Nuclear Localization and Export Signals of the Human Aryl Hydrocarbon Receptor*

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The aryl hydrocarbon receptor (Ahr) is a ligand-activated transcription factor that binds DNA in the form of a heterodimer with the Ahr nuclear translocator (hypoxia-inducible factor 1β). We found in this study that Ahr contains both nuclear localization and export signals in the NH2-terminal region. A fusion protein composed of β-galactosidase and full-length Ahr translates from the cytoplasm to the nucleus in a ligand-dependent manner. However, a fusion protein lacking the PAS (Per-Ahr nuclear translocator-Sim homology) domain of the Ahr showed strong nuclear localization activity irrespective of the presence or absence of ligand. A minimum bipartite Ahr nuclear localization signal (NLS) consisting of amino acid residues 13–39 was identified by microinjection of fused proteins with glutathione S-transferase-green fluorescent protein. A NLS having mutations in bipartite basic amino acids lost nuclear translocation activity completely, which may explain the reduced binding activity to the NLS receptor, PTAC58. A 21-amino acid peptide (residues 55–75) containing the Ahr export signal is sufficient to direct nuclear export of a microinjected complex of glutathione S-transferase-Ahr-green fluorescent protein. These findings strongly suggest that Ahr acts as a ligand- and signal-dependent nucleocytoplasmic shuttling protein.

The aryl hydrocarbon receptor (Ahr) binds a variety of environmentally important carcinogens, including polycyclic aromatic hydrocarbons and certain halogenated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-p-dioxin. Before binding ligands, Ahr is located in the cytoplasm as one component of a complex that has a molecular mass of about 280 kDa (1). This complex is composed of Ahr, two molecules of the 90-kDa heat shock protein, and possibly a 43-kDa protein (2). After ligand binding, Ahr dissociates from the complex and translocates to the nucleus (3). The heterodimer of Ahr and Ahr nuclear translocator (ARNT) constitutes a transcription factor and binds specific DNA sequences called XREs (xenobiotic-responsive elements) in the enhancer regions of the CYP1A1 and several other proteins involved in xenobiotic metabolism (4). Because these enzymes are involved in the metabolism of polycyclic aromatic hydrocarbons to active genotoxic metabolites, Ahr plays an important role in carcinogenesis caused by these compounds (5–7).

Because ARNT was first cloned as a factor required for ligand-dependent nuclear translocation of Ahr from the cytoplasm to the nucleus (8), the subcellular localization of ARNT was believed to be cytoplasmic. In fact, most ARNT was recovered in the cytosolic fraction by cell fractionation. However, immunohistochemical analysis has shown that ARNT is localized predominantly in the nucleus, regardless of the presence or absence of ligands (9, 10). This controversial subject was clarified by our recent study in which a nuclear localization signal (NLS) of the amino acid residues between 39 and 61 of human ARNT was found to be a novel bipartite type recognized by the two components of nuclear pore-targeting complex (11). Because the heterodimeric partner Ahr is present in the cytoplasm in the absence of ligands and translocates to the nucleus upon binding of ligands even in the ARNT-deficient cell line, Hepa-1 c4, these two subunits may translocate independently to the nucleus, where they may form a heterodimer to bind to the cognate DNA sequence, XRE. This finding prompted us to investigate the molecular translocation mechanism of Ahr from the cytoplasm to the nucleus in a ligand-dependent manner.

Active transport of proteins between the nucleus and cytoplasm is a major process in eukaryotic cells (12, 13). Transport of proteins across the nuclear pore is generally selective and signals-dependent. Active import of proteins into the nucleus requires the presence of NLSs. NLSs of various proteins identified so far can be classified mainly into two classes: 1) a single cluster of basic amino acids represented by the SV40 large T antigen NLS, and 2) a bipartite type, in which two sets of adjacent basic amino acids are separated by a stretch of approximately 10 amino acids (12, 13). On the other hand, nuclear export signals (NESs) have been found recently in the human immunodeficiency virus (HIV) Rev protein (14), a cAMP-dependent protein kinase (protein kinase A) inhibitor (PKI; 15), the fragile X mental retardation protein (FMRF) (16), and mitogen-activated protein kinase kinase (MAPKK) (17). The characteristic of the NESs defined was certain leucine-enriched amino acid stretches (18). Two novel signals found to direct both import and export were the M9 domain of human nuclear ribonucleoprotein A1 (19) and KNS of human nuclear ribonucleoprotein K (20).
The NLS-dependent nuclear import process requires at least four different proteins that act in a sequential manner with NLS-containing proteins. There appear to be several discrete steps in the import process which involve: 1) binding of the NLS receptors (importin α, karyopherin α, PTAC58) to an NLS; 2) complex formation in conjunction with importin β (karyopherin β, PTAC97); 3) targeting nuclear pore proteins; and 4) ATP/GTP-dependent translocation through the nuclear pore mediated by Ran (12, 13, 21). A number of NLS receptors have been identified recently, suggesting that there is a family of these NLS-binding proteins (22–28). Differential expression and secretion of amino acids 13–39, which completely overlaps the DNA binding domain of Ahr. We investigated the molecular mechanisms of the nuclear translocation of Ahr further using an in vitro system and also found signal-dependent nuclear export activity.

MATERIALS AND METHODS

Cell Cultures—Cell lines used for this study were the mouse hepatoma Hepa-1 clone Hepa 1c1c7, Hepa-1 c4 mutant, which lacks ARNT (hypoxia-inducible factor), generously provided by Dr. O. Hankinson, as well as HeLa cells, HeLa Hepa-1 clone Hepa 1c1c7, Hepa-1 c4 mutant, which lacks ARNT (hypoxia-inducible factor). Subsequent cloning into the pSV110 region of the pSV110 vector (Promega). The sequence of the construct was confirmed by sequencing using fluorescent-labeled SP6 and T7 primers, Automated Sequencing kits, and an A.L.F. II DNA sequence (Pharmacia Biotech Inc.). Subsequent cloning into the pSV-gal (Promega) was performed as described previously (11). The BglII-BglII fragment of the Ahr cDNA was ligated to the BglII site of the modified β-gal control vector to generate the β-gal/Ahr(1-848) vector.

Various portions of Ahr cDNA were amplified by polymerase chain reaction using the β-gal/Ahr(1-848) vector as a template and β-gal DNA polymerase with specific sets of primers to generate artificial BglII sites at both ends. The sequences of the primers used for the preparation of fragments of Ahr were as follows: F1, TTC AGA TCT GAC GGA AAG CCG and R6, TTC AGA TCT TCG CCT GGA AGG ATT TGA for Ahr(1-39); F36 and R54, TTC AGA TCT CCG CCT GGA AGG ATT TGA for Ahr(13-40); F36 and R54, TTC AGA TCT CCG GAT GTA TGA TGA TGA for Ahr(13-42); F36 and R54, TTC AGA TCT TCG GTC TCT ATG CCT GGA AGG ATT for Ahr(13-42)/R40A; F36 and R58, TTC AGA TCT TCG CTT GTC TCT ATG CCT GGA AGG ATT for Ahr(13-42)/R40A; F36 and R58, TTC AGA TCT TCG CTT GTC TCT ATG CCT GGA AGG ATT for Ahr(13-42)/R40A; F36 and R58, TTC AGA TCT TCG CTT GTC TCT ATG CCT GGA AGG ATT for Ahr(13-42)/R40A; F36 and R58, TTC AGA TCT TCG CTT GTC TCT ATG CCT GGA AGG ATT for Ahr(13-42)/R40A; F36 and R58, TTC AGA TCT TCG CTT GTC TCT ATG CCT GGA AGG ATT for Ahr(13-42)/R40A; F36 and R58.
cytosol. The nuclear rim targeting assay was performed by the addition of purified recombinant PTAC58 and PTAC97 proteins in the testing solution followed by incubation at 4 °C for 30 min. After incubation, the cells were fixed with 4% formaldehyde, and localization of GST-Ahr-GFP fusion proteins was examined under a fluorescent microscope.

**Binding Assay—**GST-Ahr-GFP proteins, which were adjusted with the same amounts by monitoring the absorbance at 280 nm, were incubated with glutathione-Sepharose 4B (Pharmacia) for 1 h at 4 °C and then washed three times with transport buffer. The Sepharose was incubated with purified PTAC58 and PTAC97 in transport buffer containing 1 mg/ml BSA, 1 mM of phenylmethylsulfonyl fluoride, and 0.05% CHAPS. After incubation for 1 h at 4 °C, the Sepharose was washed three times with transport buffer, added to lysozyme buffer (50 mM Tris-HCl, pH 8.3, containing 500 mM NaCl, 1 mM EDTA, 2 mM diithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride) and boiled for 10 min. The eluted proteins were separated by 8% SDS-polyacrylamide gel electrophoresis, and blotted onto a nitrocellulose membrane. After incubation with blocking buffer containing 3% gelatin in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 30 min, the filter was rinsed by TBS and incubated with anti-PTAC58 rabbit antibody (25 µL/h) for 1 h. The filter was washed three times with TBS containing 0.05% Tween 20 and then incubated with alkaline phosphatase-labeled anti-rabbit IgG (Bio-Rad) for 1 h. After washing with the same buffer followed by TBS, the bound antibodies were detected using an alkaline phosphatase conjugate substrate kit (Bio-Rad).

**Microinjection into Nuclei of MDBK Cells—**The amplified fragment of Ahr(55–75) was labeled with F57/R67 as primers encodes the 21-amino acid sequence of PFPQDVINKLDKLSVLRLSVS. GST-Ahr(55–75)wt-GFP or GST-Ahr(55–75)mut-GFP, which have the substitutions of Leu 70 and 72 for alanines, were injected into HeLa, Hepa-1, and ARNT-deficient Hepa-1 c4 mutant (Fig. 1C), showing that Arg13 and Lys14 were not essential for nuclear translocation activity. However, when the two separated basic regions were linked directly (Ahr 13–42, Ahr), the GST-Ahr-GFP fusion protein showed efficient nuclear localization within a 30-min incubation at 37 °C (Fig. 2B, d), confirming that the fragment serves as an NLS.

To determine the minimum length of NLS of Ahr, we first constructed a plasmid by insertion of the Ahr(13–42) fragment into the fusion gene of GST-GFP, using a minimum fragment containing two complete clusters of basic amino acids. A representative profile of nuclear localization of the microinjected fusion protein is shown in Fig. 3A, a). Next, GST-GFP fused proteins, which contain a series of deleted fragments from the COOH- or NH2-terminus of Ahr(13–42), were purified and microinjected into the cytoplasm of HeLa cells as described previously (11). As was seen for the transient expression of β-gal-GFP fusions (Fig. 2B, a and b), the GST-GFP fusion protein showed efficient nuclear localization in the absence of Ahr (Fig. 4A, a). When amino acid residues from Arg42 to Arg60 were deleted successively from the COOH terminus of Ahr(13–42), clear nuclear localization was observed (Fig. 3A, b-d). However, when a deletion from Arg42 to His39 was carried out, both nuclear and cytoplasmic localizations were observed (Fig. 3A, c). On the other hand, GST-Ahr(13–37)GFP did not translocate to the nucleus (Fig. 3A, f). We next constructed a deletion mutant of NH2-terminal Ahr13 and found clear cytoplasmic localization of the gene product (Fig. 3A, g), showing that Arg13 was unequivocally essential for the nuclear translocation of Ahr. Furthermore, when the two separated basic regions were linked directly (Ahr 13–42, A19–35), the fusion protein was localized in the cytoplasm (Fig. 3A, h), indicating that amino acids between 19 and 35 were also required as spacers to provide enough length between the two separated clusters of basic amino acids to interact with the receptor(s). Using an in vitro nuclear transport assay system (Fig. 3B), we also confirmed clear nuclear translocation activity in Ahr(13–39) that were incubated with cytosol (Fig. 3B, a), and mixture of purified proteins PTAC58, PTAC97, Ran, and p10 (Fig. 3B, c) in the presence of ATP at 37 °C. To conclude, the minimum length of Ahr NLS was estimated to be composed of 27 amino acid residues between Arg13 and His39.

**Essential Amino Acids Involved in Nuclear Translocation of Ahr—**To identify which amino acid residue(s) are essential for full NLS activity, mutational analysis in the region of Ahr(13–42) was performed, and the results obtained are shown in Fig. 4A. When substitutions of Arg13 to Lys14, and Arg20 to Ala were performed and the mutated gene products were microinjected into the cytoplasm of HeLa cells, they completely lost their nuclear translocation activity (Fig. 4A, b-d, respectively). The
A gene product with R16A was localized in both the nucleus and cytoplasm (Fig. 4A, e), and clear nuclear localization was observed in the case of fused protein containing K17A (Fig. 4A, f), thereby indicating that the three basic amino acids between residues 13 and 15 were essential in NLS activity as part of the basic amino acid cluster. An essential role of basic amino acids

Fig. 1. Panel A, subcellular localization of β-gal/Ahr(1–848) fusion protein in HeLa, Hepa-1, and Hepa-1 c4 mutant cells. An expression vector of β-gal/Ahr(1–848) fusion gene was delivered into the indicated cells by means of electroporation. After transfection, cells were cultured for 48 h with or without 1 μM MC. Next, the cells were fixed and stained with X-gal solution, and the subcellular localization of the fusion proteins was examined by microscopy. a, β-gal control vector; b, β-gal/SV40 NLS, a fusion of β-gal with the NLS of SV40 large T antigen; c, β-gal/Ahr(1–848). The hatched boxes represent PAS-A and PAS-B direct repeat. The dotted and solid boxes represent bHLH and clusters of basic amino acids, respectively. Panel B, identification of the region responsible for the nuclear localization of Ahr. Various portions of Ahr were synthesized using the polymerase chain reaction, and the resulting fragments were fused to the modified β-gal control vector. After transfection, cells were cultured for 48 h with or without 1 μM MC. a, β-gal/Ahr(1–848); b, β-gal/Ahr(1–427); c, β-gal/Ahr(1–119); d, β-gal/Ahr(120–386); e, β-gal/Ahr(387–848).
Lys\textsuperscript{37} and Arg\textsuperscript{38} in another part of bipartite NLS was similarly observed (Fig. 4A, i and j). The H39A mutant also had reduced the NLS activity (Fig. 4A, k), since both cytoplasmic and nuclear localizations were observed.

We developed great interest in Ser\textsuperscript{36}, which lies within a recognition sequence for phosphorylation by protein kinase C, since nuclear localization is often regulated by phosphorylation of Ser or Thr close to the cluster of basic amino acids (38). However, nuclear localization of the fused protein containing S36A and wild type protein were indistinguishable, indicating that Ser\textsuperscript{36} does not participate in the NLS activity (Fig. 4A, h).

**Mutations in the NLS of Ahr Resulted in Reduced Targeting to the Nuclear Rim**—Because only one amino acid located in the two basic regions (amino acid residues between 13 and 15, and residues 37 and 38) replaced by alanine resulted in complete loss of nuclear targeting activity of Ahr(13–42) as judged by microinjection (Fig. 4A), the effect of mutations on the nuclear transport activity was investigated in *vitro*. Fig. 4B shows a comparison of the binding activity between the wild type of GST-Ahr(13–39)\textsubscript{wt}-GFP and GST-Ahr(13–39)\textsubscript{mut}-GFP, which has the double mutation of R15A/R38A in Ahr(13–39), to PTAC58 in the presence of PTAC97. Ahr with double mutations R15A/R38A (Fig. 4B, lane 4) showed drastically reduced binding to the NLS receptor PTAC58 compared with Ahr(13–39)\textsubscript{wt} (Fig. 4B, lane 3).

Next, we analyzed the nuclear rim targeting activity of GST-Ahr(13–39)\textsubscript{wt}-GFP or GST-Ahr(13–39)\textsubscript{mut}-GFP using an *in vitro* nuclear transport assay (Fig. 4C). We observed clear targeting to the nuclear rim of GST-Ahr(13–39)\textsubscript{wt}-GFP (Fig. 4C, g) as well as the control substrate of GST-NLSc-GFP (Fig. 4C, f) incubated with purified PTAC58/PTAC97 at 4 °C. In contrast, however, there was no accumulation of GST-Ahr(13–39)\textsubscript{mut}-GFP to the nuclear rim (Fig. 4C, h). These results clearly indicate that the deficiency of nuclear localization of alanine-substituted Ahr in the two bipartite parts of NLS by microinjection (Fig. 4A) may be explained in part by reduced interaction between mutated NLS and NLS receptor PTAC58 resulting in the loss of nuclear rim targeting.

**Ahr Contains Both NLS and NES, Suggesting That It Functions as a Shuttle Protein**—During the course of experiments designed to elucidate the mechanism of nuclear translocation of Ahr, we found that Ahr has in its helix 2 region a short sequence of NES that regulates nuclear export of some proteins. The core sequence of NES, like the NES of PKI, Rev, or MAPKK, is rich in leucine residues, which were found to be crucial for NES activity (Fig. 5A). To test whether the leucine-rich sequence in Ahr can act as an NES, the fused protein of GST-GFP containing Ahr(55–75)\textsubscript{wt} was expressed in BL21 and purified by glutathione-Sepharose. When injected into the nucleus of MDBK cells, the GST-Ahr(55–75)\textsubscript{wt}-GFP was found to be present in the cytoplasm and excluded from the nucleus almost completely within 30 min (Fig. 5B, a). In contrast, co-injected Texas Red-labeled BSA localized in the nucleus (Fig. 5B, b). The nuclear export of GST-Ahr(55–75)\textsubscript{wt}-GFP was sensitive to low temperature; it remained in the nucleus at 4 °C (Fig. 5B, c). Because the two leucines in the core sequence of NES were shown to be prerequisite for NES activity (15), we introduced substitutions to the corresponding amino acids Leu\textsuperscript{70} and Leu\textsuperscript{72} for Ala. When injected into the nucleus, the GST-Ahr(55–75)\textsubscript{mut}-GFP was unable to cross the nuclear membrane and remained in the nucleus (Fig. 5B, e) as well as Texas Red-labeled BSA (Fig. 5B, f). Furthermore, when GST-Ahr(55–75)\textsubscript{mut}-GFP was injected into the nucleus together with an excess amount of NES peptide of PKI conjugated with BSA (Fig. 5B, g), the export of GST-Ahr(55–75)\textsubscript{mut}-GFP from the nucleus was inhibited completely. In contrast, the same amount of BSA alone did not inhibit the nuclear export of GST-Ahr(55–75)\textsubscript{wt}-GFP to any extent (Fig. 5B, h). Therefore, it is likely that the nuclear export of GST-Ahr(55–75)\textsubscript{mut}-GFP may be a signal-dependent active transport mediated by NES-binding protein(s) (39, 40).

**DISCUSSION**

Ahr and ARNT belong to the same bHLH/PAS family and have similar modular structures (8, 41, 42). The bHLH domains are located toward the amino termini of the proteins; the HLH regions mediate dimerization between Ahr and ARNT, whereas the basic regions are involved in DNA recognition by...
FIG. 3

Nucleocytoplasmic Shuttling of Ahr

A

a. \( R\text{-K-R-R-K-P} \quad S\text{-K-R-H-R-D-R} \)

b. \( R\text{-K-R-R-K-P} \quad S\text{-K-R-H-R-D} \)

c. \( R\text{-K-R-R-K-P} \quad S\text{-K-R-H-R} \)

d. \( R\text{-K-R-R-K-P} \quad S\text{-K-R-H} \)

e. \( R\text{-K-R-R-K-P} \quad S\text{-K-R} \)

f. \( R\text{-K-R-R-K-P} \quad S\text{-K} \)

g. \( K\text{-R-R-K-P} \quad S\text{-K-R-H-R-D-R} \)

h. \( R\text{-K-R-R-K-P} \quad S\text{-K-R-H-R-D-R} \)

i. ..........................

B

a. ..........................

b. ..........................

c. ..........................

d. ..........................

Fig. 3
the Ahr-ARNT heterodimer. The PAS regions, two repeats homologous with Drosophila Per and Sim proteins, may contribute to Ahr-ARNT dimerization and ligand/hsp90 binding. The carboxyl regions of Ahr contain a transactivation domain that contributes to transcriptional control by the Ahr-ARNT complex. The heterodimer Ahr-ARNT complex recognizes the cis-acting DNA sequence termed XREs, which acts upstream of several drug-metabolizing enzymes including the CYP1A1 gene, to induce transcription (5, 7, 43). The Ahr-ARNT system is also thought to mediate the various biological effects of

FIG. 3. Panel A, determination of minimum NLS of human Ahr by microinjection. The affinity-purified recombinant protein GST-Ahr-GFP was microinjected into the cytoplasm of HeLa cells. After incubation at 37 °C for 30 min the cells were fixed, and the localization of microinjected proteins was examined by fluorescent microscopy. a, Ahr(13–42); b, R13A; c, K14A; d, R15A; e, R16A; f, K17A; g, P18A; h, S36A; i, K37A; j, R38A; k, H39A; l, R40A. Panel B, effect of alanine substitution in the bipartite regions of Ahr on the binding to PTAC58. The same amounts of GST-Ahr(13–39)_mut-GFP and GST-Ahr(13–39)_wt-GFP, which has double mutations of R15A/R38A, were incubated with glutathione-Sepharose as described under “Materials and Methods.” GST-GFP alone or GST-NLSc-GFP was used as a negative or positive control, respectively. The binding activity to PTAC58 in the presence of PTAC97 was analyzed by Western blotting probed with antibody against PTAC58 followed by alkaline phosphatase-labeled anti-rabbit IgG. Lane 1, GST-GFP; lane 2, GST-NLSc-GFP, a fusion protein of the core sequence of NLS of SV40 large T antigen with GST-GFP; lane 3, GST-Ahr(13–39)_mut-GFP; lane 4, GST-Ahr(13–39)_wt-GFP. The arrow indicates PTAC58. Panel C, effect of alanine substitutions on the nuclear rim targeting activity. In vitro nuclear rim targeting activity was analyzed as described under “Materials and Methods.” GST-GFP (a and e), GST-NLSc-GFP (b and f), GST-Ahr(13–39)_mut-GFP (c and g), and GST-Ahr(13–39)_wt-GFP (d and h) were incubated with buffer only (a–d) or with PTAC58 and PTAC97 (e–h) at 4 °C for 30 min. After incubation, the cells were fixed, and the location of fused proteins was examined.

FIG. 4. Panel A, effect of alanine substitution in NLS of the Ahr on the nuclear translocation activity. Various positions of amino acids between residues 13 and 42 of Ahr were replaced by alanine as described under “Materials and Methods.” The affinity-purified recombinant proteins were microinjected into the cytoplasm of HeLa cells. After incubation at 37 °C for 30 min the cells were fixed, and the localization of microinjected proteins was examined by fluorescent microscopy. a, Ahr(13–42); b, R13A; c, K14A; d, R15A; e, R16A; f, K17A; g, P18A; h, S36A; i, K37A; j, R38A; k, H39A; l, R40A. Panel B, in vitro nuclear transport assay of Ahr(13–39). GST-Ahr(13–39)-GFP was incubated with buffer only (a), cytosol with ATP (b), or with a mixture of purified PTAC58, PTAC97, Ran, and p10 in the presence of ATP (d) at 37 °C for 30 min as described under “Materials and Methods.” The experimental condition in c was the same as that in b except for the incubation temperature of 4 °C.
2,3,7,8-tetrachlorodibenzo-p-dioxin-like environmental pollutants, including carcinogenesis, teratogenesis, tumor promotion, and immunosuppression (44, 45). In fact, recent reports showed that disruption of the Ahr or ARNT gene caused impairment of the immune system and liver fibrosis (46) or abnormal angiogenesis (47) in mice, respectively, suggesting that the Ahr-ARNT system participates essentially in developmental processes.

Because the first step of these biological effects seems to be caused by signal transduction mediated by activated Ahr, investigation of nuclear translocation of Ahr and its heterodimer partner ARNT should provide useful information on the regulation of gene transcription. We recently showed a clear nuclear localization of ARNT in the absence of ligands to Ahr and identified an NLS of amino acid residues 39–61 (11). These observations led to the notion that Ahr translocates ligand and NLS dependently from the cytoplasm to the nucleus irrespective of ARNT. We observed ligand-dependent nuclear translocation of full-length Ahr (Fig. 1A), but ligand-independent nuclear translocation activity was observed when the PAS domain was deleted (Fig. 1B). These observations support the notion that the NLS of Ahr is masked by hsp90 molecules in the absence of ligands, resulting in disturbance of recognition by its NLS receptor(s). On the contrary, when ligand is present in the cells, Ahr dissociates from the two molecules of hsp90 resulting in unmasking of the NLS, allowing it to translocate to the nucleus with formation of a nuclear targeting complex. A detailed analysis on the mechanisms of ligand-dependent nuclear translocation is now under investigation.

Ahr contains both NLS (13–39) and NES (55–75) in the NH2-terminal region as shown in Fig. 6, in which the previously reported DNA binding domain is also shown (48–51). In the case of ARNT, two basic amino acid clusters are separated by 30 amino acid residues; these participated in different functions with one serving as a variant bipartite core of NLS (39–61) (11) and the other (91–102) involved in binding to DNA (48, 49, 51). Because it has been reported that about 80% of the nuclear acid-binding proteins contain overlapping or flanking (less than 10-amino acid separation) NLSs and DNA or RNA binding regions (52), the distal location between NLS and the DNA binding domain in ARNT is a rare case. On the other hand, a typical bipartite NLS of Ahr (13–39) overlaps completely with its DNA binding domain of amino acid residues 10–44 (48, 49). Although ARNT and Ahr belong to the same bHLH/PAS family, the different assignment of the two basic amino acid clusters near the NH2 terminus may be explained by a different evolutionary aspect of the association between NLS and DNA binding motifs in one modular domain (52). Furthermore, it is noteworthy that some basic amino acids (Arg15, Lys37, His39) and a spacer region (residues 19–35) between the two basic amino acid clusters are required both for NLS function (Figs. 3A and 4A) and DNA binding activity (48) of the Ahr. The complete loss of nuclear targeting activity of the mutants in basic amino acids correlates with reduced nuclear rim targeting activity as shown in Fig. 4C, thereby confirming the requirement of these basic amino acids for the first step of nuclear localization as shown previously with ARNT (11). In addition, the intervening amino acids may be critical for establishing a precise protein conformation of Ahr not only for the association between NLS and NLS receptor(s), but also for the protein-DNA interaction.

The finding of a functional leucine-rich NES in Ahr (Fig. 5) might provide new insight into the mechanisms of Ahr-mediated gene regulation. It is of interest that the core sequence of the NES (residues 64–72) was localized in the helix 2 region of Ahr, which is involved essentially in heterodimer formation.

![Fig. 5. Nuclear export activity of the human Ahr. Panel A, comparison of determined NES of human Ahr with the corresponding region among various species of proteins. Bars indicate gaps to give maximum matching among these proteins. Conserved leucines are shown by bold enclosed in shaded boxes. Panel B, micrographs of MDBK cells 30 min after nuclear injection of GST-Ahr(55–75)_GFP. Mixtures of GST-Ahr(55–75)_GFP and Texas Red-labeled BSA (a–d) or GST-Ahr(55–75)_GFP and Texas Red-labeled BSA (e and f) were microinjected into MDBK cell nuclei. After injection and incubation for 30 min at 37°C (a and b; e and f) or 4°C (c and d), the cells were fixed, and the localization of microinjected proteins was examined by fluorescent microscopy. Experimental conditions were the same as in a and b except that an excess amount of NES peptide of PKI conjugated with BSA (g; final concentration of NES-BSA conjugate was 50 mg/ml) or BSA alone (h, final concentration of BSA was 50 mg/ml) was included.](http://www.jbc.org/content/280/9/2902.full)
with ARNT (53). When activated, Ahr translocated to the nucleus to induce transcription by Ahr-ARNT-XRE complex formation, hence it is likely that the NES may be subject to steric hindrance masking resulting in disturbance of interaction with NES receptor(s). When inducible gene expression of target genes, such as CYP1A1, has proceeded and Ahr dissociates from the Ahr-ARNT heterodimer complex, a presumable NES receptor(s) might recognize this common leucine-rich domain of the NES to export Ahr from the nucleus (39, 40). That the NES peptide of PKI inhibited the nuclear export of GST-Ahr(55–75)wt-GFP suggests the existence of a common mechanism of nuclear export using a leucine-rich NES as shown in MAPKK (17). Although the biological significance of the NES in Ahr should be elucidated in more detail in the future, it is important to note that no other bHLH/PAS proteins except for Ahr have been found to contain the NES motif at present. To conclude, subcellular distribution of Ahr may be regulated by masking and unmasking of the two different signals of NLS and NES in response to ligands, resulting in nucleocytoplasmic shuttling of the protein.

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