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# The Role of ARNT2 in Tumor Angiogenesis and the Neural Response to Hypoxia

Emin Maltepe,*,[1] Brian Keith,†,[1,2] Andrew M. Arsham,‡,[2]
James R. Brorson,§ and M. Celeste Simon*,¶,‖,[3]
*Pritzker School of Medicine, †Section of Hematology and Oncology, ‡Committee on Genetics, §Department of Neurology, ¶Department of Molecular Genetics and Cell Biology, and ‖Howard Hughes Medical Institute, University of Chicago, Chicago, Illinois 60637*

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**The Hypoxia-Inducible Factor-1 (HIF-1) activates the transcription of many genes required for cellular and organismal responses to oxygen deprivation. The HIF-1 complex is composed of the ubiquitously expressed basic helix-loop-helix/PAS (bHLH/PAS) proteins HIF-1$\alpha$ and Arylhydrocarbon Receptor Nuclear Translocator (ARNT). ARNT2 is a conserved ARNT homolog that is highly expressed in neurons, suggesting that ARNT2/HIF-1$\alpha$ heterodimers mediate transcriptional responses to oxygen deprivation in the nervous system. We show here that ARNT2 forms functional HIF complexes *in vivo,* and that ARNT2 restores hypoxia-induced gene expression to ARNT-deficient ES cells and hepatocytes. Formation of neural ARNT2/HIF-1$\alpha$ complexes in *Arnt*$^{-/-}$ ES cell-derived teratocarcinomas may explain why these tumors express VEGF, vascularize and grow efficiently, in contrast to ARNT-deficient hepatomas. Interestingly, all neural cell types studied accumulate both ARNT- and ARNT2-containing HIF complexes. We conclude that ARNT2 forms functional HIF complexes in neurons and plays an integral role in hypoxic responses in the CNS.**


***Key Words:*** **ARNT2; HIF-1; neuron; hypoxia; gene expression; angiogenesis; VEGF.**

Oxygen deprivation, or hypoxia, induces pleiotropic physiological responses at cellular, tissue and systemic levels. Transcriptional responses to hypoxia are mediated by HIF-1, a heterodimer comprised of the basic helix-loop-helix (bHLH)/PAS proteins HIF-1$\alpha$ and the Arylhydrocarbon Receptor Nuclear Translocator (ARNT/HIF-1$\beta$). During hypoxia, HIF-1 activates expression of a large number of genes affecting processes that range from basic cellular metabolism and glucose transport to angiogenesis and respiration (1). This response occurs via binding of HIF-1 to hypoxia response elements (HREs) located within the promoters or enhancers of these genes (2).

While methodical enumeration of various HIF-1 target genes firmly established the central role of HIF-1 in hypoxia responses, the subsequent discovery of related family members has introduced an additional level of complexity into this signaling system. Two novel HIF-1$\alpha$-like proteins, HIF-2$\alpha$ (alternatively called EPAS1 (3), MOP2 (4), HLF (5), and HRF (6)) and HIF-3$\alpha$ (7), as well as two ARNT-like proteins, ARNT2 (8, 9) and MOP3 (4) (also called BMAL1 (10)), have increased the number of possible HIF complex combinations to nine. Analysis of the expression patterns of these genes has revealed partly overlapping expression profiles, suggesting both redundant and unique functions for each (11). HIF-2$\alpha$, which exhibits nearly identical $O_2$-dependent regulation and biochemical properties to HIF-1$\alpha$ (12), is most highly expressed in endothelial cells, whereas HIF-1$\alpha$ is expressed in a nearly ubiquitous fashion. Similarly, ARNT2 is expressed at low levels in many embryonic tissues, but is highly expressed in neural tissues and kidney. In adult mice, ARNT2 expression is restricted to the CNS and kidney, whereas ARNT is expressed in an apparently ubiquitous fashion. Gene targeting experiments have begun to highlight the unique functions of these proteins (13–17).

The predominantly neural distribution of ARNT2 in adults suggests a unique neuron-specific function for this protein. ARNT2 has been shown to dimerize *in vitro* with the bHLH-PAS proteins Aryl hydrocarbon receptor (AHR) and murine SIM1 (8). However, the role of ARNT2 in the response to oxygen deprivation

[1] These authors contributed equally to this work.
[2] Present address: Abramson Cancer Research Institute, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.
[3] To whom correspondence should be addressed at Abramson Cancer Research Institute, University of Pennsylvania School of Medicine, BRB II/III Room 456, 421 Curie Boulevard, Philadelphia, PA 19104. Fax: (215) 746-5511. E-mail: celeste2@mail.med.upenn.edu.



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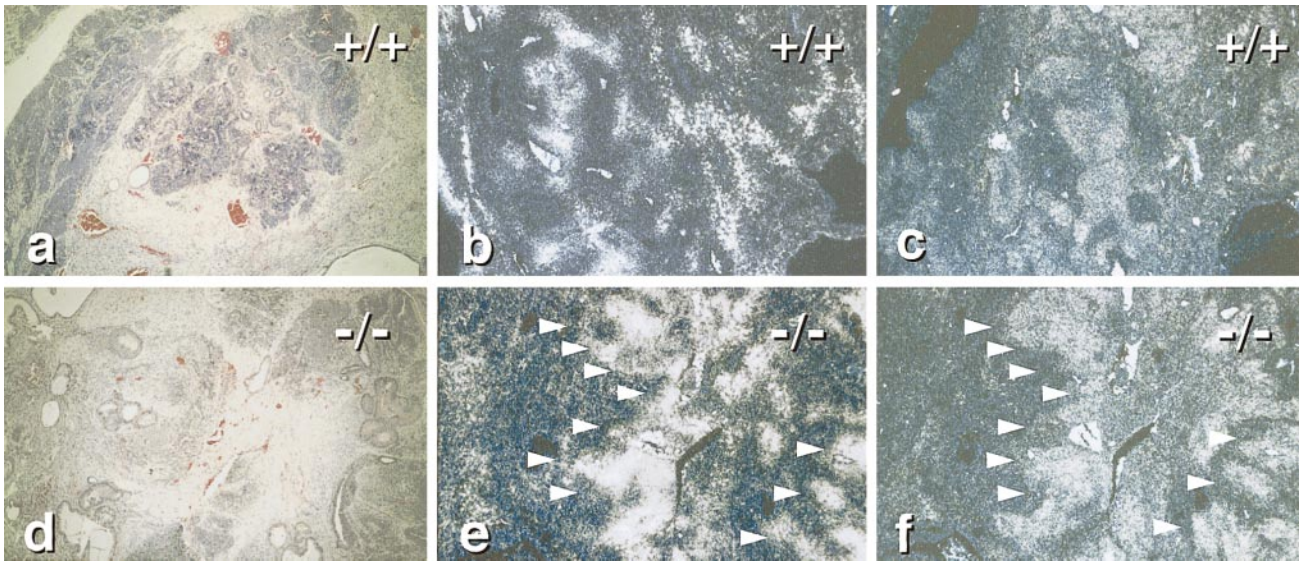


**FIG. 1.** *In situ* hybridization analysis of VEGF and ARNT2 mRNA expression in $Arnt^{+/+}$ and $Arnt^{-/-}$ ES cell-derived teratocarcinomas. Wild-type (upper row) and mutant (lower row) tumor sections were stained with hematoxylin-eosin (a,d) or hybridized with anti-sense cRNA probes specific for the 3′ UTRs of VEGF (b,e) or ARNT2 (c,f). Sense cRNA probes demonstrated only low level background hybridization (not shown). Arrowheads reveal tumor cells that appear to express both VEGF and ARNT2 (e,f). Original magnification, 40×.

has not yet been established. We describe here the ability of ARNT2 to form HIF complexes *in vitro* and in primary neuronal cultures as well as nerve growth factor (NGF)-induced PC12 cells. We also show that ARNT2 directly regulates hypoxia-induced gene expression: specifically, ARNT2 can restore hypoxia-driven gene transcription in transfected $Arnt^{-/-}$ ES cells and hepatocytes. These results are consistent with our observation that teratocarcinomas derived from $Arnt^{-/-}$ ES cells are able to vascularize and grow, apparently due to VEGF expression that correlates spatially to ARNT2-expressing neural tissues in these tumors. Together, these data indicate that ARNT2 forms functional HIF complexes *in vivo*, and may play an integral role in hypoxia responses in the CNS.

## MATERIALS AND METHODS

*Cell culture.* Mixed cortical cultures were derived from E13.5 embryonic cerebral cortices according to (18) and plated in Neurobasal medium containing B-27 supplement (Gibco/BRL) on poly L-lysine coated tissue culture dishes at a density of 0.5 hemispheres/35 mm plate. Total RNA (TRIzol) or nuclear extracts were isolated after 5–10 days in culture. Purified hippocampal neurons were prepared as previously described (19). PC12 cells were grown in DMEM, 10% fetal calf serum (FCS), 10% horse serum and induced to differentiate by culture for 5–7 days in DMEM, 1% FCS with 50 ng/ml NGF (Boerhinger Mannheim). $Arnt^{-/-}$ ES cells were generated as previously described (16), and all ES cell lines grown under standard conditions (16). Arnt-deficient Bprc1 hepatocytes (20) were maintained in DMEM with 10% fetal calf serum and penicillin/streptomycin. Hypoxic culture conditions were established by culturing cells in an atmosphere of 1.5% $O_2$/5% $CO_2$/93.5% $N_2$ generated by a gas mixer (Pro-Ox model #110, Reming Bioinstruments) or by culturing in the presence of 100 $\mu$M $CoCl_2$.

*Transient transfections.* BPRC1 hepatocytes ($10^6$ cells/35 mm well) were transfected with 1.5 $\mu$g of HRE-luciferase reporter plasmid, 0.5 $\mu$g of pSV-Beta-Galactosidase plasmid (Promega), 1.0 $\mu$g of pcDNA3 (Invitrogen), and 1.0 $\mu$g of pcDNA3-ARNT or pcDNA3-ARNT2 plasmid using Lipofectamine 2000 reagent (LifeTechnologies) according to the manufacturer's protocol. The HRE-luciferase plasmid was constructed by placing a trimer of the Epo 3′ HRE upstream of a 1.3 kb portion of the Glut1 promoter linked to luciferase in pGL2 (Promega). Full length murine ARNT cDNA (kindly provided by C. Bradfield) or ARNT2 cDNA (generated by RT-PCR using the following primers: (sense) 5′-GACTGAATTCCCC-GGCAAGATGGCAAC-3′, (antisense) 5′-GACTCTTAAGCTTCC-GAGCACACAGATAAAG-3′) were cloned into pcDNA3. Cells were transfected for 12 hours, and one-half of each sample exposed to hypoxia for 18 hours. Proteins were extracted from hypoxic and corresponding normoxic samples using reporter lysis buffer (Promega), and luciferase, $\beta$-galactosidase assays (Promega) and protein concentration (Pierce BCA) determinations were performed according to manufacturer's protocols.

*Generation of "rescued" ES cell lines.* $2 \times 10^7$ $Arnt^{-/-}$ ES cells were electroporated with 20 $\mu$g of Sal I linearized expression construct Station I generated by replacing the *LacZ* cDNA of the *LacZ* expression vector described by Kuo *et al.* (21) with full length murine ARNT or ARNT2 cDNA, followed by selection in 0.2 mg/ml hygromycin. Resistant colonies were screened by Northern analysis for expression of stably integrated transgenes and clones expressing high levels of ARNT or ARNT2 mRNA were selected for further study.

*Northern analysis.* Cells cultured for the indicated times were washed once with PBS and RNA was extracted with TRIzol reagent according to the manufacturer's instructions. 20 $\mu$g total RNA were electrophoresed in 1.0% denaturing (formaldehyde) agarose gels and blotted on Hybond $N^+$ membranes (Amersham). The following labelled probes were hybridized under standard conditions: full length murine ARNT or ARNT2 cDNAs were used as probes for screening clones. Other probes included RT-PCR products generated with 3′-UTR specific primers for VEGF (sense) 5′-GACCGACCAAG-CTGTTCAG-3′, (antisense) 5′-GATGCACTTGAGTGGTCTTG-3′;

GLUT-1 (sense) 5′-GTCCTATCTGAGCATCGTGG-3′, (antisense) 5′-CAAGGTGAAGACTACAGTGTG-3′; GLUT-3 (sense) 5′-GCCTTCTTTGAGATTGGACC-3′, (antisense) 5′-CATTGGCGATCTGGTCAACC-3′; and an 18S RNA-specific probe kindly provided by J. Walsh (U. Chicago).

*ARNT2 antiserum generation.* A DNA fragment encoding amino acids 467–624 of murine ARNT2 was cloned into the pGEX-4T-3 vector (Pharmacia) in frame with glutathione S-transferase (GST). Polyclonal rabbit antisera were generated against purified GST-ARNT2 fusion protein and affinity purified using Affi-gel 10 (Bio-Rad) resin coupled to the fusion protein.

*Western blot analysis.* 25 $\mu$g of nuclear extract prepared using a modified Dignam technique (22) were subjected to immunoblot analysis following standard protocols using anti-ARNT antibody (provided by C. Bradfield), anti-ARNT2 antibody and anti-CREB antibody provided in the PhosphoPlus CREB kit (New England Biolabs). In vitro-translated (IVT) proteins were synthesized using T7-coupled transcription/translation rabbit reticulocyte lysates (Promega). One-half of each reaction was primed with $^{35}$S-methionine, and resulting proteins quantitated using a Molecular Dynamics phosphorimager. Values were adjusted to account for the number of methionine residues in each protein. IVT proteins synthesized with unlabeled methionine were used in EMSA and western blot experiments. ARNT antiserum was obtained from Novus Biologicals.

*Electrophoretic mobility shift assays (EMSA).* EMSA was performed in binding buffer consisting of 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 50 mM KCl, 1 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM EDTA, and 5% glycerol to which 0.1 mg/ml bovine serum albumin, 5 $\mu$g nuclear extract, and $10^4$ cpm of probe were added per 20 $\mu$l reaction. The binding site sequence of the wild-type probe (W18) was 5′-GCCCTACGTGCTGTCTCA-3′. Where indicated, cold CREB competitor oligo (100× excess) containing the CREB binding site from the somatostatin promoter was added: 5′-GATCGCCTCCTTGGCTGACGTCAGAGAGCTAG-3′.

*Teratoma formation.* For injection in nude mice, $1 \times 10^7$ cells were injected subcutaneously into the dorsal area of female Beige nude/xid mice as described (23) and tumor weight was assessed after 4 weeks of growth.

*In situ hybridization.* Hybridizations using $^{35}$S-labeled cRNA were performed as described previously (24). The cRNA probes used for *in situ* hybridizations were derived from cDNA templates obtained by RT-PCR using the same primers described above for VEGF and the following 3′-UTR specific primers for ARNT2: (sense) 5′-GTTTGTCTGAGAGTGGACAG-3′, (antisense) 5′-CTTCCGAGCACACAGATAAAG-3′.

## RESULTS

### *Neural ARNT2 and VEGF Expression in Arnt$^{-/-}$ Teratocarcinomas*

Hypoxia-driven VEGF expression is considered to be an important factor governing tumor angiogenesis and growth (25). Unlike their wild-type counterparts, *Vegf*$^{-/-}$ ES cells fail to form teratocarcinomas when injected into nude mice (23). Similarly, ARNT-deficient Hepa1c1c7-c4 hepatoma cells, which are unable to mount a transcriptional response to hypoxia, also exhibit reduced VEGF expression, tumor vascularity and growth rates when introduced into mice (26). To assess whether ARNT-mediated VEGF expression is required for ES cell-derived teratocarcinoma formation, *Arnt*$^{+/+}$ and two independently generated *Arnt*$^{-/-}$ ES cell clones were injected into beige nude/xid mice and tumor growth was monitored over the course of four weeks. Surprisingly, no statistical difference in tumor mass was observed (data not shown). Histological analysis revealed that the tumors were teratocarcinomas that contained tissues derived from all three germ layers, including a large amount of primitive neuroepithelium, and anti-CD34 immunohistochemistry indicated comparable vascular densities among the three groups (data not shown). Interestingly, *in situ* hybridization analysis revealed comparable levels of VEGF mRNA expression in both wild-type and *Arnt*$^{-/-}$ ES cell-derived tumors (Figs. 1B and 1E). Moreover, the distribution of VEGF-expressing cells in the *Arnt*$^{-/-}$ tumors predominantly correlated with regions expressing the highest levels of ARNT2 (Figs. 1E and 1F, arrowheads), whereas VEGF expression was more uniformly distributed in wild-type teratocarcinomas and displayed no correlation with ARNT2 expression (Figs. 1B and 1C). Thus, ARNT2-mediated VEGF expression in primitive neuroepithelial cells may be responsible for the proper vascularization and growth of *Arnt*$^{-/-}$ tumors and suggests that ARNT2 plays a role in hypoxic gene induction.

### *Arnt and Arnt2 Form Distinct HIF Complexes*

To distinguish between ARNT2/HIF-1$\alpha$ and ARNT/HIF-1$\alpha$ complexes, we generated antiserum to an ARNT2-GST fusion protein as described in Materials and Methods. Western blot analysis of *in vitro*-translated (IVT) proteins revealed that our ARNT2 antibody and commercially available ARNT antibody are highly specific: no cross-reactivity was observed (Fig. 2A). To investigate the relative ability of ARNT and ARNT2 to form HIF complexes, equimolar amounts of IVT ARNT, ARNT2 and HIF-1$\alpha$ proteins were mixed and analysed in electrophoretic mobility shift assays (EMSA) using the EPO 3′ enhancer HRE binding site probe (Fig. 2B). ARNT/HIF-1$\alpha$ and ARNT2/HIF-1$\alpha$ complexes were readily detected, although the ARNT2/HIF-1$\alpha$ complex migrated slightly faster than the ARNT/HIF-1$\alpha$ complex. When IVT ARNT, ARNT2 and HIF-1$\alpha$ were mixed in equimolar amounts, approximately equal levels of both ARNT and ARNT2 HIF complexes were formed, suggesting no inherent difference in the affinity of HIF-1$\alpha$ for ARNT as compared to ARNT2.

### *Arnt2 Expression and the Hypoxia Response in Neurons*

Previous studies have demonstrated that ARNT2 mRNA is highly expressed in neurons (9, 11), whereas ARNT2 RNA transcripts and protein are absent in multiple glial cell types ((9), and B.K. and M.C.S., unpublished observations). To assess HIF-1 complex formation in response to oxygen deprivation, primary

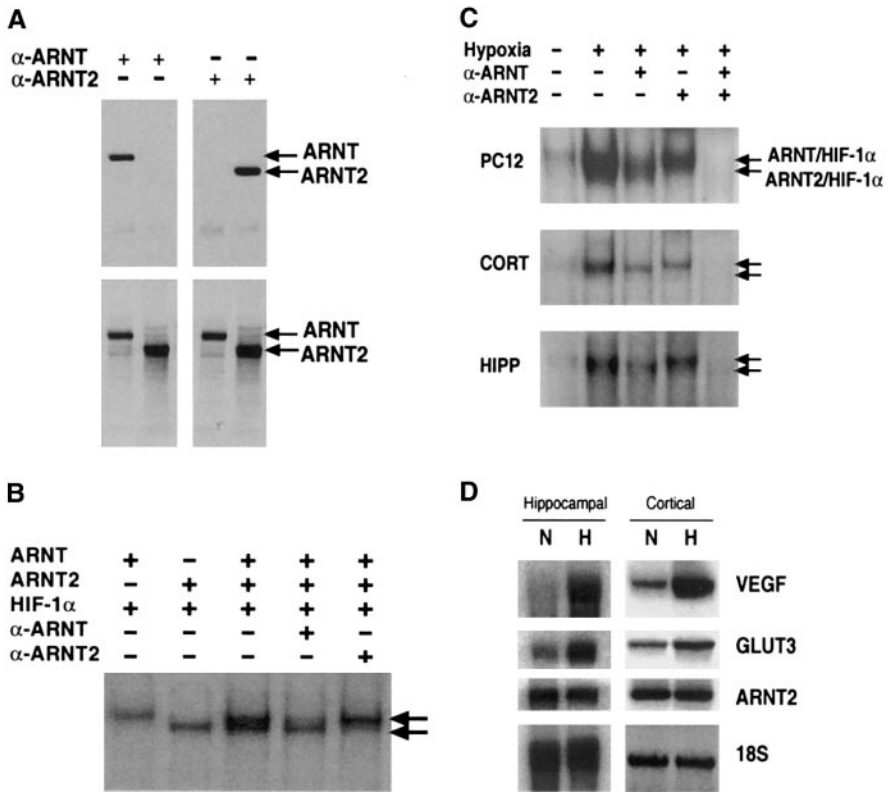


**FIG. 2.** (A) Specificity of anti-ARNT and anti-ARNT2 antibodies. $^{35}$S-methionine IVT proteins are shown in lower panels. Unlabeled IVT proteins were probed with the indicated antisera (upper panels). (B) EMSAs of IVT ARNT, ARNT2, and HIF-1$\alpha$ proteins incubated with the EPO 3′ HRE probe. The arrows indicate the various ARNT/HIF-1$\alpha$ and ARNT2/HIF-1$\alpha$ DNA complexes, specifically ablated with anti-ARNT or anti-ARNT2 antibodies. (C) HIF-1 DNA binding activity in nuclear extracts from NGF-treated PC12 cells, cortical neurons (CORT), and hippocampal neurons (HIPP). All cell types were exposed to normoxia or hypoxia for 4 hours; extracts were incubated with the EPO 3′ HRE probe in the presence of antibodies to ARNT and/or ARNT2 where indicated. (D) Northern blot analysis of RNA isolated from hippocampal and cortical neurons during normoxia (21% $O_2$/5% $CO_2$/74% $N_2$), N, or hypoxia (1.5% $O_2$/5% $O_2$/93.5% $N_2$), H, for 24 hours. The same blot was sequentially hybidized with probes for VEGF (vascular endothelial growth factor A) and Glut-3 (glucose transporter three), ARNT2 and 18S RNA.

hippocampal and mixed cortical neurons, as well as neuron-like NGF-treated PC12 cells, were exposed to 1.5% $O_2$ for 6 hours prior to extraction of nuclear proteins. EMSA analysis revealed the presence of hypoxia-inducible protein complexes in all three cell types (Fig. 2C). The neuronal hypoxia complex was partially supershifted by the ARNT antibody, revealing a faster migrating, second complex that specifically shifted with the ARNT2 antibody. Coincubation with ARNT and ARNT2 antibodies abrogated both complexes, indicating that both ARNT/HIF-1$\alpha$ and ARNT2/HIF-1$\alpha$ heterodimers can be detected in neurons. The ARNT/HIF-1$\alpha$ complexes observed are unlikely to be caused by contaminating glia as the hippocampal cultures are purely neuronal (27) and the mixed cortical cultures contain fewer than 5% glial cells (28–30). HIF-1$\alpha$ antibodies determined that HIF-1$\alpha$ and not HIF-2$\alpha$ was dimerized with both ARNTs (data not shown).

Northern blot analysis of RNA extracted from primary hippocampal neurons and mixed cortical cultures exposed to 1.5% $O_2$ for 16 hours revealed a 10-fold induction of the hypoxia inducible angiogenic agent VEGF and a 3- to 4-fold induction of the neural-specific glucose transporter GLUT-3 (Fig. 2D). ARNT2 mRNA levels were not affected by hypoxia. These data raise the possibility that hypoxic responses in neurons may require both ARNT and ARNT2, which may therefore have partly redundant functions in these cells.

### *ARNT2 Restores Hypoxic Gene Expression in Arnt$^{-/-}$ ES Cells*

To determine if ARNT2 protein activates hypoxia responses *in vivo*, *Arnt*$^{-/-}$ ES cells (16) were engineered to stably express either ARNT or ARNT2 under the control of the ubiquitous Elongation Factor-1$\alpha$ promoter and 4F2 enhancer. Clones expressing high levels of each cDNA were identified by Northern analysis (Fig. 3A). Western blot analysis indicated comparable levels of ARNT protein in both wild-type and *Arnt*$^{-/-}$ ES cells stably expressing ARNT RNA ($^{-/-}$.A) (Fig. 3B, lanes 1, 2 and 5, 6). As previously described, *Arnt*$^{-/-}$ ES

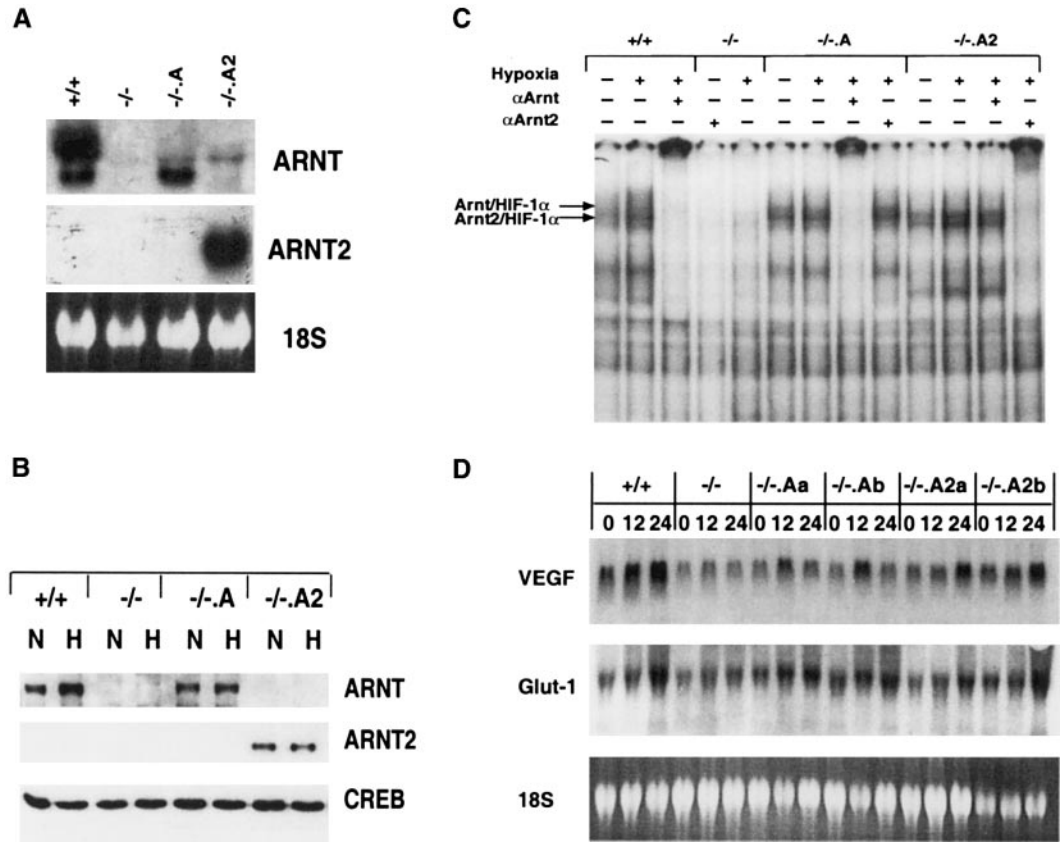


**FIG. 3.** (A) Northern analysis of mRNA isolated from $Arnt^{+/+}$ ES cells (+/+), $Arnt^{-/-}$ ES cells (−/−), and $Arnt^{-/-}$ ES cell stable transformants transfected with ARNT (−/−.A) or ARNT2 (−/−.A2). Fifteen micrograms total RNA were loaded per lane and the same blot sequentially hybridized with probes specific for ARNT and ARNT2 mRNA. 18S ribosomal RNA was stained as a loading control. (B) Western analysis of nuclear extracts prepared from $Arnt^{+/+}$ and $Arnt^{-/-}$ ES cells and the stable transformants described in (A). Ten micrograms of nuclear protein from each sample were probed with ARNT and ARNT2 antibodies, and the same Western blot reprobed with anti-CREB antibodies to control for loading. (C) EMSA of nuclear extracts prepared from $Arnt^{+/+}$, $Arnt^{-/-}$, and stably transformed ES clones grown under normoxic or hypoxic culture conditions as indicated. The slower migrating ARNT/HIF-1$\alpha$ and faster migrating ARNT2/HIF-1$\alpha$ DNA binding complexes are marked with arrows. (D) Northern analysis of mRNA isolated from ES clones grown under normoxic or hypoxic conditions for 12 and 24 hours. As described in the legend to Fig. 2D, the same blot was hybidized with probes for VEGF (vascular endothelial growth factor A) and Glut-1 (glucose transporter one).

cells express no detectable ARNT protein (Fig. 3B, lanes 3 and 4). Only $Arnt^{-/-}$ ES cells selected to stably express ARNT2 RNA ($^{-/-}$.A2) display abundant levels of ARNT2 (Fig. 3B, lanes 7 and 8), whereas wild-type or $Arnt^{-/-}$ ES cells do not express levels of ARNT2 detectable by Western blot or Northern blot assays.

$Arnt^{+/+}$, $Arnt^{-/-}$, $^{-/-}$.A, and $^{-/-}$.A2 clones were exposed to 1.5% $O_2$ for 6 hours and nuclear extracts subsequently subjected to EMSA. R1 ES cells exhibit low levels of constitutive HIF-1 complex similar to that described in the J1 ES cell line (Fig. 3C, lane 1) (14). Hypoxic wild-type ES cells induce a strong HIF-1 complex that supershifted with an ARNT, but not an ARNT2, antibody (Fig. 3C, lanes 2–6). Although they contain levels of ARNT2 mRNA and protein undetectable by standard Northern and Western blot analyses, $Arnt^{-/-}$ ES cells generate a modest hypoxia-inducible complex specifically abrogated by the anti-ARNT2 antibody, indicating the presence of very low levels of ARNT2 protein in these cells. This ARNT2 complex was detectable only in the absence of ARNT. Hypoxic $^{-/-}$.A2 cells displayed a robust HIF-1 complex with a mobility identical to the faint ARNT2/HIF-1$\alpha$ complex observed in $Arnt^{-/-}$ cells, and that supershifted with the ARNT2 antibody (Fig. 3C, lanes 10–13).

Endogenous HIF-1 target genes were also assayed for hypoxic gene induction by Northern analysis. The angiogenic agent VEGF and the facilitative glucose transporter GLUT-1 represent HIF-1 target genes induced by hypoxia in wild-type ES cells (Fig. 3D, lanes 1–3 and (13, 14, 16)). As previously described, hypoxic $Arnt^{-/-}$ ES cells exhibit only slight increases in the levels of these transcripts when compared with wild-type cells (Fig. 3D, lanes 4–6) due either to increases in mRNA stability or to low levels of ARNT2 detected by EMSA in ES cells. Furthermore, basal levels of these transcripts are also reduced in $Arnt^{-/-}$ cells due to the aforementioned constitutive HIF-1 activity in R1

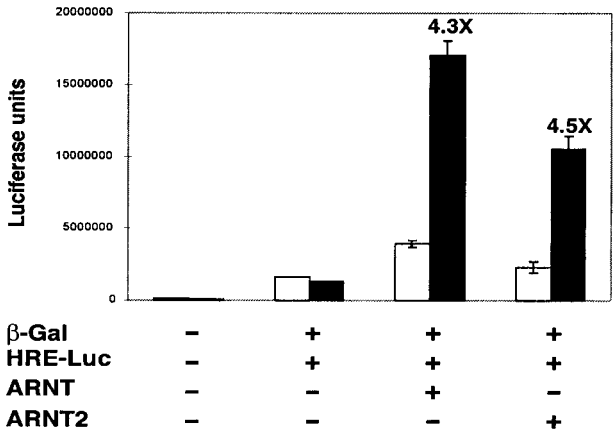


**FIG. 4.** ARNT and ARNT2 can transactivate the HRE-driven reporter plasmid in Bprc1 hepatocytes. The indicated plasmids were transiently transfected into Bprc1 cells and assayed for luciferase reporter activity. All data represent mean fold luciferase activity (normalized to protein and $\beta$-galactosidase activity) in cells grown under normoxia (21% $O_2$, open bars) or hypoxia (1.5% $O_2$, filled bars) for three independent experiments ($\pm$ S.E.).

ES cells. ARNT and ARNT2 restored maximal $O_2$-inducibility of VEGF over a 24-hour period in *Arnt*$^{-/-}$ ES cells (Fig. 3D, lanes 7–15), indicating a degree of functional redundancy for hypoxia responses within this family. Interestingly, the kinetics of VEGF and GLUT-1 oxygen regulation was consistently slower in the $^{-/-}$.A2 cells. We concluded that ARNT2 can also regulate hypoxia-induced transcription of HIF-1 target genes.

Finally, we tested the ability of ARNT2 to directly activate hypoxia-induced transcription from an HRE. Specifically, we co-transfected ARNT-deficient Bprc1 hepatocytes with an HRE-driven luciferase reporter plasmid and either ARNT- or ARNT2-expressing plasmids. Bprc1 cells contain no functional ARNT (31), and HRE-luciferase expression was accordingly not induced by hypoxia (Fig. 4). In contrast, co-transfection of either ARNT- or ARNT2-expressing plasmids restored the hypoxic transcriptional response in these cells, revealing a 4.3- and 4.5-fold hypoxic induction of HRE-luciferase expression, respectively. These data argue that the ARNT2/HIF-1$\alpha$ complex is functional, and activates transcription from the HRE as effectively as the ARNT/HIF-1$\alpha$ complex.

## DISCUSSION

The identification of HIF-1 has been critical to our understanding of the cellular and organismal response to oxygen-mediated stress (1). Our experiments formally prove that ARNT2 mediates hypoxic transcriptional responses by forming HIF complexes, and implicate ARNT2 in neural responses to hypoxia. Moreover, the presence of apparently comparable amounts of ARNT and ARNT2 in neural HIF-1 complexes suggests a functional redundancy between these proteins. The more distantly related protein MOP3 also forms HIF complexes *in vitro*, but is less likely to play a major role in general neural hypoxic responses for the following reasons: (1) the expression of MOP3 is more restricted than ARNT2 in the CNS (32) and (2) HIF complexes in three different neural cell types are completely ablated by a combination of ARNT and ARNT2 antibodies (Fig. 2). It is quite possible that MOP3 is involved in hypoxic responses in the subset of neural cells in which it is expressed.

Hypoxic VEGF expression is believed to play an important role in tumor angiogenesis, and many primary CNS neoplasms have been shown to express high levels of this angiogenic agent (33–35). The formation of oxygen and nutrient gradients within solid tumors is thought to stimulate HIF-1-mediated VEGF expression and subsequent tumor angiogenesis. As such, tumors derived from *Vegf*$^{-/-}$ ES cells, or ARNT-deficient hepatoma cells impaired in their ability to produce VEGF, are unable to vascularize and grow (26). Our data indicate that *Arnt*$^{-/-}$ teratocarcinomas circumvent a requirement for ARNT-mediated VEGF expression as they contain a large amount of primitive neuroepithelium, which express ARNT2. We propose that ARNT2/HIF-1$\alpha$ complexes in these cells initiate hypoxia/nutrient deprivation-induced VEGF expression, permitting tumor angiogenesis. The largely coincident spatial pattern of ARNT2 and VEGF gene expression in these tumors is particularly striking in this regard.

Recent reports have revealed that HIF-1 plays complex roles in tumor biology. For example, *Hif*-1$\alpha^{-/-}$ tumors express decreased levels of VEGF, and are therefore less vascularized than wild type tumors (36). Paradoxically, however, HIF-1 deficient cells are resistant to hypoxia-induced cell death, and exhibit a significant growth advantage when compared to their wild-type counterparts (36). The apparent inability of HIF-1 deficient cells to activate apoptotic pathways involving p53 and Bcl-2 family members results in a competitive advantage for tumor cells lacking HIF-1 activity. The role of HIF-1 in mediating VEGF expression, as well as the toxic consequences of hypoxia, potentially via physical interaction with the p53 tumor suppressor (37, 38), reveals the complexity of HIF-1 activity in tumor growth and homeostasis. It is an intriguing possibility, for example, that ARNT/HIF-1$\alpha$ and ARNT2/HIF-1$\alpha$ complexes may interact with p53 in different ways to modulate apoptosis in response to hypoxia/ischemia in neurons and other tissues.

An accurate determination of the unique functions conferred by ARNT2, and the degree to which ARNT2 is required for HIF-1 activity in neurons must await analyses using ARNT2 deficient animals. Recent analysis of mouse strains harboring overlapping chromo-

some deletions that remove the ARNT2 locus (39), as well as targeted mutation of the ARNT2 gene (BK and MCS, unpublished data) have suggested an essential interaction between ARNT2 and SIM1, another bHLH-PAS protein. In the absence of either ARNT2 or SIM1, embryos die perinatally from apparently identical hypothalamic defects (39, 40). The consequences of disrupted ARNT2/HIF-1$\alpha$ interactions in these strains is currently under investigation. Our findings do offer an explanation, however, for the relatively less severe phenotype of *Arnt*$^{-/-}$ mutant embryos (16, 17) as compared to *HIF*-1$\alpha^{-/-}$ mutant embryos (13, 14, 36), which show massive neuromesenchymal apoptosis and embryonic vascular malformations. Specifically, the high expression of ARNT2 in neuroepithelium, as well as lower level expression in other embryonic tissues (11) may partly compensate for the loss of ARNT-dependent HIF-1 activity through the formation of ARNT2/HIF-1$\alpha$ heterodimers.

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