THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 49, pp. 37507–37516, December 8, 2006

Unique and Overlapping Transcriptional Roles of Arylhydrocarbon Receptor Nuclear Translocator (Arnt) and Arnt2 in Xenobiotic and Hypoxic Responses*

Received for publication, July 20, 2006, and in revised form, September 13, 2006 Published, JBC Papers in Press, October 5, 2006, DOI 10.1074/jbc.M606910200

Hiroki Sekine[‡], **Junsei Mimura**[‡§], **Masayuki Yamamoto**[‡], **and Yoshiaki Fujii-Kuriyama**[‡§1]

From the [‡]*Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8577, Japan and* [§]*SORST, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, 332-0012 Japan*

Arnt and the homologous Arnt2 share a high degree of sequence similarity and are believed to function as obligate common partners for a number of basic helix-loop-helix (bHLH)-PAS transcription factors including arylhydrocarbon receptor (AhR) and HIFα. Genetic disruption of both Arnt and Arnt2 demonstrated both unique and overlapping functions in response to environmental stimuli and during mouse development. Either stably or transiently expressed Arnt/Arnt2 wild type and various mutants or chimeric constructs in Hepa1-c4 cells exhibit similar levels of hypoxic response element-driven reporter gene expression and the induction of endogenous *Glut-1* through binding with HIFα in response to hypoxia. In contrast, we observed clear functional differences in the ability of Arnt and Arnt2 to induce xenobiotic response element-driven reporter and endogenous *CYP1A1* gene expression. In contrast with Arnt, Arnt2 was practically incapable of interacting with ligand-activated AhR to induce the expression of target genes for xenobiotic-metabolizing enzymes in response to xenobiotics. The differential binding of AhR by Arnt and Arnt2 can be ascribed to a single His/Pro amino acid difference in the PASB region of Arnt and Arnt2, suggesting that the PASB/PASB interaction between bHLH-PAS transcription factors plays a selective role for their specific partner molecule.

Arylhydrocarbon receptor nuclear translocator (Arnt)[2] is a nuclear localized transcription factor that is a canonical member of a transcription factor family consisting of an N-terminal basic helix-loop-helix (bHLH) domain followed by a PAS domain, so named because it is conserved among Per, Arnt, and Sim (1–5). In contrast, the arylhydrocarbon receptor (AhR), a member of the same transcription factor family, associates with an HSP90 complex in the cytoplasm and, upon binding an exogenous inducer such as 3-methylcholanthrene (3MC) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), translocates from the cytoplasm to the nucleus. Within the nucleus, AhR dissociates from HSP90 and forms a heterodimer with Arnt. The newly formed AhR·Arnt complex binds *cis*-acting DNA enhancer sequences, known as xenobiotic response element (XRE), or dioxin response element (DRE), to enhance the expression of a number of drug-metabolizing enzyme genes including cytochrome P4501A1 (*CYP1A1*) (6). Furthermore, AhR·Arnt signaling is essential for liver and palate development as well as normal reproductive homeostasis, as shown by studies on AhR-deficient mice (7, 8).

Under normoxic conditions, expression of the bHLH-PAS transcription factors hypoxia-inducible factor (HIF)-1α and HIF2α is negatively regulated by the von Hippel-Lindau tumor suppressor protein through the 26 S proteasome (9). However, under hypoxic conditions, HIFα is stabilized and translocates into the nucleus, where it forms a heterodimer with Arnt and binds the hypoxic response element (HRE) leading to the expression of target genes involved in glycolysis, erythropoiesis, and angiogenesis (6). In addition, HIF1α and HIF2α may be involved in normal embryonic development (10–12).

Arnt2, first identified as a homologue with a high degree of sequence similarity to Arnt, undergoes heterodimerization with other bHLH-PAS transcription factors, but unlike the ubiquitously expressed Arnt, Arnt2 expression is restricted to neural tissues and the kidney (13, 14). Arnt2-deficient mice die in the perinatal period and have impaired hypothalamic development (15), but targeted disruption of the Arnt gene leads to embryonic lethality between E9.5 and E10.5 characterized by disrupted placental, hematopoietic and yolk sac vascular development (16–19). When compound heterozygous $Arnt^{2+/-}$ and $Arnt^{+/-}$ mice were mated, all homozygous double mutant mice died before E8.5, and a much smaller number of compound heterozygous $Arnt^{-/-}Arnt^{2+/-}$ or $Arnt^{+/-}$ $Arnt2^{-/-}$ mutant fetuses were found to be alive at E8.5 than expected from Mendelian genetics, suggesting a strong genetic interaction between Arnt2 and Arnt during mouse development (20). Thus, although the expression and functions of Arnt2 and Arnt are not entirely overlapping, there is a strong genetic and functional interaction during development. We wished to deter-

* This work was funded in part by Solution Oriented Research for Science and Technology, Japan Science and Technology Agency and by a grant for Scientific Research from the Ministry of Health, Labor, and Welfare of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: Center for Tsukuba Advanced Research Alliance, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8577 Japan. Tel.: 81-29-853-7323; Fax: 81-29-853-7318; E-mail: ykfujii@tara.tsukuba.ac.jp.

[2] The abbreviations used are: Arnt, arylhydrocarbon receptor nuclear translocator; mArnt, mouse Arnt; 3MC, 3-methylcholanthrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; bHLH, basic helix-loop-helix; AhR, arylhydrocarbon receptor; HIF, hypoxia-inducible factor; XRE, xenobiotic response element; HRE, hypoxic response element; E, embryonic day; PBS, phosphate-buffered saline; WT, wild type; aa, amino acid(s).

mine the biochemical and molecular basis for the observed differential roles of Arnt and Arnt2 in physiology and development. Although in our previous report on the transient DNA transfection experiments Arnt2 exhibited some 20% transactivation activity of Arnt with XRE-driven reporter gene (13), stably expressed Arnt2 in transformant cells failed to induce the expression of the XRE-reporter and endogenous *CYP1A1* genes in sharp contrast with studies of Arnt. Interestingly, the differential activity between Arnt and Arnt2 in heterodimer formation with AhR can be ascribed to a single amino acid replacement in the PASB domain.

EXPERIMENTAL PROCEDURES

Plasmids—mArnt and mArnt2 in pBSK were cleaved with NcoI and fused at their N termini with a 3xFLAG tag derived from p3xFLAG-CMV-10 (Sigma). pBSK3xFLAG-Arnt and pBSK3xFLAG-Anrt2 were cleaved with EcoRI and XbaI, and the isolated inserts were blunt-ended and subsequently cloned into XbaI-digested blunt-ended pEFBOS (21) to produce pBOS3xFLAG-Arnt and pBOS3xFLAG-Arnt2. Arnt/Arnt2 chimeras were produced and designated as follows: 3xFLAG-A1(A2TA); 3xFLAG-Arnt N-terminal region (aa 1–465) was connected with the Arnt2 C-terminal region (aa 440–712), 3xFLAG-A2(A1TA); 3xFLAG-Arnt2 N-terminal region (aa 1–439) was connected with the Arnt C-terminal region (aa 466–791), 3xFLAG-A1(A2AB), and 3xFLAG-A2(A1AB); 3xFLAG Arnt or Arnt2 PAS domain was replaced with the Arnt2 or Arnt PAS domain (Arnt-(156–465), Arnt2-(130–439)), respectively (see Fig. 2*A* for summary), 3xFLAG-A1(A2A); 3xFLAG-Arnt PASA domain was replaced with the Arnt2 PASA domain (Arnt-(156–356), Arnt2-(130–330)), respectively (see Fig. 5*A* for summary), 3xFLAG-A1(A2B); 3xFLAG Arnt PASB domain was replaced with the Arnt2 PASB domain (Arnt-(357–465), Arnt2-(331–439)) (see Fig. 5*A* for summary). These cDNA expression vectors were generated using the standard methods and confirmed by sequencing, and the inserts were then cloned into the pBOS vector. pBOS3xFLAG-ArntH378P, pBOS3xFLAG-A1(A2A)H378P, and pBOS-3xFLAG-A1(A2B)P352H were generated by site-directed mutagenesis using the Sculptor *in vitro* mutagenesis system (Amersham Biosciences) with pBOS3xFLAG-Arnt, pBOS3xFLAG-A1(A2A), and pBOS3xFLAG-A1(A2B), as templates, respectively (See Fig. 5*A* for summary).

For construction of pBOSGAL4DBD-Arnt-bHLHPAS-(91–465) and pBOSGAL4DBD-Arnt2-bHLHPAS-(65–439), mArnt-bHLHPAS and mArnt2-bHLHPAS region fragments were produced by PCR, using pBOS3xFLAG-Arnt and pBOS3xFLAG-Arnt2 as templates, and confirmed by sequencing. These fragments were cloned into the pBOSGAL4DBD vector (22). Chimeric constructs were produced and designated as follows: pBOSGAL4DBD-A1A2-bHLHPAS; pBOSGAL-4DBD-Arnt-bHLHPAS N-terminal region (aa 91–257) fused with the Arnt2-bHLHPAS C-terminal region (aa 232–439), pBOSGAL4DBD-A1A2A1-bHLHPAS, A1A1A2-bHLHPAS, A1A2A1–2-bHLHPAS, and A1A1A2–2-bHLHPAS. The sequences of the following regions (aa 258–333, 334–465, 334–397, and 398–465) from pBOSGAL4DBD-Arnt-bHLHPAS were exchanged, respectively, with the corresponding regions of Arnt2-(232–307), -(308–439), -(308–371), and -(372–439). pBOSGAL4DBD-ArntH378P-bHLHPAS was generated by site-directed mutagenesis using pBOSGAL4DBD-Arnt-bHLHPAS as a template.

To produce pBOSVP16AD-mHIF1αΔC, the mHIF1αΔC-(1–613) fragment was generated by PCR using cDNA from Hepa1 RNA as a template and cloned into pGEM-T-Easy vector (Promega). After sequencing, the excised cDNA fragment was inserted into pBOSVP16AD multi-cloning sites (22).

Cell Culture—Hepa1-c4 (an Arnt-defective cell line of Hepa1c1c7 cells (23)), Hepa1, 293T, and HeLa cells were maintained, respectively, in high or low glucose Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Sigma) and penicillin/streptomycin (Invitrogen) under 5.0% CO_2 at 37 °C.

Reporter Assay—All luciferase assays were performed using the Dual-Luciferase reporter assay system according to the manufacturer's protocol (Promega) with some modifications. Hepa1-c4 cells (2.0×10^4 cells/well) were plated in 24-well plates 24 h prior to transfection. Cells were co-transfected with pXRE4-SV40-Luc (22) or pHRE6-SV40-Luc reporter (24) using 1 ng of *Renilla* luciferase as an internal control and pBOS3xFLAG-Arnt, pBOS3xFLAG-Arnt2, or the chimeric constructs using FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer's protocol. All cells were incubated for 24 h at 37 °C after transfection. pXRE4-SV40-Luc-transfected cells were treated with 1 μM 3MC, a potent inducer of XRE-driven transcription, or with Me_2SO and then incubated for an additional 18 h. To measure hypoxia-induced transcription, cells transfected with pHRE6-SV40-Luc were incubated under normoxic or hypoxic (1.0% O_2) conditions for an additional 16 h.

HeLa cells (2.5×10^4 cells/well) were cultured in 24-well plates for 24 h prior to transfection and then were cotransfected with pXRE4-SV40-Luc or pHRE6-SV40-Luc, 1 ng of *Renilla* luciferase as an internal control, effector plasmid (pBOS3x-FLAG-Arnt, pBOS3xFLAG-Arnt2, chimeric or mutant constructs), and partner molecule plasmid (either pBOSmAhR (22) or pBOShHif1α (24)) using Lipofectamine Plus (Invitrogen) according to the manufacturer's protocol. After an additional 24 h of incubation, cells were treated with 3MC or placed under hypoxic condition as described above.

For the Arnt-AhR two-hybrid assay, 293T cells (2.0×10^4 cells/well) were cultured in 24-well plates 24 h prior to transfection and cotransfected with 100 ng of pG3-Luc (22), 0.1 ng of *Renilla* luciferase reporter as an internal control, 10 ng of the bait plasmid (either pBOSGAL4DBD-Arnt, -Arnt2-bHLHPAS, or chimeric or mutant constructs), and 60 ng of prey plasmid (either pBOSVP16AD-mAhRΔC (22) or pBOSVP16AD-mAhRΔBΔC) using Lipofectamine Plus (Invitrogen). For Arnt-HIF1α two-hybrid assay, cultured 293T cells were cotransfected with 100 ng of pG3-Luc (22), 0.1 ng of *Renilla* luciferase reporter as an internal control, 2 ng of the bait plasmid (either pBOSGAL4DBD-mArnt, pBOSGAL4DBD-mArnt2-bHLHPAS, or chimeric and mutants constructs), and 5 ng of pBOSVP16AD-mHIF1αΔC as described above. 3MC and hypoxia treatment were performed as described above.

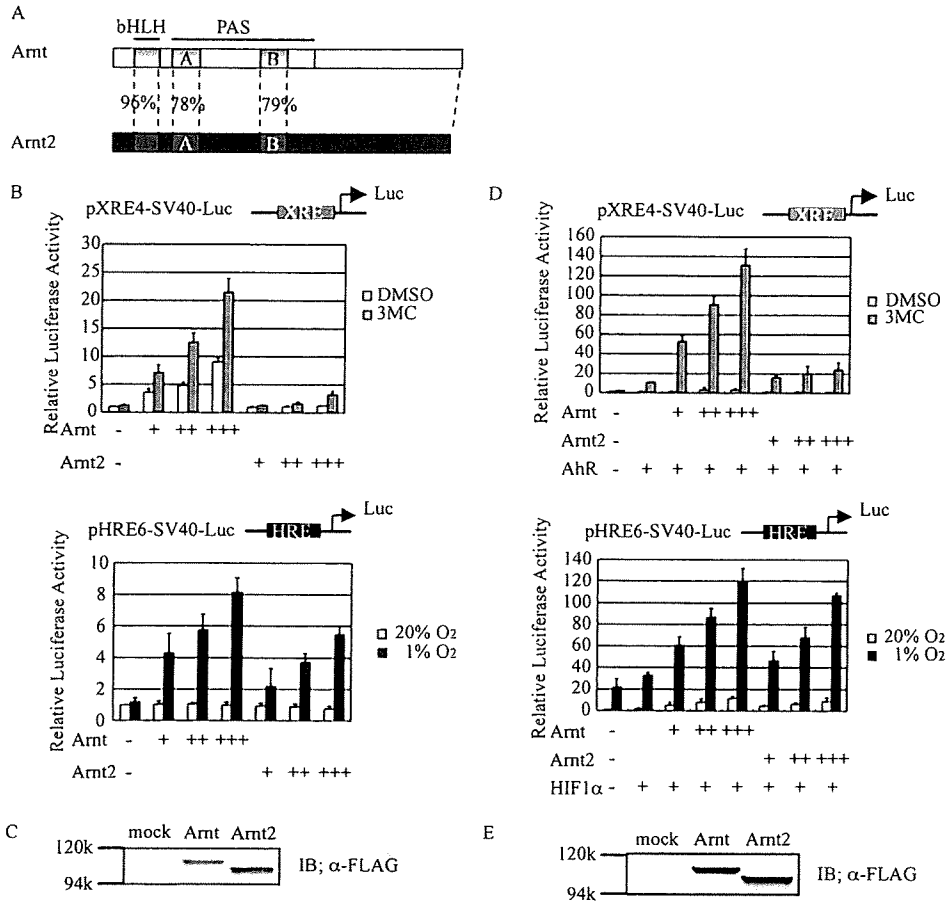

FIGURE 1. **Transcriptional activity of Arnt and Arnt2 on XRE- and HRE-driven reporter genes in Hepa1-c4 and HeLa cells.** *A*, amino acid identities between Arnt and Arnt2. *B*, transcriptional activity of Arnt and Arnt2 in Hepa1-c4 cells. Hepa1-c4 cells were transfected with 100 ng of pXRE4-SV40-Luc and increasing amounts (1, 10, and 100 ng) of 3xFLAG-Arnt or 3xFLAG-Arnt2 expression plasmids, incubated for 24 h, and treated with 1 μM 3MC or Me_2SO (dimethyl sulfoxide (*DMSO*)) for 18 h (*top panel*). For analysis of the hypoxic response, Hepa1-c4 cells were transfected with 100 ng of pHRE6-SV40-Luc and increasing amounts (1, 10, and 100 ng) of 3xFLAG-Arnt or 3xFLAG-Arnt2 expression plasmids. After 24 h of incubation, the cells were treated for 16 h under conditions of normoxia (20% O_2) or hypoxia (1% O_2) (*bottom panel*). The cell extracts were prepared from the treated cells and used for luciferase assays. Values are represented by mean ± S.D. of the results of three independent experiments normalized to *Renilla* luciferase activity used as an internal control. *C*, expression of Arnt and Arnt2 in Hepa1-c4 cells. Hepa1-c4 cells were transfected with 0.5 μg of the indicated expression construct in 6-well plates. The protein levels were evaluated by Western blotting using anti-FLAG antibody. Equal amounts of cell lysates were used for Western blot analysis. The *mock* lane represents cell lysate transfected with empty vector alone. *D*, transcriptional activity of Arnt and Arnt2 in HeLa cells. *Top panel*, XRE-driven reporter activity. HeLa cells were transfected with 10 ng of pXRE4-SV40-Luc, increasing amounts (0.1, 0.5, and 2 ng) of 3xFLAG-Arnt or -Arnt2 expression plasmids, and 10 ng of pBOSmAhR, and the reporter gene expression assays were performed as described in *B*. *Bottom panel*, HRE-driven reporter activity. HeLa cells were transfected with 10 ng of pHRE6-SV40-Luc, increasing amounts (0.1, 0.5, and 2 ng) of 3xFLAG-Arnt or -Arnt2 expression plasmids, and 20 ng of pBOShHIF1α, and the reporter gene expression assays were performed as described in *B*. Values are represented by mean ± S.D. of the results of three independent experiments normalized to *Renilla* luciferase activity used as an internal control. *E*, expression of Arnt and Arnt2 in HeLa cells. HeLa cells were transfected with 10 ng of the indicated expression construct in 6-well plates. *IB*, immunoblot.

Generation of Stable Transformant Cell Lines—Hepa1-c4 cells were cotransfected with pSVneo and effector plasmid (either pBOS3xFLAG-Arnt, pBOS3xFLAG-Arnt2, or pBOS3x-FLAG-ArntH378P). After transfection, the cells were replated and incubated with selection medium containing 0.5 mg/ml Geneticin (Invitrogen).

Western Blot Analysis—Cells were dissolved in SDS sample buffer, and proteins were separated by SDS-PAGE for Western blot analysis. Proteins were then transferred to polyvinylidene difluoride membranes and blocked in 3% skim milk for 30 min. A rabbit anti-Arnt antiserum (25) or anti-FLAG (Sigma), anti-AhR (Biomol), or anti-actin or anti-VP16AD (Santa Cruz Biotechnology) antibodies were used as primary reagents. After being washed three times in TBS (25 mM Tris/HCl (pH 7.5), 150 mM NaCl) containing 0.1% Triton X-100, membranes were incubated with species-specific horseradish peroxidase-conjugated secondary antibody (Zymed Laboratories Inc.). The protein-antibody complexes were visualized by the enhanced chemiluminescence detection system (Amersham Biosciences) according to the recommendations of the manufacturer.

Whole Cell Lysate Preparation for Coimmunoprecipitation—Hepa1-c4 cells stably transfected with pBOS-3xFLAG-Arnt, pBOS3xFLAG-Arnt2, or pBOS3xFLAG-ArntH-378P were incubated with 1 μM 3MC or Me_2SO for 2 h and washed with ice-cold phosphate-buffered saline followed by TBS. The cells were harvested by scraping, centrifuged at 5,000 rpm at 4 °C for 5 min, and suspended in TBS containing 1 mM $CaCl_2$, 1% Triton X-100, and protease inhibitor mixture (Roche Applied Science). The cells were vortexed and placed on ice for 5 min. The samples were then centrifuged at 15,000 rpm for 5 min at 4 °C, and the supernatants were reserved as whole cell lysates.

Coimmunoprecipitation Assay—The prepared whole cell lysate (200 μl) was added to 200 μl TBS with 1 mM $CaCl_2$ and protease inhibitor mixture and incubated with anti-FLAG antibody for 1 h at 4 °C. The reaction mixture was supplemented with 15 μl of protein A-agarose beads (Amersham Biosciences). After incubating for an additional 1 h at 4 °C, the beads were washed three times with TBS and resuspended in SDS sample buffer. The coimmunoprecipitated proteins were resolved by SDS-PAGE, and Western blot analysis was performed.

Immunohistochemistry—Hepa1-c4 cells stably transfected with pBOS3xFLAG-Arnt, pBOS3xFLAG-Arnt2, or pBOS3x-FLAG-ArntH378P were washed, fixed in 4% paraformaldehyde at room temperature for 10 min, and treated with cold acetone for 1 min on ice. After washing with PBS, the cells were incubated with 3% skim milk for 1 h at room temperature and treated with 10 μg/ml mouse anti-FLAG antibody for 16 h at 4 °C. After washing with PBS, the cells were subsequently

treated with biotinylated anti-mouse IgG antibody (Vector) for 3 h at 4 °C, washed with PBS, and treated with streptavidin-Alexa Fluor 488 (Invitrogen) and Hoechst solution (Dojindo) for 30 min at room temperature. After washing with PBS, a drop of fluorescent mounting medium (Dako) was placed on the cells, which were then examined by fluorescence microscopy.

Real-time PCR—Total RNA samples were prepared from the treated cells using Isogen (Nippon Gene) as described. First-strand cDNA was synthesized from 1 μg of total RNA using SuperScript reverse transcriptase (Invitrogen). Real-time PCR was performed in triplicate for each sample with the ABI Prism 7700 sequence detector (PE Applied Biosystems) using primers designed against mouse *GLUT1* (26) and *CYP1A1* (primer sequences GGTACAGAGAAAGATCCAGGAGGA and CGAAGGATGAATGCCGGAAGGTCT and probe sequence 6-FAM-CTAGACACAGTGATTGGCAGAGATCGGCA-TAMRA) or rRNA genes (PE Applied Biosystems).

RESULTS

Comparison between Transcription Activities of Arnt and Arnt2 for Expression of the XRE- and HRE-driven Reporter Genes—It has been reported that the bHLH and PAS domains of Arnt and Arnt2 are very similar and mediate homo- and heterodimerization (Fig. 1*A*), but Arnt2 showed only 20% transcription activity of Arnt in the expression of the XRE-driven reporter gene (13).

To clarify whether the low transcription activity of Arnt2 for the expression of the XRE reporter gene is because of a low level of expression of Arnt2 or a low affinity of Arnt2 to AhR as compared with Arnt, we investigated the transcription activity of the XRE- and HRE-driven reporter genes by Arnt2 and Arnt in a dose-dependent manner using the transient transfection assay. In an experiment using either Hepa1-c4 or HeLa cells (the same result was obtained with NIH3T3 cells, data not shown), a highly inducible expression of the XRE-driven reporter gene was observed by Arnt in response to 3MC. In marked contrast, Arnt2 exhibited only a low level of inducible expression of the reporter gene even at the highest dose (Fig. 1, *B* and *D*). On the other hand, the HRE-driven reporter gene was induced similarly by Arnt and Arnt2 in response to hypoxia. Taken together with the results that Arnt and Arnt2 were similarly expressed at a protein level as shown in Fig. 1, *C* and *E*, all of these results suggest that Arnt2 is much less efficient than Arnt in conjunction with AhR for the inducible expression of the XRE reporter gene, whereas both Arnt and Arnt2 work equally well with HIFα to regulate the HRE reporter gene expression.

PAS Domain Is Responsible for Differential Activities between Arnt and Arnt2 for XRE-driven Reporter Gene Expression—We were interested in the molecular mechanisms responsible for the differences in transcriptional activity between Arnt and Arnt2. To investigate which part of the Arnt and Arnt2 molecules is responsible for the differential activity, we divided Arnt and Arnt2 into three parts based on the N-terminal bHLH, PAS, and C-terminal activation domains and generated several chimeric constructs by swapping the respective domains for Arnt and Arnt2 (Fig. 2*A*). As shown in the *upper panel* of Fig. 2*B* (*column 1 versus 3*), the luciferase activity is the same in cells

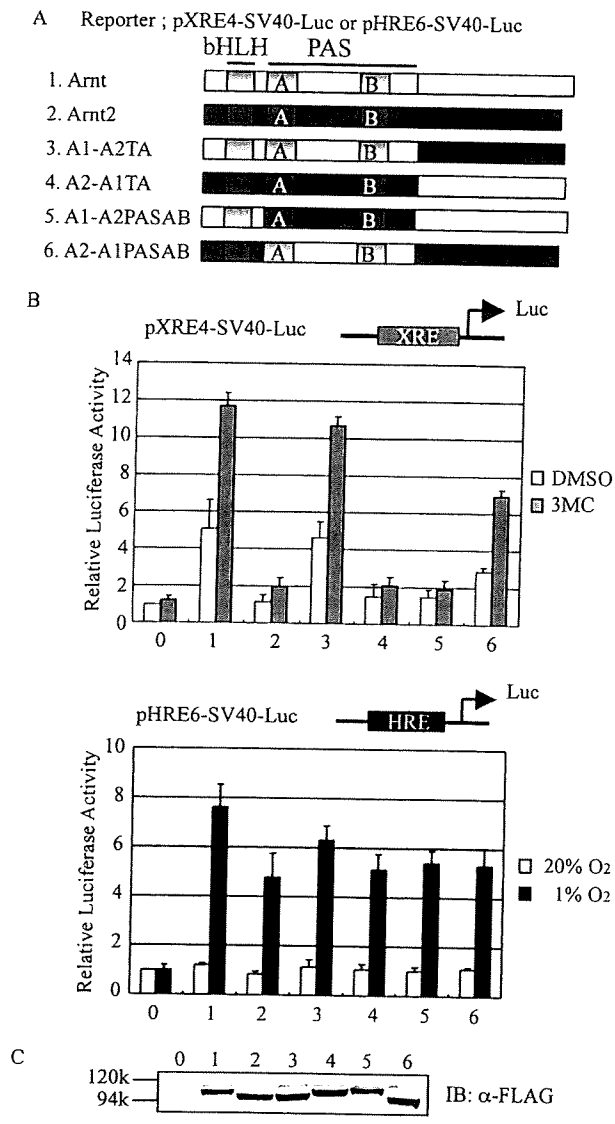

FIGURE 2. **Transcriptional activity of Arnt/Arnt2 chimeric proteins on XRE- and HRE-driven reporter genes in Hepa1-c4 cells.** *A*, Arnt and Arnt2 structures and their domain-swapping constructs. *B*, transcriptional activity of Arnt/Arnt2 chimeric constructs. Hepa1-c4 cells transfected with 100 ng of pXRE4-SV40-Luc and 10 ng of the indicated Arnt/Arnt2 chimeric constructs were incubated for 24 h and then treated with 1 μM 3MC or Me_2SO (dimethyl sulfoxide (*DMSO*)) for 18 h (*top panel*). For analysis of the hypoxic response, Hepa1-c4 cells were transfected with 100 ng of pHRE6-SV40-Luc and 10 ng of the Arnt/Arnt2 chimeric constructs. After 2 h of incubation, the cells were treated for 16 h under normoxia or hypoxia (1% O_2) (*bottom panel*). The cell extracts were prepared from the treated cells and used for luciferase assays. Values are represented by mean ± S.D. of the results of three independent experiments normalized to *Renilla* luciferase activity used as an internal control. *C*, expression of Arnt, Arnt2, and chimeric constructs. The cells were transfected with 50 ng of the indicated expression construct in 6-well plates. The protein levels of all constructs were evaluated by Western blotting using anti-FLAG antibody. Equal amounts of cell lysates were used for Western blot analysis. *Columns* in *B* and *C*: *0*, pBOS; *1*, pBOS3xFLAG-Arnt; *2*, pBOS3xFLAG-Arnt2; *3*, pBOS3xFLAG-A1(A2TA); *4*, pBOS3xFLAG-A2(A1TA); *5*, pBOS3xFLAG-A1(A2AB); *6*, pBOS3xFLAG-A2(A1AB). *IB*, immunoblot.

expressing WT Arnt and a chimera composed of the N-terminal bHLH and PAS domains of Arnt and the C-terminal region of Arnt2. In contrast, a chimera composed of the bHLH and C-terminal domains of Arnt and the PAS domain of Arnt2 activates only a low level of luciferase expression, as observed with WT Arnt2 for the expression of XRE-driven reporter gene (Fig.

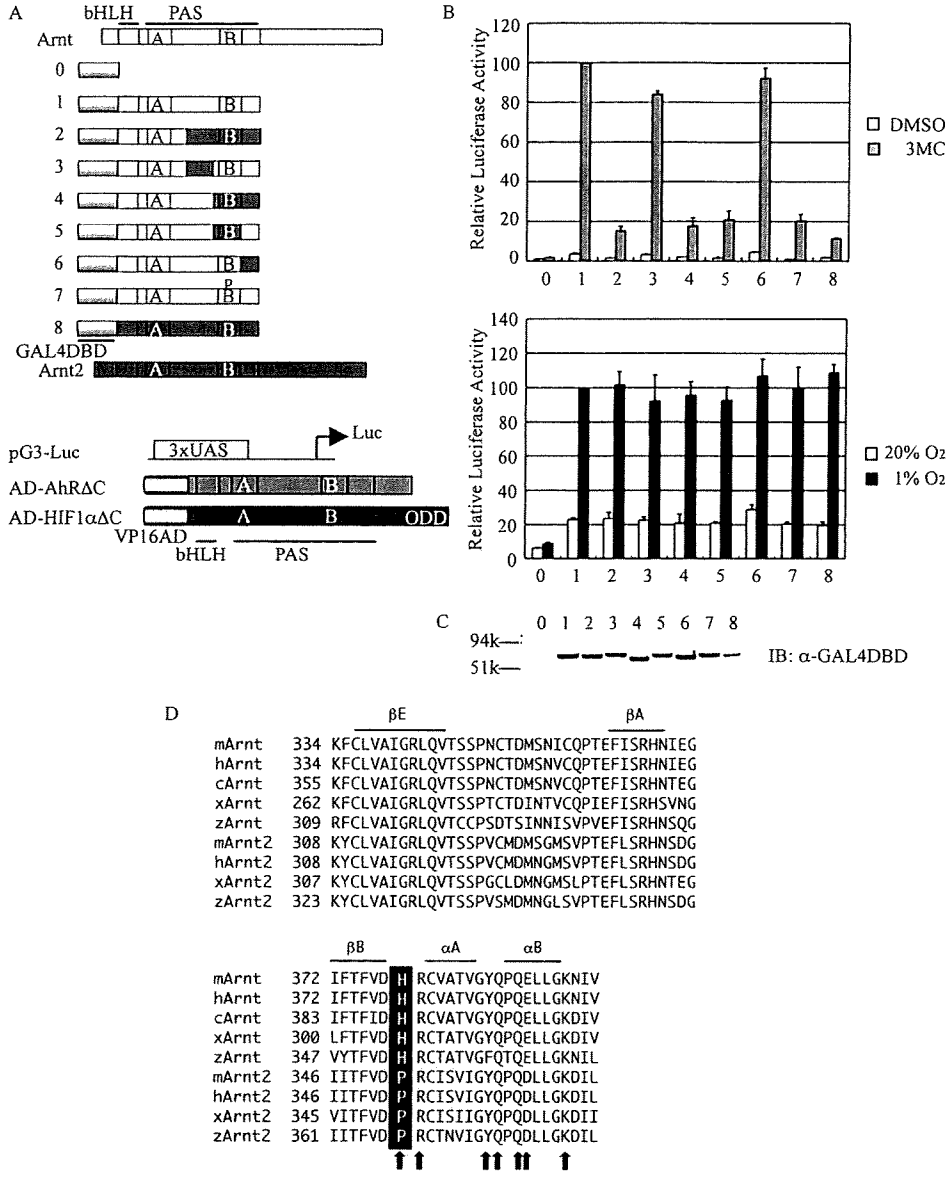

FIGURE 3. **Two-hybrid analysis of transcriptional activities of Arnt and Arnt2 and their chimeric and mutant derivatives.** *A*, constructs of bait proteins of Arnt and Arnt2, their chimeras and mutant, and prey molecules of AhR and HIF1α. *B*, transcriptional activity of various Arnt/Arnt2-bHLHPAS chimeric proteins in the two-hybrid system. 293T cells were transfected with pG3-Luc, the indicated Arnt/Arnt2 construct, and pBOSVP16AD-mAhRΔC (*AD-AhRΔC*) or pBOSVP16AD-mHIF1αΔC (*AD-HIF1αΔC*) (see "Experimental Procedures"). The transfected cells were treated as described in the legend to Fig. 1*B*. *Top panel*, relative luciferase activities induced by 3MC treatment. *Bottom panel*, relative luciferase activities induced by hypoxic treatment. Values are represented by mean ± S.D. of the results of three independent experiments normalized to *Renilla* luciferase activity used as internal control. *DMSO*, dimethyl sulfoxide (Me_2SO). *C*, expression of the bait proteins. The cells were transfected with 50 ng of the indicated expression construct in 6-well plates. Protein levels of all constructs were evaluated by Western blot analysis using α-GAL4DBD antibody. The cells were homogenized, and supernatants were subjected to SDS-PAGE for Western blot analysis (*IB*, immunoblot). *Columns*: *0*, pBOSGAL4DBD; *1*, pBOSGAL4DBD-Arnt-bHLHPAS; *2*, pBOSGAL4DBD-Arnt2-bHLHPAS; *3*, pBOSGAL4DBD-A1A2-bHLHPAS; *4*, pBOSGAL4DBD-A1A2A1–1-bHLHPAS; *5*, pBOSGAL4DBD-A1A1A2–1-bHLHPAS; *6*, pBOSGAL4DBD-A1A2A1–2-bHLHPAS; *7*, pBOSGAL4DBD-A1A1A2–2-bHLHPAS; *8*, pBOSGAL4DBD-ArntH378P-bHLHPAS (see "Experimental Procedures"). The full activity of *column 1* was taken as a standard to calculate relative activities. *D*, amino acid sequences of a part of PASB region of Arnt and Arnt2 responsible for differential transcriptional activities in association with AhR. Predicted exposed side chains of amino acids (27) are indicated by *arrows*. *m*, mouse; *h*, human; *c*, chicken; *x*, *Xenopus*; *z*, zebrafish.

2*B*, *upper panel*, *columns* 2 and 5). Thus, it is not the highly variable C-terminal domain but the PAS domain that is responsible for the differences in XRE-driven luciferase expression. In contrast, any combination of Arnt and Arnt2 domains was able to activate luciferase expression to the same extent in response to hypoxia. Taken together, these results suggest that only the PAS domain of Arnt is capable of efficient heterodimerization with AhR to activate the XRE, but HIFα interacted equally with the PAS domains of Arnt and Arnt2 leading to HRE-mediated transcription.

Interaction of the PAS Domains of Arnt or Arnt2 with Those of AhR and HIF1α—To further investigate the interaction of the PAS domains of Arnt and Arnt2 with AhR, we used a mammalian two-hybrid system. Arnt/Arnt2 bHLH-PAS chimeric constructs fused with the GAL4DBD were used as bait, and AhRΔC or HIF1αΔC fused with the VP16 activation domain were used as preys (Fig. 3*A*). Replacement of only a small portion of the PASB domain of Arnt with that of Arnt2 almost completely abrogated 3MC-induced luciferase expression (Fig. 3*B*, *upper panel*, *columns 2, 4*, and *5 versus 1, 3*, and *6*). All constructs were equally expressed except for a slightly lower expression of construction 8 (Fig. 3*C*). Taken together, these data indicate that the critical region for determining the differential interaction of Arnt and Arnt2 with AhR covers the sequence of amino acids 334–397 of Arnt and 308–371 of Arnt2. This region is predicted to form the N-terminal cap and a part of the PAS core of the PASB fold and is thought to be solvent-exposed, forming an interface to interact with partner proteins (27). In this region there are seven amino acids with side chains that are predicted to be solvent-exposed (27) (indicated by *arrows* in Fig. 3*D*), and six are conserved or conservative amino acid replacements between Arnt and Arnt2. Only the replacement of His with Pro was considered to be significant for the functional difference between Arnt and Arnt2. These amino acids, His and Pro, are conserved, respectively, in Arnt and Arnt2 of various animal species (Fig. 3*D*). When we generated a His-to-Pro point mutation in Arnt, this construct was not able to interact efficiently with AhR to activate luciferase expression in response to 3MC (Fig. 3*B*, *upper panel*, *column 7*). On the other hand, all chimeric constructs, WT Arnt and Arnt2, and

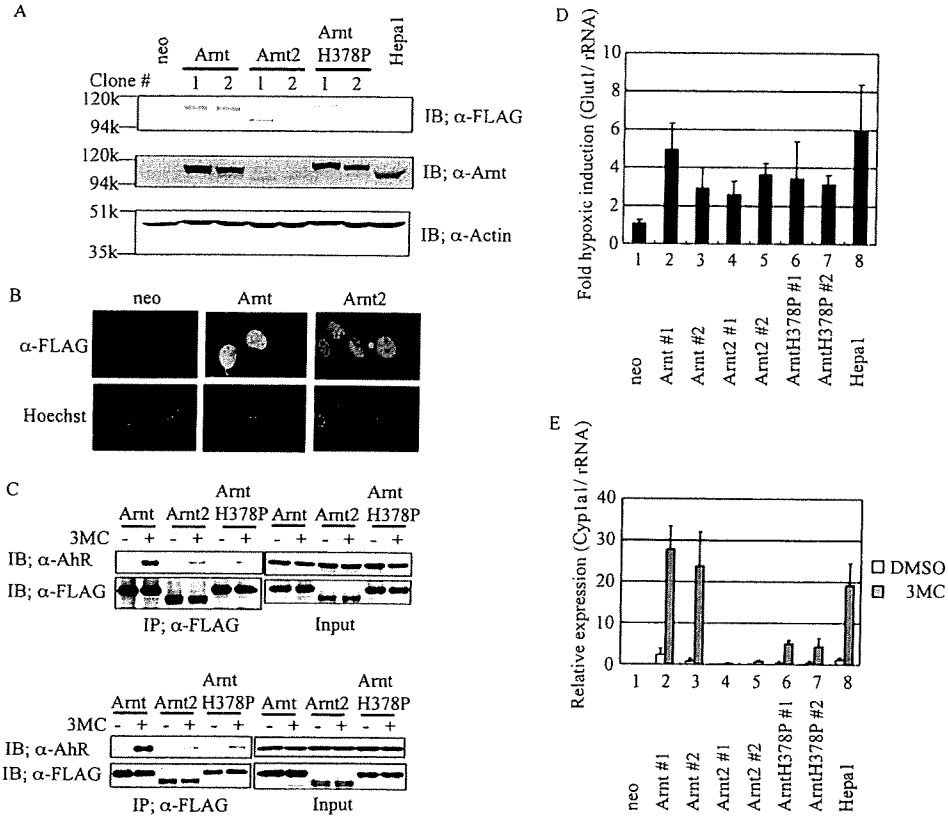

FIGURE 4. **Transcriptional activities of Arnt, Arnt2 and ArntH378P mutant in stably transformed Hepa1-c4 cells.** *A*, expression of 3xFLAG-Arnt, -Arnt2, and -ArntH378P. Expressed proteins were assessed by Western blotting (*IB*, immunoblot) using anti-Arnt, anti-FLAG, and anti-actin antibodies. *B*, cellular localization of 3xFLAG-Arnt and 3xFLAG-Arnt2. Hepa1-c4 cells stably expressing 3xFLAG-Arnt and 3xFLAG-Arnt2 were fixed and immunostained for Arnt and Arnt2 with anti-FLAG antibody. *C*, coimmunoprecipitation of AhR with Arnt, Arnt2, and ArntH378P in response to 3MC. Whole cell extracts of transformants expressing 3xFLAG-Arnt, 3xFLAG-Arnt2, and 3xFLAG-ArntH378P treated with 3MC or Me_2SO (dimethyl sulfoxide (*DMSO*)) were coimmunoprecipitated by anti-FLAG antibody. Coimmunoprecipitation and Western blotting were performed by the methods described under "Experimental Procedures." The *top* and *bottom left panels* show AhR and 3xFLAG-Arnts proteins coimmunoprecipitated by anti-FLAG antibody. *Input* is shown in the *right panels*. The *upper* and *lower panels* represent clone 1 and clone 2, respectively. *D*, expression of endogenous *Glut-1* gene in transformants expressing 3xFLAG-Arnt, -Arnt2, and -ArntH378. Transformed Hepa1-c4 cells were cultured for 16 h under conditions of normoxia or hypoxia (1% O_2), and cell extracts were prepared and used for determination of Glut-1 mRNA expression by real-time PCR analysis. Values are normalized against those for rRNA, and the results are expressed as induction ratios of hypoxic to normoxic activities. *E*, Expression of endogenous *CYP1A1* gene in the transformants. Stably transformed Hepa1-c4 cells were treated with Me_2SO or 3MC for 18 h, and the cell extracts were prepared and used for determination of CYP1A1 mRNA by real-time PCR analysis.

the Arnt His-to-Pro mutant induced luciferase expression to the same extent in response to hypoxia (Fig. 3*B*, *lower panel*). These data indicate that a single amino acid change is mainly responsible for the differential binding of Arnt and Arnt2 with AhR, whereas both Arnt and Arnt2 are equally capable of binding with HIFα.

Physical Interaction and Transcriptional Activity of Arnt and Arnt2 with AhR in Stable Transformants—Up to this point, we have studied the activity and interactions of WT Arnt and Arnt2 and their mutants with AhR and HIFα using relatively artificial transient transfection and two-hybrid assays. We wanted to examine the behavior of these proteins in a more physiologic setting, and we generated Hepa1-c4 cells stably expressing 3xFLAG-Arnt, -Arnt2, and -ArntH378P as described under "Experimental Procedures."

For each construct we isolated two clones with 3xFLAG-Arnt/Arnt2 expression levels comparable with endogenous Arnt in unmodified Hepa1 cells (Fig. 4*A*) and the stably expressed 3xFLAG-Arnt, -Arnt2, and -ArntH378P (data not shown) proteins localized to the nucleus (Fig. 4*B*) (28). To evaluate the interaction between endogenous AhR and these stably expressed 3xFLAG-Arnt, -Arnt2, and -ArntH378P proteins, AhR-3xFLAG-Arnt coimmunoprecipitation assays were performed. In the absence of cellular treatment with 3MC, AhR was not coimmunoprecipitated with 3xFLAG-tagged Arnt, Arnt2, or ArntH378P by an anti-FLAG antibody. However, following incubation of cells with 3MC, AhR was coimmunoprecipitated with 3xFLAG-Arnt by using anti-FLAG antibody, but only a small amount of AhR was detected in the anti-FLAG immunoprecipitates from two transformant cell lines expressing 3xFLAG-Arnt2 or 3xFLAG-ArntH378P (Fig. 4*C*). These results are consistent with the reduced affinity of Arnt2 or the ArntH378P mutant for AhR as revealed in the mammalian two-hybrid system (Fig. 3*B*, *top panel*, *columns 1*, *7*, and *8*), but they are at odds with a previous study from our laboratory (13). In that report, we incubated *in vitro* translated Arnt and Arnt2 with AhR produced in Sf2 cells. It is likely that the relatively high concentrations of Arnt2 and AhR produced in the *in vitro* incubation system gave rise to a misleadingly significant band.

We next investigated the transcriptional activity of Arnt, Arnt2, and the ArntH378P mutant in the generated stable transformant cells, and we chose to examine Glut-1 and CYP1A1 induction by quantitative real-time PCR as target genes for HIF1α and AhR, respectively (29, 30). In cells expressing 3xFLAG-Arnt, -Arnt2, or -ArntH378P, Glut-1 was induced to a similar extent under hypoxic conditions when mRNA levels were normalized to normoxic cells (Fig. 4*D*). In contrast, treatment of cells stably expressing 3xFLAG-Arnt with 3MC dramatically increased CYP1A1 mRNA levels compared with untreated cells, and little induction was seen in cells expressing 3xFLAG-Arnt2 (Fig. 4*E*). Cells expressing 3xFLAG-ArntH378P slightly, but significantly, increased CYP1A1 mRNA levels following 3MC treatment, indicating that His-378 in the Arnt PASB domain strongly influences Arnt binding to AhR but that other regions of Arnt also contribute to AhR binding.

The Arnt PASA and PASB Domains Cooperatively Bind AhR—We saw some degree of *CYP1A1* transcription in response to 3MC in cells expressing 3xFLAG-ArntH378P, and we were

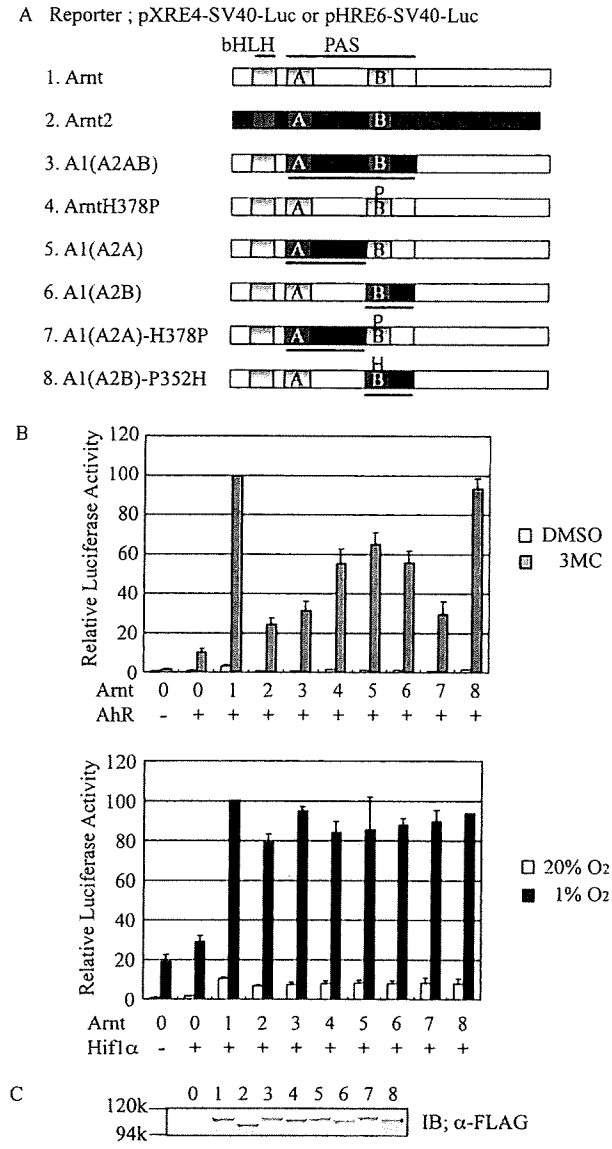

FIGURE 5. **Contribution of PASA and PASB sequences of full-length Arnt and Arnt2 to the expression of XRE-driven and HRE-driven reporter genes.** *A*, constructs of the expression plasmids of Arnt and Arnt2 and their chimeric and mutant proteins. *Underlines* show the Arnt domain replaced by the corresponding Arnt2 domain (*constructs 3* and *5–8*). *B, top*, expression of the XRE-driven reporter gene. HeLa cells were transfected with 10 ng of pXRE4-SV40-Luc, 2 ng of the indicated expression plasmid, and 20 ng of pBOSmAhR, and the reporter gene expression assay was performed as described in the legend to Fig. 1*B*. *DMSO*, dimethyl sulfoxide (Me_2SO). *Bottom*, expression of the HRE-driven reporter gene. HeLa cells were transfected with 10 ng of pHRE6-SV40-Luc, 2 ng of the indicated expression plasmid, and 20 ng of pBOShHIF1α, and the reporter gene expression assay was performed as described in the legend to Fig. 1*B*. Values are represented by mean ± S.D. of the results of three independent experiments normalized to *Renilla* luciferase activity used as an internal control. The full activity of *column 1* was taken as a standard to calculate relative activities. *C*, concentrations of the expressed effector proteins. The cells were transfected with 10 ng of the indicated expression construct in 6-well plates. Equal amounts of cell lysates were used for determination of the expressed proteins. *Columns* in *B* and *C*: *0*, pBOS; *1*, pBOS3xFLAG-Arnt; *2*, pBOS3xFLAG-Arnt2; *3*, pBOS3xFLAG-A1(A2AB); *4*, pBOS3xFLAG-ArntH378P; *5*, pBOS3x-FLAG-A1(A2A); *6*, pBOS3xFLAG-A1(A2B); *7*, pBOS3xFLAG-A1(A2A)H378P; *8*, pBOS3xFLAG-A1(A2B)P352H. *IB*, immunoblot.

interested in determining other regions of Arnt that support its interaction with AhR. Toward this end, we generated several additional Arnt/Arnt2 chimeric constructs (Fig. 5*A*) and investigated their ability to interact with AhR by luciferase reporter assay. As shown above, all WT and chimeric Arnt and Arnt2 constructs were able to induce luciferase expression in response to hypoxia when coexpressed with HIF1α (Fig. 5*B*, *lower panel*). As expected, Arnt induced XRE-driven luciferase expression to a greater extent than Arnt2, but some luciferase expression was seen in Arnt2-expressing cells compared with untreated cells as reported in our previous study (13) (Fig. 5*B*, *upper panel*, *columns 0*, *1*, and *2*). The physiologic significance of this induction is questionable, however, given the inability of stably expressed Arnt2 to induce the expression of the endogenous *CYP1A1* gene (Fig. 4*E*, *columns 4* and *5*); the ability of Arnt2 to induce luciferase in response to 3MC may be an overexpression artifact. Substitution of the Arnt PASA and PASB domains with those of Arnt2 led to reduced luciferase expression comparable with WT Arnt2 (Fig. 5*B*, *upper panel*, *columns 2*, and *3*). Additionally, the H378P mutation as well as swapping the PASA or PASB domain of Arnt2 led to intermediate levels of luciferase expression (Fig. 5*B*, *upper panel*, *columns 4*, *5*, and *6*), indicating that the PASA domain is largely responsible for the additional AhR binding activity of Arnt. Interestingly, replacement of the Arnt PASA domain with that of Arnt2 and mutation of His-378 to Pro led to luciferase expression comparable with that of WT Arnt2 (column 7), thus confirming the importance of the two regions for Arnt·AhR binding. In contrast, when the Pro residue of the Arnt2 PASB domain was mutated to His, and this mutant PASB domain replaced the corresponding one in Arnt, luciferase expression in response to 3MC was restored to WT Arnt levels (Fig. 5*B*, *upper panel*, *column 8*). As expected from the two-hybrid assay, all WT and chimeric Arnt/Arnt2 constructs behaved similarly in response to hypoxia (Fig. 5*B*, *lower panel*).

The PASB Domain of AhR Is Responsible for Differential Binding to Arnt and Arnt2—A single amino acid change largely impaired the ability of Arnt to bind AhR, and we determined the region of AhR that interacts with Arnt PASB. We used a mammalian two-hybrid system with GAL4DBD-fused Arnt, Arnt2, and the Arnt mutant as bait (Fig. 3*A*, *constructs 1*, *7*, and *8*). When we used VP16AD-AhRΔC (Fig. 6*A*) as prey together with GAL4DBD-Arnt-bHLHPAS, the reporter gene was strongly induced following 3MC treatment (Fig. 6*B*, *column 4*). Expression of a VP16AD-AhRΔBΔC construct (Fig. 6*A*) further lacking the AhR PASB region constitutively expressed a high degree of luciferase activity, although a slight inducibility remained with 3MC (Fig. 6*B*, *column 5*). The PASB region of AhR binds the HSP90 complex, and in its absence, AhR is not retained in the cytoplasm and mediates transcription in the absence of stimuli (31–33). In contrast, in cells expressing both GAL4DBD-Arnt2-bHLHPAS and VP16AD-AhRΔC, reporter gene expression was reduced remarkably and was only slightly inducible (Fig. 6*B*, *column 10*). In stark contrast, coexpression of the PASB-deleted construct VP16AD-AhRΔBΔC with GAL4DBD-Arnt2-bHLHPAS led to constitutive luciferase activity comparable with that seen with GAL4DBD-Arnt-bHLHPAS (Fig. 6*B*, *column 5 versus 11*). These results indicate that the bHLH and PASA domains of AhR are able to interact with Arnt and Arnt2 to an equal extent to mediate constitutive transcriptional activity. Addition of the PASB region of AhR, how-

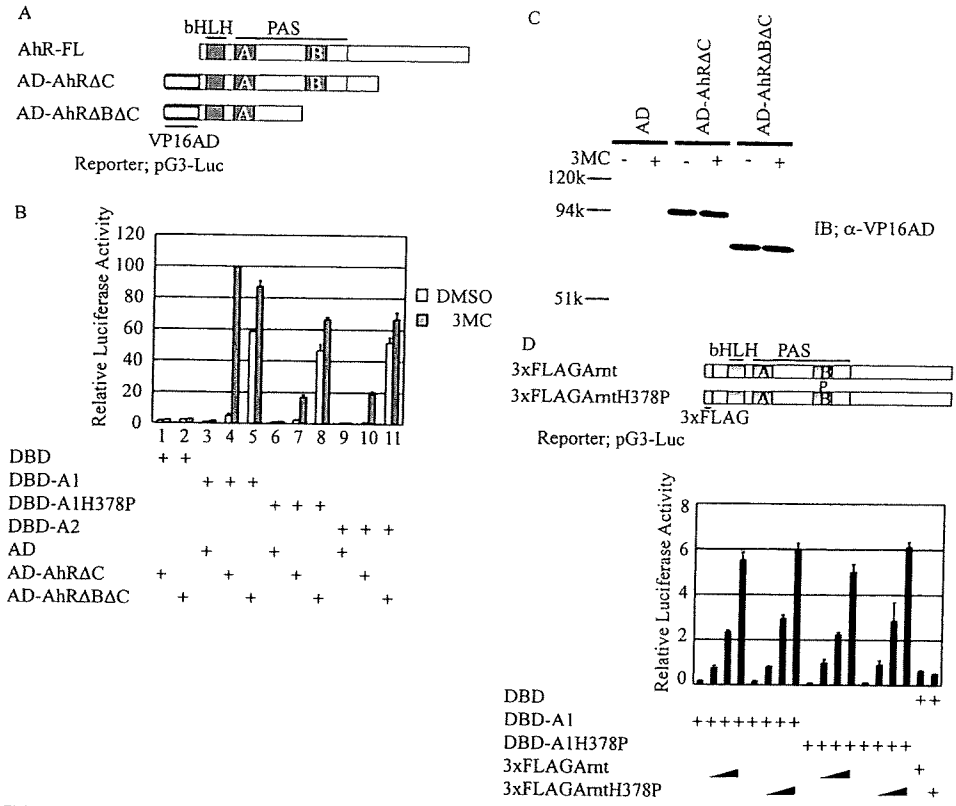

FIGURE 6. **PASB/PASB interaction is important for specific AhR/Arnt heterodimerization.** *A*, constructs of VP16AD-AhRΔC (*AD-AhRΔC*) and VP16AD-AhRΔBΔC (*AD-AhRΔBΔC*) used as prey. *B*, reporter gene expression. 293T cells were transfected with pG3-Luc, the indicated bait plasmid (pBOSGAL4DBD (*DBD*), pBOSGAL4DBD-Arnt-bHLHPAS (*DBD-A1*), pBOSGAL4DBD-ArntH378P-bHLHPAS (*DBD-A1H378P*), or pBOSGAL4DBD-Arnt2-bHLHPAS (*DBD-A2*)), and the indicated prey plasmid (pBOSVP16AD (*AD*), pBOSVP16AD-mAhRΔC, or pBOSVP16AD-mAhRΔBΔC), and after 24 h of incubation, the cells were treated with 1 μM 3MC or Me_2SO (dimethyl sulfoxide (*DMSO*)) for 18 h. Values are represented by mean ± S.D. of the results of three independent experiments normalized to *Renilla* luciferase activity used as an internal control. The full activity of *column 1* was taken as a standard to calculate relative activities. *C*, protein expression. The cells were transfected with 50 ng of the indicated expression construct in 6-well plates. The protein levels of all constructs were evaluated by Western blotting (*IB*, immunoblot) using anti-VP16AD antibody. Equal amounts of cell lysates were used for gel electrophoresis. *Columns 1* and *2*, pBOSVP16AD (*AD*); *columns 3* and *4*, pBOSVP16AD-mAhRΔC (*AD-AhRΔC*); *columns 5* and *6*, pBOSVP16AD-mAhRΔBΔC (*AD-AhRΔBΔC*). In *columns 1*, *3*, and *5*, cells were treated with Me_2SO for 18 h; in *columns 2*, *4*, and *6*, cells were treated with 1 μM 3MC for 18 h. *D*, effect of H378P mutation on Arnt/Arnt homodimerization. *Top*, 3xFLAG-Arnt and 3xFLAG-ArntH378P were used as activators. *Bottom*, expressed luciferase activities from Arnt/Arnt interaction. 293T cells were transfected with 100 ng of pG3-Luc and 10 ng of the indicated bait vectors (pBOSGAL4DBD, pBOSGAL4DBD-Arnt-bHLHPAS, or pBOSGAL4DBD-ArntH378P-bHLHPAS) and the indicated prey vector (50 ng of pBOS or 10, 20, or 50 ng of pBOS3xFLAG-Arnt or pBOS3xFLAG-ArntH378P). After 24 h of culture, the medium was changed, and the cells were further cultured for 24 h. The cell extracts were prepared from the cultured cells and used for luciferase assays. Values are represented by mean ± S.D. of the results of three independent experiments normalized to *Renilla* luciferase activity used as an internal control.

ever, specifically inhibited its interaction with Arnt2, leading to reduced reporter gene expression. Thus, the PASB domain of AhR determines its binding specificity for Arnt.

We next examined the ability of the His-378 to Pro Arnt mutant to interact with AhR; it behaved virtually identically to Arnt2 (Fig. 6*B*, *columns 7* and *8 versus 10* and *11*), indicating that Pro-352 in Arnt2 is primarily responsible for its reduced affinity for AhR. In these experiments, the prey proteins were expressed almost equally (Fig. 6*C*).

Homodimerization of Arnt and Arnt H378P—Arnt can form a heterodimer with AhR and HIF1α, but it also homodimerizes and binds E-box sequences (25, 34, 35). We investigated the ability of ArntH378P to homodimerize with either WT or mutant Arnt, using the mammalian two-hybrid assay with GAL4DBD-Arnt-bHLHPAS or GAL4DBD-ArntH378P-bHLHPAS as bait (Fig. 3*A*, *constructs 1* and *7*) and a full-length 3xFLAG-Arnt or its H378P mutant as prey (Fig. 5*A*, *constructs 1* and *4*). In any combination of bait and prey, both WT and H378P mutant Arnt activated similar levels of reporter gene expression (Fig. 6*D*), indicating that Arnt homodimerization is not sensitive to the H378P mutation. This result also suggests that Arnt PASB His-to-Pro mutation influences specifically AhR-Arnt dimerization, not the general dimerization.

DISCUSSION

Arnt and Arnt2 are structurally very similar and are believed to be common obligate dimerization partners for a number of bHLH-PAS transcription factors including AhR, HIFα, and Sims (13). We found no differences in the ability of Arnt, Arnt2, or any mutant or chimeric constructs used to activate gene expression in response to hypoxia in both transient and stable expression systems, suggesting that both proteins are equally able to bind HIFα, the key transcription factor mediating the hypoxic response. Thus, Arnt and Arnt2 play functionally interchangeable and overlapping roles in glycolysis, erythropoiesis, and angiogenesis in response to hypoxic conditions. The compensatory effects of Arnt and Arnt2 are noticeable in central nervous system development where Arnt2 is expressed abundantly (36, 37). Whole-mount PECAM (platelet endothelial cell adhesion molecule) immunohistochemistry on the embryonic central nervous system revealed no obvious differences between the WT and $Arnt2^{-/-}$ embryos, whereas $Arnt^{-/-}$ embryos had clear disruptions of the vascular endothelial network (20). This observation suggests that the expression of *VEGF* and other HIFα target genes in $Arnt2^{-/-}$ embryos is effectively normal, presumably because of the compensatory effects of Arnt. In the later stage of central nervous system development, however, several unique functions of Arnt2 become apparent, and $Arnt2^{-/-}$ embryos die perinatally with impaired hypothalamic development (15).

In contrast to their overlapping ability to bind HIFα, and also in apparent contrast with a previous report (13), clear differences were seen in the ability of Arnt and Arnt2 to interact with AhR. XRE-driven luciferase activity in cells expressing Arnt2 was much less than that seen in cells expressing Arnt following

3MC treatment, and the transcription of an XRE-responsive endogenous gene, *CYP1A1*, was completely absent in Hepa1-c4 cells stably expressing Arnt2 after treatment with 3MC (Fig. 4*E*, *columns 4* and *5*). In contrast, Hepa1-c4 cells stably expressing Arnt induced high levels of *CYP1A1* transcripts following 3MC treatment (Fig. 4*E*, columns 2 and 3). Some activation of the XRE-driven reporter gene by Arnt2 (about one-fifth of the Arnt activation) in the previous report (13) is misleading, most probably because of overexpression of Arnt2 in the transient DNA transfection experiments. Arnt2 expressed in these transformant cells was clearly functional, as shown by its ability to induce HRE-responsive genes in conjunction with HIFα (Fig. 4*D*), suggesting that Arnt2 plays little or no role in the cellular response to xenobiotics to induce the XRE-inducible genes in conjunction with AhR. This conclusion is further supported by the observation that zebrafish injected with a zfArnt2 antisense morpholino induced zfCYP1A1 in response to TCDD- as well as sham-treated animals, and there were no differences in TCDD-induced cytotoxicities requiring Arnt/AhR dimerization, such as pericardial edema, reduced trunk blood flow, and shortened lower jaw between these animals (38).

The bHLH domain of AhR is thought to be essential for heterodimer formation between bHLH-PAS transcription factors, and the bHLH domain alone of AhR is capable of dimerizing with the unrelated bHLH-Zip transcription factor USF (39). When the PASA domain is added to the AhR bHLH domain, however, AhR becomes much less promiscuous and stably binds Arnt (39). We showed that a construct consisting of the AhR bHLH and PASA domains binds both Arnt and Arnt2 (Fig. 6*B*, *columns 5* and *11*), but the addition of the PASB domain restricts AhR binding exclusively to Arnt (Fig. 6*B*, *columns 4* and *10*). Taken together, these data indicate that Arnt2 is not likely involved in the induction of drug-metabolizing enzymes following exposure to exogenous aromatic chemicals and other biological processes requiring AhR as a transcription factor.

A recent study reported that both the PASA and PASB domains of HIFα are necessary for heterodimer formation with Arnt (40). The minimal PAS domain structure consists of a 3-stranded β sheet, designated the β-scaffold, and a central α-helical PAS core region containing three short stretches of α-helices and β-sheets connected by a single α-helix called the helical connector. Mutational analysis revealed that hydrophobic interactions between conserved amino acids on the surface of the β-scaffold of HIFα and Arnt are important for heterodimerization, and replacement of hydrophilic amino acids (Q322E, M338E, and Y342T in HIF2α) disrupted this interaction leading to reduced transcription activity in response to hypoxia (40–42). Replacement of His-378 with Pro in Arnt, a residue a little away from the β-scaffold, disrupted its interaction with AhR but had no effect on the interaction of Arnt with HIFα. This suggests that the PASB/PASB-interacting surfaces or modes of AhR and Arnt are different from those of HIFα and Arnt. Additionally, the structural basis for the inhibition of AhR binding by Pro-352 of Arnt2, without affecting HIFα binding, should be examined. Such studies could provide valuable insight into the molecular regulation of the xenobiotic response as well as the diversification of a highly conserved family of transcription factors.

Acknowledgments—We thank Dr. O. Hankinson for kindly providing Hepa1-c4 mutant and Y. Nemoto for clerical work.

REFERENCES

1. Jackson, F. R., Bargiello, T. A., Yun, S. H., and Young, M. W. (1986) *Nature* **320,** 185–188
2. Reddy, P., Jacquier, A. C., Abovich, N., Petersen, G., and Rosbash, M. (1986) *Cell* **46,** 53–61
3. Hoffman, E. C., Reyes, H., Chu, F. F., Sander, F., Conley, L. H., Brooks, B. A., and Hankinson, O. (1991) *Science* **252,** 954–958
4. Crews, S. T., Thomas, J. B., and Goodman, C. S. (1988) *Cell* **52,** 143–151
5. Nambu, J. R., Lewis, J. O., Wharton, K. A., Jr., and Crews, S. T. (1991) *Cell* **67,** 1157–1167
6. Kewley, R. J., Whitelaw, M. L., and Chapman-Smith, A. (2004) *Int. J. Biochem. Cell Biol.* **36,** 189–204
7. Schmidt, J. V., Su, G. H., Reddy, J. K., Simon, M. C., and Bradfield, C. A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 6731–6736
8. Baba, T., Mimura, J., Nakamura, N., Harada, N., Yamamoto, M., Morohashi, K., and Fujii-Kuriyama, Y. (2005) *Mol. Cell. Biol.* **25,** 10040–10051
9. Huang, L. E., Gu, J., Schau, M., and Bunn, H. F. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 7987–7992
10. Ryan, H. E., Lo, J., and Johnson, R. S. (1998) *EMBO J.* **17,** 3005–3015
11. Iyer, N. V., Kotch, L. E., Agani, F., Leung, S. W., Laughner, E., Wenger, R. H., Gassmann, M., Gearhart, J. D., Lawler, A. M., Yu, A. Y., and Semenza, G. L. (1998) *Genes Dev.* **12,** 149–162
12. Tian, H., Hammer, R. E., Matsumoto, A. M., Russell, D. W., and McKnight, S. L. (1998) *Genes Dev.* **12,** 3320–3324
13. Hirose, K., Morita, M., Ema, M., Mimura, J., Hamada, H., Fujii, H., Saijo, Y., Gotoh, O., Sogawa, K., and Fujii-Kuriyama, Y. (1996) *Mol. Cell. Biol.* **16,** 1706–1713
14. Drutel, G., Kathmann, M., Heron, A., Schwartz, J. C., and Arrang, J. M. (1996) *Biochem. Biophys. Res. Commun.* **225,** 333–339
15. Hosoya, T., Oda, Y., Takahashi, S., Morita, M., Kawauchi, S., Ema, M., Yamamoto, M., and Fujii-Kuriyama, Y. (2001) *Genes Cells* **6,** 361–374
16. Maltepe, E., Schmidt, J. V., Baunoch, D., Bradfield, C. A., and Simon, M. C. (1997) *Nature* **386,** 403–407
17. Kozak, K. R., Abbott, B., and Hankinson, O. (1997) *Dev. Biol.* **191,** 297–305
18. Adelman, D. M., Gertsenstein, M., Nagy, A., Simon, M. C., and Maltepe, E. (2000) *Genes Dev.* **14,** 3191–3203
19. Abbott, B. D., and Buckalew, A. R. (2000) *Dev. Dyn.* **219,** 526–538
20. Keith, B., Adelman, D. M., and Simon, M. C. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 6692–6697
21. Mizushima, S., and Nagata, S. (1990) *Nucleic Acids Res.* **18,** 5322
22. Mimura, J., Ema, M., Sogawa, K., and Fujii-Kuriyama, Y. (1999) *Genes Dev.* **13,** 20–25
23. Numayama-Tsuruta, K., Kobayashi, A., Sogawa, K., and Fujii-Kuriyama, Y. (1997) *Eur. J. Biochem.* **246,** 486–495
24. Ema, M., Taya, S., Yokotani, N., Sogawa, K., Matsuda, Y., and Fujii-Kuriyama, Y. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 4273–4278
25. Sogawa, K., Nakano, R., Kobayashi, A., Kikuchi, Y., Ohe, N., Matsushita, N., and Fujii-Kuriyama, Y. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 1936–1940
26. Park, S. K., Dadak, A. M., Haase, V. H., Fontana, L., Giaccia, A. J., and Johnson, R. S. (2003) *Mol. Cell. Biol.* **23,** 4959–4971
27. Pellequer, J. L., Wager-Smith, K. A., Kay, S. A., and Getzoff, E. D. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 5884–5890
28. Eguchi, H., Ikuta, T., Tachibana, T., Yoneda, Y., and Kawajiri, K. (1997) *J. Biol. Chem.* **272,** 17640–17647
29. Wood, S. M., Gleadle, J. M., Pugh, C. W., Hankinson, O., and Ratcliffe, P. J. (1996) *J. Biol. Chem.* **271,** 15117–15123
30. Ko, H. P., Okino, S. T., Ma, Q., and Whitlock, J. P., Jr. (1996) *Mol. Cell. Biol.* **16,** 430–436
31. Coumailleau, P., Poellinger, L., Gustafsson, J. A., and Whitelaw, M. L. (1995) *J. Biol. Chem.* **270,** 25291–25300
32. Whitelaw, M. L., Gottlicher, M., Gustafsson, J. A., and Poellinger, L. (1993) *EMBO J.* **12,** 4169–4179

33. McGuire, J., Okamoto, K., Whitelaw, M. L., Tanaka, H., and Poellinger, L. (2001) *J. Biol. Chem.* **276,** 41841–41849
34. Antonsson, C., Arulampalam, V., Whitelaw, M. L., Pettersson, S., and Poellinger, L. (1995) *J. Biol. Chem.* **270,** 13968–13972
35. Huffman, J. L., Mokashi, A., Bachinger, H. P., and Brennan, R. G. (2001) *J. Biol. Chem.* **276,** 40537–40544
36. Jain, S., Maltepe, E., Lu, M. M., Simon, C., and Bradfield, C. A. (1998) *Mech. Dev.* **73,** 117–123
37. Aitola, M. H., and Pelto-Huikko, M. T (2003) *J. Histochem. Cytochem.* **51,** 41–54
38. Prasch, A. L., Heideman, W., and Peterson, R. E. (2004) *Toxicol. Sci.* **82,** 250–258
39. Pongratz, I., Antonsson, C., Whitelaw, M. L., and Poellinger, L. (1998) *Mol. Cell. Biol.* **18,** 4079–4088
40. Yang, J., Zhang, L., Erbel, P. J., Gardner, K. H., Ding, K., Garcia, J. A., and Bruick, R. K. (2005) *J. Biol. Chem.* **280,** 36047–36054
41. Erbel, P. J., Card, P. B., Karakuzu, O., Bruick, R. K., and Gardner, K. H. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 15504–15509
42. Card, P. B., Erbel, P. J., and Gardner, K. H. (2005) *J. Mol. Biol.* **353,** 664–677