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Nucleocytoplasmic Shuttling of the Aryl Hydrocarbon Receptor[1]

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The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that acts in concert with the AhR nuclear translocator (ARNT), and alters gene expression in response to environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). We have previously shown that AhR contains both a nuclear localization signal (NLS), AhR(13-39), and a nuclear export signal (NES), AhR(55-75), in its NH_2-terminal region. In this study, we obtained direct evidence for the nucleocytoplasmic shuttling of AhR and show the biological significance of the shuttling in terms of the transcriptional activation of its target gene, *CYP1A1*. When AhR(13-75) fused with glutathione *S*-transferase (GST)–green fluorescent protein (GFP) was microinjected into the nucleus of a polykaryotic of BHK21 cell, the GST-AhR(13-75)-GFP migrated from one nucleus to the other. This event, nucleocytoplasmic shuttling, was completely inhibited in the presence of leptomycin B (LMB). The interaction between chromosome region maintenance 1 (CRM1) and endogenous AhR was shown by immunoprecipitation with antibodies to AhR followed by immunoblot analysis with antibodies to CRM1. The inhibition of the nuclear export of AhR by LMB repressed the transcriptional activation of the *CYP1A1* gene. The findings suggest that nuclear-cytoplasmic shuttling of AhR is essential for the inducible expression of the CYP1A1 protein.

Key words: aryl hydrocarbon (TCDD) receptor, nucleocytoplasmic shuttling, CYP1A1, AhR nuclear translocator (ARNT), leptomycin B.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is one of the most potent toxic contaminants in the environment, and has various biological effects upon animals, including carcinogenicity, teratogenicity, immune suppression, liver toxicity, and developmental or reproductive toxicity. In humans, TCDD exposure causes not only chloracne, but also tumor promotion, immunotoxicity and birth defects (*1–3*). Recent studies on aryl hydrocarbon receptor (AhR) knockout mice indicated that most TCDD-induced toxicity is mediated by AhR (*4*, *5*). This receptor is a ligand-activated transcription factor, and acts in concert with the AhR nuclear translocator (ARNT) to bind the xenobiotic responsive element (XRE). The AhR-ARNT complex bound to the XRE motif results in the transcriptional activation of certain drug-metabolizing enzyme genes such as *CYP1A1* (*6*). Because the first step in these biological effects seems to be signal transduction by activated AhR, investigation of cytoplasmic-nuclear trafficking of AhR should provide useful information about the mechanism of TCDD toxicity (*7*).

Prior to ligand binding, AhR locates in the cytoplasm as one component of a macromolecular complex, comprising AhR, HSP90 (*8*, *9*) and ARA9, which closely resembles the FK506 binding protein (*10*). After ligand binding, AhR translocates into the nucleus (*11*) with HSP90 attached (*12*). In order to elucidate the molecular mechanism of nuclear transport of AhR, we previously investigated the subcellular localization of AhR by means of the transient expression of chimeric constructs of β-galactosidase (β-gal) and full-length AhR in the presence or absence of ligand, and found the ligand-dependent nuclear translocation of the fused protein (*7*). Subsequent analysis of various portions of AhR using β-gal fusions as well as the fusion protein with GST-GFP (green fluorescent protein) gave a minimum bipartite nuclear localization signal (NLS) comprising amino acids 13–39, which overlaps with the DNA binding domain (*13*, *14*). On the other hand, we found clear nuclear localization of ARNT in the absence of exogenous ligands for AhR (*15*), and identified its NLS as amino acid residues

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Abbreviations: AhR, aryl hydrocarbon receptor; ARNT, AhR nuclear translocator; NLS, nuclear localization signal; NES, nuclear export signal; bHLH, basic-helix-loop-helix; XRE, xenobiotic responsive element; PAS, Per-ARNT-Sim homology region; TCDD, 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin; MC, 3-methylcholanthrene; GST, glutathione *S*-transferase; GFP, green fluorescent protein; LMB, leptomycin B; β-gal, β-galactosidase; PCR, polymerase chain reaction; CRM1, chromosome region maintenance 1.

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39–61, the bipartite core of which is distinct from the DNA binding domain (*13*, *14*, *16*). As in the case of the well-characterized NLS of the SV40 T-antigen, the NLSs for both AhR (*7*) and ARNT (*15*) are recognized by two components of the nuclear pore targeting complex, importin α and importin β, and are targeted to the nuclear pore. These findings strongly suggest that AhR and ARNT translocate to the nucleus, where they may form a heterodimer to bind to the XRE sequence in target genes.

We also found a nuclear export signal (NES) in AhR (*7*), providing new insight into the mechanism of AhR-mediated TCDD toxicity. A 21–amino acid region, AhR(55-75), was sufficient to direct the nuclear export of a microinjected complex, GST-AhR-GFP, and should contain NES; NES is often a leucine-rich sequence in which the leucine residues are critical for targeting proteins out of the nucleus (*17*). Molecular mechanisms governing NES-dependent nuclear export have recently been reported: a chromosome region maintenance 1 protein (CRM1), which shares sequence homology with the importin β family, has been shown to participate in nuclear protein export as an NES receptor, and an antifungal antibiotic leptomycin B (LMB) specifically blocks the export of NES-containing proteins (*18–20*). Since the nuclear export activity of AhR(55-75) can be inhibited by replacing leucines with alanines in the core sequence of NES (*7*), we postulated that CRM1 is involved in the nuclear export of AhR and that AhR shuttles between the cytoplasm and nucleus.

In this study, we obtained direct evidence for the nucleocytoplasmic shuttling of AhR by microinjection of GST-AhR(13-75)-GFP into polykaryotic of BHK21 cells (*21*). Furthermore, we showed that CRM1 is involved in the nuclear export of AhR. The biological role of this shuttling in terms of transcription of the *CYP1A1* gene is also shown.

MATERIALS AND METHODS

Cell Culture—The human leukemic cell line U937 was maintained in RPMI-1640 supplemented with 10% fetal calf serum. Mouse hepatomas Hepa 1c1c7 and Hepa-1 c12, and BHK21 derived from hamster kidney were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C under a 5% CO_2 atmosphere.

Preparation of the GST-AhR-GFP Fusion Protein—To construct a GST-AhR-GFP fusion gene, a GST-GFP2 cassette vector was prepared as described previously (*15*). AhR(13-75) was amplified by means of a polymerase chain reaction (PCR) using the β-gal/AhR(1-848) vector as a template and pfu DNA polymerase (*7*). The sequences of the primers used were as follows: F36, TAT AAG ATC TGC CGC AAG CGG CGG AAG CCG, and R67, TTC AGA TCT ACT GAC GCT GAG CCT AAG AAC. After cleavage with *Bgl*II, the resultant fragment was ligated into the *Bam*HI site of the GST-GFP2 vector as described previously (*15*). The GST-AhR(13-75)-GFP vector was introduced into *Escherichia coli* strain BL21. Purification of the expressed protein was carried out as described previously (*15*).

Cell Fusion with the Hemagglutinating Virus of Japan (HVJ, Sendai Virus)—BHK21 cells grown on a coverglass (2×10^4/35 mm dish) were washed with ice-cold BSS-Ca (10 mM Tris-HCl, pH 7.6, 140 mM NaCl, 5.4 mM KCl, and 2 mM $CaCl_2$), and then incubated with UV-inactivated HVJ (500–1,000 hemagglutinating units/ml) in BSS-Ca on ice for 30 min. After washing with BSS-Ca, the cells were transferred to fresh medium and incubated at 37°C. The cells started to fuse within 20 min after transfer (*21*).

Microinjection Experiments Involving Fused BHK21 Cells—The purified GST-AhR(13-75)-GFP protein was microinjected into the cytoplasm or nucleus of fused BHK21 cells along with Texas Red–labeled BSA, which was coinjected to the site of injection. The number of cells injected was about 30. After injection, the cells were incubated at 37°C for 2 h and then fixed in 4% formaldehyde. The localization of the injected protein was investigated under a fluorescence microscope.

Immunoprecipitation and Immunoblotting—Cells were washed with PBS and then suspended in MENG buffer (25 mM MOPS, pH 7.5, 2 mM EDTA, 0.02% NaN_3, 10% glycerol) supplemented with 2% NP-40 and 0.5 M NaCl. The cell suspension was homogenized and then incubated on ice for 1 h. After centrifugation at 12,000 rpm for 5 min, the supernatant was incubated with anti-AhR antibodies (ABR, CO) or normal mouse IgG, followed by the addition of protein A Sepharose. The immunocomplex was washed with MENG buffer and the final pellet was dissolved in lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% lithium dodecyl sulfate, 12.5% glycerol, 2 mM EDTA, 0.005% bromophenol blue). The protein samples were separated by SDS-PAGE in an 8% acrylamide gel and then transferred electrophoretically to a nitrocellulose membrane (BIO-RAD). The membrane was washed with TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) and then immersed in TBS containing 3% gelatin for 30 min at room temperature. The membrane was incubated for 1 h with anti-CRM1 antibodies (*22*) diluted with TTBS (TBS containing 0.05% Tween 20) containing 1% gelatin. The membrane was washed three times with TTBS, and then incubated with a 1:1,000 dilution of alkaline-phosphatase conjugated anti–rabbit IgG antibodies for 1 h at room temperature. Following incubation the membrane was washed twice with TTBS and then with TBS, and then color development was performed with an AP Conjugate Substrate Kit (BIO-RAD).

DNA Transfection and CAT Assaying—phMC carrying the 5′ upstream region of the human *CYP1A1* gene fused to the CAT gene (*23*) was transfected into Hepa 1c1c7 cells by the calcium phosphate-DNA precipitation method. Hepa-1 c12 cells were cotransfected with phMC and the pSVL-AhR expression plasmid by the same method. Cells were collected 48 h after transfection. Cellular protein was extracted into 25 mM Tris-HCl, pH 8.0, by 3 cycles of freeze-thawing using dry ice-ethanol, and the same amount of protein (150–200 μg) was used. The CAT assay was carried out as described previously (*24*).

Site-Directed Mutagenesis—We used the PCR-based site-directed mutagenesis approach. Mutagenesis of Leu-70 to Ala and Leu-72 to Ala in human AhR cDNA was performed with Mutan-Super Express Km (TaKaRa). The sequences of the primers used were 5′-CTTTCAGTTGCTAGGCTCAG-3′ and 5′-GTTCTTAGGGCCAGCGTCAG-3′ for the replacement of Leu-70 and Leu-72 by Ala, respectively. The synthesized PCR fragment was used to replace the corresponding wild type fragment, and then the full-length AhR mutant was transferred into the pSVL expression vector (Amersham Pharmacia Biotech).

Gel Mobility Shift Assay—Human wild or mutant type AhR and murine ARNT cDNAs in pRc/CMV were tran-

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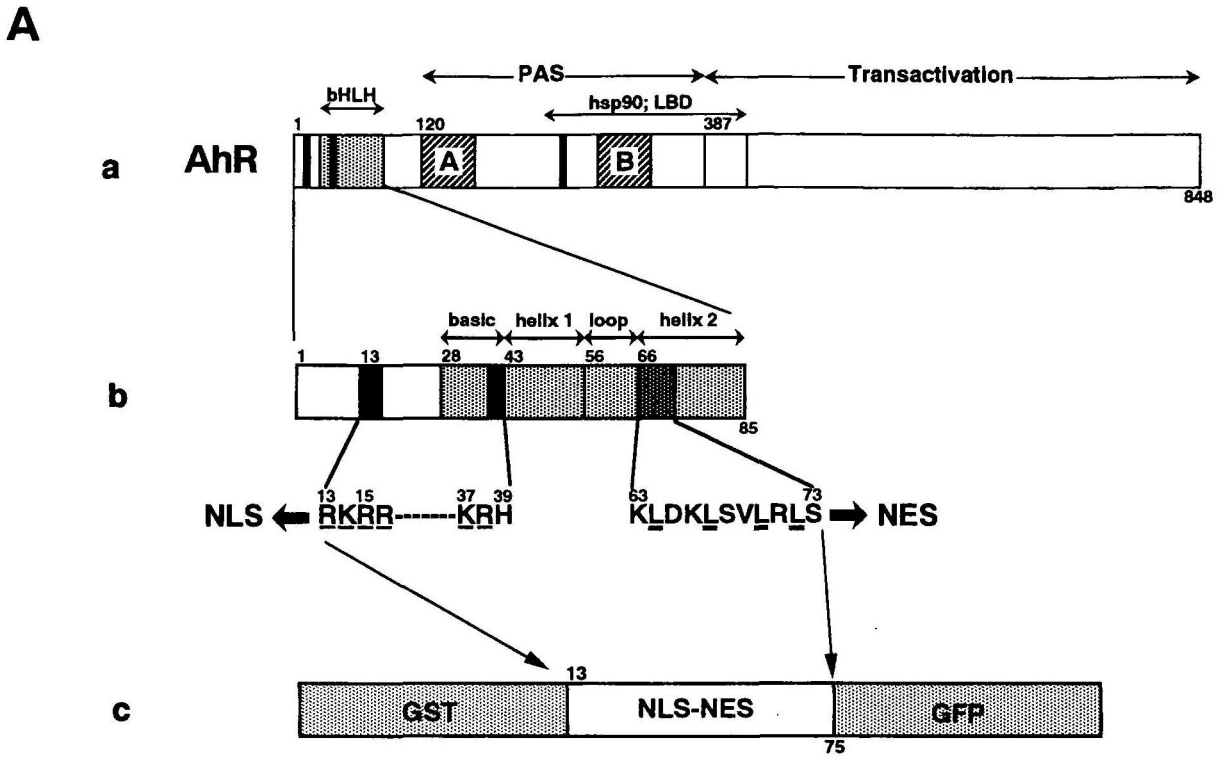

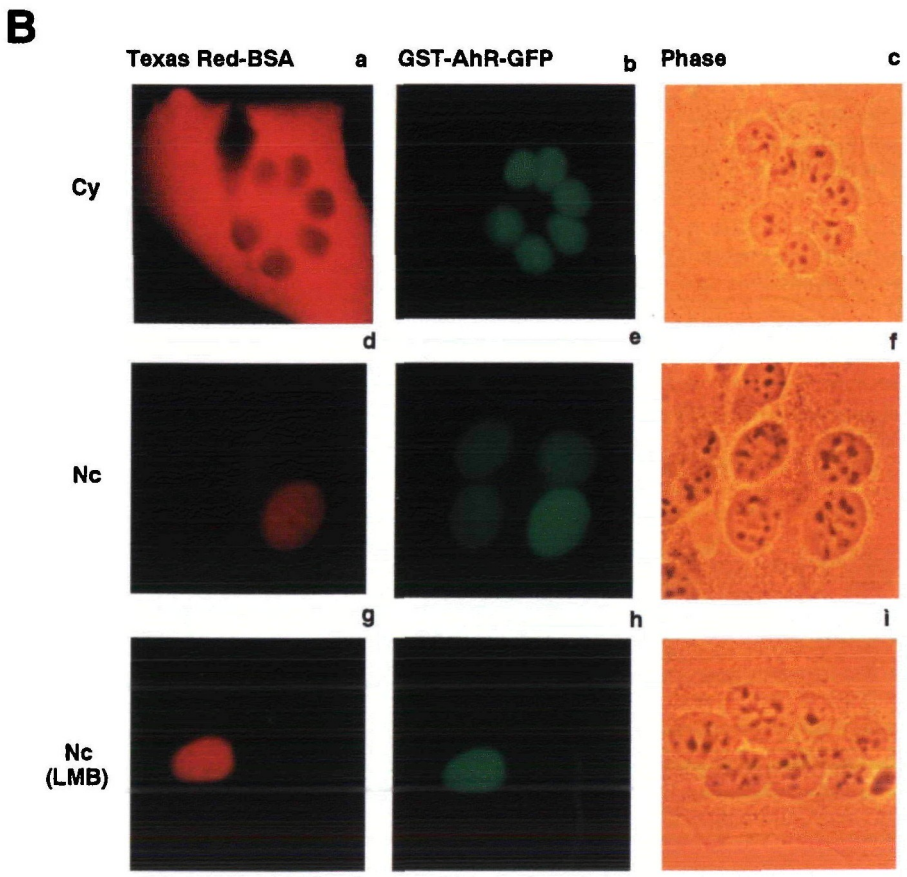

Fig. 1. **(A) Schematic representation of the AhR(13-75) region.** a: Domain structure of AhR. b: N-terminal structure of AhR. The dotted and solid boxes represent bHLH and clusters of basic amino acids, respectively. The dark dotted region in helix 2 is the core sequence of NES. The amino acids essential for NLS and NES activity are cited from Ikuta *et al.* (7). c: GST-AhR(13-75)-GFP fused protein used for microinjection. **(B) Nucleocytoplasmic shuttling of AhR(13-75) in polykaryotic BHK cells.** Representative subcellular localization profiles are shown. A mixture of GST-AhR(13-75)-GFP and Texas Red-labeled BSA was microinjected into the cytoplasm (a–c) or nuclei (d–i) of polykaryotic of BHK cells. After injection and incubation with (g–i) or without (a–f) 10 ng/ml LMB for 2 h at 37°C, the cells were fixed and the localization of the injected proteins was examined under a fluorescent microscope. LMB was added to the culture medium 2 h before microinjection. (a, d, and g) Texas Red-labeled BSA, (b, e, and h) GST-AhR(13-75)-GFP, and (c, f, and i) Phase.

scribed and translated *in vitro* from the T7 promotor using TNT-coupled rabbit reticulocyte lysate (Promega). In order to confirm the protein synthesis *in vitro*, a small volume reaction was performed in the presence of ^{35}S-Met and de-

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tected by SDS-PAGE followed by autoradiography.

Wild or mutant AhR (5 μl) was mixed with ARNT (5 μl) and incubated for 2 h at 30°C in the absence or presence of 1 μM MC. The reaction mixtures were diluted by adding 10 μl of 2 × binding buffer (20 mM Hepes, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 6 mM $MgCl_2$, 2 mM DTT, 0.2 mg/ml salmon sperm DNA, 12% glycerol). After incubation for 20 min at 30°C, an end-labeled XRE probe (*6*) was added and the mixture was further incubated at 30°C for 20 min. Protein-DNA complexes were resolved by 4.5% PAGE followed by autoradiography.

RESULTS AND DISCUSSION

AhR (13-75) Mediates Nucleocytoplasmic Shuttling—The active transport of proteins between the nucleus and the cytoplasm is a major process in eukaryotic cells (*25*). Bidirectional transport of proteins across the nuclear membrane occurs through nuclear pore complexes, and is generally energy- and signal-dependent. The active import of proteins into the nucleus requires the presence of basic amino acid clusters, NLS, while the nuclear export of proteins usually involves NES comprising stretches of hydrophobic residues. Elucidation of the molecular mechanism underlying the cytoplasmic-nuclear trafficking of proteins, *e.g.* transcription factors and ligand-dependent nuclear receptors, is essential for understanding not only the signal transduction cascade but also the regulation of gene expression.

We previously reported that AhR contains both an active NLS (13–39) and NES (55–75) in its N-terminal region (*7*). To determine whether AhR is a nucleocytoplasmic shuttling protein or not, microinjection analysis was performed using AhR(13-75) fused with GST-GFP as a substrate (Fig. 1A) and BHK cells made polykaryotic by a cell fusion technique. Two hours after GST-AhR(13-75)-GFP was injected into the cytoplasm of a BHK cell (Fig. 1B, a–c), the fusion protein was found in all of the nuclei, while co-injected Texas Red–labeled BSA remained in the cytoplasm. On the other hand, when GST-AhR(13-75)-GFP was injected into one nucleus in a polykaryocytic BHK cell (Fig. 1B, d–f), the protein was observed in all of the nuclei in the cell, while Texas Red–labeled BSA was found only in the injected nucleus. These results indicate that GST-AhR(13-75)-GFP is exported from the nucleus into the cytoplasm, and then imported into other nuclei; AhR most likely shuttles from one nucleus to another through the cytoplasm. This nucleocytoplasmic shuttling activity was completely inhibited by leptomycin B (LMB) (Fig. 1B, g–i), which interacts with CRM1 (*26*). These results indicate the involvement of CRM1 in the nuclear export of AhR.

AhR Interacts with CRM1—It has been shown that

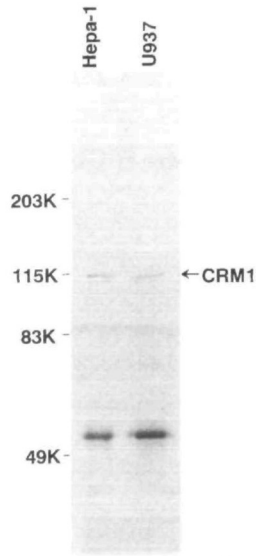

Fig. 2. **AhR interacts with CRM1.** The immunoprecipitates obtained from Hepa 1c1c7 and U937 lysates using anti-AhR antibodies or normal mouse IgG (not shown) were separated by SDS-PAGE, then immunoblotted with anti-CRM1 antibodies (*21*) as described under "MATERIALS AND METHODS." Neither cell line was treated with exogenous ligand of AhR. CRM1 was not precipitated when normal mouse IgG was used for immunoprecipitation (data not shown). The band at about 55 kDa represents the immunoglobulin heavy chain.

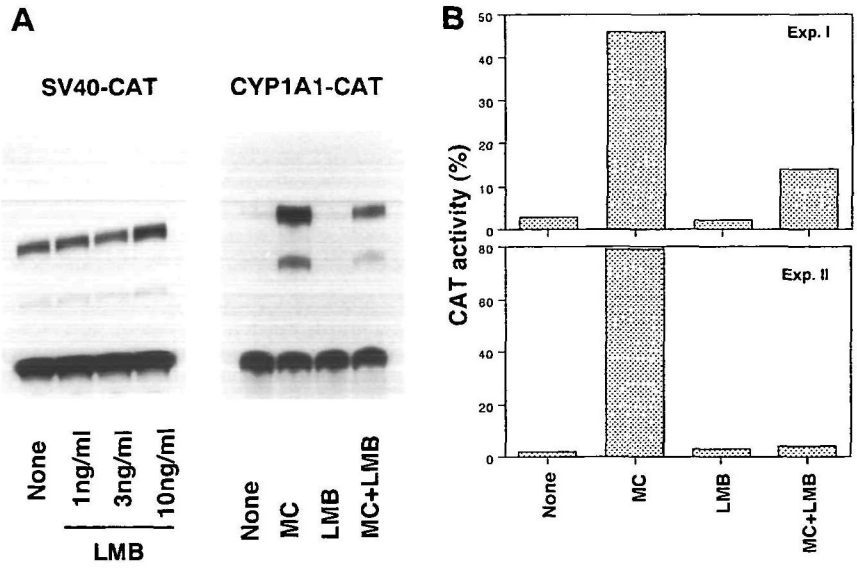

Fig. 3. **Treatment of Hepa 1c1c7 cells with LMB results in the repression of *CYP1A1* gene transcription.** Hepa 1c1c7 cells were transfected with phMC by the calcium phosphate–DNA precipitation method. Twenty-four hours after transfection, MC (1 μM), LMB (10 ng/ml), or MC in combination with LMB was added to the culture medium. The cells were collected 48 h after transfection. (A) Autoradiograms obtained from thin layer chromatographic analysis of SV40-CAT and CYP1A1-CAT (Exp. I) are shown. In the case of SV40-CAT, the concentration of LMB was varied as indicated. (B) The activity (%) of CAT obtained from CYP1A1-CAT as described in panel A was determined by autoradiography with a Fuji Bio-Image Analyzer BAS 2000. The results of two separate experiments are shown. Transfection efficiency was examined by β-galactosidase assay.

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CRM1 interacts with leucine-rich NES in a number of molecules (*20*, *26*, *27*). To study whether the NES of AhR also interacts with CRM1, immunoprecipitation was carried out using Hepa 1c1c7 and U937 as AhR-expressing cell lines. The immunoprecipitates of Hepa 1c1c7 and U937 cells with anti-AhR antibodies were separated by SDS-PAGE, and immunoblotted with anti-CRM1 antibodies.

As shown in Fig. 2, CRM1 was detected in the immunoprecipitates obtained from both of the cell lines tested, while it was not detected with normal mouse IgG (data not shown). These data are consistent with the notion that AhR interacts with the CRM1 protein, and suggests that endogenously expressed AhR is exported from the nucleus to the cytoplasm in a process mediated by CRM1.

Inhibition of Nuclear Export of AhR Results in the Repression of CYP1A1 Gene Transcription—In this study, we clearly showed that AhR, a ligand-dependent transcription factor belonging to the bHLH/PAS family (*28*, *29*), is a nucleocytoplasmic shuttling protein. As already mentioned, AhR acts in concert with ARNT and alters the expression of some genes, such as *CYP1A1*, in response to environmental contaminants including TCDD or MC. We tested whether the nucleocytoplasmic shuttling of AhR is involved in the regulation of such genes using the reporter assay. We first examined the effect of LMB on the transient expression of CYP1A1-CAT activity in wild type Hepa 1c1c7 cells (Fig. 3). Hepa 1c1c7 cells were transfected with a plasmid construct, phMC, which contained the 5′-flanking region of the human *CYP1A1* gene fused to the CAT reporter gene (*22*). Treatment with MC resulted in an approximately 10-fold increase in the CYP1A1-CAT activity in Hepa 1c1c7 cells compared with untreated cells. Treatment with LMB alone did not influence CAT activity. However, when MC treatment was carried out in the presence of LMB, the enhancement of CAT activity by MC was largely inhibited (Fig. 3). The repressing effect of LMB on CAT activity was reproducibly observed. These results suggest that the export of AhR is necessary for the regulation of CYP1A1 transcription. In contrast, a CAT reporter plasmid regulated by the SV40 promoter did not alter in the presence of LMB (Fig. 3). Repression (*30*, *31*) or activation (*32*) of transcription by inhibiting the nuclear export of some other transcription factors with LMB has been reported. Thus, the supressive effect of LMB on CYP1A1-CAT activity is unlikely to be non-specific.

Next, we examined the effect of the disruption of AhR-NES on the CYP1A1-CAT activity. We used the NES-disrupted AhR (L70A/L72A) prepared by site-directed mutagenesis that had no nuclear export activity as judged by microinjection assay (*7*). Hepa-1 c12, an AhR-deficient mutant cell line, was cotransfected with the CYP1A1-CAT plasmid and an expression plasmid, *i.e.* wild type or NES-disrupted AhR cDNA (Fig. 4). There was no difference between the stabilities of the wild and mutant type proteins since the two gene products of AhR were detected in almost equal amounts by immunoblotting (data not shown). When wild-type AhR was cotransfected with the CYP1A1-CAT fused gene into Hepa-1 c12 cells, an 18-fold increase in CAT activity was observed compared with the vector alone; the high CYP1A1-CAT activity might be explained by the existence of endogenous ligand(s) in CYP1A1-deficient cells as reported by Chang *et al.* (*33*). In contrast, when AhR with mutations in the NES motif was transfected, the expression of CYP1A1-CAT activity decreased drastically.

Since the NES of AhR exists in the bHLH domain, a region that participates in AhR-ARNT heterodimer formation, amino acid replacement in NES may affect the formation of the AhR-ARNT-XRE complex. We tested this possibility by gel shift assay. Wild type AhR formed heterodimers with ARNT in the presence of MC (Fig. 5) as previously reported by Matsushita *et al.* (*6*). In contrast, mutant AhR showed only weak XRE-binding activity even in the presence of MC. These results indicate that the low CYP1A1-CAT activity obtained by mutant AhR may be attributed not only to the inhibition of nuclear export but also to the inhibition DNA-binding activity. It is interesting that replacement of the two amino acid residues (L70A/

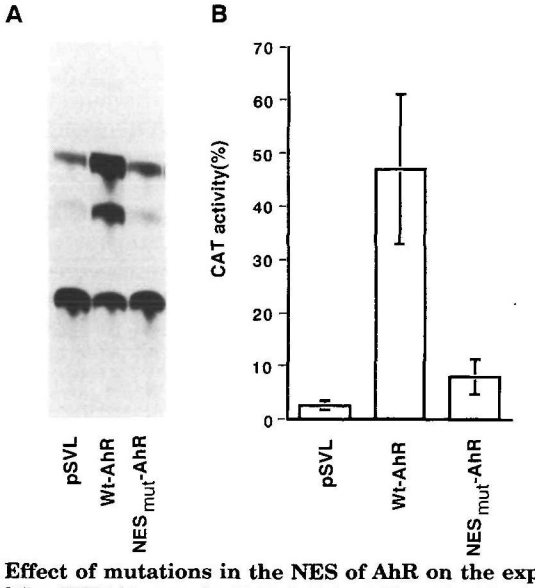

Fig. 4. **Effect of mutations in the NES of AhR on the expression of the *CYP1A1* gene in AhR-deficient Hepa-1 c12 mutant cells.** Hepa-1 c12 cells were cotransfected with the CYP1A1-CAT plasmid (phMC) and a pSVL expression plasmid, pSVL-Wt-AhR or pSVL-NES_{mut}-AhR. The cells were not treated with the exogenous ligand of AhR (described in "RESULTS AND DISCUSSION"). After 48 h of transfection, the cells were harvested and the CAT assay was carried out as described under "MATERIALS AND METHODS." (A) Representative autoradiogram of a thin layer chromatograph. (B) CAT activity (%) was determined with a Fuji Bio-Image Analyzer BAS 2000. Values presented are means; bars, SE; $n = 3$.

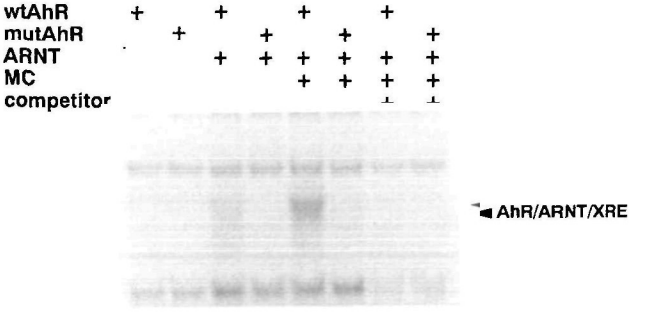

Fig. 5. **Comparison of the XRE binding activities of wild and mutant AhR.** *In vitro* interaction of the wild or NES-disrupted AhR/ARNT heterodimer with the XRE sequence was carried out. Components of the reaction mixture are indicated with (+). Non-labeled XRE (250-fold) was used as a competitor.

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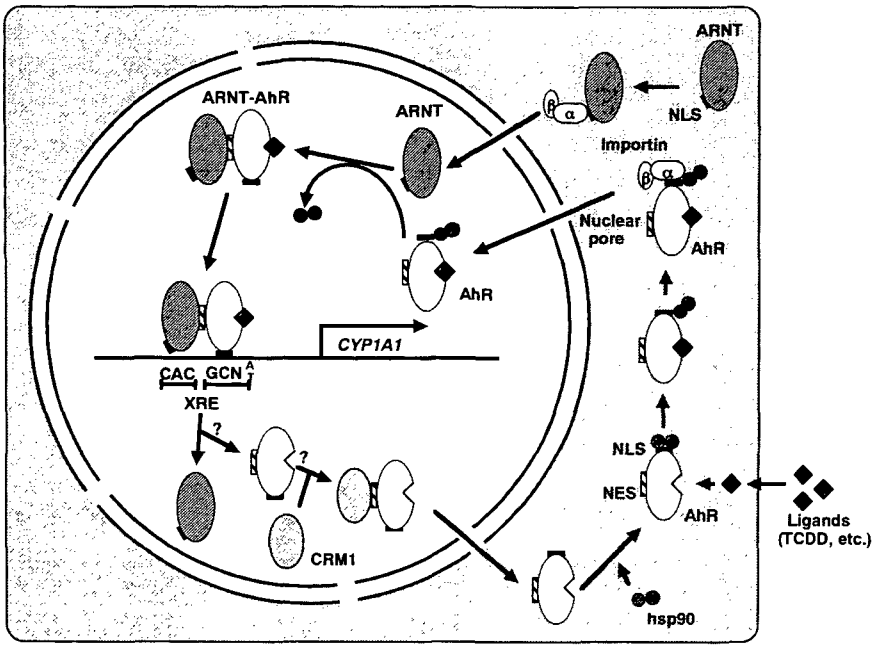

Fig. 6. **A model for signal transduction mediated by the AhR/ARNT system.**

L72A) inactivates both nuclear export and DNA-binding. Nuclear export and DNA-binding appear to require the same region of AhR for their activities. Although the exact mechanism remains to be elucidated, nuclear export may be regulated by steric-hindrance masking by XRE and ARNT.

It has been reported that the nuclear export signal of p53 locates in the tetramerization domain (*34*). A model in which the regulated p53 tetramerization occludes the NES, thereby ensuring the nuclear retention of the DNA-binding form, was proposed. Once p53 is tetrameric, the masking of its NES should lead to nuclear retention and an increase in the DNA-binding form of p53. Inactivating modifications or dissociation factors may separate the subunits of the p53 tetramer, exposing the NESs to CRM1, which then mediates the removal of the subunits from the nucleus. In the case of AhR, nuclear export appears to be regulated by a mechanism similar to the case of p53. When AhR is complexed with ARNT and XRE, AhR remains in the nucleus because CRM1 cannot reach the NES in the complexed form. When AhR is released from the complex, CRM1 may reach the NES, resulting in the export of AhR to the cytoplasm. In addition to the regulation by DNA-binding, ARNT may regulate the function of AhR. The NES of AhR is localized in the helix 2 region, which has been shown to participate in heterodimer formation with ARNT (*35*). Thus, it is conceivable that ARNT may form an AhR-ARNT-XRE complex and cause steric-hindrance that prevents CRM1 from reaching the NES.

Based on the results of our investigation and a recent report (*12*), we here propose a model for the nucleo-cytoplasmic shuttling of AhR (Fig. 6). First, ligands bind to AhR associated with HSP90. The liganded AhR translocates into the nucelus with the aid of importins α and β (*7*), while ARNT moves independently to the nucleus (*15*). In the nucleus, AhR and ARNT dimerize to from the AhR-ARNT heterodimer, which binds to the XRE ($CACGCN^{A}/_{T}$) motif in the promoter region of specific genes such as *CYP1A1*. After transactivating such genes, the dimer dissociates to make the NES of AhR accessible; CRM1 accesses and recognizes the NES. Then, AhR bound to CRM1 is exported from the nucleus to the cytoplasm.

The reason for the repression of *CYP1A1* transcription by inhibition of the nuclear export of AhR found in this study (Figs. 3 and 4) remains to be elucidated: In the normal state, after transactivation of the target gene, the inactivated AhR may be exported to the cytoplasm. However, when nuclear export is inhibited, the inactive AhR may remain in the nucleus and repress transcription. Alternatively, LMB may hamper the export of an inhibitor of AhR activity. Consistent with this notion, Mimura *et al.* (*36*) reported an AhR repressor (AhRR) that negatively regulates AhR-mediated *CYP1A1* gene expression. This might be involved in the transcriptional repression of *CYP1A1* expression seen in these experiments if the subcellular localization of AhRR is affected by LMB treatment.

Together with our previous data (*7*), the present study reveals that the subcellular localization of AhR is regulated by masking and unmasking of NLS and NES in response to ligands. With the aid of these signals, AhR shuttles between the nucleus and cytoplasm in a cell. The biological role of the NES in AhR should be elucidated in more detail with respect to endogenous ligands and the physiological function of AhR (*37–40*).

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REFERENCES

1. Poland, A. and Knutson, J.C. (1982) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related aromatic hydrocarbons: examination of the mechanism of toxicity. *Annu. Rev. Pharmacol. Toxicol.* **22**, 517–

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2. Safe, S.H. (1986) Comparative toxicology and mechanism of action of polychlorinated dibenzo-*p*-dioxins and dibenzofurans. *Annu. Rev. Pharmacol. Toxicol.* **26**, 371–399
3. Schmidt, J.V. and Bradfield, C.A. (1996) Ah receptor signaling pathways. *Annu. Rev. Cell Dev. Biol.* **12**, 55–89
4. Fernandez-Salguero, P.M., Hilbert, D.M., Rudikoff, S., Ward, J.M., and Gonzalez, F.J. (1996) Aryl hydrocarbon receptor-deficient mice are resistant to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced toxicity. *Toxicol. Appl. Pharmacol.* **140**, 173–179
5. Mimura, J., Yamashita, K., Nakamura, K., Morita, M., Takagi, T.N., Nakao, K., Ema, M., Sogawa, K., Yasuda, M., and Fujii-Kuriyama, Y. (1997) Loss of tetragenic response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in mice lacking Ah(dioxin) receptor. *Genes Cell* **2**, 645–654
6. Matsushita, N., Sogawa, K., Ema, M., Yoshida, A., and Fujii-Kuriyama, Y. (1993) A factor binding to the xenobiotic responsive element (XRE) of P-450 1A1 gene consists of at least two helix-loop-helix proteins, Ah receptor and ARNT. *J. Biol. Chem.* **268**, 21002–21006
7. Ikuta, T., Eguchi, H., Tachibana, T., Yoneda, Y., and Kawajiri, K. (1998) Nuclear localization and export signals of the human aryl hydrocarbon receptor. *J. Biol. Chem.* **273**, 2895–2904
8. Perdew, G. H. (1988) Association of the Ah receptor with the 90-kDa heat shock protein. *J. Biol. Chem.* **263**, 13802–13805
9. Pongratz, I., Mason, G.G.F., and Poellinger, L. (1992) Dual roles of the 90-kDa heat shock protein hsp90 in modulating functional activities of the dioxin receptor. *J. Biol. Chem.* **267**, 13728–13734
10. Carver, L.A. and Bradfield, C.A. (1997) Ligand-dependent interaction of the arylhydrocarbon receptor with a novel immunophilin homolog *in vivo*. *J. Biol. Chem.* **272**, 11452–11456
11. Pollenz, R.S., Sattler, C.A., and Poland, A. (1994) The aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein show distinct subcellular localization in Hepa 1c1c7 cell by immunofluorescence microscopy. *Mol. Pharmacol.* **45**, 428–438
12. Lees, M.J. and Whitelaw, M.L. (1999) Multiple roles of ligand in transforming the dioxin receptor to an active basic helix-loop-helix/PAS transcription factor complex with the nuclear protein ARNT. *Mol. Cell. Biol.* **19**, 5811–5822
13. Bacsi, S.G. and Hankinson, O. (1996) Functional characterization of DNA-binding domains of the subunits of the heterodimeric aryl hydrocarbon receptor complex imputing novel and canonical basic helix-loop-helix protein-DNA interactions. *J. Biol. Chem.* **271**, 8843–8850
14. Dong, L., Ma, Q., and Whitlock, J.P. (1996) DNA binding by the heterodimeric Ah receptor. Relationship to dioxin-induced *CYP1A1* transcription *in vivo*. *J. Biol. Chem.* **271**, 7942–7948
15. Eguchi, H., Ikuta, T., Tachibana, T., Yoneda, Y., and Kawajiri, K. (1997) A nuclear localization signal of human ARNT/HIF-1β is a novel bipartite type recognized by the two components of nuclear pore-targeting complex. *J. Biol. Chem.* **272**, 17640–17647
16. Swanson, H.I., Chan, W., and Bradfield, C.A. (1995) DNA binding and pairing rules of the Ah receptor, ARNT, and SIM proteins. *J. Biol. Chem.* **270**, 26292–26302
17. Wen, W., Meinkoth, J.L., Tsien, R.Y., and Taylor, S.S. (1995) Identification of a signal for rapid export of proteins from the nucleus. *Cell* **82**, 463–473
18. Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I.W. (1997) CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* **90**, 1051–1060
19. Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and Nishida, E. (1997) CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* **390**, 308–311
20. Ossareh-Nazari, B., Bachelerie, F., and Dargemont, C. (1997) Evidence for a role of CRM1 in signal-mediated nuclear protein export. *Science* **278**, 141–144
21. Tachibana, T., Imamoto, N., Seino, H., Nishimoto, T., and Yoneda, Y. (1994) Loss of RCC1 leads to suppression of nuclear protein import in living cells. *J. Biol. Chem.* **269**, 24542–24545
22. Kudo, N., Khochbin, S., Nishi, K., Kitano, K., Yanagida, M., Yoshida, M., and Horinouchi, S. (1997) Molecular cloning and cell cycle-dependent expression of mammalian CRM1, a protein involved in nuclear export of proteins. *J. Biol. Chem.* **272**, 29742–29751
23. Kawajiri, K., Watanabe, J., Gotoh, O., Tagashira, Y., Sogawa, K., and Fujii-Kuriyama, Y. (1986) Structure and drug inducibility of the human cytochrome P450c gene. *Eur. J. Biochem.* **159**, 219–225
24. Watanabe, J., Hayashi, S-I., and Kawajiri, K. (1994) Different regulation and expression of the human CYP2E1 gene due to the RsaI polymorphism in the 5′-flanking region. *J. Biochem.* **116**, 321–326
25. Nigg, E.A. (1997) Nucleocytoplasmic transport: signals, mechanisms and regulation. *Nature* **386**, 779–787
26. Kudo, N., Wolff, B., Sekimoto, T., Schreiner, E.P., Yoneda, Y., Yanagida, M., Horinouchi, S., and Yoshida, M. (1998) Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1. *Exp. Cell Res.* **242**, 540–547
27. Zhu, J. and McKeon, F. (1999) NF-AT activation requires suppression of Crm1-dependent export by calcineurin. *Nature* **398**, 256–260
28. Burbach, K.M., Poland, A., and Bradfield, C.A. (1992) Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proc. Natl. Acad. Sci. USA* **89**, 8185–8189
29. Ema, M., Sogawa, K., Watanabe, N., Chujo, Y., Matsusita, N., Gotoh, O., Funae, Y., and Fujii-Kuriyama, Y. (1992) cDNA cloning and structure of mouse putative Ah receptor. *Biochem. Biophys. Res. Commun.* **184**, 246–253
30. Freedman, D.A. and Levine, A.J. (1998) Nuclear export is required for degradation of endogenous p53 by MDM2 and human papilloma virus E6. *Mol. Cell. Biol.* **18**, 7288–7293
31. Rodoriguez, M.S., Thompson, J., Hay, R.T., and Dargemont, C. (1999) Nuclear retention of IκBα protects it from signal-induced degradation and inhibits nuclear factor κB transcriptional activation. *J. Biol. Chem.* **274**, 9108–9115
32. Kudo, N., Taoka, H., Toda, T., Yoshida, M., and Horinouchi, S. (1999) A novel nuclear export signal sensitive to oxidative stress in the fission yeast transcription factor pap1. *J. Biol. Chem.* **274**, 15151–15158
33. Chang, C. and Puga, A. (1998) Constitutive activation of the aromatic hydrocarbon receptor. *Mol. Cell. Biol.* **18**, 525–535
34. Stommel, J.M., Marchenko, N.D., Jimenez, G.S., Moll, U.M., Hope, T.J., and Wahl, G.M. (1999) A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. *EMBO J.* **18**, 1660–1672
35. Fukunaga, B.N., Probst, M.R., Reisz-Porszasz, S., and Hankinson, O. (1995) Identification of functional domains of the aryl hydrocarbon receptor. *J. Biol. Chem.* **270**, 29270–29278
36. Mimura, J., Ema, M., Sogawa, K., and Fujii-Kuriyama, Y. (1999) Identification of a novel mechanism of regulation of Ah (dioxin) receptor function. *Genes Dev.* **13**, 20–25
37. Abbott, B.D., Birnbaum, L.S., and Perdew, G.H. (1995) Developmental expression of two members of a new class of transcription factors: I. Expression of aryl hydrocarbon receptor in the C57BL/6N mouse embryo. *Dev. Dynamics* **204**, 133–143
38. Hayashi, S-I., Okabe-Kado, J., Honma, Y., and Kawajiri, K. (1995) Expression of Ah receptor (TCDD receptor) during human monocytic differentiation. *Carcinogenesis* **16**, 1403–1409
39. Fernandez-Salguero, P., Pineau, T., Hilbert, D.M., McPhail, T., Lee, S.S.T., Kimura, S., Nebert, D.W., Rudikoff, S., Ward, J.M., and Gonzalez, F.J. (1995) Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor. *Science* **268**, 722–726
40. FitzGerald, C.T., Fernandez-Salguero, P., Gonzalez, F.J., Nebert, D.W., and Puga, A. (1996) Differential regulation of mouse Ah receptor gene expression in cell lines of different tissue origins. *Arch. Biochem. Biophys.* **333**, 170–178

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