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cDNA Cloning and Tissue-Specific Expression of a Novel Basic Helix-Loop-Helix/PAS Factor (Arnt2) with Close Sequence Similarity to the Aryl Hydrocarbon Receptor Nuclear Translocator (Arnt)

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We isolated mouse cDNA clones (Arnt2) that are highly similar to but distinct from the aryl hydrocarbon receptor (AhR) nuclear translocator (Arnt). The composite cDNA covered a 2,443-bp sequence consisting of a putative 2,136-bp open reading frame encoding a polypeptide of 712 amino acids. The predicted Arnt2 polypeptide carries a characteristic basic helix-loop-helix (bHLH)/PAS motif in its N-terminal region with close similarity (81% identity) to that of mouse Arnt and has an overall sequence identity of 57% with Arnt. Biochemical properties and interaction of Arnt2 with other bHLH/PAS proteins were investigated by coimmunoprecipitation assays, gel mobility shift assays, and the yeast two-hybrid system. Arnt2 interacted with AhR and mouse Sim as efficiently as Arnt, and the Arnt2-AhR complex recognized and bound specifically the xenobiotic responsive element (XRE) sequence. Expression of Arnt2 successfully rescued XRE-driven reporter gene activity in the Arnt-defective c4 mutant of Hepa-1 cells. RNA blot analysis revealed that expression of Arnt2 mRNA was restricted to the brains and kidneys of adult mice, while Arnt mRNA was expressed ubiquitously. In addition, whole-mount in situ hybridization of 9.5-day mouse embryos showed that Arnt2 mRNA was expressed in the dorsal neural tube and branchial arch 1, while Arnt transcripts were detected broadly in various tissues of mesodermal and endodermal origins. These results suggest that Arnt2 may play different roles from Arnt both in adult mice and in developing embryos. Finally, sequence comparison of the currently known bHLH/PAS proteins indicates a division into two phylogenetic groups: the Arnt group, containing Arnt, Arnt2, and Per, and the AhR group, consisting of AhR, Sim, and Hif-1α.

The aryl hydrocarbon (Ah) receptor nuclear translocator (Arnt) is a member of a novel transcription factor family consisting of a basic helix-loop-helix (bHLH) structural motif contiguous with a PAS domain, a designated region conserved among Per (29, 38), Arnt (28), Ah receptor (AhR) (2, 13), and Sim (7, 34). Recent molecular cloning and biochemical studies have demonstrated that upon binding with an exogenous inducer such as 3-methylcholanthrene (3-MC) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, the AhR is translocated from the cytoplasm to the nucleus. During this process, association of the AhR with heat shock protein 90 (HSP90) (8, 35, 52) is disrupted and a heterodimer with Arnt is formed (32, 40). The AhR-Arnt complex recognizes *cis*-acting DNA enhancer sequences, known as xenobiotic responsive elements (XREs), which function upstream of the cytochrome P-4501A1 (CYP1A1) gene to induce transcription (17, 21). In addition to the induction of drug-metabolizing enzymes including CYP1A1, the AhR-Arnt system is considered to mediate the various biological effects of dioxin-like environmental pollutants, which include teratogenesis, tumor promotion, epithelial dysplasia, and immunosuppression (37, 46, 51). It has recently been reported that AhR gene disruption caused impairment of the liver and immune system in mice (15). Taken together, these results suggest that the AhR-Arnt system is involved in critical developmental processes. *Drosophila* Sim, another bHLH/PAS protein, is known to act as a master regulator in the development of the central nervous system (7, 34, 47) and is also capable of forming a heterodimer with human Arnt (44). On the basis of these results, we have made an attempt to find a mouse counterpart of Sim by cDNA cloning. We have isolated cDNA clones encoding a polypeptide highly homologous to *Drosophila* Sim, and the encoded product shows a high affinity for dimerization with Arnt (14).

Transcriptional regulatory factors with bHLH or bHLH/leucine zipper (LZ) structural motifs such as MyoD, myogenin, Myc, and Max constitute a superfamily with many constituents capable of forming homo- or heterodimers, and these factors are involved in key cellular mechanisms of differentiation and proliferation (1, 49, 54). It is therefore important to identify the full complement of bHLH/PAS factors and investigate how these factors influence cellular processes. We have screened a cDNA library prepared from mRNA of mouse 11.5-day embryos with segments of cDNAs coding for the bHLH/PAS domains of AhR and Arnt and isolated a novel cDNA clone (Arnt2) encoding a peptide with high sequence similarity to Arnt.

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Here we describe an entire primary sequence encoded by the isolated cDNA, tissue distribution of the mRNA, and some biochemical properties of the encoded Arnt2 product.

MATERIALS AND METHODS

Oligonucleotides. The following oligonucleotides were chemically synthesized and used in the experiments described below: Alike 1, 5′-CTCGAGCGCTTTCC TCCTCGGGCAGGCATGG-3′; Alike 2, 5′-CTCAATCTCACTGTGGTTCTC TCTT-3′; Alike 3, 5′-GTGGACCCCAGATGCATCAGTGTGA-3′; Alike 4, 5′-ATTTTGCCACCCTGAGGATCAGAGC-3′; and oligo(dT)$_{17}$, 5′-GAATTC GAGCTCTTTTTTTTTTTTTTTT-3′.

Cloning and sequencing of cDNA for mouse Arnt2. A 5′-stretch cDNA library from mouse 11.5-day embryos (Clontech) was screened with a mixture of ^{32}P-labeled mouse AhR (0.6 kb) and human Arnt (1.1 kb) cDNAs, both of which encode a part of the bHLH and PAS domains. Seven positive clones out of approximately 10^6 recombinant plaques were isolated by a standard screening procedure (42). Four of them had an identical 1.24-kb fragment and were designated Arnt2 cDNA, which is similar to but distinct from mouse Arnt cDNA, while the others contained parts of mouse Arnt cDNA. To obtain a full-length Arnt2 cDNA, rapid amplification of 5′ and 3′ cDNA ends (5′ and 3′-RACE) (18) was performed by using two pairs of Arnt2-specific primers (Alike 1 and 2 and Alike 3 and 4), oligo(dT)$_{17}$ primer, and poly(A) RNA isolated from 11.5-day embryos. The 5′-AmpliFINDER RACE kit (Clontech) was used for all RACE procedures according to the manufacturer's instructions. Total RNA from mouse 11.5-day embryos was applied to oligo(dT)-cellulose (Pharmacia) to obtain poly(A) RNA.

Nucleotide sequence was determined by the fluorescence dye-labeling cycle sequencing system (Applied Biosystems). Three independent clones each for 5′- and 3′-RACE were sequenced to check for PCR errors in the RACE products.

RNA preparation and RNA blot analysis. Total RNAs were isolated from various tissues of adult C57BL/6J mice and mouse 11.5-day embryos either by the acid guanidinium thiocyanate-phenol-chloroform method (6) or by extraction with guanidinium thiocyanate followed by centrifugation in CsCl solutions (42). Total RNA (20 μg) for each sample was electrophoresed on a 0.8% agarose gel in the presence of 2.2 M formaldehyde (42) and then blotted to a nylon membrane. The membrane was hybridized for 2 h at 65°C with a ^{32}P-labeled probe of a 1.15-kb *Acc*I-*Eco*81I fragment of mouse Arnt cDNA in ExpressHyb hybridization solution (Clontech) and then washed twice at 65°C for 30 min with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate (SDS). This membrane was dehybridized in a solution containing 50% formamide and 10 mM Tris-HCl (pH 7.5) at 65°C and reprobed with a ^{32}P-labeled 0.82-kb *Eco*O109I fragment of mouse Arnt2 cDNA.

Whole-mount in situ hybridization. Whole-mount in situ hybridization was performed essentially as described by Wilkinson (53). Mouse embryos (9.5 days postcoitum [dpc]) were treated as previously described. Color reactions were conducted for 4 h. A highly variable part (3′ half) of the cDNA sequence between Arnt and Arnt2 was used for hybridization experiments. For Arnt, a fragment of the 3′ coding sequence (0.62 kb) of Arnt cDNA was amplified by using a pair of primers, 5′-TTGTAGAATTTTGTCATCCT-3′ and 5′-AGACCA CTATTCCTGAAATT-3′, and inserted into pBluescriptSK(+) (Stratagene). For the Arnt2 probe, an *Eco*O109I fragment (0.82 kb) of Arnt2 cDNA was excised and inserted into pBluescriptSK(+). Antisense- and sense-strand digoxigenin-labeled probes of the mRNA were synthesized in vitro from the cDNA-containing pBluescripts by using either T3 or T7 promoter and the cognate T3 or T7 RNA polymerase.

Construction of plasmids. The 5′- and 3′-RACE products were joined to the previously isolated cDNA fragment (1.24 kb) at common *Nco*I and *Eco*81I sites, respectively, to obtain the complete coding sequence of Arnt2 which was then subcloned into pBluescriptSK(+). This plasmid (pBSKmArnt2) was digested with *Eco*RI-*Hin*dIII, blunt-ended by the Klenow fragment of DNA polymerase I, and subsequently inserted into the pCMSV expression vector (13) at the unique *Hin*dIII site, which had also been blunt-ended by the Klenow fragment. The resultant plasmid was named pCMArnt2 and used for the chloramphenicol acetyltransferase (CAT) assay.

Expression plasmids for the yeast two-hybrid system encoding the GAL4 DNA binding domain (pGBT9) and the GAL4 activation domain (pGAD424) were kindly provided by S. Fields. *Eco*RI (2.45 kb), *Eco*T14I-*Eco*RI (0.87 kb), and *Eco*RI-*Sac*I (1.56 kb) fragments excised from pBSKmArnt2 were blunt-ended and inserted into either the *Sma*I site or the blunt-ended *Bam*HI site of pGBT9 in an appropriate direction in frame to obtain pGBTmArnt2(1–712), pGBTmArnt2(524–712), and pGBTmArnt2(1–510), respectively. The blunt-ended 2.45-kb *Eco*RI fragment of pBSKmArnt2 was also inserted into pGAD424 at the *Sma*I site to make pGADmArnt2(1–712). pGBThAhR(1–424) was constructed by inserting *Xba*I termination linkers (New England Biolabs) at the *Bam*HI site of the plasmid encoding the GAL4 DNA binding domain-AhR, which was made by cloning the blunt-ended 2.6-kb *Xho*I-*Sph*I fragment from pBSAhR encoding the entire human AhR into the *Sma*I site of pGBT9. pGADhArnt(1–789) was constructed by cloning a blunt-ended 2.4-kb *Xho*I-*Bam*HI fragment from pR SETArnt encoding the entire human Arnt sequence into the blunt-ended *Pst*I site of pGAD424. The same fragment was also inserted into the blunt-ended *Pst*I site of pGBT9, digested with *Pst*I, blunt ended, and ligated with the *Xba*I termination linkers to obtain pGBTArnt(1–617). pGBTmSim(1–325) was constructed similarly and will be described elsewhere (11).

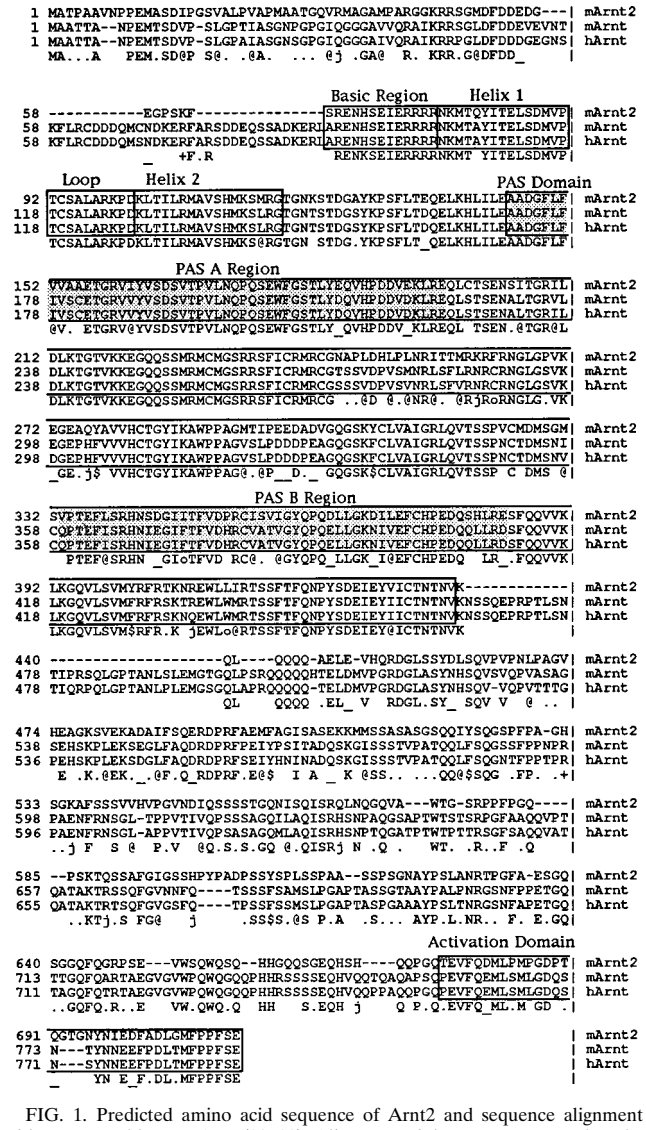

FIG. 1. Predicted amino acid sequence of Arnt2 and sequence alignment with mouse and human Arnt (28, 39). Alignment of the sequences was done by the method of Gotoh (24). Completely conserved sites are indicated by the amino acid letters beneath the sequences, and conserved hydrophobic (o), hydrophilic (j), small aliphatic (.), large aliphatic (@), aromatic ($), positive (+), and negative (−) sites are also indicated.

In vitro binding assay. Arnt2 and Arnt proteins were synthesized by the TNT T7-coupled reticulocyte lysate system (Promega) containing [^{35}S]methionine (ICN). The ^{35}S-labeled mouse Arnt2 or human Arnt was incubated with cell extracts from Sf21 cells infected with recombinant baculovirus expressing human AhR (45) and then immunoprecipitated with anti-AhR antibody (32). The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) as described previously (44).

Gel mobility shift assay. In vitro-synthesized mouse Arnt2 and human Arnt were incubated with the cell extracts from Sf21 cells infected with the recombinant baculovirus at 25°C for 2 h in a solution containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.9]), 6 mM $MgCl_2$, 50 mM KCl, 6% glycerol, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.5% aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 500 μg of salmon sperm DNA per ml in the presence or absence of 3-MC (1 μM). To the reaction mixture, ^{32}P-labeled XRE probe (22) was added with or without a 100-fold excess of unlabeled wild-type or mutated XRE (32), and the incubation was carried out for an additional 30 min. The reaction mixture was electrophoresed in a nondenaturing 4.5% polyacrylamide gel and autoradiographed.

Cell maintenance, DNA transfection, and CAT assay. A mutant cell line, c4, derived from Hepa-1c1c7 but defective in Arnt function (27, 31), kindly provided by O. Hankinson, was maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 0.35% glucose and seeded at a density of 10^6 cells per 6-cm dish 1 day before the transfection. An effector plasmid, pCMArnt (32) or pCMArnt2, was transfected into the cells with a reporter plasmid, pMC6.3k (20), by the calcium phosphate coprecipitation method (25). Four hours later, the cells were treated with glycerol and incubated for another 40 h in the presence or absence of 3-MC (1 μM) before harvest. Expressed CAT activities were assayed as described previously (23).

Yeast two-hybrid system. The yeast two-hybrid system was used essentially as described in reference 16. *Saccharomyces cerevisiae* SFY526 (*MAT***a** *ura3 his3 ade2 lys2 trp1 leu2 can*^{r} *gal4 gal80 URA3*::*GAL1-lacZ*) was transformed with various sets of expression plasmids constructed from pGBT9 and pGAD424, which express fusion proteins consisting of the GAL4 DNA binding domain (1 to 147 amino acids) and the GAL4 activation domain (768 to 881 amino acids), respectively. Transformants were incubated overnight in the selecting medium for both leucine and tryptophan requirements. The overnight culture (~5 μl) was inoculated into YPAD medium (3 ml) and incubated until the cell growth reached the mid-log phase in the presence or absence of 3-MC (10 μM). The optical densities of the cell suspensions at 600 nm (OD_{600}) were measured, and the cells were pelleted by centrifugation and resuspended in Z buffer (0.1 M phosphate buffer [pH 7.0], 10 mM KCl, and 1 mM $MgSO_4$). Subsequently, the cells were broken by rapid freezing in liquid nitrogen and thawing in a 37°C water bath. To the cell extract (100 μl), 700 μl of Z buffer supplemented with 0.27% 2-mercaptoethanol and 160 μl of *o*-nitrophenylgalactoside (4 mg/ml of phosphate buffer [pH 7.0]) was added and the mixture was incubated at 30°C. The reaction was stopped by the addition of 400 μl of 1 M Na_2CO_3, and the OD_{420} of the solution was measured after removal of the cell debris by centrifugation. β-Galactosidase activities were expressed in the units calculated by the following equation (33): β-galactosidase units = 1,000 × (OD_{420}/OD_{600})/reaction time (minutes)/amount of culture used for the reaction (milliliters).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number D63644.

RESULTS

Cloning and sequence of mouse Arnt2 cDNA. To investigate whether there are additional transcriptional regulators with the bHLH/PAS motif, we screened a cDNA library prepared from 11.5-day embryos with parts of Arnt and AhR cDNA encoding the characteristic bHLH/PAS structural motifs used as probes. cDNA clones covering a total length of about 1.24 kb with sequences highly similar to but distinct from those of Arnt were isolated. Subsequently, missing parts of the 5′- and 3′-terminal sequences in the cloned cDNA were isolated by RACE as described in Materials and Methods to obtain a full coding sequence. Combination of the three cloned cDNAs led to elucidation of a total 2,443-bp sequence. There are several ATG codons preceded by one stop codon in the upstream part of the sequence. The most upstream ATG is putatively assigned to be an initiation codon for several reasons: (i) it is followed by the longest open reading frame (2,136 bp); (ii) the sequence surrounding it fits the consensus (GCC[A/G]CCAT GG) for translational initiation (30) in 7 of 10 nucleotides; and (iii) the nucleotide sequence surrounding this ATG reasonably agrees with the beginning of the coding sequence for Arnt. However, since we could not isolate cDNA clones which extend further upstream, probably because of the GC-rich content of the sequence, it cannot be rigorously ruled out that the predicted sequence has a further N-terminal coding sequence. The predicted coding sequence encodes a polypeptide of 712 amino acids with an overall sequence identity of 57% with Arnt. In the conserved regions of the bHLH (55 amino acids) and PAS (294 amino acids) domains, the predicted primary structure has 95 and 78% amino acid identity, respectively, with Arnt (Fig. 1). Thus, the cDNA cloned here was tentatively designated Arnt2 cDNA.

Amino acid sequences of the bHLH/PAS regions for members of this protein family that have been determined so far were aligned together with that of Arnt2 in Fig. 2A. These six proteins are Arnt2, Arnt (28), Per (29, 38), AhR (2, 13), Hif-1α

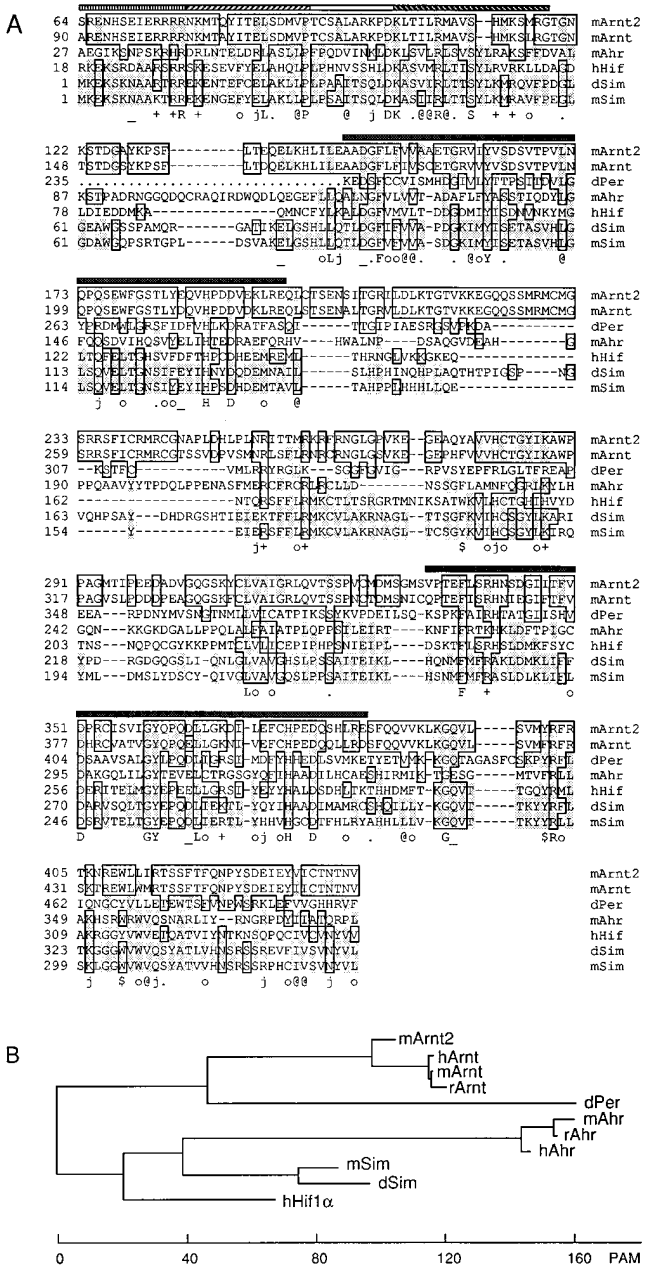

FIG. 2. Alignment of bHLH/PAS and PAS protein sequences in the bHLH motif and PAS domain (A) and phylogenetic tree constructed from the sequences of PAS regions (B). (A) Alignment of the sequences was done by the method of Gotoh (24). Basic region (▥), helix 1 (▨), loop (▭), helix 2 (▩), and two of the repeated sequences (▩) in the PAS domain are shown by the bars above the alignment. Completely conserved amino acids are indicated by the single-letter code beneath the sequences, and conserved hydrophobic (o), hydrophilic (j), small aliphatic (.), large aliphatic (@), aromatic ($), positive (+), and negative (_) sites are also indicated. Amino acids identical to those of mouse Arnt2 and mouse Sim are enclosed by solid lines and shadowed, respectively. (B) The phylogenetic tree was constructed by the method of Saitou and Nei (41). Sequences have been reported so far for human Arnt (28), mouse Arnt (39), rat Arnt (3), *Drosophila* Per (29), human AhR (9, 12), mouse AhR (2, 13), rat AhR (3), *Drosophila* Sim (7, 34), mouse Sim (14), and human Hif-1α (48). Abbreviations: m, mouse; h, human; r, rat; d, *Drosophila*; PAM, accepted point mutations per 100 sites.

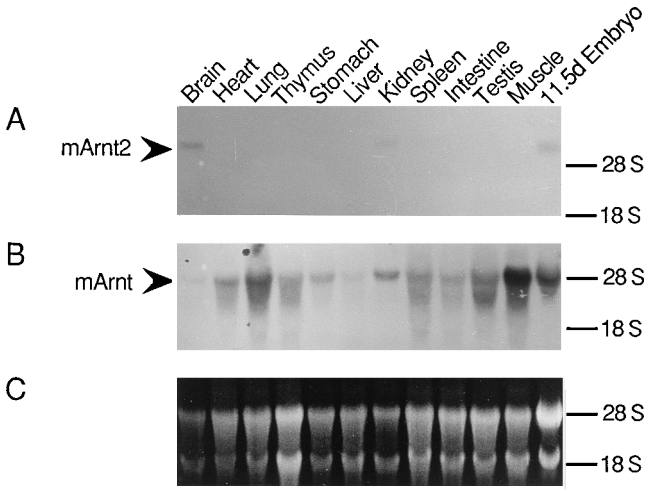

FIG. 3. RNA blot analysis of total RNAs from various tissues of adult mice and mouse embryos. Total RNAs (20 μg) were electrophoresed on a 0.8% agarose gel in the presence of 2.2 M formaldehyde and blotted to a nylon membrane. The membrane was probed with 0.82-kb Arnt2 (A) and 1.15-kb Arnt (B) cDNA probes and washed twice in 0.1× SSC–0.1% SDS for 30 min at 65°C. The positions of 28S and 18S rRNAs are shown on the right. The membrane was hybridized with the 1.15-kb Arnt cDNA probe and then subjected to dehybridization before hybridization with the 0.82-kb Arnt2 cDNA probe. (C) rRNAs stained with ethidium bromide as a control.

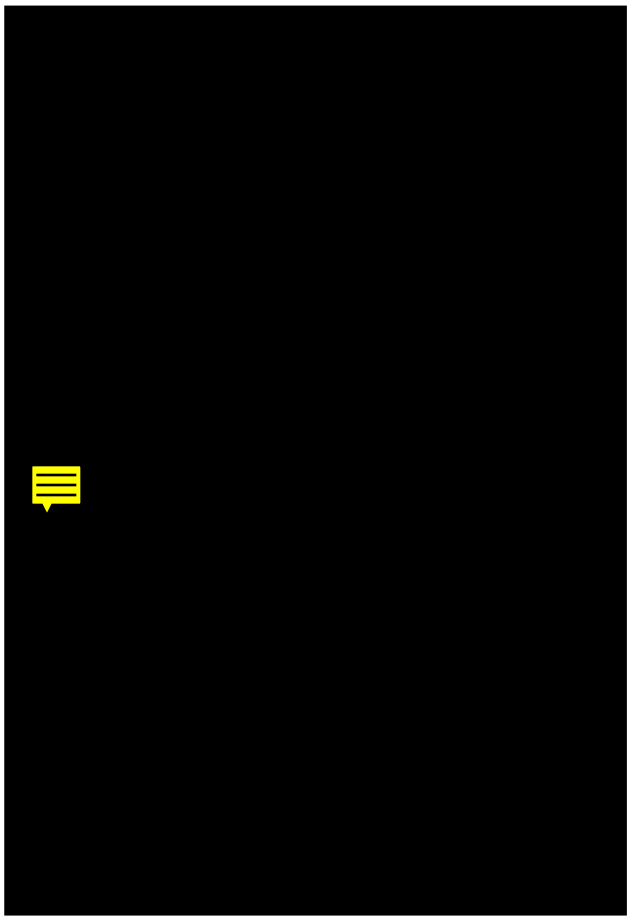

FIG. 4. Whole-mount in situ hybridization of 9.5-dpc embryos with Arnt2 and Arnt probes. Mouse embryos were taken out and treated as described by Wilkinson et al. (53). Color reactions were conducted for 4 h. A highly variable part (3′ half) of the cDNA sequence between Arnt and Arnt2 was used for the templates to synthesize antisense- and sense-strand digoxigenin-labeled RNA probes. Stainings with the Arnt2 antisense-strand (A) and sense-strand (B) probes and with the Arnt antisense-strand (C) and sense-strand (D) probes are shown.

(48), and Sim (7, 34). This family could be divided into two subgroups by sequence similarity; one includes Arnt2, Arnt, and Per, while the other comprises AhR, Hif-1α, and Sim. Sequence relatedness in the PAS region is summarized in the phylogenetic tree in Fig. 2B.

Tissue distribution of mouse Arnt2. Tissue distribution of Arnt2 mRNA was studied by RNA blot analysis using total RNAs isolated from brains, hearts, lungs, thymuses, stomachs, livers, kidneys, spleens, intestines, testes, muscles, and 11.5-day embryos. Arnt2 mRNA was detected only in brains, kidneys, and embryos, while Arnt mRNA was detected in all tissues and embryos examined (Fig. 3). The expression pattern of Arnt2 is also very different from that of AhR (3, 13, 36). The length of the Arnt2 transcript was estimated to be about 7 kb, which is much longer than that of the cDNA isolated, suggesting that the intact transcript would extend further to the 5′ end and/or to the 3′ end by alternative polyadenylation.

Whole-mount in situ hybridization. Different distributions of the Arnt and Arnt2 mRNA were also clearly observed in developing embryos by whole-mount in situ hybridization. Figure 4 presents representative results of 9.5-dpc embryos. Expression of Arnt2 mRNA was restricted to the dorsal spinal cord and branchial arch 1 (Fig. 4A), while Arnt mRNA was expressed broadly in various tissues of mesodermal and endodermal origins (Fig. 4C). On the other hand, the Arnt2 and Arnt sense-strand probes gave essentially no staining (Fig. 4B and D). These results suggest that Arnt2 has specific functions distinct from those of Arnt.

Interaction with AhR and XRE in vitro. Arnt is reported to form a heterodimer with liganded AhR and then bind the XRE sequence, a *cis*-acting DNA element found upstream of the CYP1A1 gene or other drug-metabolizing genes, resulting in activation of gene transcription (19, 46). Since Arnt2 is highly similar to Arnt in the primary structure as shown in Fig. 1, we tested if Arnt2 can functionally substitute for Arnt. First, we tested the ability of Arnt2 to heterodimerize with AhR in vitro by coimmunoprecipitation. Either ^{35}S-labeled Arnt2 or ^{35}S-labeled Arnt was synthesized in vitro and used in dimerization reactions with AhR which had been produced in Sf21 cells with the baculovirus expression system. Arnt2 and Arnt were separately incubated with Sf21 extracts containing AhR in the presence or absence of 3-MC and subsequently immunoprecipitated with an anti-AhR antibody. As shown in Fig. 5, both Arnt2 and Arnt were coimmunoprecipitated with an anti-AhR antibody in the presence or absence of 3-MC, although increased amounts of ^{35}S-labeled Arnt2 or Arnt seemed to be precipitated in the presence of 3-MC (Fig. 5, compare lane 3 with 4 and lane 8 with 9). Neither Arnt2 nor Arnt was precipitated above the background level when nonimmune serum or control cell extract prepared from uninfected Sf21 cells was used (Fig. 5, lanes 2, 5, 7, and 10). Thus, Arnt2 forms a heterodimer with AhR in vitro in an analogous fashion to Arnt.

Secondly, we examined by gel mobility shift assay whether the Arnt2-AhR heterodimer could recognize the XRE sequence in a similar manner to the Arnt-AhR heterodimer. Figure 6 shows that the Arnt2-AhR complex specifically bound to the XRE in the presence or absence of 3-MC, although the retarded signals were slightly enhanced in the presence of 3-MC (Fig. 6, compare lane 4 with 5 and lane 9 with 10).

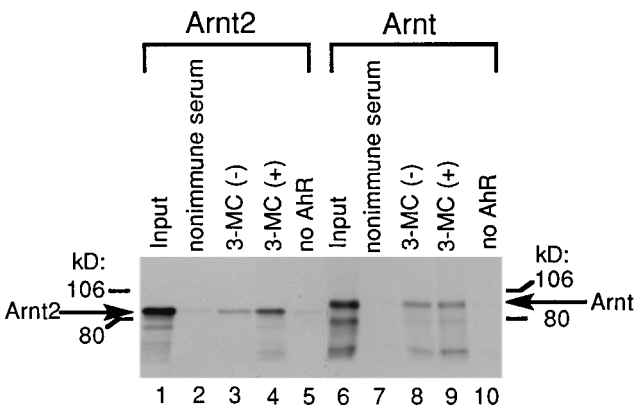

FIG. 5. Heterodimerization of AhR with Arnt2 and Arnt in vitro. ^{35}S-labeled Arnt2 (lanes 1 to 5) and Arnt (lanes 6 to 10) were synthesized by in vitro transcription-translation, incubated with extracts from Sf21 cells infected with recombinant baculovirus expressing human AhR (lanes 3, 4, 8, and 9) or uninfected Sf21 whole-cell extract (lanes 5 and 10) in the presence (lanes 4 and 9) or absence (lanes 3 and 8) of 3-MC (1 μM), and coimmunoprecipitated with anti-AhR antibody (lanes 3 to 5 and 8 to 10) or nonimmune serum (lanes 2 and 7). Precipitated proteins were denatured in an SDS gel-loading buffer by boiling and then subjected to SDS-PAGE on an 8% gel. Lanes 1 and 6 contained 1/100 volume of ^{35}S-labeled proteins used in those reactions.

Inducibility of heterodimer formation of Arnt2-AhR and Arnt-AhR, as revealed by the coimmunoprecipitation and gel mobility shift assays, was smaller than that reported previously because of a relatively high level of constitutive dimer formation without inducer. The reason for the high background level of inducible dimer formation is unknown. Since it has also been observed in a substantial quantity in previous studies (9, 10, 44), the level of inducible dimer formation may be variable, depending upon the method of the AhR preparation.

Competition experiments demonstrated that the retarded bands were inhibited efficiently by competition with an excess amount of unlabeled XRE (Fig. 6, compare lane 5 with 6 and lane 10 with 11), while no competition was observed with a mutated XRE sequence (Fig. 6, lanes 7 and 12).

Transcriptional activity in vivo of Arnt2 in Hepa-1 mutant (c4) cells. Since it was shown that Arnt2 heterodimerizes with AhR to bind the XRE sequence in vitro, we tested whether Arnt2 can substitute for Arnt function in vivo by using the transient-expression plasmid of Arnt2 in a mutant Hepa-1 cell line, c4, which is defective in the Arnt function (26, 27, 31). Arnt2 and Arnt expression plasmids were separately transfected into c4 cells together with a reporter plasmid, pMC6.3k, which contains the CAT gene under control of a 6.3-kb promoter sequence from the rat CYP1A1 gene (20). Transfected c4 cells were incubated for 40 h in medium with or without 3-MC. Transient expression of either Arnt2 or Arnt enhanced CAT activity in the c4 cells in response to the added 3-MC (Fig. 7B, lanes 3 through 6). As previously reported (32), some constitutive CAT activity without inducer is apparent (Fig. 7B, lanes 3 and 5). Expressed CAT activity in response to transfection of the Arnt2 plasmid was always lower than that with the Arnt plasmid, possibly because of differences of affinity between Arnt and Arnt2 for AhR (as demonstrated with the yeast two-hybrid system), different affinities of the heterodimers for the XRE sequence, and/or variances in the amounts of expressed products. In any event, these results showed that Arnt2 essentially substitutes for Arnt in the transcriptional activation driven by the XRE sequence.

Activation domain of Arnt2 and interaction between bHLH/PAS proteins revealed by the yeast two-hybrid system. The

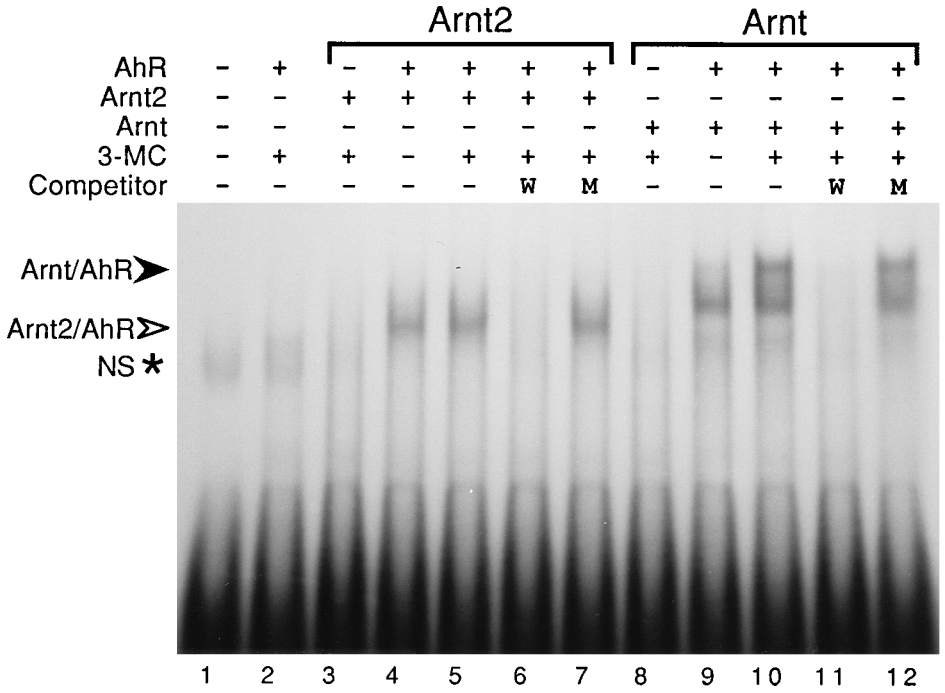

FIG. 6. Gel mobility shift assay of the XRE and the heterodimeric complexes of AhR with Arnt2 and Arnt. Human AhR produced in Sf21 cells with a baculovirus expression system was incubated in the presence or absence of 3-MC (1 μM) with either Arnt2 (lanes 3 to 7) or Arnt (lanes 8 to 12) synthesized in vitro. A ^{32}P-labeled XRE probe was added to the solution with or without a 100-fold excess of unlabeled XRE probes (W) or mutated XRE probes (M) and then electrophoresed on a 4.5% nondenaturing polyacrylamide gel after an additional 30 min of incubation. Only the labeled probe was electrophoresed in lane 1. One of the heterodimeric subunits was used in lanes 2, 3, and 8. Arrowheads indicate the retarded bands. NS, nonspecific bands.

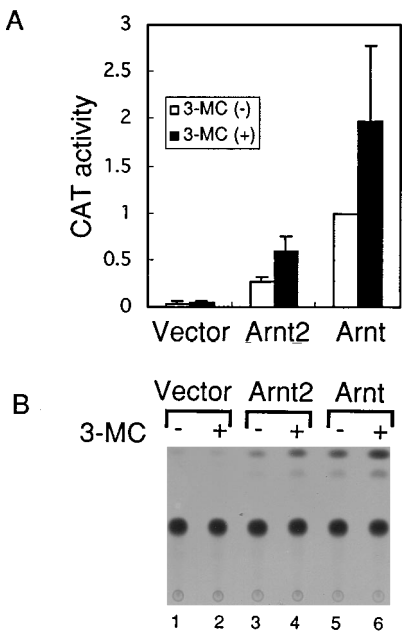

FIG. 7. Transfection of Arnt2 and Arnt expression plasmids in c4, an Arnt-deficient mutant of the Hepa-1 cell line. (A) The c4 cell line was cotransfected with the CAT reporter plasmid pMC6.3K (4 μg), which carries a 6.3-kb upstream sequence of the CYP1A1 gene, together with one of the three expression plasmids (4 μg) constructed from pCMSV: vector alone, pCMSVArnt2, or pCMSVArnt. CAT activities were assayed after a 2-day incubation in the presence or absence of 3-MC (1 μM) and normalized on the basis of the CAT activity of the c4 cells transfected with the Arnt expression plasmid without 3-MC. The columns show average CAT activities from three independent experiments, with standard deviations. (B) A representative result of the CAT assay.

bHLH/PAS proteins identified so far function as hetero- or homodimers. As shown in Fig. 5, Arnt2 can heterodimerize with AhR in an analogous fashion to Arnt; however, its tissue distribution pattern is different from that of either Arnt or AhR. Thus, there is a possibility that Arnt2 forms a dimer with itself or another member of the bHLH/PAS class of proteins. We therefore examined the interactions of Arnt2 with other known mammalian bHLH/PAS proteins by a yeast two-hybrid system (5, 16). cDNAs for mouse Arnt2, human Arnt, human AhR, and mouse Sim were fused with a set of the vectors pGAD424 and pGBT9 to express the hybrid proteins.

TABLE 1. Interaction of bHLH/PAS proteins with Arnt2 examined by the yeast two-hybrid system

DNA binding domain hybrid	3-MC (10 μM)	Enzyme activity of activation domain hybrid[a]		
		GAD424	GADmArnt2 (1–712)	GADhArnt (1–789)
GBT9	−	0.038	0.061	0.088
GBThAhR(1–424)	−	0.046	3.4	8.2
GBThAhR(1–424)	+	0.051	7.8	10
GBTmSim(1–325)	−	0.050	180	160
GBThArnt(1–617)	−	0.059	0.82	0.18
GBTmArnt2(1–510)	−	0.043	2.5	0.36

[a] Results were obtained and expressed in β-galactosidase units (33) as described in Materials and Methods.

When the hybrid protein of full-length Arnt2 that was fused to the GAL4 DNA binding domain [pGBTmArnt2(1–712)] was expressed in yeast cells, a high level of β-galactosidase activity was observed regardless of the presence or absence of a partner expression plasmid consisting of the GAL4 activation domain (Fig. 8). Therefore, to investigate the interaction of Arnt2 with other bHLH/PAS proteins, we deleted the activation domain of Arnt2 from this construct. The activation domain of Arnt was found to be located at its very C-terminal sequence of 34 amino acids (43), and the amino acid sequence of this region is conserved between Arnt and Arnt2 (Fig. 1). Thus, we created a C-terminal deletion construct, pGBT mArnt2(1–510), which was cotransformed with the vector pGAD424 into yeast cells. Activity of this C-terminally truncated Arnt2 decreased to background levels. In contrast, a deletion construct containing the C-terminal sequence, pGBT mArnt2(524–712), retained a high level of β-galactosidase activity. Therefore, the activation domain of Arnt2 is localized in the C-terminal sequence. Since similar results were obtained with other bHLH/PAS protein hybrids (data not shown), we deleted a part of the cDNAs encoding the C-terminal half for construction of the bait vectors.

As shown in Table 1, when either the bait or prey vector was transfected into yeast cells together with the empty partner vector, the expressed β-galactosidase activities were around

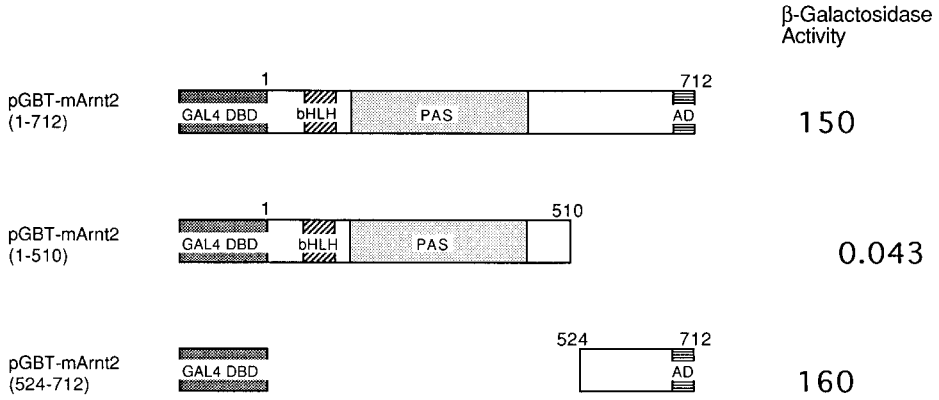

FIG. 8. Activation domain of Arnt2. Expression plasmids of hybrid genes coding for the GAL4 DNA binding domain and full-length Arnt2 or the C- or N-terminal half of Arnt2 were constructed and transfected into yeast strain SFY526 together with pGAD424 vector. β-Galactosidase activities were measured for the transformants selected for both leucine and tryptophan and expressed in β-galactosidase units as described in Materials and Methods. Activities are averages of results obtained from at least three experiments with independent transformants. GAL4 DBD, GAL4 DNA binding domain; AD, putative activation domain of Arnt2 estimated from that of Arnt.

the background level, in contrast to the remarkably enhanced activities seen when the products generated from the bait and the prey vectors interacted with each other. Combinations of mouse Arnt2 or human Arnt with mouse Sim showed strong enhancing activities for expression of β-galactosidase, while those of mouse Arnt2 or human Arnt with human AhR gave rise to a moderate expression of β-galactosidase. As shown in Table 1, the inducibility of β-galactosidase activity in response to 3-MC was rather small, compared with previously reported results (4, 50), because of the high level of constitutive expression of β-galactosidase activity without the inducer. Although the reason for this high level of constitutive expression is unknown, it could be partly explained by the different construction of the expression plasmids. The combinations of mouse Arnt2-mouse Arnt2, human Arnt-human Arnt, and mouse Arnt2-human Arnt showed weak but significant enhancing activities. The interaction properties with other bHLH/PAS proteins are very similar for Arnt2 and Arnt, although Arnt seemed to show a higher affinity for AhR than did Arnt2.

DISCUSSION

It has been reported that an Arnt-related gene would not exist unless it was highly homologous because low-stringency genomic Southern hybridization with human Arnt cDNA could detect no extra band in a human hepatoma cell line (28). In this study, however, we isolated a novel cDNA clone from a cDNA library of 11.5-day mouse embryos and determined its sequence to be highly similar to, but distinct from, that of Arnt. This discrepancy could be due either to the species difference between mice and humans or to the failure of Hoffman et al. to detect a human Arnt2 counterpart in their genomic hybridization experiment because of the stringent conditions used. In any event, this will be clarified by screening human genomic libraries with mouse Arnt2 cDNA as a probe. Notably, the bHLH regions of 55 amino acids are identical between Arnt2 and Arnt except for three replacements (Fig. 1), and the PAS A regions are highly homologous to each other. The C-terminal amino acid sequences beyond the PAS region are considerably variable between the two proteins, while the very C-terminal sequences of 37 amino acids are relatively conserved. Interestingly, the very C-terminal region of Arnt has been clarified to be a major region of transcriptional activation (43). Consistent with these results, Arnt2 also exhibited a potent transcription-enhancing activity in the C-terminal sequence. As expected from their close similarity in the bHLH/PAS regions, the affinity of Arnt2 for dimer formations closely resembles that of Arnt, as revealed by coimmunoprecipitation assays and analysis by a yeast two-hybrid system. Arnt2 rescued activity of an Arnt-dependent CAT reporter gene in the Arnt-deficient c4 mutant cells in DNA transfection experiments. Levels of expressed CAT activity, however, were lower in the cells transfected with the Arnt2 plasmid than with the Arnt. It remains to be investigated whether the difference in expressed CAT activities was due to the different amounts of expressed Arnt and Arnt2 proteins or their different affinities for AhR. Since almost equal amounts of Arnt and Arnt2 were used in the gel mobility shift assay, it could be suggested from the intensity of the retarded bands that the optimal binding sequence for Arnt2-AhR is different from the XRE sequence which is most suitable for AhR-Arnt. Despite these similar molecular properties at the protein level, the two Arnt genes display a striking contrast in their expression patterns as revealed by RNA blot analysis and whole-mount in situ hybridization. In RNA blot analysis, Arnt2 was specifically expressed only in the brains and kidneys of adult mice, in contrast to the ubiquitous expression of Arnt (Fig. 3). A striking contrast in the distribution of the mRNA between Arnt2 and Arnt was also observed in the whole-mount in situ hybridization experiments using 9.5-day mouse embryos. Arnt2 was expressed exclusively in the dorsal region of the spinal cord and branchial arch 1, while Arnt transcript was distributed rather broadly in the ventral portion of mesodermal and endodermal tissues (Fig. 4). These results suggest that Arnt2 may perform different biological functions from Arnt, and investigations towards finding possible target genes and a natural partner molecule are in progress. From a structural viewpoint, bHLH/PAS transcription factors whose primary sequences have been elucidated so far are classified into two groups. Group 1 is the Arnt group, including Arnt, Arnt2, and Per, and group 2 is the AhR group, consisting of AhR, Sim, and Hif-1α. The members of group 1 form homodimers with themselves as well as heterodimers with group 2 proteins, while those of the AhR group form only heterodimers with the proteins of group 1 (11). In general, the formation of heterodimers is more stable than that of homodimers. In this context, a partner molecule of Arnt2 may be AhR or Sim because both these proteins are expressed in early embryos. However, their detailed tissue distribution in embryos needs to be clarified. Needless to say, an unknown bHLH/PAS protein, probably of the AhR group, could be a partner of Arnt2. Identification of the target genes and the natural partner molecule of Arnt2 is important for elucidation of its possible function in neurogenesis.

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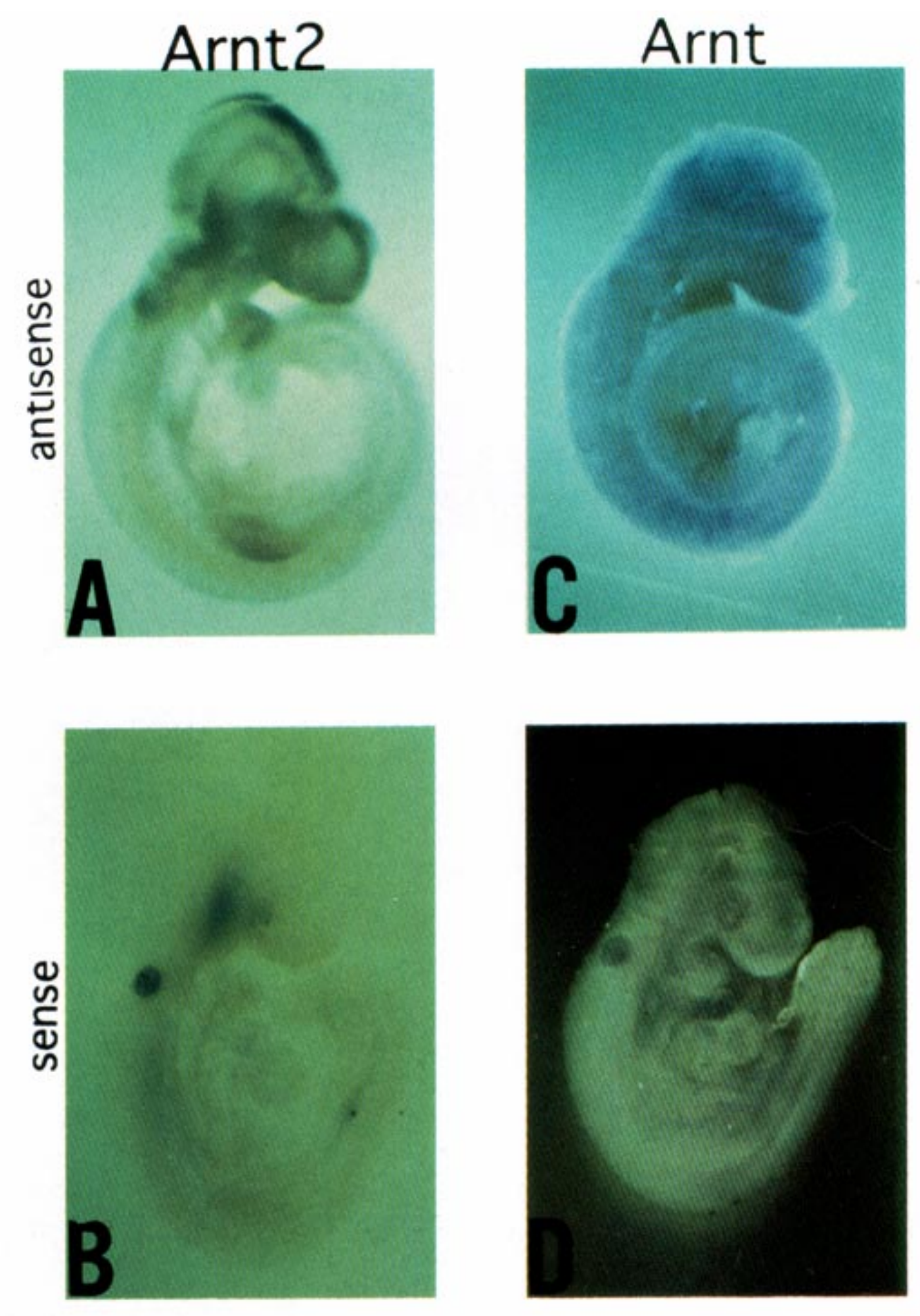
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