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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Zhong-xun Zhou†, Madhabananda Sar‡, Jorge A. Simental†, Malcolm V. Lane†, and Elizabeth M. Wilson†

From the Laboratories for Reproductive Biology and the Departments of §Pediatrics, §Cell Biology and Anatomy, and ¶Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599

The amino acid sequence requirements for androgen-dependent androgen receptor nuclear import were determined by immunostaining transiently transfected 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, RKCYEAGMTLGar-KL-KK. 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 transcriptional 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, a nine-amino-acid sequence of predominantly basic residues (4). 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). Studied on the androgen receptor (AR) 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 acid need not be present in 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).

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† To whom correspondence should be addressed: Lab. for Reproductive Biology, CB#7500 MacNider Bldg., University of North Carolina, Chapel Hill, NC 27599. Tel.: 919-966-5159; Fax: 919-966-2203.
acids similar to the bipartite nuclear targeting signal of nucleo-
plasm was required for predominant nuclear localization of
AR. It spans two exons and includes portions of the DNA bind-
ing 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 NH2-
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 Collec-
tion; Dulbecco's modified essential medium with high glucose, with
or without phenol red from JRH Biosciences; bovine calf serum from Hy-
Clone Laboratories, Inc.; ECL Western blotting detection kit from Am-
ersham Corp.; unlabelled deoxynucleotide triphosphates from Pharma-
cia LKB Biotechnology Inc.; Sequenase from United States Biochemical
Corp.; unlabeled deoxynucleotide triphosphates from Pharma-
cia LKB Biotechnology Inc.; Sequenase from United States Biochemical
Corp.; deep-Vent polymerase and T4 DNA ligase from New England Biolabs; restriction en-
donucleases from Life Technologies, Inc., Promega, and New England Biolabs; protein
molecular weight standards from Life Technologies, Inc.; W-
OMAT-AR diagnostic x-ray film from Kodak; 6-mer in diabetic
Luminescence; Immobilon from Millipore; buffers and chemicals from
Fisher, EM Science, and Sigma.

Cell transfections—AR nuclear import was assessed
immunocytochemically in COS cells transfected with wild type or mu-
ant 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 pCMV-AR previously described (15) con-
tained the full-length coding sequence of human AR cloned in the
pCMV5 expression vector containing the cytoplasminovirus promoter
(26). For determination of transcriptional activation, CV1 cells were
used because low level plasmid expression enhances androgen sensitiv-
ity. Cells were plated at 4.5 x 104 cells/ml and cotransfected with
0.01-0.05 pg of wild type or mutant AR expression vectors unless
indicated otherwise, and 0.5 pg of luciferase reporter vector. The lucifer-
ase reporter vector, kindly provided by Ronald M. Evans, the Sask
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 pCMV-AR and luciferase reporter vectors in 0.5 ml of
0.25 M CaCl2 together with 0.5 ml of 0.28 M NaCl, 1.5 M NaHPO4,
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-hm 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, 1.0 mM CaCl2, 0.5 MX MgCl2, 0.9 M NaHPO4,
25 M 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 lytic buffer (Ligand Pharma-
cuticals) and supplemented with 8 mM MgCl2, 1.0 M dithiothreitol, and
0.4 M 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 adding 2.0 ml of reaction buffer (1 mg/ml T4 DNA ligase, 5 M
mg/ml bovine serum albumin, 15 mM glycylglycine, pH 7.8), 100 M
ml of cell lysate, and 100 M of 6-mer luciferin injected automatically.

Immunoblot Analysis—COS cells plated in 10-cm dishes were tran-
siently transfected with 10 pg of wild type or mutant AR expression
vector DNA using DEAE-dextran (5). 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 M R1881 was
added 24 h prior to harvest. Forty-eight h after transfection, cells were
washed in phosphate-buffered saline and harvested in 200 ml of 2% SDS
(5 M glycylglycine, 10 M Tris, pH 6.8, mercaptoethanol (4%) and
bromphenol blue (1%) were added and the samples boiled 5 min.
Forty-ml aliquots were analyzed by electrophoresis in 8% acrylamide
gels as described previously using AR52 (28) or AR32 (29) rabbit poli-
clonal anti-peptide antibodies. Protein bands were calibrated using
molecular weight markers phosphorylase b, bovine serum albumin, and
ovalbumin.

Immunocytochemistry—COS cells were transfected in two chamber

containing mutations in the dibasic domain of the DNA/hinge region. The 919-amino-acid human AR is comprised of the NH₂-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 conversion to basic residues to methionine either independently and together.

androgen 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, RKCYEAGMTLGARKKK (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. 2A, unliganded AR is perinuclear in the cytoplasm, and with added androgen, is nuclear (Fig. 2B). 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. 2B). 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. 2H) 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. 3A), 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-
pressed in expression in COS cells and for determination of apparent molecular weight markers.

Five extra amino acids GPLGS at amino acid position 625-629 were added to AR1-660 (A-CID', lune 3); K632,633M (2KM, lune 4); and AR1-771 (A-E, lune 8). 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 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, NH2-terminal peptide AR1-503, migrated as a doublet between 71 and 76 kDa on immunoblots (Fig. 3C) 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 NH2-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. NH2-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

**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.

**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.
expressed in each construct, followed by letter notation to create a series of carboxyl-terminal deletion mutants, all of which lack androgen binding activity. Indicated at the left are amino acid residues predominantly cytoplasmic, but with significant nuclear staining were investigated by deleting exon E (724-771) from AR1-815. Interestingly, A-FAE, which encodes a protein about the size of the predominantly cytoplasmic A-E (106 kDa), displayed partial nuclear staining and absence of transactivation 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-C, 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-FAE suggests, in addition, that sequences within exons E and F contribute to transport and transcriptional inhibition.

**Influence of NH2-terminal Domain Deletions**—We examined the effects of NH2-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 NH2-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 NH2- and carboxyl-terminal regions block target signal activity in the absence of androgen. An alternative possibility is that the effects of the NH2-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. 8A). In control experiments, chimeric constructs containing nuclear import signals, c-mycPK (Figs. 7 and 8B) and SV40 large T antigen/PK (Figs. 7 and 8D), 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 8E), indicating that this region alone was...
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 8C). It was reported previously that this sequence was sufficient to target PK to the nucleus (9). The activity of the AR-bipartite signal is dependent on additional AR sequences was tested by expressing AR/PK chimeric sequences that include NH2- 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, NH2-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. 8J) 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. 8C). 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. 8N). 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 and 8C).
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, AR538-614, 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 KpM site NH₂-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 NH₂-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, PKKRRKVEF, 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 mediate an adjacent sequence at the N-terminal end of the 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 prerequisite 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 carboxy-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 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 NH2-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 NH2-terminal region, raising the possibility that ER lacks sequences in its NH2 terminus required for transport inhibition, and that in the absence of androgen, a region within the AR NH2-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 NH2-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 NH2-terminal sequence in full-length AR. The apparent lowering in androgen binding activity by the PK chimeras suggests an intramolecular interaction between AR and the PK 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 NHR-terminal domain. Nuclear targeting chimeras 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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