3*B*). Thus, AR undergoes phosphorylation during the export reaction in the presence of ERS/OA/ARE. Time course analysis of AR export suggested that there are multiple pools of exported AR under this reaction condition (Fig. 3*C*). The faster migrating form of AR is exported within 4 min of the start of the assay. Within 8–12 min, a slower migrating form of AR appears in the exported fraction; the amount of this form of AR increases as a function of time (Fig. 3*C,* compare *lanes 3–8*).

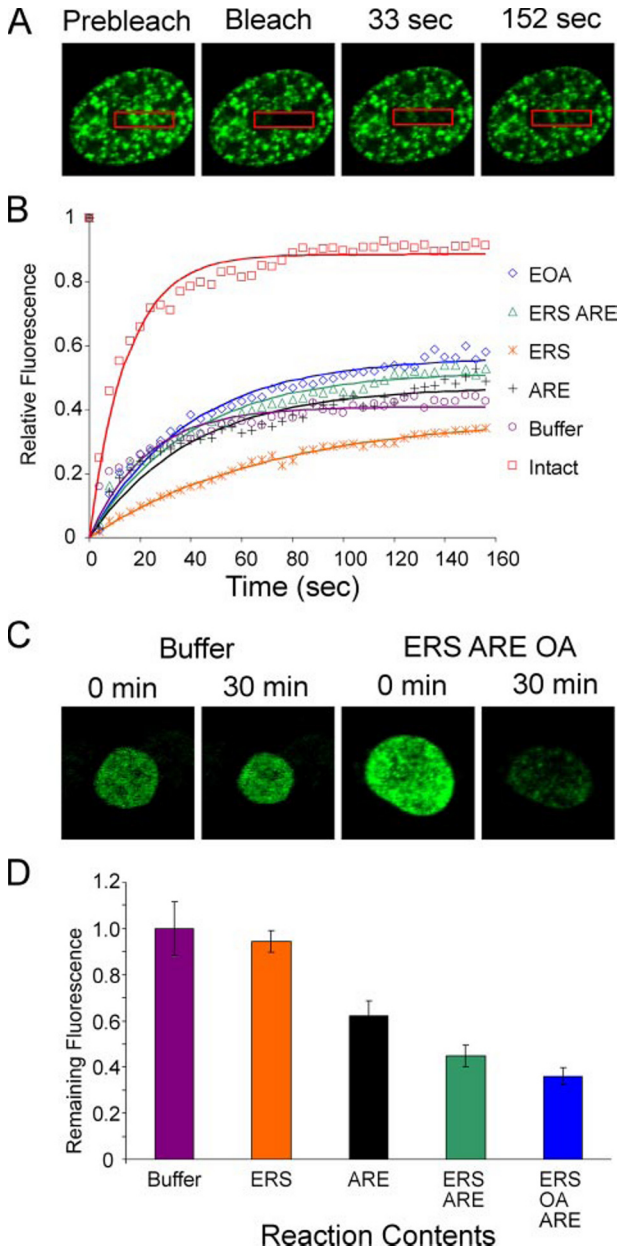


FIGURE 4. **Single-cell analysis of GFP-AR in permeabilized LNCaP cells.** *A,* LNCaP cells expressing GFP-AR were treated with 10 nM R1881, permeabilized, and subjected to FRAP analysis in the presence of standard export reaction mixes. In the representative cell shown here, recovery of GFP-AR into the bleached zone was visualized in the presence of ERS/OA/ARE (see "Experimental Procedures" for further details). *B,* FRAP recovery curves of intra-nuclear GFP-AR were generated using the different export reaction mixes shown. *Intact* cells were not permeabilized; *EOA* signifies an export reaction mix of ERS/OA/ARE. Each recovery curve was generated from a single cell that represents the behavior of all cells sampled under a particular reaction condition. *C,* LNCaP cells expressing GFP-AR were treated with 10 nM R1881, permeabilized, and subjected to standard *in vitro* export assays. Individual cells expressing GFP-AR were monitored for the loss of nuclear GFP fluorescence as a measure of GFP-AR nuclear export. Nuclear GFP fluorescence is shown in representative cells before (0 min) and after (30 min) an export reaction in the presence of buffer alone (*Buffer*) or in the presence of ERS/OA/ARE. *D,* LNCaP cells expressing GFP-AR were treated as in *C*. The amount of GFP fluorescence remaining in the nucleus after an *in vitro* export assay, compared with total nuclear GFP fluorescence prior to the assay, was calculated for individual cells in the presence of different export reaction mixes. The average value of this fraction is shown for all cells under a particular reaction condition.

**TABLE 1**

| Reaction condition | $t_{½}$ | Mobile AR fraction | Exported AR fraction |
|---|---|---|---|
| | *s* | % | % |
| Buffer | 15.8 ± 2.1 | 40 ± 5 | 0 ± 12 |
| ERS | 51.0 ± 8.3 | 36 ± 3 | 6 ± 5 |
| ARE | 25.6 ± 5.1 | 46 ± 4 | 38 ± 7 |
| ERS/ARE | 26.3 ± 2.5 | 59 ± 3 | 55 ± 5 |
| ERS/OA/ARE | 24.7 ± 5.6 | 43 ± 5 | 64 ± 4 |

*FRAP Analysis Suggests That AREs Do Not Promote Export Simply by Relief of Retention*—Theoretically, AREs might promote AR export by relief of AR nuclear retention on chromatin-binding sites or by induced changes in AR structure and/or subnuclear compartmentalization. To investigate this question further, we carried out export assays in single LNCaP cells to establish that the requirements for GFP-AR export are the same as for unfused, endogenous AR (Fig. 4*D*, compare with Fig. 3*A*). To address whether AR is subject to nuclear retention in our assay, we analyzed the intranuclear mobility of GFP-AR using FRAP. LNCaP cells expressing GFP-AR were permeabilized with digitonin, supplemented with different export reaction mixes, subjected to photobleaching, and monitored for fluorescence recovery (Fig. 4*B*). The recovery rate of GFP-AR (expressed as $t_{½}$ values) did not correlate with the level of GFP-AR nuclear export under different reaction conditions. For example, maximal intranuclear mobility of GFP-AR was observed in the presence of ERS alone; however, this condition promoted only near-background levels of GFP-AR export (Table 1). These data suggest that ARE-dependent AR export does not simply reflect relief of nuclear retention.

*Nuclear Export of Androgen-bound AR*—NRs including GR (35), AR (11), and the progesterone receptor (PR) (36) undergo ligand-dependent nuclear import. Some NRs undergo nuclear export following ligand exposure and subsequent withdrawal (11, 37–39), but whether ligand dissociation is required for NR export is unknown. To address this question, we pretreated LNCaP cells in culture with radiolabeled androgen ([$^3$H]R1881, 2 nM) to provide a readout for androgen-bound AR. Scintillation counting revealed that ARE stimulated the release of [$^3$H]R1881 into the export reaction supernatant (Fig. 5*A*). Furthermore, the amounts of [$^3$H]R1881 released into the supernatant under different export conditions were similar to the amounts of AR exported from the nucleus in nonradioactive assays (compare Fig. 5*A* with Fig. 3*A*). We established by immunoprecipitation and scintillation counting that the [$^3$H]R1881 released into the supernatant was bound to AR (Fig. 5*B*). These data indicate that androgen dissociation is not a prerequisite for AR nuclear export and that AR remains ligand-bound during export via the dsDNA-dependent export pathway.

*Inhibition of Phosphatidylinositol 3-Kinase Reduces AR Export and AR Phosphorylation*—ARE stimulation of both AR export and AR phosphorylation suggested that a nuclear kinase might be activated by ARE in the permeabilized cell assay. The DNA-dependent protein kinase (DNA-PK) merited consideration for two reasons. DNA-PK kinase activity is stimulated by dsDNA *in vitro* (40–42), and DNA-PK has been reported to associate with AR in LNCaP cells (19). To address whether DNA-PK is involved in ARE-dependent AR export, we tested

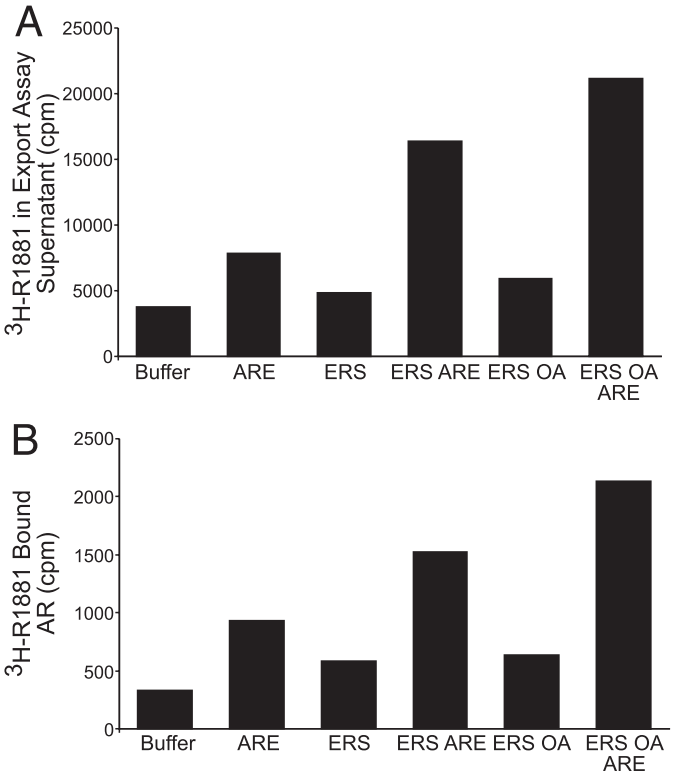


FIGURE 5. **Ligand dissociation is not a prerequisite for AR nuclear export.** *A,* LNCaP cells were supplied with [$^3$H]R1881 (2 nM) prior to standard *in vitro* export assays under the reaction conditions shown. Reaction supernatants containing the exported AR fraction were harvested and subjected to scintillation counting to determine the amount of [$^3$H]R1881 liberated from permeabilized cells during the export assay. Values from a representative experiment are shown. *B,* LNCaP cells were treated as in *A*, except that reaction supernatants containing the exported AR fraction were immunoprecipitated with anti-AR AR-21 antibody. Immunoprecipitates were then washed, extracted (as described under "Experimental Procedures"), and subjected to scintillation counting to determine the amount of [$^3$H]R1881 associated with AR in the exported fraction. Values from a representative experiment, in which background [$^3$H]R1881 binding to control resin has been subtracted to yield corrected values of [$^3$H]R1881 binding to the anti-AR resin, are shown.

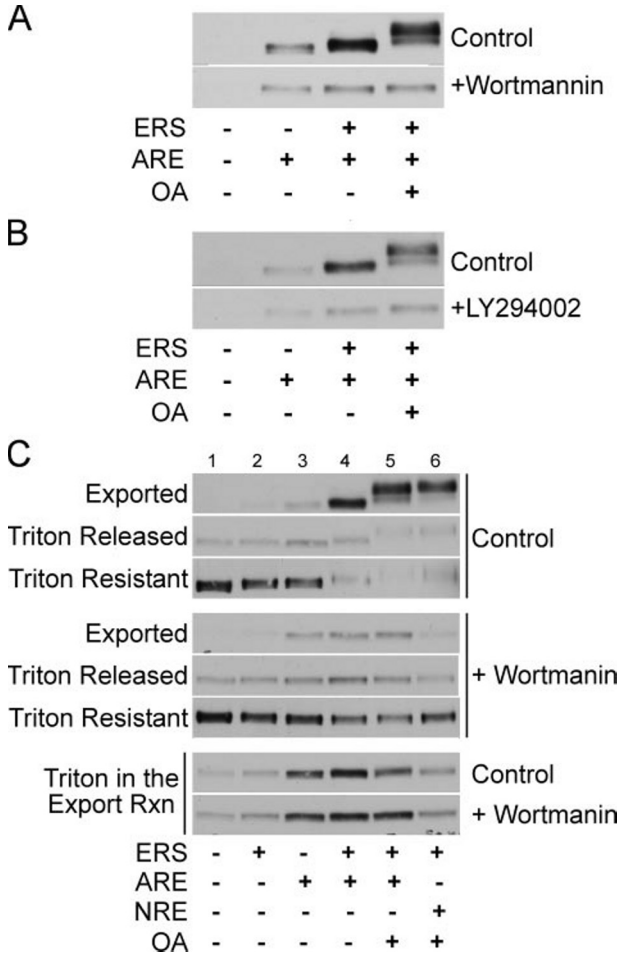


FIGURE 6. **Wortmannin and LY294002 inhibit ARE-dependent AR export and phosphorylation.** *A,* LNCaP cells were treated with 10 nM R1881 alone or in addition to 20 $\mu$M wortmannin prior to standard *in vitro* export assays in the presence of ERS, OA, and/or ARE as shown. Exported AR fractions were immunoblotted with the PG-21 antibody. *B,* LNCaP cells were treated with 10 nM R1881 alone or in addition to 50 $\mu$M LY294002 prior to standard *in vitro* export assays. *C,* LNCaP cells were treated with 10 nM R1881 alone or in combination with 20 $\mu$M wortmannin. Cells were then subjected to standard *in vitro* export assays under the conditions shown, and reaction supernatants containing the exported AR fraction were harvested. Cell pellets containing residual nuclear, nonexported AR were then subjected to extraction with 0.5% Triton X-100 on ice for 20 min. After centrifugation, the extracted (*Triton Released*) and nonextracted (*Triton Resistant*) AR fractions were harvested and subjected to SDS-PAGE along with the exported AR fractions and detected by immunoblotting with the PG-21 antibody. For *Triton in the Export Rxn* samples, LNCaP cells were subjected to standard *in vitro* export assays in the presence of 0.5% Triton X-100, *i.e.* detergent was added at the start of the export reaction rather than at a post-export extraction step.

wortmannin and LY294002 in the AR export assay. These inhibitors are known to target phosphatidylinositol 3-kinase family members, including DNA-PK (43, 44). In the presence of ERS/OA/ARE, wortmannin and LY294002 each reduced AR export to a level similar to that obtained in the presence of ARE alone (Fig. 6, *A* and *B, rows 1* and *2*, compare *2nd* and *4th lanes*). Wortmannin and LY294002 also blocked the mobility shift of exported AR observed in the presence of ERS/OA/ARE (Fig. 6, *A* and *B*).

We next performed biochemical extraction to gain insight into the role of AR compartmentalization in ARE-stimulated AR export. We harvested the supernatants from export reactions (Fig. 6*C*, *Exported*) and then treated the same LNCaP nuclei with Triton X-100 to generate Triton-released and Triton-resistant fractions. In permeabilized cells, AR is initially found in a Triton-resistant compartment (Fig. 6*C*, *lane 1, Control*). The amount of AR in the Triton-resistant compartment changes little with the addition of ERS alone or ARE alone (Fig. 6*C*, *lanes 2* and *3, Control*). However, ERS and ARE in combination significantly reduce the amount of AR in the Triton-resistant compartment (Fig. 6*C*, *lanes 4* and *5, Control*). In this assay, wortmannin addition increases the amount of AR in the Triton-resistant fraction (Fig. 6*C*, *lanes 4* and *5*, compare *Control* and *+Wortmannin*), and it blocks the upward electrophoretic mobility shift of AR. These data suggest that a wortmannin-sensitive kinase regulates an early step in the AR export pathway. Together with findings presented below, these results implicate DNA-PK in the regulation of dsDNA-dependent AR export.

*Evidence That DNA-PK Activation Promotes AR Export in Vitro*—We sought to characterize what oligonucleotide sequence and/or structure is necessary for AR export stimulation (Fig. 7). Our panel of oligonucleotides included duplexes similar to ones used by other laboratories to activate DNA-PK *in vitro* (40–42, 45). An ARE oligonucleotide synthesized with

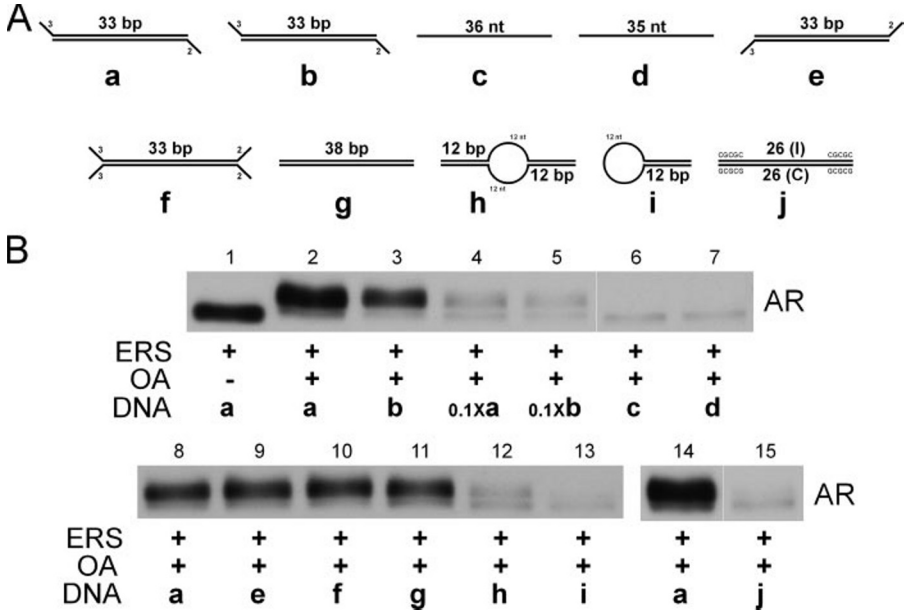


FIGURE 7. **Oligonucleotides with differing sequence and structure can stimulate AR export and phosphorylation.** *A,* schema of different DNA oligonucleotides used in *B* to stimulate AR export *in vitro*. dsDNA oligonucleotide (*a*) is the standard ARE; *b* is a mutant ARE containing four point mutations within the pair of ARE half-sites (30); *c* and *d* are the sense and antisense strand components, respectively, of the standard ARE; *e* is a modified ARE possessing two 3′ overhangs; *f* is a modified ARE possessing two 3′ overhangs and two 5′ overhangs; *g* is a modified ARE possessing two blunt ends; *h* is a modified ARE with a noncomplementary bubble between both half-sites of the ARE; *i* is a hairpin oligonucleotide containing within its complementary dsDNA region one of the two half-sites of the ARE; and *j* is a dsDNA oligonucleotide containing an internal stretch of 26 inosine:cytosine pairs flanked by five C:G pairs on each end of the oligonucleotide. *B,* LNCaP cells were pretreated with 10 nM R1881 and subjected to standard *in vitro* export assays in the presence of the oligonucleotides described in *A*. Oligonucleotides were included at 1 $\mu$M except in *lanes 4* and *5* (100 nM) and *lanes 6* and *7* (2 $\mu$M). Exported AR fractions were immunoblotted with the PG-21 antibody.

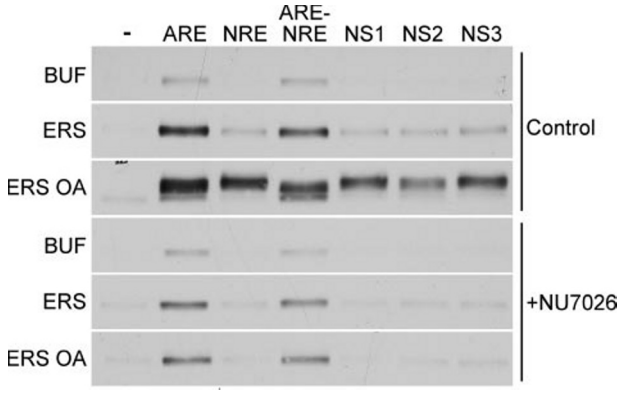


FIGURE 8. **A DNA-PK inhibitor blocks ARE-dependent AR export and phosphorylation.** LNCaP cells were treated with 10 nM R1881 alone or in combination with 2.5 $\mu$M NU7026 prior to permeabilization and standard *in vitro* export assays in the presence of ERS, OA, and/or dsDNA as shown. Exported AR fractions were immunoblotted using anti-AR PG-21. In the panel shown, the *left-hand column* indicates the reaction mix components that are combined with the dsDNA oligonucleotide (1 $\mu$M) in the *top row* to stimulate AR export. *NRE* is a modified ARE in which the ARE has been exchanged for a negative regulatory element-1 sequence (26). *ARE-NRE* is a modified ARE containing a negative regulatory element-1 sequence in *cis* to the ARE. *NS1*, *NS2*, and *NS3* are dsDNA oligonucleotides that do not contain an ARE. All gel lanes shown are from the same film exposure. Therefore, although absolute amounts of exported AR cannot be compared directly between the two cell populations, the amounts of exported AR can be compared between different rows within the same cell population.

four point mutations in the ARE half-sites (Fig. 7*A*, duplex *b*) (30) stimulated AR export and phosphorylation (Fig. 7*B*, *lane 3*), although the level of export was slightly reduced when compared with a wild type ARE oligonucleotide (duplex *a*, Fig. 7*B*, *lane 2*). The observation that AR export can be stimulated by a mutant ARE suggested the reaction is not sequence-specific, but a duplex comprised predominantly of inosine and cytosine had no activity in the assay (duplex *j*, Fig. 7*B*, *lane 15*). Reducing the concentration of ARE and mutant ARE 10-fold (100 nM final) resulted in significantly less AR export (Fig. 7*B*, *lanes 4* and *5*). Neither of the single-stranded oligonucleotides that together comprise the ARE duplex were alone able to stimulate AR export or AR mobility shift (Fig. 7*B*, duplexes *c* and *d*, *lanes 6* and *7*). As the ARE we used contains 5′ overhangs, we tested whether oligonucleotide end structure is important for ARE-stimulated AR export and phosphorylation. ARE duplexes possessing two 5′ and two 3′ overhangs (Fig. 7*B*, duplex *f*, *lane 10*) or blunt ends (duplex *g*, *lane 11*) stimulated AR export and mobility shift similarly to ARE (duplex *a*, *lane 2*). Finally, a stem-loop structure (Fig. 7*B*, oligo *i*, *lane 13*) and a duplex containing an internal bubble (duplex *h*, *lane 12*) both failed to stimulate AR export. Our results suggest ARE treatment stimulates export both by direct binding to AR and by activation of DNA-PK.

As an additional test for the involvement of DNA-PK in dsDNA-stimulated AR export, we employed the chemical inhibitor NU7026. When used at a concentration (2.5 $\mu$M) to inhibit DNA-PK but not phosphoinositide 3-kinase-related protein kinase subfamily kinases ATM or ATR (44, 46), NU7026 inhibited AR export and abolished the electrophoretic mobility shift of exported AR induced by ERS/OA/ARE (Fig. 8). We also tested NU7026 in the presence of a modified ARE in which the two ARE half-sites were exchanged for a sequence element that binds the Ku regulatory dimer of the DNA-PK complex (denoted NRE; see Ref. 26). The NRE duplex stimulated AR export in the presence of ERS and OA, and it also induced the electrophoretic mobility shift observed in the presence of ARE (Fig. 8). We also examined whether AR export could be stimulated by an oligonucleotide duplex containing AR- and Ku-binding sequences in *cis*, reasoning that this duplex might function as a scaffold and facilitate an interaction between AR and DNA-PK. This oligonucleotide (denoted ARE-NRE) stimulated AR export and mobility shift with about the same efficiency as the ARE oligonucleotide. Interestingly, the NRE oligonucleotide induced AR export without generating the faster migrating form of AR routinely observed in the presence of the ARE oligonucleotide. Three dsDNA oligonucleotides unrelated to the ARE also stimulated NU7026-sensitive

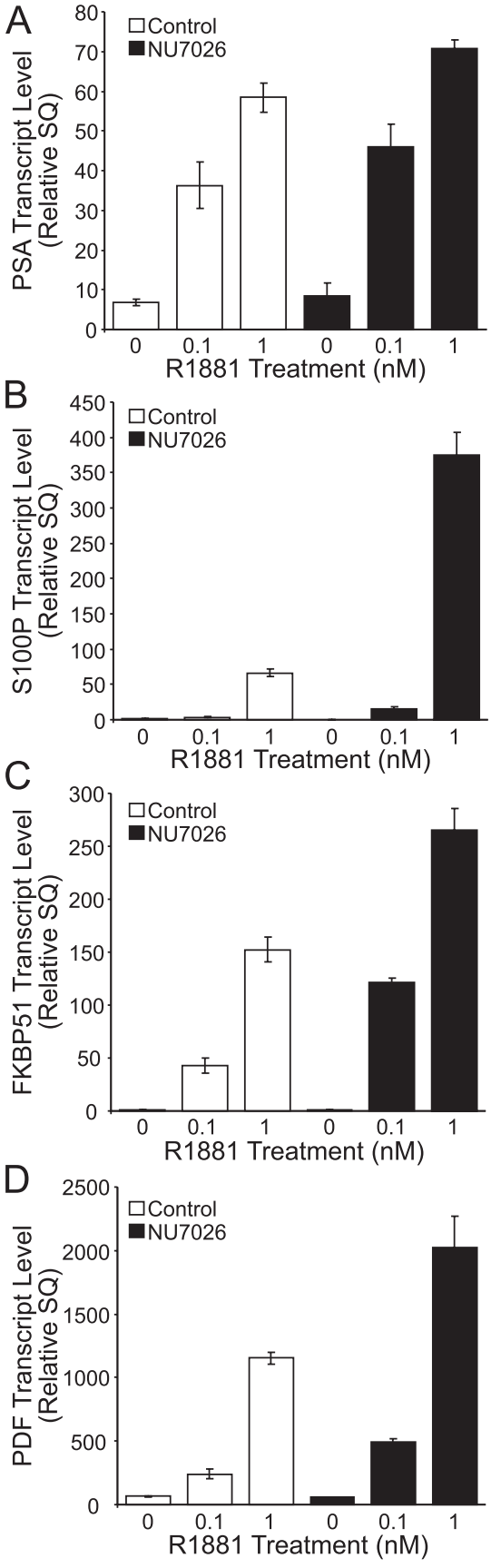


FIGURE 9. **NU7026 enhances transcription of androgen-responsive genes in LNCaP cells.** LNCaP cells were treated with 10 $\mu$M NU7026 for 16 h and then treated with or without R1881 for an additional 24 h at the concentrations shown. Transcription of *PSA* (*A*), *S100P* (*B*), *FKBP51* (*C*), and *PDF* (*D*) genes was then monitored using real time RT-PCR. Transcript levels were normalized to those of $\beta$-glucuronidase, and this ratio is plotted as the means ± S.E. *SQ*, starting quantity.

AR export (Fig. 8, see *NS1–3* in the presence of ERS/OA, *Control versus +NU7026*). These data indicate that nuclear export promoted by dsDNA is mediated through DNA-PK.

*NU7026 Enhances Androgen-dependent Transcription in Prostate Cancer Cells*—To address whether DNA-PK inhibition affects AR transcriptional activity, we treated LNCaP cells with NU7026 and assayed the transcript levels of four genes whose expression is regulated by androgen and AR. Real time PCR analysis revealed that NU7026 slightly enhanced (20% increase) AR-dependent transcription from the *PSA* gene (Fig. 9*A*). NU7026 increased AR-dependent transcription of the *S100P* gene to a greater extent (~5-fold; Fig. 9*B*). Intermediate effects were measured for the *PDF* and *FKBP51* genes, as NU7026 enhanced the androgen-induced transcript levels of these genes ~2-fold (Fig. 9, *C* and *D*). These results, together with our *in vitro* biochemical data and previous work showing that DNA-PK binds to AR (19), indicate a functional link between DNA-PK and AR.

*Exportin-mediated AR Export*—Nuclear import and export of most proteins is mediated by transport receptors belonging to the importin $\beta$ superfamily (47). We tested exportins 4–7 (prepared by *in vitro* translation) for RanGTP-dependent binding to the GST-DBD-Hinge protein immobilized on glutathione beads. All four exportins displayed Ran-sensitive binding to the AR DBD-Hinge (data not shown), but only one of these receptors, exportin 5, stimulated AR export from the nucleus of digitonin-permeabilized LNCaP cells (Fig. 10*A*). Exportin 5-dependent export was inhibited by including excess AR GST-DBD-Hinge protein in the permeabilized cell assay, which indicates the DBD-hinge contains the signal for exportin 5-mediated AR export (Fig. 10*B*). Unexpectedly, AR export in this setting did not require the addition of recombinant Ran, but this may indicate our permeabilized cells retain sufficient Ran to facilitate exportin 5-mediated AR export. Exportin 5 and DNA-PK appear to function in distinct export pathways because NU7026 blocked AR export and AR phosphorylation (based on gel shift), but the inhibitor failed to block exportin 5-dependent export in the permeabilized cell setting (Fig. 10*C*).

## DISCUSSION

We developed a digitonin-permeabilized cell assay to analyze nuclear export of AR from LNCaP cell nuclei. Permeabilized cell assays have been used to purify and characterize cytosolic transport factors that function in a variety of nuclear import and export pathways. Unexpectedly, we found that an energy-regenerating system together with short dsDNA oligonucleotides, like those typically used for electrophoretic mobility shift assay, stimulated efficient AR export. The fact that AR export in this assay does not require the addition of cytosol may indicate that nuclei of digitonin-permeabilized cells retain sufficient levels of transport factors needed for this export pathway. A similar observation was made in the context of the *in vitro* nuclear export of NFAT (31). Alternatively, AR may not require a clas-

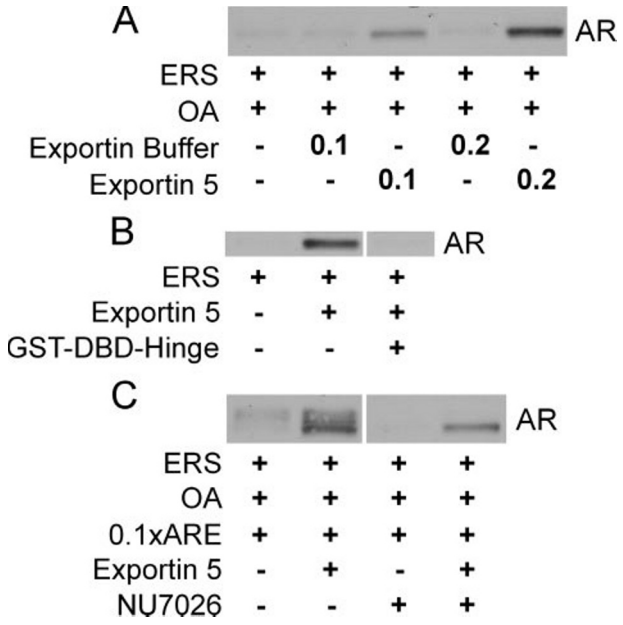


FIGURE 10. **Exportin 5 stimulates AR export *in vitro*.** *A,* LNCaP cells were treated with 10 nM R1881 prior to standard *in vitro* export assays in the presence of ERS, OA, and/or recombinant human exportin 5 (0.1 or 0.2 mg/ml) as shown. Exported AR fractions were immunoblotted with PG-21. In *lanes 2* and *4*, dialysate obtained from the purification of recombinant exportin 5 was added to the export reaction mix as a negative (buffer) control. *B,* standard *in vitro* export reactions were conducted in the presence of ERS, recombinant exportin 5 (1 $\mu$M), and recombinant GST-DBD-Hinge (10 $\mu$M) as shown. *C,* LNCaP cells were treated with 10 nM R1881 alone or in combination with 2.5 $\mu$M NU7026 prior to standard *in vitro* export assays in the presence of ERS, OA, ARE (100 nM), and/or recombinant exportin 5 as shown.

sical export receptor for nuclear export via this pathway. The permeabilized cell assay enabled us to identify several biochemical features of AR export *in vitro*. These include the following, 1) dsDNA oligonucleotides potently stimulate AR export. 2) This AR export pathway requires ATP hydrolysis. 3) dsDNA-dependent export is blocked by the DNA-PK inhibitor NU7026. 4) Ligand dissociation is not a prerequisite for AR export. 5) AR export can also be stimulated by the export receptor exportin 5.

*Energy Requirements for dsDNA-dependent AR Export*—dsDNA-dependent AR nuclear export requires nucleotide hydrolysis based on our finding that ATP, but not the nonhydrolyzable analog AMP-PNP, supports AR export. In our assay, ATP may serve primarily as a substrate for DNA-PK, another nuclear kinase(s), and/or a molecular chaperone(s). Yang *et al.* (32) found that ATP hydrolysis is necessary for robust GR export in digitonin-permeabilized cells when tyrosine phosphorylation is stabilized, consistent with an ATP requirement by a nuclear tyrosine kinase involved in GR export. ATP is also rate-limiting for the *in vitro* export of NFAT (31) and the human immunodeficiency virus, type 1, protein Rev (29). However, the identities of the ATP-utilizing enzyme(s) in these systems remain undefined. Elbi *et al.* (48) found that ATP is necessary for the chaperone-mediated restoration of the intranuclear mobility of GR and PR in permeabilized cells, but in that study the relationship between ATP utilization, nuclear receptor mobility, and nuclear export was not examined. Stavreva and co-workers (49) found that GFP-GR recycling on a mouse mammary tumor virus promoter array in live cells is sensitive to energy depletion and to the hsp90 inhibitor geldanamycin. These findings with GR imply that ATP may also be utilized by a chaperone(s) for successful interactions with AR prior to AR nuclear export. However, hsp90 is not the most likely candidate because geldanamycin does not inhibit AR export in our assay.[3]

GTP does not support, and GMP-PNP does not inhibit, dsDNA-dependent AR export in permeabilized cells. At face value these data suggest that dsDNA-dependent AR export is independent of GTP-utilizing enzymes such as Ran, a stoichiometric component of numerous export receptor-cargo complexes. But GTP hydrolysis by Ran is not required for efficient export of a classical NES-containing cargo (33, 50), and Ran-GMP-PNP can stimulate NES export by promoting export complex assembly. It is possible that the level of Ran-GTP remaining in our cells following permeabilization is sufficient to promote formation of AR export complexes. Alternatively, dsDNA-dependent AR export may not require either a classical export receptor or RanGTP. Ran has been reported to bind AR and enhance AR transactivation in luciferase reporter assays (51), but the relationship between these observations and our export data is not clear.

*Ligand Occupancy and AR Export*—NR export is often studied in the context of receptor movement from nucleus to cytoplasm after cognate ligand has been added and subsequently withdrawn (11, 37, 38, 52). Using our permeabilized cell assay, we were able to test whether ligand dissociation is a prerequisite for AR export. Using a radiolabeled synthetic androgen, we found that AR can remain agonist-bound during dsDNA-dependent nuclear export. Thus, AR can undergo both import (11, 53) and export (this study) while bound to agonist, and agonist dissociation is not an absolute prerequisite for AR export.

*AR Export and Phosphorylation*—Defranco *et al.* (34) observed that the phosphatase inhibitor okadaic acid (100 nM) promotes the cytoplasmic accumulation of GR in cultured cells, which implies that a kinase/phosphatase cycle influences the nucleocytoplasmic localization of GR. We applied OA to our permeabilized cell assay to determine whether AR export is regulated by a kinase/phosphatase cycle, and we observed a modest (~2-fold) enhancement of ERS/ARE-stimulated AR export. Under this condition AR is clearly phosphorylated because it undergoes an electrophoretic mobility shift that can be reversed by alkaline phosphatase treatment. This suggests that direct phosphorylation of AR could be one of the rate-limiting steps for AR export *in vitro*. AR is phosphorylated on multiple serines (54, 55) and on at least one tyrosine (56). Phosphorylation of Ser-650 in the hinge region positively regulates AR internuclear migration in a heterokaryon assay (27). However, Ser-650 phosphorylation does not appear to regulate dsDNA-dependent export *in vitro*, because AR containing an alanine substitution at this position still undergoes nuclear export and electrophoretic mobility shift in response to ARE addition in permeabilized PC-3 cells (data not shown). Alterna-

[3] L. C. Shank and B. M. Paschal, unpublished observations.

tively, phosphorylation of a factor(s) other than AR may be rate-limiting for dsDNA-dependent AR export.

Phosphorylation is thought to regulate the nuclear export of nuclear receptors in addition to AR, but thus far a common mechanism has not emerged. GR undergoes export in response to UV irradiation in a pathway that depends on JNK phosphorylation of Ser-226 in the AF-1 region (57). GR export in response to UV treatment can be inhibited by leptomycin B, which provides evidence that the export receptor Crm1 mediates GR export in this setting. GR lacks a prototypical NES of the type recognized by Crm1, suggesting the interaction either is mediated by an adaptor protein(s) and/or involves a unique export signal in GR. As GR export can be leptomycin B-insensitive in nonirradiated cells (52) and can be mediated by the chaperone calreticulin (37), NR export probably occurs by more than one pathway (reviewed in Ref. 58). PR export appears to be mediated by Crm1 via a mechanism that is dependent on ERK (extracellular signal-regulated kinase) phosphorylation of Ser-294 in the AF-1 region of PR (59). It has also been reported that cytoplasmic localization of the estrogen receptor is regulated by phosphorylation of Thr-311 in the AF-1 region of the receptor (60). It is interesting that AF-1 phosphorylation appears to play a role in the nucleocytoplasmic localization of multiple NRs, given that AF-1 sequences exhibit a high degree of variability among different nuclear receptors.

*DNA Oligonucleotide Stimulation of AR Export in Vitro*—One of the major findings of this study is that short dsDNA oligonucleotides potently stimulate nuclear export of AR. The logic behind the introduction of the ARE oligonucleotide (containing an ARE) into the permeabilized cell assay was based on the assumption that AR is subject to nuclear retention through DBD-mediated binding to chromatin, and that titration of this reaction by ARE addition should enhance AR export. The absence of a correlation between GFP-AR nuclear mobility ($t_{1/2}$ values) and the amount of GFP-AR nuclear export, across different export reaction conditions, suggests that ARE do not merely relieve AR nuclear retention. Theoretically, ARE could bind directly to the DBD of AR and induce changes in AR conformation and/or subnuclear localization to promote AR export. ARE could also stimulate AR export by activating a nuclear kinase that phosphorylates AR and/or another component(s) of this AR export pathway. Nuclear kinases that can be activated by DNA include the phosphoinositide 3-kinase-related protein kinase subfamily members ATM, ATR, and DNA-PK. We addressed the potential contributions of these kinases to ARE-induced AR export by pharmacological inhibition. Wortmannin and LY294002 inhibit ATM, ATR, and DNA-PK, and both inhibitors were highly effective at reducing AR export in permeabilized cells. We next utilized the inhibitor NU7026 because the $IC_{50}$ values of this compound allow discrimination between the involvement of DNA-PK (0.23 $\mu$M), ATM (>100 $\mu$M), and ATR (>100 $\mu$M) (44). NU7026 inhibited AR export and phosphorylation induced by ERS/OA/ARE (Fig. 8), indicating that DNA-PK activity is limiting for this pathway of AR export. Although ARE and the other dsDNA oligonucleotides used in this study provide means for the artificial activation of DNA-PK, it is possible that this form of activation mimics an activation step that occurs when DNA-PK holoenzyme is bound to chromatin in the context of live cells (see below). There is also a pathway for the activation of DNA-PK end-joining activity that involves inositol phosphate binding to the Ku regulatory dimer (61), an interesting finding in light of the fact that inositol phosphates co-regulate certain export pathways (62, 63).

The activities of oligonucleotides of differing sequence and structure in our export assay appeared to corroborate results obtained with NU7026. Neither single-stranded DNA component of the ARE dsDNA oligonucleotide stimulated AR export, consistent with the inability of single-stranded DNA to stimulate DNA-PK activity (40, 45, 64). dsRNA oligonucleotides failed to stimulate AR export (data not shown) and do not stimulate DNA-PK activity (42, 45). Perhaps most compelling was our finding that AR export and phosphorylation were stimulated by nonspecific dsDNA that do not contain an ARE, and these effects were inhibited by NU7026 (Fig. 8). As discussed below, the effects of dsDNA on AR export could be a mimetic of DNA exposed during transcription, but it is also possible that in our system dsDNA acts as a biochemical surrogate for some other DNA-PK activation mechanism that has not been described.

DNA-PK is best known for roles in DNA damage response and V(D)J recombination (20), but multiple studies have implicated DNA-PK in pathways of nuclear receptor function. DNA-PK has been shown to physically interact with estrogen receptor $\alpha$ (22), vitamin $D_3$ receptor (23), PR (24), and AR (19). Subunits of the DNA-PK complex have been localized by chromatin immunoprecipitation to promoters regulated by vitamin $D_3$ receptor (23) and AR (19). Furthermore, DNA-PK can phosphorylate GR (25) and PR (24). It is interesting to note that DNA-PK phosphorylates PR (24) and GR (25) in the DBD/hinge region, and AR contains a DNA-PK consensus phosphorylation site in its hinge region (Ser-656). In an early study of PR phosphorylation it was noted that HeLa nuclear extract contains a kinase that phosphorylates PR in response to addition of plasmid DNA harboring a progesterone-response element (65). Together, these data suggest that at least one general mechanism of transcriptional regulation by DNA-PK involves direct phosphorylation of nuclear receptors; this function appears to extend to other transcription factors and RNA polymerase II (66, 67). Exactly how DNA-PK regulates transcription has not been elucidated, but it has been proposed to promote recycling of AR by directing re-initiation (19). Knockdown of the Ku70 or Ku80 subunit of the DNA-PK complex caused a modest reduction in androgen-dependent transcription of the *PSA* gene (19). Using a pharmacological approach, we found that inhibiting DNA-PK catalytic activity with NU7026 increased AR-dependent transcription from several genes. It is possible that DNA-PK has both positive and negative roles in AR-dependent transcription, and that reducing Ku70/80 levels by knockdown and inhibiting catalytic activity with NU7026 are not equivalent in terms of DNA-PK inactivation.

*Exportin 5 Stimulates AR Export*—Virtually all proteins that undergo nuclear transport rely on transport receptors to mediate their translocation through the NPC (47). Nuclear localization sequences have been defined in most NRs, and several import receptors are able to mediate the nuclear import of GR,

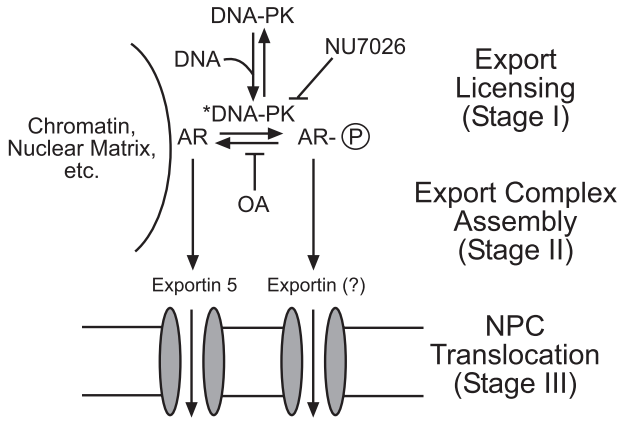


FIGURE 11. **A model of how DNA-PK may regulate AR nuclear export.** In this model, DNA-PK becomes activated (*DNA-PK) via interaction with DNA and mediates a phosphorylation licensing of AR. This licensing can be blocked by the DNA-PK inhibitor NU7026 or stabilized by the phosphatase inhibitor OA. AR licensing could then facilitate engagement with an exportin, resulting in formation of an export complex that translocates through the NPC. Cells may also contain a DNA-PK-independent pathway that operates through exportin 5. See the text for further details.

including importin $\alpha$ and importin 7 (68). Our previous work on GR showed that the DBD contains the information that is sufficient to specify export of a reporter protein to the cytoplasm (16). This observation, together with the finding that mutations in the DBD of GR and AR interfere with reporter protein nuclear export (16), leads us to propose that the NES in nuclear receptors is encoded by the DBD. Using binding assays we found that several exportins bind to a fragment of AR containing its DBD and hinge domains (data not shown). We tested recombinant exportins 4–7 in our permeabilized cell assay and found that recombinant exportin 5 can stimulate AR export. Furthermore, this effect can be inhibited by excess AR GST-DBD-Hinge protein. These results are consistent with exportin 5 mediation of AR export in permeabilized cells via binding to the DBD.

*Working Model for AR Export*—In this study we have shown that DNA-PK activity is critical for dsDNA-dependent AR export. The primary function of exogenous dsDNA oligonucleotides in our permeabilized cell assay may be to provide a surrogate activation mechanism for DNA-PK in place of activation mechanism(s) normally operational in live LNCaP cells. Alternatively, dsDNA may both activate DNA-PK and bind directly to AR as a means of enhancing AR export (see Ref. 29). DNA-PK appears to function at an early step in the dsDNA-dependent AR export pathway (see Fig. 6*C*). Furthermore, nonspecific oligonucleotides that do not contain an ARE can stimulate AR export (see Fig. 8), Together, these results suggest that if dsDNA both activate DNA-PK and bind directly to AR, DNA-PK activation would occur before direct AR:oligonucleotide binding.

Our current and previous findings together with data from other laboratories prompted us to speculate that phosphorylation may provide AR with a license to undergo export from the nucleus in the dsDNA-dependent export pathway (Fig. 11, *Stage I*). Based on the fact that DNA-PK and AR can be localized to the same regions of the *PSA* promoter by chromatin immunoprecipitation (19), DNA-PK could phosphorylate AR when both proteins are engaged with chromatin. We speculate that the signal for DNA-PK activation could be DNA that is exposed during chromatin remodeling. This would provide a means of coupling AR participation in transcription with subsequent AR export. AR could receive its license to leave the nucleus after a single round or after multiple rounds of transcription, depending on the gene involved and the influence of OA-sensitive phosphatases. In this model, phosphorylated AR is expected to co-assemble with export factors after receiving its export license (Fig. 11, *Stage II*). Following assembly, an AR-exportin complex would translocate through the central channel of the NPC and enter the cytoplasm where the complex is disassembled (Fig. 11, *Stage III*). Exportin 5 does not appear to function within this dsDNA-dependent export pathway, as a DNA-PK inhibitor did not block exportin 5-mediated export. Finally, the model emphasizes direct phosphorylation of AR as the event that provides the license to leave the nucleus, but it remains possible that DNA-PK phosphorylates an AR-interacting factor that, in turn, regulates AR export.

The fact that dsDNA-dependent AR export can be stimulated without adding export factors clearly indicates that permeabilized cells retain sufficient quantities of these factors, such that they are not rate-limiting in our assay. It is formally possible that AR engages directly with the NPC without the aid of transport factors, but given the mobility of AR in the nucleus, it is difficult to envision how AR export regulation would be achieved. Finally, neither the DNA-PK-dependent nor the exportin 5-dependent export pathways required the addition of RanGTP. In addition, GTP was not required for AR export stimulated by the addition of dsDNA. These data imply these pathways are Ran-independent or that residual levels of RanGTP are sufficient to promote export. The fact that the highly specific DNA-PK inhibitor NU7026 enhances AR-dependent transcription in LNCaP cells can be viewed as evidence that DNA-PK negatively regulates AR activity. A simple interpretation of this result is that inhibition of DNA-PK increases the nuclear dwell time of AR or that it increases the pool of AR that can reinitiate transcription. The extent of the NU7026 effect on AR transcription is gene-dependent, which is interesting in light of our recent finding that the nuclear concentration of AR required for efficient transactivation is not the same for all AR-regulated genes (69). Rigorous evaluation of the export license model will require defining the DNA-PK phosphorylation site(s) on AR and determining how these modifications contribute to interactions with the nuclear export machinery.

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