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# A Ligand-dependent Bipartite Nuclear Targeting Signal in the Human Androgen Receptor

## REQUIREMENT FOR THE DNA-BINDING DOMAIN AND MODULATION BY $NH_2$-TERMINAL AND CARBOXYL-TERMINAL SEQUENCES*

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**The amino acid sequence requirements for androgen-dependent androgen receptor nuclear import were determined by immunostaining transiently expressed full-length wild type and mutant human androgen receptors (AR) in monkey kidney COS cells and measuring transcriptional activity by cotransfection with a luciferase reporter vector in monkey kidney CV1 cells. Mutagenesis studies revealed a bipartite nuclear targeting sequence in the DNA binding and hinge regions at amino acids 617–633, consisting of two clusters of basic amino acids separated by 10 amino acids, RKCYEAGMTLGARKLKK. In a series of deletion mutants, AR $NH_2$-terminal fragments (residues 1–639 through 1–723) displayed constitutive nuclear import, and transcriptional activity was similar to that of the ligand-activated full-length wild type AR. In contrast, nuclear import and transcriptional activation were inhibited by sequence extensions into the steroid-binding domain (1–771). Constitutive nuclear import was regained in part by $NH_2$-terminal deletions of full-length AR. Expression of AR/pyruvate kinase chimeras defined a sequence required for predominant nuclear localization as residues 580–661, comprised of the second zinc finger region of the DNA-binding domain, the 17-amino-acid putative targeting sequence, and 28 residues of flanking carboxyl-terminal sequence. These studies suggest that the bipartite nuclear targeting sequence of AR includes flanking sequence and is modulated by interactions between the $NH_2$- and carboxyl-terminal regions.**

Selective transport of proteins to the nucleus represents a potential site of regulation unique to eukaryotic cells (1). A key step in nuclear import of transcription regulatory proteins is the activation of nuclear targeting signals required for proteins to traverse nuclear pore complexes (2, 3). The first nuclear targeting signal identified was that for SV40 large T antigen (4); a nine-amino-acid sequence of predominantly basic residues was sufficient to direct a heterologous protein to the nucleus (4, 5). Similar studies revealed a single domain nuclear targeting signal in c-*myc* (6–8). In contrast, nucleoplasmin, a histone binding protein, requires two interdependent domains of basic amino acids separated by a 10-amino-acid spacer sequence (9).

The concept of ligand activated nuclear transport (10, 11) has persisted for certain members of the steroid receptor family, shown most definitively for the glucocorticoid receptor (12–14). Studies on the androgen receptor (AR)[1] in transiently transfected cells support a ligand-activated transport mechanism (15, 16). However, endogenous AR, while nuclear in the presence of androgen, becomes undetectable with prolonged hormone withdrawal (17, 18) due in part to an enhanced rate of degradation in the absence of androgen (19). Overexpression by transient transfection in eukaryotic cells provides a high rate of synthesis to facilitate immunocytochemical detection in the absence of androgen.

Initial reports characterizing nuclear targeting signals of steroid receptors included the two signals, NL1 and NL2, of the rat glucocorticoid receptor: NL1 is a 28-amino-acid region associated with the DNA-binding domain and NL2 comprises the hormone-binding domain (13). For the rabbit progesterone receptor, a targeting signal sequence in the region of amino acids 638–642 was homologous to the SV40 large T antigen targeting sequence, and when deleted, caused the progesterone receptor to be cytoplasmic in the absence of hormone (20). Nuclear translocation was associated with dimerization mediated through the steroid-binding domain. The progesterone receptor nuclear targeting signal was postulated to reside in two domains: a constitutive signal in the hinge region and a hormone-dependent signal in the second zinc finger region of the DNA-binding domain (21). The specific residues required for nuclear import of the progesterone receptor are as yet unknown. Exposure of cells to inhibitors of energy synthesis indicated that nuclear residency is dynamic with receptors diffusing into the cytoplasm and being actively transported back to the nucleus (21). In striking contrast is immunocytochemical and biochemical evidence that the estrogen receptor is nuclear independent of hormone status (22, 23), a concept also supported by some studies on the progesterone receptor (20, 24, 25).

In this report, we investigate not only the minimal sequence required for predominant nuclear localization of AR, but also those regions required for complete nuclear import as observed with wild type AR in the presence of androgen. Mutagenesis studies indicated that a signal sequence rich in basic amino

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[1] The abbreviations used are: AR, androgen receptor; PK, pyruvate kinase; IgG, immunoglobulin; PCR, polymerase chain reaction; Hsp, heat shock protein; R1881, methyltrienolone; DHT, dihydrotestosterone; T, testosterone; WT, wild type; N, nuclear; C, cytoplasmic.

acids similar to the bipartite nuclear targeting signal of nucleoplasmin was required for predominant nuclear localization of AR. It spans two exons and includes portions of the DNA binding and hinge regions. Mutagenesis of the wild type receptor and analysis of chimeric proteins expressing different regions of AR provided insight into the modulatory effects of the $NH_2$- and carboxyl-terminal domains. Transcriptional activity was monitored, in addition, since nuclear import is a requirement for transactivation.

## EXPERIMENTAL PROCEDURES

*Materials*—The following cells and reagents were obtained: monkey kidney COS-1 and CV-1 cells from the American Type Culture Collection; Dulbecco's modified essential medium with high glucose, with or without phenol red from JRH Biosciences; bovine calf serum from HyClone Laboratories, Inc.; ECL Western blotting detection kit from Amersham Corp.; unlabeled deoxynucleotide triphosphates from Pharmacia LKB Biotechnology Inc.; Sequenase from United States Biochemical Corp.; *Taq* polymerase from Promega; deep-*Vent* polymerase and T4 DNA ligase from New England Biolabs; restriction endonucleases from Life Technologies, Inc., Promega, and New England Biolabs; prestained protein molecular weight standards from Life Technologies, Inc.; W-OMAT-AR diagnostic x-ray film from Kodak; D-luciferin from Analytical Luminescence; Immobilon from Millipore; buffers and chemicals from Fisher, EM Science, and Sigma.

*Cell Culture and Transfections*—AR nuclear import was assessed immunocytochemically in COS cells transfected with wild type or mutant AR expression vector DNA or pyruvate kinase (PK) chimeric DNA. COS cells allow for high level AR expression due to SV40 transformed-T antigen-induced amplification of the expression vector. The wild type human AR expression vector pCMVhAR previously described (15) contained the full-length coding sequence of human AR cloned in the pCMV5 expression vector containing the cytomegalovirus promoter (26). For determination of transcriptional activation, CV1 cells were used because low level plasmid expression enhances androgen sensitivity. Cells were plated at $0.45 \times 10^6$ cells/6-cm dish and cotransfected with 0.01–1 μg of wild type or mutant AR expression vectors unless indicated otherwise, and 5 μg of luciferase reporter vector. The luciferase reporter vector, kindly provided by Ronald M. Evans, the Salk Institute, La Jolla, CA, is under the control of the mouse mammary tumor virus promoter. DNA precipitates were prepared by vortexing wild type or mutant pCMVhAR and luciferase reporter vectors in 0.5 ml of 0.25 M $CaCl_2$ together with 0.5 ml of 0.28 M NaCl, 1.5 mM $Na_2HPO_4$, 0.05 M HEPES, pH 7.12, and incubated for 30 min at room temperature. The mixture was added to the aspirated plates and 3 ml of 10% bovine serum-containing media added and the cells incubated for 4 h at 37 °C. Following a 4-min treatment at room temperature with 15% glycerol in 10% fetal calf serum-containing media, cells were placed in 0.2% calf serum and returned to the 37 °C incubator after two washes with TBS (0.14 M NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.9 mM $Na_2HPO_4$, 25 mM Tris, pH 7.4). After 24 and 48 h, media was replaced with serum-free media with or without the indicated concentrations of the synthetic androgen, methyltrienolone (R1881). Seven h after the last media change, cells were harvested in 0.6 ml of lysis buffer (Ligand Pharmaceuticals) and supplemented with 8 mM $MgCl_2$, 1 mM dithiothreitol, and 0.4 mM phenylmethylsulfonyl fluoride, and either stored overnight at −74 °C or assayed immediately. Relative light units were determined on a Monolight 2010 Analytical Luminescence Laboratory luminometer after combining 0.4 ml of reaction buffer (15 mM $MgCl_2$, 5 mM ATP, 0.5 mg/ml bovine serum albumin, 15 mM glycylglycine, pH 7.8), 100 μl of cell lysate, and 100 μl of 1 mM D-luciferin injected automatically.

*Immunoblot Analysis*—COS cells plated in 10-cm dishes were transiently transfected with 10 μg of wild type or mutant AR expression vector DNA using DEAE-dextran (27). Cells were maintained in 10% serum, Dulbecco's modified Eagle's-H media, and for cells expressing AR mutants containing the hormone-binding domain, 50 nM R1881 was added 24 h prior to harvest. Forty-eight h after transfection, cells were washed in phosphate-buffered saline and harvested in 200 μl of 2% SDS, 10% glycerol, and 10 mM Tris, pH 6.8. Mercaptoethanol (4%) and bromphenol blue (1%) were added and the samples boiled 5 min. Forty-μl aliquots were analyzed by electrophoresis in 8% acrylamide gels as described previously using AR52 (28) or AR32 (29) rabbit polyclonal anti-peptide antisera. Protein bands were calibrated using molecular weight markers phosphorylase *b*, bovine serum albumin, and ovalbumin.

*Immunocytochemistry*—COS cells were transfected in two chamber glass slides as previously described (15) and were either untreated or treated with 100 nM R1881 or dihydrotestosterone for 24 h, and again in fresh media 1 h prior to fixation. Results were similar using either androgen. Expressed proteins were immunostained using AR antipeptide antibodies AR52 (28) or AR32 (29), or chicken PK antibody kindly provided by Chi V. Dang and Gary S. Hayward, Johns Hopkins University School of Medicine. Cells were fixed in 2% paraformaldehyde, 10% sucrose, and 0.1 M sodium phosphate, pH 7.2, treated with 5% bovine serum albumin in phosphate-buffered saline for 30 min, and incubated with AR IgG (1–2 μg/ml) or PK IgG (2–4 μg/ml) overnight at 4 °C. Cells were washed in phosphate-buffered saline and incubated with fluorescein or Texas Red$^R$ conjugated goat anti-rabbit IgG (1:500; Organon Technika, Cochranville, PA or Molecular Probes, Inc., Eugene, OR) for 40–60 min at room temperature. Cells incubated with PK antibody were also processed for immunofluorescence using Ultraavidin$^{TM}$-conjugated fluorescein or Texas Red$^{TM}$ (Leinco Technologies, Inc., St. Louis, MO). Fixed cells were incubated with biotinylated goat anti-rabbit IgG (1:400; 60 min) followed by incubation with Ultraavidin-conjugated fluorescein or Texas Red$^{TM}$ (1:1000; 30 min) at room temperature. In an alternative method, cells were fixed in 95% ethanol for 10 min at −20 °C and processed for immunofluorescence as described above. Results were similar using either fixation method with some variations; immunostaining following ethanol fixation was of stronger intensity and therefore more effective at revealing low levels of receptor. Slides were examined using a Nikon Optiphot 2 microscope with an EPI-fluorescence attachment. Each AR and PK mutant was analyzed at least three times and the cells shown best represent the overall immunocytochemical staining pattern.

*PCR Mutagenesis*—AR mutants were constructed by PCR mutagenesis of the pCMVhAR expression vector (15) using outside primers containing unique restriction sites in AR and internal primers with the appropriate mutations. Primers ranged in size from 15–35 nucleotides and were synthesized on a automated Applied Biosystems DNA synthesizer model 394 using standard cyanoethyl phosphoramidite chemistry. DNA amplification was performed using Taq or deep-Vent polymerase. All PCR amplified DNA was sequenced by the dideoxynucleotide method to verify error-free amplification. For amplified regions greater than 200 base pairs, use of deep-Vent polymerase with its 3′ exonuclease proof-reading activity was critical to avoid random PCR errors. PCR was performed in five 100-μl reactions using 1 unit of polymerase/reaction. Standard PCR conditions for 500-base pair regions were 1 cycle of 94 °C for 5 min, 56 °C for 2 min, 72 °C for 3.5 min; 11 cycles of 95 °C for 1.5 min, 56 °C for 2 min, 72 °C for 3 min; and 1 cycle of 95 °C for 1.5 min, 56 °C for 2 min, 72 °C for 5 min. Amplified fragments and enzyme-digested vectors were gel purified in agarose, cleaved with restriction enzymes, and ligated using T4 DNA ligase, typically overnight at 16 °C. Competent DH5 *Escherichia coli* cells were transformed and colonies selected on ampicillin containing agar plates.

Plasmids RLPK12 and myc317–335/PK, kindly provided by Chi V. Dang, Johns Hopkins University, contained PK coding sequence with and without the *c-myc* nuclear targeting sequence (residues 317–335) and were constructed from cDNA clones originally provided by Bruce L. Roberts, Integrated Genetics, MA. Both coding regions were cloned into the pCMV5 expression vector. AR/PK chimeric vectors were constructed using PCR where the primers contained flanking *Xho*I/*Eco*RI restriction sites. Insertions were after amino acid 16 in PK as previously described (8). For insertion of the known nuclear targeting signals, SV40 (5) and nucleoplasmin (9), and the 617–633/PK mutant, oligonucleotides of 30, 52, and 57 residues, respectively, containing flanking *Xho*I/*Eco*RI sites, were annealed and cloned into the pCMVPK expression vector.

Specific high affinity binding of [$^3$H]R1881 was verified using a whole COS cell binding assay previously described (27). All AR mutants containing an unaltered and complete steroid-binding domain were shown to retain high affinity [$^3$H]R1881 binding. All carboxyl-terminal deletions, including deletion of the final exon (Δ869–919), resulted in loss of androgen binding.

## RESULTS

*A Bipartite Nuclear Targeting Signal in Human AR*—Two methodological approaches were taken to investigate the sequence requirements for AR nuclear import. Overexpression in COS cells allowed for immunocytochemical detection of wild type and mutant receptors. The immunucytochemical method is advantageous because it can reveal incomplete nuclear transport indicating partial loss of targeting activity. The sec-

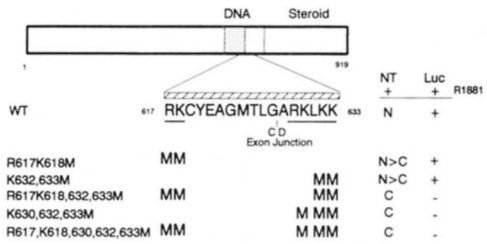


FIG. 1. **Nuclear transport and transcriptional activity of AR containing mutations in the dibasic domain of the DNA/hinge region.** The 919-amino-acid human AR is comprised of the $NH_2$-terminal transactivation region (amino acids 1–558, *open rectangle*), central DNA-binding domain (amino acids 559–624, *stippled area*), hinge region (amino acids 625–676, *small open box*), and carboxyl-terminal steroid-binding domain (amino acids 677–919). Spanning the junctions of exon C (DNA-binding domain) and D (hinge region) are two basic motifs (*underlined*) separated by 10 amino acids at residues 617–633. PCR mutagenesis converted basic residues to methionine in the full-length coding region as follows: R617K618M (indicates R617 and K618 converted to M); K632,633M; R617K618,632,633M; K630,632,633M; R617K618,630,632,633M. Immunocytochemical evaluation of nuclear transport (*NT*) was determined in transiently transfected COS cells assayed in the presence of 100 nM dihydrotestosterone or R1881. In the absence of androgen, wild type and the five mutants showed cytoplasmic perinuclear staining. Transcriptional activity (*Luc*) was determined in the presence of androgen by transient cotransfection of CV1 cells using 5 μg of luciferase reporter and 0.1 μg of AR expression vector DNA as described under "Experimental Procedures." *N* denotes predominantly nuclear staining, *N>C* more nuclear than cytoplasmic staining, *C* predominantly cytoplasmic staining, (+) greater than 20-fold induction of luciferase at 0.1 nM R1881, (–) no significant induction of luciferase activity assayed at 0.1 nM R1881. Mutations are shown relative to the coding sequence numbering of hAR previously reported (60).

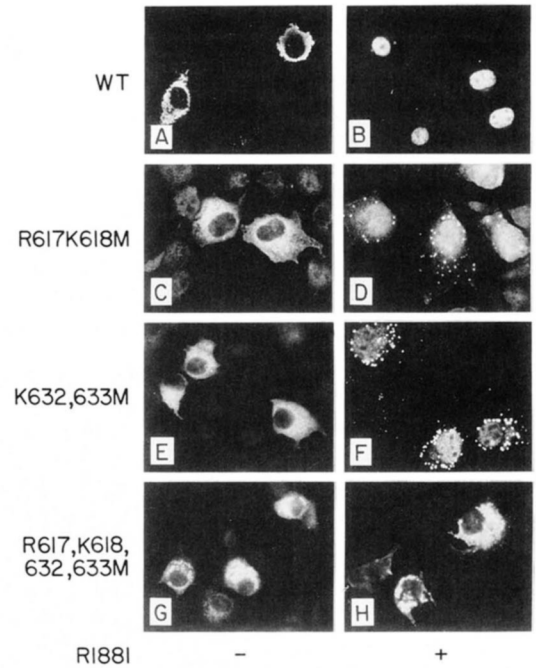


FIG. 2. **Immunocytochemical localization of wild type AR and targeting mutants expressed in COS cells in the presence and absence of androgen.** COS cells were transfected with wild type and mutant AR expression vectors described in Fig. 1 using the DEAE-dextran precipitation method. Twenty-four h after transfection, cells were placed in serum-free media and 100 nM dihydrotestosterone or R1881 was added. Androgen was readded the next day, 1 h prior to fixation. Cells were fixed and stained using the AR52 antibody described under "Experimental Procedures." Immunostaining is shown for cells either untreated (*A*, *C*, *E*, and *G*) or treated (*B*, *D*, *F*, and *H*) with androgen for wild type AR (*A* and *B*), R617K618M (*C* and *D*), K632,633M (*E* and *F*), and R617K618,632,633M (*G* and *H*). Magnification × 100.

ond approach is measurement of transcriptional activity in CV1 cells, the parent cell line for COS cells, since nuclear transport is a prerequisite for transcriptional activation.

Spanning the DNA-binding domain and hinge region of human AR at amino acid residues 617–633 are two clusters of basic amino acids separated by 10 amino acids, encoded by exons C and D, RKCYEAGMTLGARKLKK (Fig. 1). Based on homology with the nuclear targeting signal of nucleoplasmin (9), we investigated whether this region functions as a targeting sequence of the activated ligand-bound AR. PCR mutagenesis was performed to change the 5′ or 3′ basic residues to methionine either independently and together.

As shown in Fig. 2*A*, unliganded AR is perinuclear in the cytoplasm, and with added androgen, is nuclear (Fig. 2*B*). Mutants R617K618M and K632,633M, shown schematically in Fig. 1, were detected in the nucleus and as cytoplasmic granules in the presence of androgen (Fig. 2, *D* and *F*) indicating a reduction in nuclear import activity relative to wild type AR (Fig. 2*B*). The nature of the intensely stained cytoplasmic granules is currently under investigation. In a dose-response study, a reduction in transcriptional activity by the partial transport mutants was noted only at very low amounts of transfected DNA (0.5–5 ng of DNA); however, androgen-dependent transcriptional activity was equivalent to or greater than wild type AR with amounts of AR DNA between 10–50 ng (data not shown). Optimal transcriptional activity by wild type AR occurs between 0.05–0.1 μg of transfected AR DNA (data not shown).

When both right and left domains were mutated simultaneously, *i.e.* R617K618,632,633M, there was no detectable nuclear staining in the presence of androgen (Fig. 2*H*) and no transcriptional activity using 0.01–1 μg of transfected DNA (Fig. 1), indicating that both basic motifs contribute in targeting AR to the nucleus. In control studies in the absence of androgen, wild type AR and the three transport mutants were perinuclear in the cytoplasm (Fig. 2, *A*, *C*, *E*, and *G*).

The region of the targeting signal most similar to that of SV40 large T antigen is the right basic motif, consisting of four basic amino acids. Mutagenesis of 3 residues in the right domain, K630,632,633M alone, or in combination with R617K618M in the left domain, resulted in loss of androgen-dependent nuclear transport. Transcriptional activity was undetectable when assayed at 0.1 μg of transfected AR expression vector DNA (Fig. 1) or over a range of transfected DNA concentrations between 0.01–1 μg of DNA (data not shown). Thus, although replacing 2 residues in the left or right domains did not eliminate nuclear transport activity, the mutation of 3 residues in the right domain resulted in loss of transport activity. A similar predominance of the right basic domain was reported for the bipartite signal of nucleoplasmin (9). The results, summarized in Fig. 1, suggest the presence of a nuclear targeting sequence in human AR similar to that reported for nucleoplasmin, containing two basic amino acid clusters separated by 10 amino acids. Moreover, deletion of amino acids 615–633 containing this putative targeting region resulted in loss of nuclear transport and transcriptional activity in the presence of androgen (see Fig. 6).

Expression of wild type and mutant AR vectors was confirmed by immunoblot analysis (Fig. 3). While single or multiple point mutations described above did not alter the apparent 120-kDa molecular mass of AR (Fig. 3*A*), major deletions described below resulted in smaller forms of the receptor (Fig. 3, *B* and *C*). Approximate molecular weights of each mutant estimated from immunoblots are indicated. In addition, high affinity androgen binding was observed in COS cells expressing AR mutants that contained the full steroid-binding domain (data not shown).

*A Spacer Sequence Separating the Basic Motifs*—The effects of spacing and amino acid changes in the 10 amino acids be-

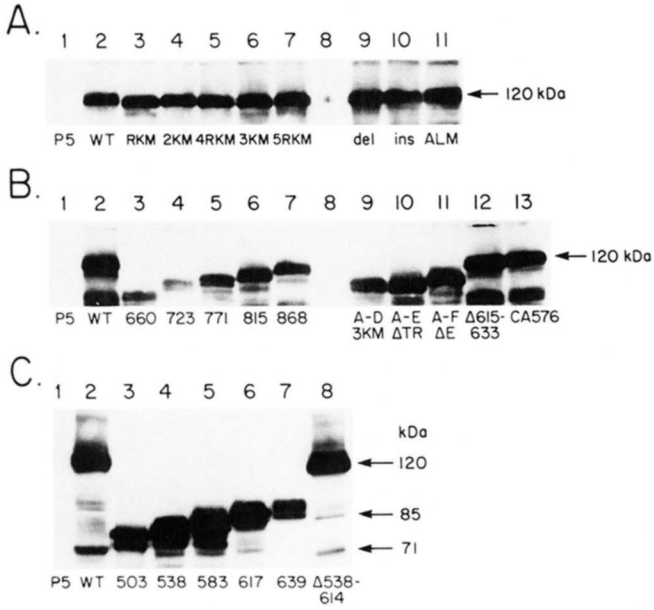


FIG. 3. **Immunoblot analysis of wild type and mutant ARs expressed in COS cells.** Wild type and mutant vectors were verified for expression in COS cells and for determination of apparent molecular weights. COS cells were transfected as described under "Experimental Procedures," and aliquots were analyzed on 8% polyacrylamide gels. Molecular weights were estimated relative to the migration of standard marker proteins. The following expressed wild type and mutant proteins are shown: *A*, pCMV5 parent expression vector lacking AR sequence (*P5*, *lane 1*); full-length pCMVhAR (*WT*, *lane 2*); R617K618M (*RKM*, *lane 3*); K632,633M (*2KM*, *lane 4*); R617K618,632,633M (*4RKM*, *lane 5*); K630,632,633M (*3KM*, *lane 6*); R617K618,630,632,633M (*5RKM*, *lane 7*); molecular weight markers (*lane 8*); deletion mutant lacking amino acids 621–625 (*del*, *lane 9*); insertion mutant containing five extra amino acids GPLGS at amino acid position 625–629 (*ins*, *lane 10*); A622L626M (*ALM*, *lane 11*). *B*, pCMV5 parent expression vector lacking AR sequence (*P5*, *lane 1*); full-length pCMVhAR (*WT*, *lane 2*); AR1–660 (*A-C/D'*, *lane 3*); AR1–723 (*A-D*, *lane 4*); AR1–771 (*A-E*, *lane 5*); AR1–815 (*A-F*, *lane 6*); AR1–868 (*A-G*, *lane 7*); molecular weight markers (*lane 8*); AR1–723,K630,632,633M (*A-D3KM*, *lane 9*); AR1–771Δ199–239 (*A-EΔTR*, *lane 10*); AR1–815Δ724–771 (*A-FΔE*, *lane 11*); ARΔ615–633 (*lane 12*); C576A (*lane 13*). *C*, pCMV5 parent vector (*P5*, *lane 1*); full-length human pCMVhAR (*WT*, *lane 2*); AR1–503 (*lane 3*); AR1–538 (*lane 4*); AR1–583 (*lane 5*); AR1–617 (*lane 6*); AR1–639 (*lane 7*); Δ538–614 (*lane 8*).

tween the basic clusters was investigated by analyzing three mutants. Mutant A622L626M, in which alanine 622 and leucine 626 were changed to methionine, displayed androgen-dependent nuclear transport and transcriptional activity indistinguishable from wild type AR (Fig. 4). Insertion of five amino acids, GPLGS (ins), after methionine 624 did not significantly alter transcriptional activity and resulted in predominantly nuclear staining in the presence of androgen with few cytoplasmic granules staining, suggesting that the insertion only slightly affected nuclear transport (Fig. 4). That one of the inserted amino acids was proline argues strongly against a strict sequence or structural requirement of the intervening region in nuclear targeting. Deletion of amino acid residues 621–625 (del) resulted in immunostaining like that observed for the insertion mutant, with predominantly nuclear staining in the presence of androgen, indicating that the five-amino-acid deletion also only partially disrupted transport (Fig. 4). However, the five-amino-acid deletion caused complete loss of transcriptional activity, the explanation for which is presently unclear (Fig. 4). With all three spacer mutants, immunostaining was cytoplasmic and transcriptional activity undetectable in the absence of androgen (not shown). Thus, none of the mutations within the 10-amino-acid spacer region resulted in significant loss of AR nuclear transport activity, suggesting that

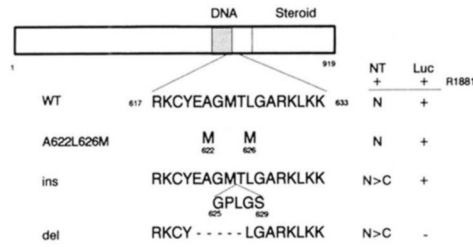


FIG. 4. **Nuclear localization and transcriptional activity of AR mutants in the intervening spacer region.** PCR mutagenesis of full-length AR (wild type, *WT*) was performed to create a double point mutation (*A622L626M*), an insertion mutation of amino acids GPLGS following M624 (*ins*), and a deletion mutation of amino acids 621–625 EAGMT (*del*). Nuclear localization of wild type and mutant ARs was determined in transfected COS cells in the presence of 100 nM DHT or R1881, and transcriptional activity in the presence of R1881 by cotransfection with a luciferase reporter plasmid in CV1 cells as described under "Experimental Procedures." The immunocytochemical evaluation of nuclear transport (*NT*) in the presence of androgen with *N* indicating predominantly nuclear and *N>C* more nuclear than cytoplasmic staining. Luciferase reporter gene activity (*Luc*) is indicated by (+) for greater than 40-fold induction of luciferase activity at 0.01 nM R1881, and (–) for no significant induction of luciferase activity. The mutations are shown relative to their position in the AR sequence.

the nuclear targeting signal is not strictly dependent on the number or sequence of amino acids within this spacer region.

*Inhibition by the Carboxyl-terminal Domain*—A series of deletion mutants lacking different regions of the carboxyl-terminal domain was created, several truncated at exon boundaries (see Fig. 5), to investigate the influence of this region on nuclear import in the absence of ligand binding. The smallest mutant, $NH_2$-terminal peptide AR1–503, migrated as a doublet between 71 and 76 kDa on immunoblots (Fig. 3*C*) likely due to differing degrees of phosphorylation.[2] Cytoplasmic immunostaining and absence of transcriptional activity (summarized in Fig. 5) was not unexpected since AR1–503 lacks the DNA binding, targeting signal, and steroid-binding domains.

Mutants that lacked the DNA-binding domain and targeting signal (AR1–538), or ones that included the first (AR1–583) or both zinc finger regions of the DNA-binding domain (AR1–617), but excluded the targeting region, were also predominantly cytoplasmic. However, punctate nuclear staining was detected in a pattern quite atypical for AR, indicating that, to a limited extent, these $NH_2$-terminal polypeptides are transported to the nucleus independent of the transport signal, perhaps through associations with other nuclear-bound proteins. Absence of transcriptional activity by these truncation mutants (Fig. 5) is consistent with their lack of a complete DNA-binding domain (30).

AR mutants that extended to include the putative targeting signal, AR1–639, and beyond, AR1–660 and AR1–723, were nuclear with intense staining and exhibited constitutive transcriptional activity similar to androgen-activated wild type AR (Fig. 5). However, sequence extension to include exon E in the steroid-binding domain, AR1–771, or further, AR1–815 (A-F) and AR1–868 (A-G), resulted in predominantly cytoplasmic immunostaining and loss of transcriptional activity (Fig. 5). When the inactive signal, K630,632,633M (see Fig. 1), was included in AR1–639 (AR1–639 3KM), immunostaining was predominantly cytoplasmic, but some punctate nuclear immunostaining persisted (data not shown). At transfected DNA concentrations of 0.1–1 μg of DNA, 1–639 3KM displayed transcriptional activity similar to ligand-activated wild type AR. $NH_2$-terminal AR fragments that lacked the targeting signal (1–538, 1–583, and 1–617, see Fig. 5) or contained a defective signal (1–639

[2] Z.-X. Zhou, and E. M. Wilson, unpublished studies.

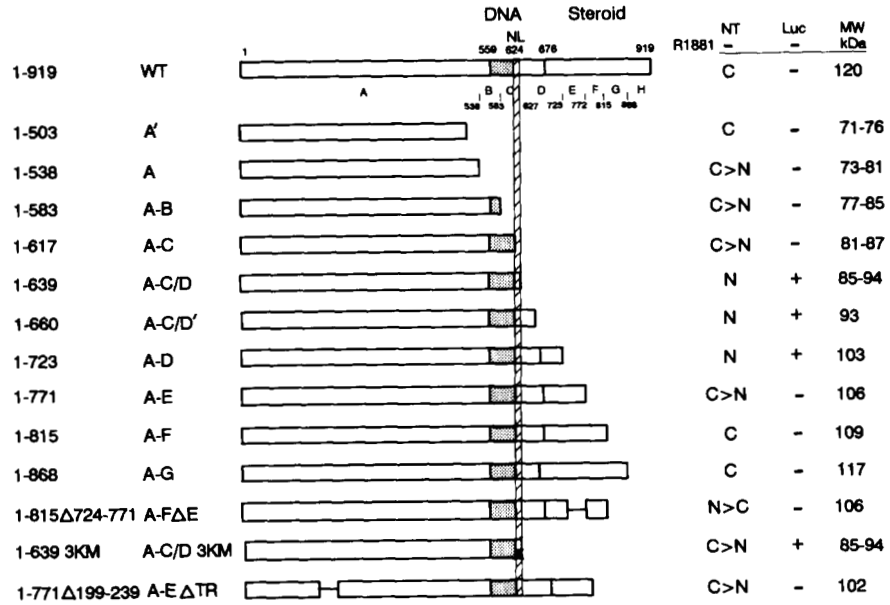


FIG. 5. **Nuclear localization and transcriptional activity of carboxyl-terminal AR deletion mutants.** PCR mutagenesis was performed to create a series of carboxyl-terminal deletion mutants, all of which lack androgen binding activity. Indicated at the left are amino acid residues expressed in each construct, followed by letter notation to reflect the encoded exons. Full-length AR is encoded by eight exons, *A-H* (61), where *A* is the $NH_2$-terminal domain, *B/C* the DNA-binding domain, part of *D* the hinge region, and *D-H* the steroid-binding domain. The mutants are diagrammed relative to wild type AR (*WT*) sequence. Nuclear transport (*NT*) was determined in the absence of androgen by transfection and immunostaining of COS cells as described. Luciferase activity (*Luc*) was determined in the absence of androgen by cotransfection with a reporter plasmid in CV1 cells as described. Approximate molecular weights (*MW*) were determined by immunoblot analysis (see Fig. 3). Those indicated as a range reflect the presence of multiple bands likely resulting from differing degrees of phosphorylation. *C* denotes predcminantly cytoplasmic staining, *N* predominantly nuclear staining, *C>N* more cytoplasmic than nuclear staining, and *N>C* more nuclear than cytoplasmic staining. Constitutive transcriptional activity assessed in the absence of androgen is indicated, where (+) implies activity essentially equivalent to wild type AR determined in the presence of androgen, and (–) implies no significant activity. The abbreviation *3KM* denotes mutation K630,632,633M. The putative nuclear targeting region is highlighted by the *hatched vertical column*.

3KM) were transported to nuclei at levels sufficient for transcriptional activation to occur if they retained a functional DNA binding domain (1–639 3KM).

The sequence requirement of the steroid-binding domain region and the effect of receptor size in inhibiting nuclear import were investigated by deleting exon E (724–771) from AR1–815 (A-F) to form A-FΔE, and by deleting residues 199–239 in the $NH_2$-terminal transactivation domain from AR1–771 (A-E) to form A-EΔTR. Interestingly, A-FΔE, which encodes a protein about the size of the predominantly cytoplasmic A-E (106 kDa), displayed partial nuclear staining and absence of transcriptional activity (Fig. 5), supporting a role for exon E in transport inhibition and exon F in transcriptional inhibition. To test whether the increase in size over that of A-D (see above) could account for transport inhibition by A-E, A-EΔTR, approximately the size of the predominantly nuclear A-D, when expressed, was predominantly cytoplasmic. The results suggest that exon E in the steroid-binding domain has a major role in inhibiting target signal activity in the absence of androgen binding. Comparison of results with A-E, A-F, and A-FΔE suggests, in addition, that sequences within exons E and F contribute to transport and transcriptional inhibition.

*Influence of $NH_2$-terminal Domain Deletions*—We examined the effects of $NH_2$-terminal domain deletions on AR nuclear targeting. As diagrammed in Fig. 6, deletion of 70 amino acids (Δ14–83, including the glutamine repeat region) did not significantly alter androgen-dependent nuclear targeting or transcriptional activation. However, deletion mutants Δ14–150 or Δ14–337 of approximately 81 and 61 kDa, respectively, were predominantly cytoplasmic, but with significant nuclear staining (15–25%) in the absence of androgen (Fig. 6). AR507–919, a 41-kDa receptor fragment, was predominantly nuclear in the absence of androgen (Fig. 6). Androgen-dependent transcriptional activity was retained by those mutants that contained the transactivation domain located between amino acids 151–338 (15). Thus, partial or complete deletion of the $NH_2$-terminal region resulted in AR nuclear transport in the absence of androgen despite the presence of the ligand-free, steroid binding region, raising the possibility that interactions between the $NH_2$- and carboxyl-terminal regions block target signal activity in the absence of androgen. An alternative possibility is that the effects of the $NH_2$-terminal deletions resulted from mutation-induced changes in protein folding.

We considered whether the shortest, 41 and 61 kDa, deletion mutants described above, AR507–919 and Δ14–337, were nuclear without hormone because of their size. To test this possibility, the defective targeting signal, R617K619,632,633M (see Fig. 1), was introduced into each expression vector. Both expressed mutant AR proteins were excluded from the nucleus in the absence or presence of androgen (Fig. 6) indicating that, at least for these two constructs, the target signal was required for nuclear import despite their relatively small size and loss of strict hormonal control of nuclear uptake.

*Chimeras of AR and Pyruvate Kinase*—To establish whether the putative AR nuclear targeting region 617–633 was sufficient to effect nuclear transport, chimeric proteins containing either known nuclear targeting sequences or portions of AR linked to the cytoplasmic protein, PK, were expressed from the pCMV expression vector (Fig. 7). Rabbit polyclonal PK antiserum was used to localize the chimeras in COS cells and revealed cytoplasmic staining of wild type PK (Fig. 8*A*). In control experiments, chimeric constructs containing nuclear import signals, c-*myc*/PK (Figs. 7 and 8*B*) and SV40 large T antigen/PK (Figs. 7 and 8*D*), were completely nuclear as previously reported (4, 6, 8).

The minimal putative AR targeting sequence linked to PK, 615–633/PK, resulted in predominantly cytoplasmic immunostaining (Figs. 7 and 8*E*), indicating that this region alone was

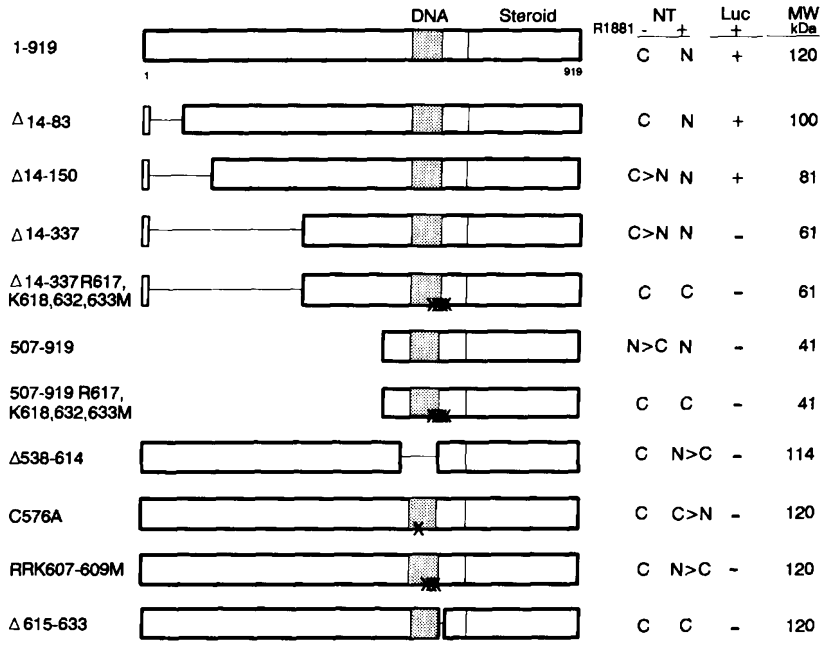


FIG. 6. **Nuclear localization and transcriptional activity of $NH_2$-terminal AR deletion mutants.** A series of $NH_2$-terminal deletion mutants was created by PCR mutagenesis. On the *left* are indicated amino acid residues expressed or deleted, where Δ denotes a deletion; on the *right* is summarized immunocytochemical nuclear transport (*NT*) determined in the absence and presence of 100 nM DHT or R1881. *N* denotes predominantly nuclear staining, *N>C* more nuclear than cytoplasmic staining, *C>N* more cytoplasmic than nuclear staining, and *C* predominantly cytoplasmic staining. Luciferase transcriptional activity (*Luc*) was determined in the presence of 0.1 nM R1881, and the approximate molecular weights by immunoblot analysis. Molecular weights were as previously reported (15) or shown in Fig. 3. x denotes the approximate position of single or multiple base mutations.

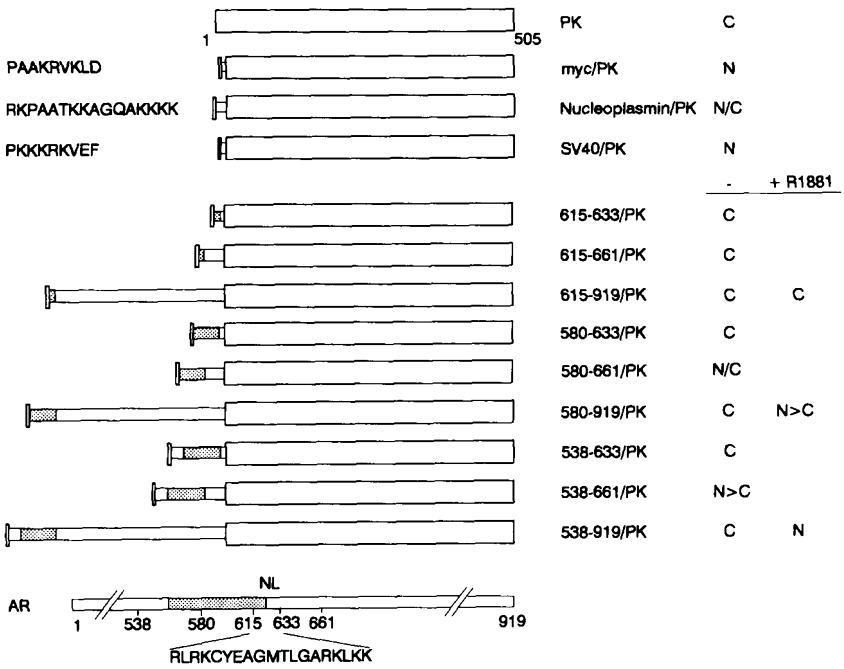


FIG. 7. **Nuclear targeting of AR/PK chimeras.** A series of AR/PK chimeric and control expression vectors was created in pCMV5 by inserting the regions of AR indicated on the right after amino acid codon 16 in PK at an internal *XhoI/Eco*RI site. Control vectors, myc/PK, nucleoplasm/PK and SV40/PK, were constructed to contain the sequences shown. Nuclear localization was determined by expression in COS cells and immunostaining using a PK antibody, where *N* denotes predominantly nuclear, *C* predominantly cytoplasmic, *N>C* more nuclear than cytoplasmic staining, and *N/C* a variable mixture of nuclear and cytoplasmic staining. For constructs that contained the full steroid-binding domain (through amino acid 919), immunostaining was determined in the presence and absence of 100 nM DHT or R1881. PK sequence is designated by the *open rectangle* and includes 505 amino acids. Sixteen $NH_2$-terminal PK amino acids precede the *Xho/Eco*RI insertion site (*small vertical rectangle*). The AR sequence is denoted by the *narrow rectangles*, portions of which in some constructs are *stippled* to indicate the DNA-binding domain. The schematic at the bottom denotes AR sequence.

not sufficient to function as an independent target sequence. In contrast, linking the bipartite nuclear targeting signal of nucleoplasmin caused varying degrees of nuclear and cytoplasmic staining (Figs. 7 and 8*C*). It was reported previously that this sequence was sufficient to target PK to the nucleus (9). The results suggest that AR and nucleoplasmin bipartite sequences may be less efficient nuclear import signals than the single domain sequences of c-*myc* and SV40 T antigen. The possibility that the activity of the AR bipartite signal is dependent on additional AR sequences was tested by expressing AR/PK chimeras that included $NH_2$- and carboxyl-terminal sequences flanking 615–633 (Fig. 7).

Extending the AR bipartite nuclear target sequence into the carboxyl-terminal domain to include flanking sequence (615–661) or the entire steroid-binding domain (615–919, Fig. 7) resulted in cytoplasmic immunostaining in the presence or absence of androgen (Figs. 7 and 8, *F-H*) as observed for 615–633/PK. In contrast, $NH_2$-terminal extension to include the second zinc finger region of the DNA-binding domain resulted in partial nuclear import for 580–661/PK in the absence of hormone (Fig. 8*J*) and for 580–919/PK upon androgen addition (Fig. 8, *K* and *L*). The extent of nuclear import was similar to that observed with nucleoplasmin/PK (Fig. 8*C*). Predominant nuclear staining (>85%) was also observed when the entire DNA-binding domain and flanking carboxyl-terminal sequence was included (amino acids 538–661) (Fig. 8*N*). The results suggest that the minimal AR sequence that promotes predominant nuclear staining like that observed with nucleoplasmin/PK is amino acids 580–661, comprised of the second zinc finger region of the DNA-binding domain, the nuclear targeting signal, and 28 amino acids toward the carboxyl terminus. Essentially complete nuclear staining like SV40/PK was observed when the entire DNA-binding domain and carboxyl-terminal region (538–919/PK) was tested in the presence of androgen (Figs. 7

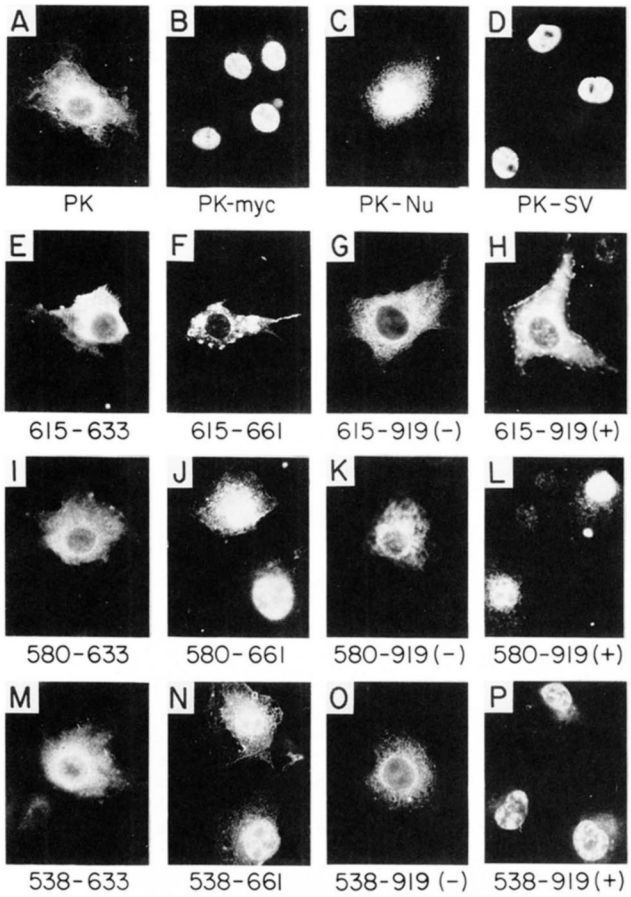


FIG. 8. **Immunocytochemistry of AR/PK chimeras and nuclear import control PK vectors expressed in COS cells.** The AR/PK and control constructs were expressed in COS cells by transient transfection. Immunostaining was determined in the absence of androgen for mutants lacking the steroid-binding domain, and for those constructs that contained the steroid-binding domain, in the absence and presence of 200 nM dihydrotestosterone or R1881. The chimeric proteins were localized using the PK antibody. Shown is the immunostaining for PK (*A*), myc/PK (*B*), nucleoplasmin/PK (*C*), SV40/PK (*D*), 615–633/PK (*E*), 615–661/PK (*F*), 615–919/PK (*G*), 615–919/PK with androgen (*H*), 580–633/PK (*I*), 580–661/PK (*J*), 580–919/PK (*K*), 580–919/PK with androgen, 538–633/PK (*M*), 538–661/PK (*N*), 538–919/PK (*O*), 538–919/PK with androgen. The constructs are diagrammed in Fig. 7. Magnification × 215.

and 8, *O* and *P*) indicating that the ligand-bound hormone-binding domain enhances, but is not essential, for nuclear transport.

As shown above, androgen promoted nuclear import of 580–919/PK and 538–919/PK containing the steroid-binding domain, but not 615–919/PK, which lacks the DNA-binding domain (Fig. 7). Immunostaining intensity (Fig. 8) and Western blot analysis (data not shown) indicated that expression levels of the chimeric proteins were sufficient for the measurement of ligand binding affinity. However, for all three constructs, androgen binding was only slightly above background in whole COS cell binding assays, suggesting that the binding affinity was near or above $10^{-8}$ M, the limit of detection of the assay. Previous androgen binding studies of AR deletion mutants indicated that $NH_2$-terminal deletions through amino acid residue 627 retain high affinity androgen binding activity (15). Mutant 580–919/PK was transported to the nucleus despite low androgen binding similar to that of 615–919/PK. It appears, therefore, that 615–919/PK failed to enter the nucleus in the presence of androgen because its lacks the second finger region of the DNA-binding domain.

*Role of DNA Binding in Nuclear Targeting*—Results from the AR/PK chimeras and AR mutants suggest that the DNA-binding domain contributes to nuclear targeting. To investigate whether loss of DNA binding activity interferes with nuclear import, we constructed three DNA-binding domain mutants, each of which caused loss of transcriptional activity. Deletion of the DNA-binding domain region, ARΔ538–614, resulted in partial disruption of nuclear import, but nuclear staining remained prominent in the presence of androgen (diagrammed in Fig. 6). Changing cysteine 576 to alanine in the first zinc finger, C576A, showed both cytoplasmic and nuclear staining in the presence of androgen, indicating some interference in nuclear import (Fig. 6). The C576A mutant lacked DNA binding activity when expressed as a truncated fragment in *E. coli* and assayed in the mobility shift assay with androgen response element DNA (data not shown). Conversion of RRK 607–609 to M in the second zinc finger region also resulted in both nuclear and cytoplasmic immunostaining in the presence of androgen. The results suggest that while the DNA binding region is involved in nuclear import, DNA binding *per se* is not an absolute requirement for transport activity.

The proximity of the DNA-binding domain to the targeting signal and its apparent role in nuclear import raised the question whether the target region could function in AR if displaced to another domain. To test this hypothesis, wild type nuclear targeting residues 615–633 were inserted into the *KpN*I site $NH_2$-terminal of the DNA-binding domain at residue 504 in mutant R617K618,632,633M (see Fig. 1) that contained a defective targeting signal. This insertion mutant, containing a nuclear targeting signal in the $NH_2$-terminal domain, failed to target to the nucleus in the presence of androgen (data not shown), suggesting further that its natural orientation and close proximity to the DNA-binding domain and 3′-flanking sequence are required for its function.

## DISCUSSION

In the present study, we establish the minimal amino acid sequence required to detect nuclear import of AR and identify those regions necessary for complete transport. Site-directed mutagenesis of human AR revealed a bipartite sequence that spans the DNA binding and hinge regions encoded by exons C and D. The sequence, RKCYEAGMTLGARKLKK, consists of two basic motifs that facilitate AR nuclear import. Two mutations in each domain or three mutations in the right basic motif resulted in undetectable nuclear import of the receptor. In contrast, single amino acid changes or small insertions within the spacer region did not block nuclear targeting, suggesting that the intervening sequence is not crucial to target signal function. However, using AR/PK chimeras, the 17-amino-acid signal sequence alone was not sufficient to target the cytoplasmic protein, PK, to the nucleus. To effect partial nuclear transport like that seen for nucleoplasmin/PK required the 17-amino-acid bipartite region together with sequences in the second zinc finger region of the DNA-binding domain in addition to 28 amino acid residues of flanking carboxyl-terminal sequence, *i.e.* residues 580–661. Including the entire DNA-binding domain, 538–661/PK, also resulted in predominantly nuclear immunostaining. It cannot be distinguished, however, whether the target sequence extends to include the DNA-binding domain and the flanking carboxyl-terminal sequence or whether the flanking sequences impose conformational constraints necessary for target sequence activity. Complete transport like that observed for SV40/PK and wild type AR in the presence of androgen required 538–919/PK, including the ligand-bound AR steroid-binding domain.

The putative AR targeting signal sequence differs from that of the SV40 large T antigen in that the latter consists of a single basic domain, PKKKRKVEF, which in our control studies and

those previously reported (4), efficiently targets PK to the nucleus. However, even with the SV40 T antigen targeting signal, the importance of protein context is evident in chimeric constructs of pyruvate kinase, where functional activity depends on the point of insertion (32). That these two types of signals, the single strong basic sequence of SV40 *versus* a weaker, more interdependent sequence of AR, differ in their effectiveness is supported by studies on the chicken progesterone receptor in transient heterokaryons. The progesterone receptor shuttles between nucleus and cytoplasm, whereas SV40 large T antigen remains nuclear, raising the possibility of a different type of interaction with the nuclear transport machinery (33). Bipartite targeting sequences of the AR type appear to rely on cooperation among different receptor domains to facilitate both nuclear/cytoplasmic shuttling and cellular regulation of the target signal. In the case of AR and the glucocorticoid receptor, nuclear targeting is regulated by steroid binding, suggesting that a conformational change secondary to ligand binding places the targeting signal in a functional orientation.

One mechanism that could modulate an adjacent sequence and thereby influence a nuclear targeting signal is phosphorylation. It was recently shown that the rate of nuclear import of SV40 large T antigen was determined by a flanking casein kinase II phosphorylation site (34, 35). The targeting signal of *S. cerevisiae* transcription factor SW15 harbors 3 nearby serine residues which undergo phosphorylation in a cell cycle-dependent manner and are phosphorylated when SW15 is cytoplasmic and dephosphorylated when nuclear (36). Phosphorylation was also implicated in nuclear transport and recycling of the glucocorticoid receptor (37). While hormone-induced phosphorylation was not a prerequiste for nuclear transport, inhibition of protein phosphatases trapped the glucocorticoid receptor in the cytoplasm (37). Several phosphorylation sites have been identified in AR, one of which is in the 28-amino-acid carboxyl-terminal region of the nuclear targeting signal sequence (38). As yet, changes in phosphorylation have not been correlated with changes in the function of the AR nuclear targeting signal.

Despite a similarity in putative nuclear targeting sequences among the steroid receptors, little is known about their functional dependence on other receptor domains. The estrogen receptor shares the arrangement of basic domains but differs from AR by being nuclear in the absence of hormone binding. Even the unliganded glucocorticoid receptor is observed as nuclear when overexpressed in certain cell lines (39, 40), invoking perhaps a role for cell-specific factors. Estrogen and progesterone receptors apparently contain multiple targeting signals, including the hormone-binding domain and regions rich in basic amino acids, that cooperate to effect receptor nuclear transport (41). In contrast to AR, the estrogen receptor targeting signal is not inhibited by its ligand-free hormone-binding domain (41). In AR the 48 amino acids of exon E contributed significantly to transport inhibition. However, deletions of the $NH_2$-terminal domain of AR resulted in partial release from the nuclear transport inhibitory effect of the unliganded steroid-binding domain. The estrogen receptor, which is constitutively nuclear, contains a short $NH_2$-terminal region, raising the possibility that ER lacks sequences in its $NH_2$ terminus required for transport inhibition, and that in the absence of androgen, a region within the AR $NH_2$-terminal domain interacts with the steroid-binding domain to effect transport inhibition. In this regard, it is noteworthy that the unliganded steroid-binding domain in the AR/PK chimeras inhibited nuclear targeting in the absence of the AR $NH_2$-terminal region. It is possible that in the AR/PK chimera, the targeting signal becomes inhibited by interaction with PK in a manner similar to the inhibition imposed by $NH_2$-terminal sequence in full-length AR. The apparent lowering in androgen binding activity by the PK chimeras suggests an intramolecular interaction between PK and the AR steroid-binding domain.

Specific protein/protein interactions might be involved in the inhibition or activation of nuclear transport. The unliganded hormone-binding domain might be recognized as improperly folded, promoting an association with Hsp70 (42). ATP-dependent selective transport through nuclear pores (43–46) requires the interaction of import proteins with nuclear targeting signals (43, 44, 47), including Hsp70 (48) and a number of nuclear localization signal-binding proteins (49–51). It is noteworthy, therefore, that Hsp70 (39, 52, 53) and Hsp90 (54–56) reportedly interact with members of the steroid receptor family (52, 57). Heat shock proteins may also function as anchors or negative regulators (58) to inhibit the activity of the targeting signal.

In conclusion, human AR contains a ligand-dependent nuclear targeting signal positioned immediately carboxyl-terminal of the DNA-binding domain consisting of two basic motifs and adjacent sequence in the DNA binding and hinge regions. The activity of the targeting signal is regulated by the ligand-binding domain and by sequences within the $NH_2$-terminal domain. Nuclear targeting could represent an important control point in gene regulation, where escape from nuclear import control might have a number of consequences, including possibly oncogenic activation (59).

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