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Expression of Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT) Isoforms in Juvenile and Adult Rainbow Trout Tissues

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Abstract: Whether and where rainbow trout aryl hydrocarbon receptor nuclear translocator (rtARNT) isoforms are expressed in juvenile and adult tissues of rainbow trout is unknown. Using reverse transcriptase polymerase chain reaction (RT-PCR), expression of the rtARNT$_b$ isoform messenger RNA was identified in day 19 and 23 embryos, in day 27, 35, 39, and 42 sac fry, and in all adult tissues investigated. The rtARNT$_a$ isoform mRNA was expressed in all juvenile trout except day 42 sac fry and in all adult tissues except skeletal muscle. Western blot analysis and immunohistochemistry demonstrated that the rtARNT$_b$ protein was present in all juvenile trout and adult tissues investigated, except skeletal muscle, and was primarily localized to the nucleus. In contrast, rtARNT$_a$ protein was not detected at any developmental stage but was expressed in the adult gill. These results imply that rtARNT$_b$ is involved in signaling events at many developmental stages, while the functionality of the dominant negative rtARNT$_a$ is restricted.

Key words: ARNT, rainbow trout, dominant negative, signal transduction, isoforms.

INTRODUCTION

The aryl hydrocarbon receptor nuclear translocator (ARNT) is a member of the basic helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) family of proteins and functions as a transcription factor in numerous signaling pathways (reviewed in Rowlands and Gustafsson, 1997; Wilson and Safe, 1998). In mammals, ARNT has been found to be involved in multiple signaling pathways including response to hypoxia, neurological and other areas of development, and reaction to exposure to aryl hydrocarbon environmental contaminants (reviewed in Rowlands and Gustafsson, 1997; Wilson and Safe, 1998). The first mammalian ARNT discovered was found to be expressed ubiquitously, while ARNT2 was found only in the brain and kidney (Hoffman et al., 1991; Drutel et al., 1996; Hirose et al., 1996). More recently, ARNT3 (brain and muscle ARNT-like protein, BMAL-1) has been shown to be expressed in the brain and skeletal muscle of mice (Takahata et al., 1998). In general, these three mammalian ARNT isoforms have been shown to exhibit a positive effect on gene regulation, and gene-targeted deletion of ARNT in the mouse results in a lethal phenotype (Kozak et al., 1997; Maltepe et al., 1997). Thus, given the importance of ARNT in numerous cellular functions, there is growing interest in the expression of ARNT in

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nonmammalian systems and the isolation of proteins that exert negative action on bHLH/PAS signaling.

Previous studies have shown that rainbow trout express two ARNT isoforms through alternative messenger RNA splicing (Pollenz et al., 1996; Necela and Pollenz, 1999). The two proteins differ by the insertion of a 373-nucleotide exon and distinct carboxy-terminal ends. Both proteins have an identical 533 amino acid amino-terminal region, but owing to the extra exon, the rtARNT$_b$ isoform contains a carboxy-terminal region of 190 amino acids rich in glutamine and asparagine (QN-rich). In contrast, the rtARNT$_a$ isoform has a carboxy-terminal region of 104 amino acids that is rich in proline, serine, and threonine (PST-rich). At the functional level, it was demonstrated that both rtARNTs could form a heterodimer with the murine aryl hydrocarbon receptor protein (AHR), but that only the rtARNT$_b$ isoform was capable of complementing AHR-mediated gene induction (Pollenz et al., 1996; Necela and Pollenz, 1999). Thus the rtARNT$_b$ protein appears to exert positive influence on gene regulation, while rtARNT$_a$ appears to be a dominant negative protein.

Recent work in zebrafish has identified multiple ARNT complementary DNAs that are the result of alternative splicing (Tanguay et al., 2000; Wang et al., 2000). The zfARNT2a protein appears to be similar to rtARNT$_a$ with regard to reduced function in AHR-mediated signaling in cell culture (Tanguay et al., 2000). A truncated ARNT has also been identified in the MDA-MB-231 breast cancer cell line that is resistant to aryl hydrocarbon exposure (Wilson et al., 1997). Thus these studies show that negatively acting ARNT proteins are expressed, and may provide an important aspect of regulation within the bHLH/PAS family.

The purpose of the current study was to determine the expression pattern of rtARNT$_a$ and rtARNT$_b$ in embryos, sac fry, and adult rainbow trout tissues at both the RNA and protein level. Such analysis should provide insight into the function of ARNT in rainbow trout that can be contrasted to previous work in mammals.

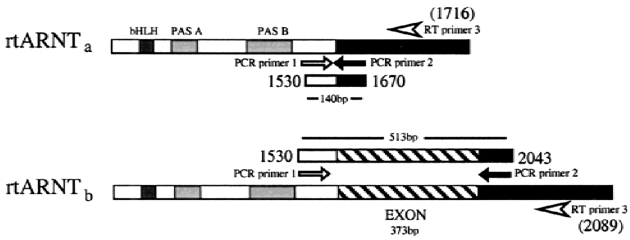

Figure 1. PCR strategy. Schematics of the rtARNT open reading frames are shown. All shading indicates identical nucleotide sequences between the two rtARNT isoforms. The alignment of the primers, size of the PCR product, and numbers of the nucleotide sequences are indicated. Note that the only sequence not found in rtARNT$_a$ is the spliced exon sequence indicated by hatching.

Materials and Methods

Buffers

Phosphate-buffered saline (PBS) is 0.8% NaCl, 0.02% KCl, 0.14% Na_2HPO_4, and 0.02% KH_2PO_4, pH 7.4. The 2× gel sample buffer is 125 mM, Tris, pH 6.8, 4% sodium dodesylsulfate (SDS), 25% glycerol, 4 mM EDTA, 20 mM dithiothreitol, and .005% bromphenol blue. TBS is 50 mM Tris and 150 mM NaCl, pH 7.5. TTBS is 50 mM Tris, 0.2% Tween-20, and 150 mM NaCl, pH 7.5. TTBS+ is 50 mM Tris, 0.5% Tween-20, and 300 mM NaCl, pH 7.5. BLOTTO is 5% dry milk in TTBS. Lysis buffer is 25 mM Hepes, pH 7.4, 20 mM sodium molybdate, 10 mM EGTA, 10 mM EDTA, and 10% glycerol. MENG is 25 mM MOPS, pH 7.4, 1 mM EDTA, 0.002% NaN_3, and 10% glycerol.

Reverse Transcriptase Polymerase Chain Reaction and Southern Blot Analysis

Oligonucleotide primers are written 5′–3′. RT primer 3 is CAGGGAAGATGTCAGG; PCR primer 1, CGGAGTACCCATCTACCC; PCR primer 2, TGCCCTGTTCTGAGCTTGC (Figure 1). Total RNA was isolated from fish using the Qiagen RNEasy kit, as detailed by the manufacturer (Valencia, Calif.). The integrity of the RNAs and their concentrations were verified by gel electrophoresis and analysis of the ribosomal RNA bands. A schematic of the PCR strategy is shown in Figure 1. One gene-specific primer (RT primer 3) was used to reverse transcribe ARNT RNA from the total RNA samples using the Gibco BRL SuperScript Preamplification System for first-strand cDNA synthesis (Rockville, Md.). The rtARNT$_a$ and rtARNT$_b$ were amplified using 2 μl of the first-strand cDNA synthesis reaction with PCR primer 1 and PCR primer 2 in a PCR reaction (95°C 1 minute; 58°C 2 minutes; 75°C 3 minutes, for 25 cycles). Each reaction contained 20 pmol/μl of each primer, 50 μM dNTPs, 5 ng/μl of $MgCl_2$, and 0.5 μl of *Taq* polymerase. The size of the fragments on a Southern blot distinguished rtARNT$_a$ (140 bp) and rtARNT$_b$ (513 bp) PCR products. The PCR prod-

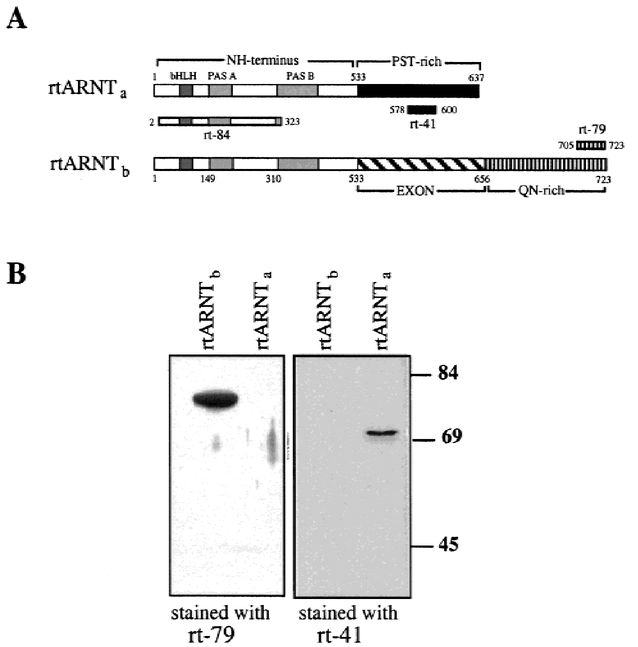

Figure 2. Alignment and specificity of rtARNT antibodies. **A:** The domain structures of the rtARNT amino acid sequences are shown. The location of the antigens used to produce rt-84, rt-41, and rt-79 are indicated. **B:** $rtARNT_a$ and $rtARNT_b$ proteins were produced in vitro and analyzed by Western blot analysis with 1 μg/ml rt-41 or rt-79, followed by GAR-HRP (1:10,000) or RAC-HRP (1:10,000). Each antibody only reacted with the appropriate protein and showed no reactivity with the alternative isoform.

ucts were detected by Southern blot analysis (Sambrook et al., 1989) with a probe derived from the entire $rtARNT_a$ cDNA. The blots were exposed to film for 10 to 36 hours.

Antibodies

The rainbow trout ARNT-84 (rt-84) polyclonal IgG was generated in rabbits against a bacterially expressed section of the protein consisting of amino acids 2 to 353 (Pollenz et al., 1996; Pollenz and Necela, 1998). The rainbow trout ARNT-41 (rt-41) antibody was generated in rabbits against a multiple antigenic peptide (MAP) of the $rtARNT_a$ isoform, consisting of amino acids 578 to 600, and was affinity purified (Figure 2, A). The rainbow trout ARNT-79 (rt-79) antibody was generated in chickens against a MAP consisting of amino acids 705 to 723 from the $rtARNT_b$ protein and was affinity purified (Figure 2, A). Both rt-41 and rt-79 antibodies recognize the appropriate in vitro expressed ARNT by Western blot analysis and do not cross-react with the other isoform (Figure 2, B). In addition, both proteins detect a nuclear protein in culture cells transfected with the appropriate rtARNT expression construct (RSP and MKS, unpublished data). The polyclonal actin antibody, nonspecific rabbit IgG, and nonspecific chicken IgG were purchased from Sigma Immuno Chemicals (St. Louis, Mo.). Peroxidase-conjugated goat antirabbit IgG (GAR-HRP) or peroxidase-conjugated rabbit antichicken IgG (RAC-HRP) were used as the secondary antibody and purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, Pa.).

Preparation of rtARNT Protein and Total Tissue Lysate

The rtARNT proteins were produced in vitro using the TNT Coupled Rabbit Reticulocyte Lysate Kit essentially as detailed by the manufacturer (Promega, Madison, Wis.). Upon completion of the 90-minute transcription and translation reaction, the sample was combined with an equal volume of 2× gel sample buffer and boiled for 5 minutes. Total tissue lysates were prepared from embryo, sac fry, and adult tissue essentially as previously described for rat tissues (Pollenz et al., 1998); however, the trout lysate samples were centrifuged 5000 rpm for 5 seconds to pellet pigment. A small aliquot was removed for protein determination (Coomassie Plus protein Assay, Pierce, Rockford, Ill.). The samples were then heated at 95°C for 5 minutes in an equal volume of 2× gel sample buffer.

Gel Electrophoresis and Western Blotting

Samples were resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose and visualized by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, Ill.), as previously described (Pollenz et al., 1994). Proteins were quantified as detailed previously (Holmes and Pollenz, 1997; Pollenz et al., 1994, 1998, 1999; Sommer et al., 1999).

Animal Sectioning and Staining

Embryos and sac fry were fixed on embryonic day 23 and sac fry days 27 and 35 in Bouin's fixative for 6 hours. The animals were then embedded in paraffin and 5-μm sagittal sections were cut. Paraffin sections were hydrated in xylene and 100%, 95%, 70%, and 50% EtOH, and washed in PBS. Endogenous peroxidase activity was squelched with a 3% hydrogen peroxide wash for 30 minutes and a 5-minute PBS wash. The sections were blocked in 4% bovine serum albumin (BSA) and 5% normal goat serum in PBS for 2 hours. The rt-84 antibody and nonspecific IgG were used on juvenile trout sections at a concentration of 4 μg/ml. The rt-41 antibody and nonspecific IgG were used on adult trout sections at a concentration of 6 μg/ml. The sections were incubated in the filtered primary antibodies overnight at 4°C. They were washed in TTBS+ 5 times for a total of 25 minutes and 10 minutes in PBS. The sections were incubated in secondary antibody and diluted in blocking solution 1:500, at room temperature for 2 hours. They were washed in TTBS+ 5 times for a total of 25 minutes and 10 minutes in PBS. The staining was visualized using the ImmunoPure Metal Enhanced DAB Substrate Kit purchased from Pierce. It is important to note that because the fish were all fixed for 6 hours, the time to develop the DAB substrate was kept constant, and all sections were stained simultaneously, the staining figures presented in this report can be compared.

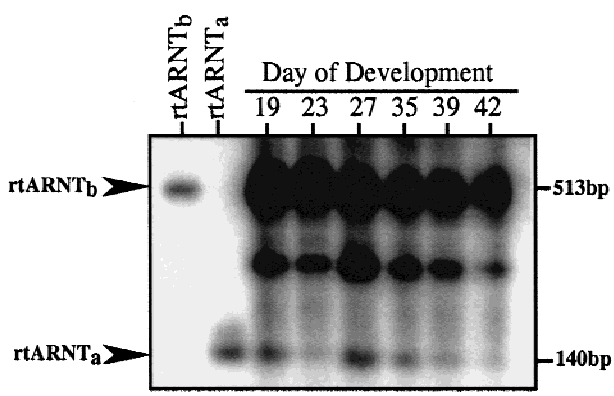

Figure 3. RT-PCR analysis of juvenile trout. Total RNA was isolated from all trout stages, reverse transcribed, and amplified by PCR as detailed in Materials and Methods. Samples were resolved by agarose gel electrophoresis, blotted to nitrocellulose, and hybridized with ^{32}P-labeled rtARNT$_a$ cDNA to visualize the bands. Standards were prepared by amplifying rtARNT$_b$ (first lane) or rtARNT$_a$ (second lane) cDNA with PCR primer 1 and PCR primer and resulted in fragments of 513 bp and 140 bp, respectively. Each trout stage is represented with one sample and is indicated on the top of the lane.

Results

Both rtARNT$_a$ and rtARNT$_b$ Messages Are Expressed in Juvenile Rainbow Trout

Gene-specific primers were used to amplify rtARNT$_a$ and rtARNT$_b$ message from trout at various developmental stages as detailed in Materials and Methods and Figure 1. A representative Southern blot is shown in Figure 3. An intense band corresponding to the expected size of the amplified rtARNT$_b$ fragment (513 bp) was detected in all samples. In addition, a band corresponding to the expected size of the amplified rtARNT$_a$ fragment (140 bp) was detected in all samples. The RT-primer 3 used for reverse transcription is a gene-specific primer and has 100% sequence identity to both the rtARNT$_b$ and rtARNT$_a$ mRNA sequences. Thus the levels of expression of the two rtARNT messages are directly comparable. In this regard, the data in Figure 3 suggest that the expression of the rtARNT$_a$ message is much lower than rtARNT$_b$ at all time points and nearly below the level of detection at days 23 and 42. The middle band observed on the blot is most likely a heteromeric complex that consists of one rtARNT$_a$ and one rtARNT$_b$ strand of cDNA since both sequences have complementary sequence outside the spliced exon (Figure 1). This band was observed previously when PCR was used to amplify the two rtARNT messages and could not be isolated by conventional molecular procedures (Pollenz et al., 1996). In addition, the band was observed when pure samples of rtARNT$_a$ and rtARNT$_b$ cDNA were amplified by PCR; thus it does not represent an additional ARNT isoform and does not affect the trend of the data.

Rainbow Trout ARNT$_b$, but Not ARNT$_a$ Is Expressed in Juvenile Trout

To determine whether the expression of the rtARNT messages correlated to protein expression, total tissue lysates were prepared from 2 fish each at days 19 and 23 (EMd19 and EMd23) and sac fry at days 27, 35, 39, and 42 (SFd27, etc.). Equal amounts of protein were then resolved by SDS-PAGE, and the expression of the ARNT isoforms was determined by Western blotting as detailed in Materials and Methods. A representative blot is shown in Figure 4. When the blots were stained with the rt-84 antibody that recognizes amino acids 2 to 373 of both rtARNT$_a$ and rtARNT$_b$ (Figure 2), a triplet band was observed in the range of 79 to 89 kDa (Figure 4, A). The lowest band of the triplet resolved at a molecular mass of approximately 79 kDa and comigrated with the protein standard for the rtARNT$_b$ protein. The level of rtARNT$_b$ expression appeared to be variable in fish of the same age and throughout the developmental series, but appeared to be highest at SFd27, and was clearly detected at all time pointed tested. The identities of the

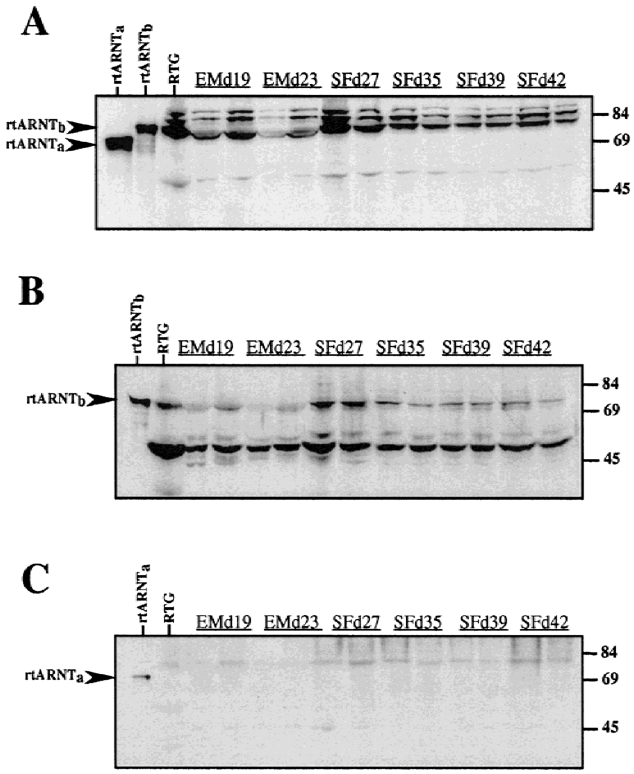

Figure 4. Western blot analysis of $rtARNT_b$ and $rtARNT_a$ protein expression in juvenile trout. Total tissue lysates were prepared as described from two rainbow trout embryos or sac fry, and 20 μg of protein was resolved by SDS-PAGE. All blots were visualized via ECL. The molecular mass of standard proteins is indicated on the right (kDa). **A:** Blots were stained with 1 μg/ml of rt-84 IgG followed by GAR-HRP IgG (1:10,000). Lane 1 contains in vitro expressed $rtARNT_a$, and lane 2 contains in vitro expressed $rtARNT_b$ protein. Lane 3 represents cell lysate from the rainbow trout gonad (RTG) cell line. Lanes 4 to 15 each represent samples from independent fish. **B:** Identical samples as detailed in A stained with 2 μg/ml of rt-79 IgG followed by RAC-HRP IgG (1:10,000). Lane 1 contains in vitro expressed $rtARNT_b$ protein, and lane 2 represents RTG cell lysate. **C:** Identical samples as detailed in A stained with 3 μg/ml of rt-41 IgG followed by GAR-HRP IgG (1:10,000). Lane 1 contains in vitro expressed $rtARNT_a$ protein, and lane 2 represents RTG cell lysate. Molecular mass markers are shown on the right.

higher molecular mass bands are unknown, but may represent additional ARNT isoforms or splice variants since the specificity of the rt-84 is to the highly conserved amino-terminal region of the protein and the expression pattern of the bands seems to mimic that of the 79-kDa band.

To confirm the trend of the $ARNT_b$ expression pattern, an identical blot was stained with the rt-79 antibody. This antibody was produced against amino acids 705 to 723 of the $rtARNT_b$ protein and does not react with $rtARNT_a$ isoform (Figure 2). A reactive band observed at approximately 79 kDa comigrates with the $rtARNT_b$ standard and does not react with the higher molecular mass proteins detected by rt-84 (Figure 4, B). However, the trend of $rtARNT_b$ expression is similar to that observed with the rt-84 antibody, with the highest level of $ARNT_b$ expression observed at SFd27. Unfortunately, the rt-79 also reacted with a band of unknown origin at approximately 50 kDa, and thus could not be used in histological staining experiments. To determine if the elevated expression of $rtARNT_b$ protein detected at SFd27 was representative of an increase in ARNT signaling at the level of DNA binding, nuclear extracts were generated from EMd23, SFd27, and SFd35 fish and evaluated for $rtARNT_b$ expression by Western blotting. The nuclear extract samples derived from SFd27 did not show a significant change in ARNT protein levels when compared with EMd23 and SFd35 samples (data not shown).

It was next pertinent to investigate the expression of the $rtARNT_a$ protein. It has been previously determined that the rt-84 antibody has high levels of reactivity with the $rtARNT_a$ protein (Pollenz et al., 1996; Necela and Pollenz, 1999); however, rt-84 did not detect a band at 70 kDa in Figure 4, A. To confirm this result, an identical blot was stained with antibody rt-41. This antibody, produced against amino acids 578 to 600 of the $rtARNT_a$ protein, does not react with the $rtARNT_b$ isoform (Figure 2, B). Figure 4, C, shows that rt-41 detects the $rtARNT_a$ standard but fails to detect a band at 70 kDa in any of the trout lysates, even though some minor reactivity is evident at approximately 80 kDa. Collectively, the results show that the different levels of rtARNT protein expression are consistent with the levels of expression of the different mRNAs. A low level of expression of the $rtARNT_a$ message and protein is consistent with the function of this protein as a dominant negative transcription factor (Pollenz et al., 1996; Necela and Pollenz, 1999).

Rainbow Trout ARNT Protein Is Ubiquitously Expressed in Tissues of Juvenile Trout

The next set of studies focused on the expression of rtARNT in tissues of juvenile trout. ARNT has been studied over developmental time in mouse, rat, and chicken (Abbott and Probst, 1995; Bryant et al., 1997; Walker et al., 1997; Hushka et al., 1998; Jain et al., 1998; Sommer et al., 1999; Sojka et al., 2000); however, this protein has yet to be investigated in young fish. Therefore, EMd23, SFd27, and

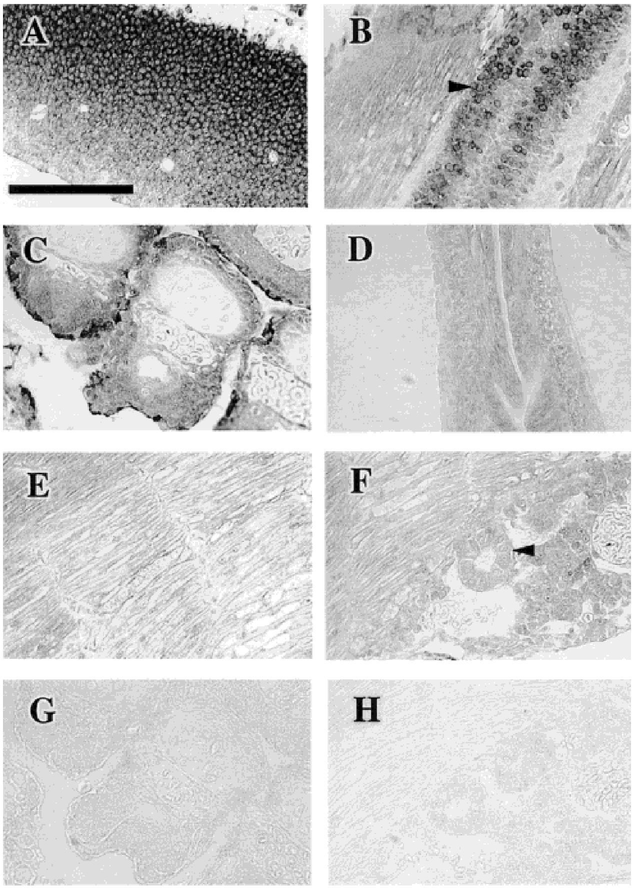

Figure 5. Immunohistochemical analysis rtARNT$_b$ in EMd23 trout. Sagittal sections were prepared, as described, and stained with rt-84 IgG (4 μg/ml) followed by GAR-HRP IgG (1:500). The staining was visualized using the ImmunoPure Metal Enhanced DAB Substrate Kit (Pierce). Brain **(A)**, spinal cord (indicated by arrow, **B**), gill **(C)**, gut **(D)**, skeletal muscle **(E)**, and kidney (indicated by arrow, **F**) were stained for rtARNT$_b$. Gill **(G)** and kidney **(H)** are shown as representative controls stained with preimmune IgG at 4 μg/ml, and minimal background staining was seen. Bar = 100 μm.

SFd39 fish were embedded in paraffin, sectioned, and stained with rt-84 to investigate the localization of rtARNT protein. Figure 5 shows the results from EMd23. Overall, the intensity of the staining was light, consistent with the low level of ARNT protein expression observed in the Western blots (Figure 4). The brain and spinal cord showed the heaviest staining, and it appeared to be predominantly nuclear (Figure 5, A and B). The epithelial cells of the developing gill also showed high levels of ARNT staining, while other portions of the organ showed light nuclear staining and some cytoplasmic reactivity (Figure 5, C). The gut, muscle, and kidney exhibited very light to no ARNT staining (Figure 5, D–F). Sections were also stained with preimmune mouse IgG. Gill and kidney tissues are shown in Figure 5, G and H, respectively, to illustrate the negligible level of background reactivity.

In contrast to the staining observed at SFd23, ARNT was expressed at a higher level throughout all tissues at SFd27. Intense nuclear staining was observed in the brain and spinal cord (Figure 6, A and B). The gill also showed heavy nuclear staining, but the epithelial cells did not show a difference in intensity of ARNT staining when compared with other cells within the gill, as observed at EMd23 (Figure 6, C). The gut and epithelial cells of the intestine showed moderate nuclear and light cytoplasmic staining (Figure 6, D–G). The muscle showed predominant nuclear staining (Figure 6, E), while the kidney exhibited moderate nuclear staining and an intense border on the luminal side of the cells (Figure 6, F). Finally, the ventricle of the heart showed strong nuclear staining and some cytoplasmic reactivity, while the bulbus arteriosus showed intense staining in both nuclear and cytoplasmic compartments (Figure 6, H). Sections were also stained with preimmune mouse IgG. Muscle and spinal cord tissues are shown in Figure 6, I and J, respectively, to illustrate the negligible level of background reactivity. To better observe the intense nuclear staining, Figure 7 shows higher-magnification sections of SFd27 muscle. (A), spinal cord (C), and gut (E); representative sections of the same tissues stained with preimmune mouse IgG are also shown (7B, D, and F).

On SFd35, the brain and spinal cord exhibited intense nuclear staining, as was seen with the previous two stages (Figure 8, A and B). The gill showed a similar pattern of expression to EMd23 with light nuclear and cytoplasmic staining and higher intensity in epithelial cells (Figure 8, C). The gut and intestine showed light nuclear and cytoplasmic staining, while the muscle exhibited moderate nuclear staining (Figure 8, D, G, and E). Finally, the kidney, liver, and heart showed heavy nuclear staining with some light cytoplasmic staining (Figure 8, F, H, and I). Heart sections were also stained with preimmune mouse IgG (Figure 8, J) to illustrate the negligible evel of background reactivity.

In summary, rtARNT expression was highest in brain and spinal cord at each time point analyzed, consistent with a role of ARNT in neural development (Nambu et al., 1991; Ema et al., 1996; Probst et al., 1997; Sonnenfeld et al., 1997). However, rtARNT was also expressed in most other tissues (especially at SFd27), which is also consistent with the function of this protein in numerous signaling cascades (reviewed in Rowlands and Gustafsson, 1997; Wilson and Safe,

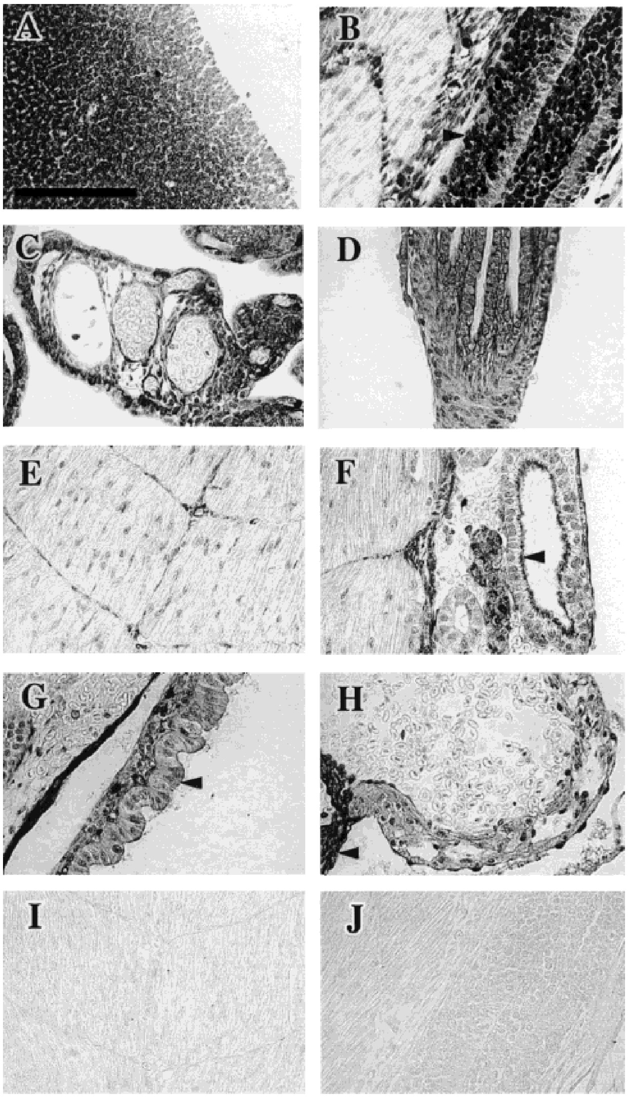

Figure 6. Immunohistochemical analysis rtARNT$_b$ in SFd27 trout. Sagittal sections were prepared, as described, and stained with rt-84 IgG (4 μg/ml) followed by GAR-HRP IgG (1:500). The staining was visualized using the ImmunoPure Metal Enhanced DAB Substrate Kit (Pierce). Brain **(A),** spinal cord (indicated by arrow, **B**), gill **(C),** gut **(D),** skeletal muscle **(E),** kidney (arrow indicates intense staining on the luminal side of cells, **F**), intestine (arrow indicates epithelial cells, **G**), and heart (arrow indicates bulbus arteriosus of heart, **H**) were stained for rtARNT$_b$. Muscle **(I)** and spinal cord **(J)** are shown as representative controls stained with preimmune IgG at 4 μg/ml and minimal background staining was seen. Bar = 100 μm.

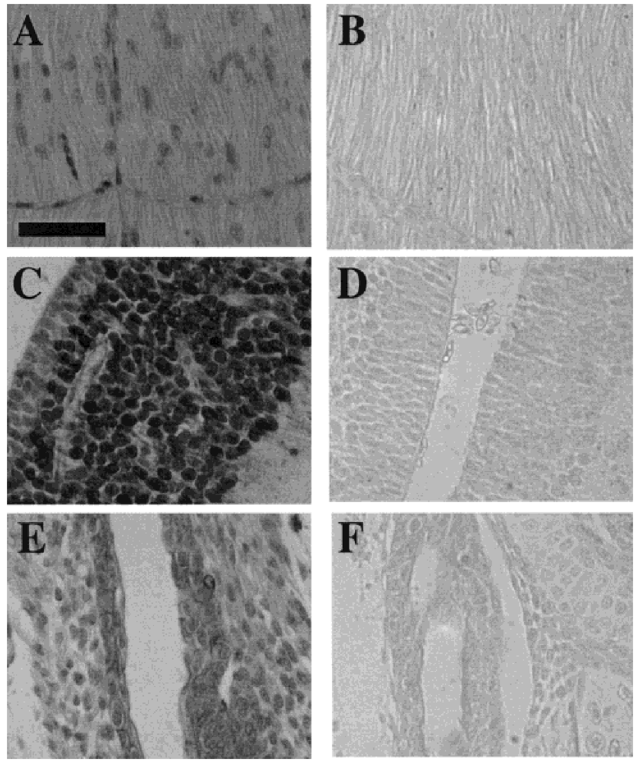

Figure 7. High magnification of muscle, brain, and gut from SFd27 trout. Sagittal sections were prepared, as described, and stained with rt-84 IgG (4 μg/ml) followed by GAR-HRP IgG (1:500). The staining was visualized using the ImmunoPure Metal Enhanced DAB Substrate Kit (Pierce). Muscle **(A),** brain **(C),** and gut **(E)** were stained for rtARNT$_b$. Muscle **(B),** brain **(D)** and gut **(F)** stained with preimmune IgG at 4 μg/ml are shown as controls. Bar = 100 μm.

1998). Since the fish were all fixed for 6 hours, the time to develop the DAB substrate was kept constant, and all sections were stained simultaneously, the staining intensities at the different developmental times can be compared. Thus the staining intensities of the sections at EMd23, SFd35, and SFd35 exhibited a similar trend to the Western blot data (Figure 4, A and B), with EMd23 staining being very light, SFd27 staining intense, and SFd35 staining moderate (Figures 5–8).

Both rtARNT$_a$ and rtARNT$_b$ Messages Are Expressed in Adult Rainbow Trout Tissues

Studies next turned to the expression of rtARNT message in tissues of adult rainbow trout. Gene-specific primers were used to amplify rtARNT$_a$ and rtARNT$_b$ message from various tissues of trout as detailed in Materials and Methods and in Figure 1. A representative Southern blot is shown in Figure 9. An intense band corresponding to the expected size of the amplified rtARNT$_b$ fragment (513 bp) was detected in the liver, gill, gonad, brain, kidney, intestine, heart, spleen, and muscle, with the highest expression present in

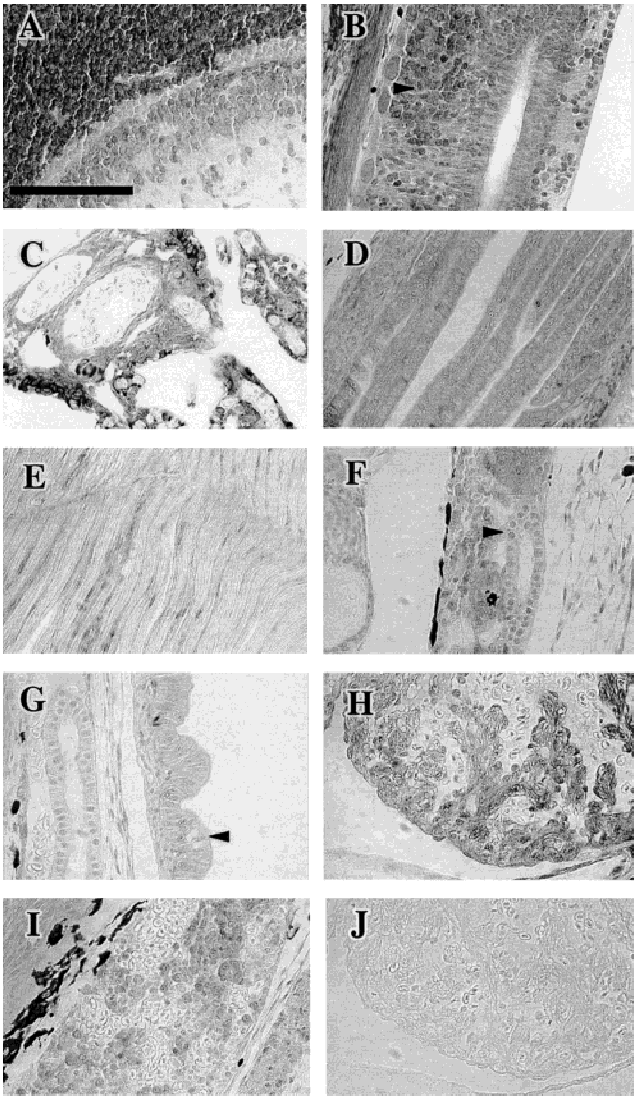

Figure 8. Immunohistochemical analysis rtARNT$_b$ in SFd35 trout. Sagittal sections were prepared, as described, and stained with rt-84 IgG (4 μg/ml) followed by GAR-HRP IgG (1:500). The staining was visualized using the ImmunoPure Metal Enhanced DAB Substrate Kit purchased from Pierce. Brain **(A),** spinal cord (indicated by arrow, **B**), gill **(C),** gut **(D),** skeletal muscle **(E),** kidney (indicated by arrow, **F**), intestine (arrow indicates epithelial cells, **G**), heart **(H),** and liver **(I)** were stained for rtARNT$_b$. Heart **(J)** is shown as a representative control stained with preimmune IgG at 4 μg/ml, and minimal background staining was seen. Bar = 100 μm.

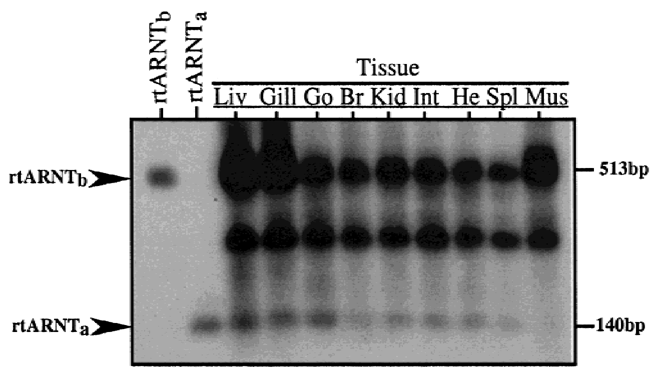

Figure 9. RT-PCR analysis of adult trout. Total RNA was isolated from the indicated trout tissues, reverse transcribed, and amplified by PCR as detailed in Materials and Methods. Samples were resolved by agarose gel electrophoresis, blotted to nitrocellulose, and hybridized with ^{32}P-labeled rtARNT$_a$ cDNA to visualize the bands. Standards were prepared by amplifying rtARNT$_b$ (lane 1) or rtARNT$_a$ (lane 2) cDNA with PCR primer 1 and PCR primer and resulted in fragments of 513 bp and 140 bp, respectively. Lanes 3 to 11 represent liver (LV), gill, gonad (Go), brain (Br), kidney (Kid), intestine (Int), heart (He), spleen (Spl), muscle (Mus).

the liver, gill, and muscle. In addition, a band corresponding to the expected size of the amplified rtARNT$_a$ fragment (140 bp) was detected in all samples except muscle. As observed in Figure 3, the expression of rtARNT$_a$ message appeared to be much lower than the rtARNT$_b$ in this semi-quantitative assay and was at the level of detection in brain and muscle. The heteromeric complex that runs midway between rtARNT$_a$ and rtARNT$_b$ was also observed in this experiment and may contribute to the reduced level of rtARNT$_a$ message detected in the brain and muscle. However, even if the rtARNT$_a$ strands present in the middle band could be quantified, the level of rtARNT$_a$ expression would be significantly less than that of the rtARNT$_b$.

Rainbow Trout ARNT$_b$ Protein Is Ubiquitously Expressed in Tissues of Adult Trout While rtARNT$_a$ Appears Confined to the Gill

To determine whether the expression of the rtARNT messages correlated to protein expression, total tissue lysates were prepared from liver, gill, gonad, brain, kidney, heart, spleen, and muscle. The expressions of the ARNT isoforms were then determined by Western blotting as detailed in Materials and Methods. Representative blots are shown in Figure 10. Blots stained with the rt-79 antibody exhibited a band that comigrated with the rtARNT$_b$ standard in all tissues investigated except skeletal muscle (Figure 10, A). Unlike the single band observed in the blots of juvenile fish, the gill and muscle showed another band that migrated at approximately 84 kDa. The detection of rtARNT$_b$ in all tissues is consistent with the expression of the mRNA and the results observed in the juvenile fish (Figures 3, 4, and 9).

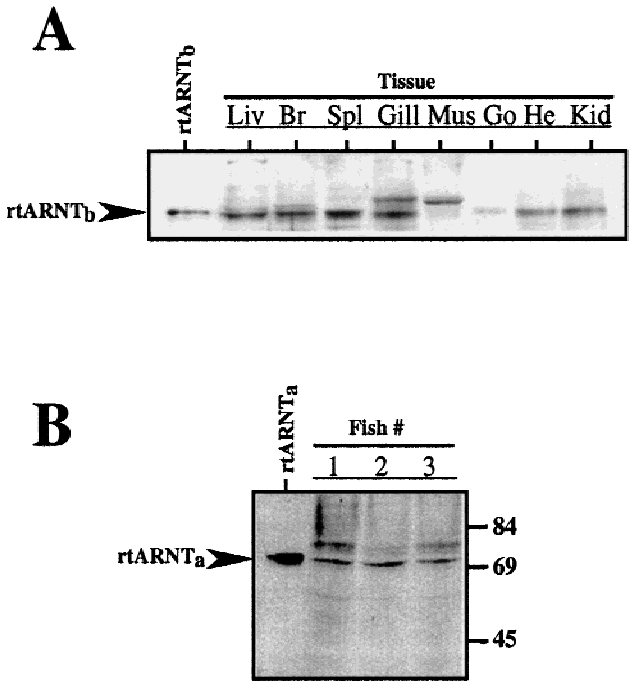

Figure 10. Western blot analysis of $rtARNT_b$ and $rtARNT_a$ and protein expression in adult trout tissues. Total tissue lysates were prepared as described from adult rainbow trout tissues, and 20 µg of protein was resolved by SDS-PAGE. All blots were visualized via ECL. **A:** Blots were stained with 2 µg/ml of rt-79 IgG followed by RAC-HRP IgG (1:10,000). Lane 1 contains in vitro expressed $rtARNT_b$ protein. Lanes 2 to 9 represent liver (LV), brain (Br), spleen (Spl), gill, skeletal muscle (Mus), gonad (Go), heart (He), and kidney (Kid), respectively. **B:** Blots were stained with 3 µg/ml of rt-41 IgG followed by GAR-HRP IgG (1:10,000). Lane 1 contains in vitro expressed $rtARNT_a$ produced in a TNT reaction. Lanes 2 to 4 represent a trout gill from 3 individual animals. The molecular mass of standard proteins is indicated on the right (kDa).

To investigate the expression of $rtARNT_a$ in adult tissues, blots containing total tissue lysates derived from liver, gill, gonad, brain, kidney, heart, spleen, and muscle were stained with the rt-41 antibody. Only gill exhibited an immunoreactive band that matched that of the $rtARNT_a$. To better evaluate the expression, gill tissue was removed from 3 different fish and used to prepare total tissue lysates. A representative Western blot is shown in Figure 10, B. A band is present at approximately 70 kDa that comigrates with the $rtARNT_a$ protein standard. In addition, a reactive band of approximately 80 kDa is observed in sample 1 (as seen in the juvenile blot in Figure 4, C). This band cannot represent $rtARNT_b$, as the rt-41 antibody does not react with this protein (Figure 2). To determine which cells in the gill were expressing $rtARNT_a$ protein, gill tissue was stained with the rt-41 as detailed. Figure 11, A, shows that all cells of the gill do not react equally with rt-41. Indeed, the staining appears to be heaviest in the epithelial cells surrounding the gill lamellae. Importantly, adjacent sections stained with nonspecific IgG showed little to no background staining (Figure 11, B). These results represent the first demonstration of the expression of the $rtARNT_a$ protein and place the protein in a location to affect environmental signals.

Discussion

The conclusions drawn from these studies are that $rtARNT_b$'s RNA and protein are ubiquitously expressed in juvenile and adult trout tissues emphasizing its importance in growing and adult trout, and that the dominant negative $rtARNT_a$ protein is present in the adult gill, which may be an important organ for multiple ARNT signaling pathways. These data represent the first account of fish ARNT proteins in vivo. The ubiquitous expression of $rtARNT_b$ protein is consistent with the localization of ARNT in mouse and chicken over developmental time and in a range of adult rat tissues (Abbott and Probst, 1995; Pollenz et al., 1998; Roman et al., 1998; Sommer et al., 1999; Sojka et al., 2000). However, unlike previous rodent and chick models, the level of ARNT staining did not remain constant over the developmental time course studied.

Rainbow trout $ARNT_b$ protein was present in all juvenile stages examined, but the intensity of the staining pattern varied. The $rtARNT_b$ staining was highest in the SFd27 samples; however, nuclear extracts did not show an elevated level of tightly bound $rtARNT_b$ during this stage. Therefore, day 27 sac fry possess elevated levels of ARNT, but do not appear to be utilizing that ARNT to signal under normal environmental conditions. These 2-day-posthatch fish may sustain elevated ARNT to help cope with changes in oxygen levels that can arise from the mobility of hatching. For example, ARNT is known to form a dimer with hypoxia-inducible factor (HIF) in mammals (Wang et al., 1995; Wang and Semenza, 1995) and may serve a similar function in fish, although HIF has yet to be characterized in fish. In addition, the removal of the chorion may leave the fish more susceptible to toxins in the environment. The elevated levels of ARNT could be stores available for the AHR signal transduction pathway, especially since rainbow trout are known to possess two forms of AHR (Abnet et al., 1999a, 1999b). Regardless of which signaling pathway the ARNT

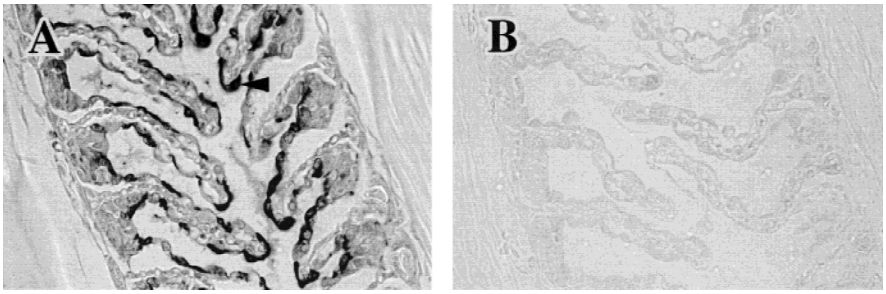

Figure 11. Immunohistochemical analysis rtARNT$_a$ in adult trout gill. Sagittal sections were prepared, as described, and stained with rt-41 IgG (4 μg/ml) followed by GAR-HRP IgG (1:500). The staining was visualized using the ImmunoPure Metal Enhanced DAB Substrate Kit purchased from Pierce **A:** The adult gill was stained for rtARNT$_a$. The arrow indicates an intensely stained epithelial cell. **B:** The gill was also stained with preimmune IgG at 4 μg/ml, and minimal background staining was seen. Bar = 100 μm.

protein participates in, the expression in juvenile trout suggests important functions for this protein as have been observed in mammalian models (Kozak et al., 1997; Maltepe et al., 1997).

At least 3 distinct ARNT genes have been discovered in mammalian species, and the juvenile trout Western blots exhibited additional bands above the rtARNT$_b$ band (Figure 2, A). These reactive proteins may represent undiscovered forms of ARNT in trout since the rtARNT is more homologous to mammalian ARNT, while other fish ARNTs, such as fundulus and zebrafish, are more similar to mammalian ARNT2 (Wang et al., 1998; Powell et al., 1999; Tanguay et al., 2000). These additional ARNT species may be reacting with the rt-84 polyclonal antibody since it was generated against a large section of rtARNT, including a portion of the basic helix-loop-helix region (Figure 2; Pollenz et al., 1996). The search for additional ARNT-like proteins in trout will be the focus of future experiments.

Immunohistology was used to determine if rtARNT proteins were present in specific organ systems of juvenile fish during the various stages examined. The brain and spinal cord exhibited the heaviest ARNT staining in EMd23 and SFd27 and 3SFd5. In mammals and *Drosophila,* ARNT forms a heterodimer with the single-minded (SIM) protein to signal during neurological development (Nambu et al., 1991; Ema et al., 1996; Probst et al., 1997; Sonnenfeld et al., 1997). Intense staining in the juvenile trout brain and spinal cord may indicate that rtARNT$_b$ is involved in a similar pathway in rainbow trout, although fish SIM has yet to be identified. Despite the strong neural staining, rtARNT$_b$ appeared to be expressed in most other tissues such as muscle, heart, and gill and to have a predominant nuclear localization, consistent with its location in cultured cells derived from trout (Pollenz and Necela, 1998). The presence of rtARNT$_b$ in multiple organ systems in the trout is consistent with studies in mammalian systems.

The expression pattern of the rtARNT$_a$ protein was more restrictive, however, even though the message was detected in nearly all tissues examined. The restriction is not unexpected given the potential negative function of rtARNT$_a$ (Pollenz et al., 1996; Necela and Pollenz, 1999). Southern blot analysis was required to detect the ARNT messages, which shows the very low level of expression of this gene. It is possible that the sensitivity of the rt-41 antibody was not sufficient to detect the presence of the rtARNT$_a$ protein in tissue lysates compared with the expression of the rtARNT$_b$. This does not mean that the rtARNT$_a$ protein is absent, but rather that at this time and under normal environmental conditions it may simply be below the detection limit of the antibodies.

However, rtARNT$_a$ was detected in epithelial cells of the adult gill, which is appropriate considering the gill has the potential to function in multiple signal transduction pathways utilizing ARNT. For example, the gill could be involved in response to hypoxia and would likely be the first site of exposure to aryl hydrocarbons. A large influx of these contaminants into a cell could activate high levels of AHR that would then have the potential to heterodimerize with ARNT. Such a scenario could possibly saturate the rtARNT$_b$ pool, thus altering AHR-independent pathways. The expression of rtARNT$_a$ in the context of this signaling pathway would bind AHR in a nonfunctional dimer, thus modulating gene regulation and maintaining the availability of rtARNT$_b$ for other pathways (Pollenz et al., 1996; Necela and Pollenz, 1999). Alternatively, rtARNT$_a$ could have a positive function in non-AHR signaling pathways, as suggested for the zfARNT2$_a$ isoform (Tanguay et al., 2000). This hypothesis is currently difficult to test in a fish model, as ARNT partners such as SIM and HIF have not yet been isolated from trout.

In summary, the presence of $rtARNT_b$ in various tissues in growing and adult trout demonstrates not only the importance of this protein, but also the potential for multiple ARNT signal transduction pathways in fish. In addition, the detection of the dominant negative $rtARNT_a$ protein in the adult gill suggests that there may be a need to modulate multiple signaling cascades in nonmammalian models. Future research will focus on the expression of other ARNT dimerization partners in the trout and protein-protein interactions. For example, analysis of the presence and localization of SIM and HIF proteins is critical to fully understanding the impact of dimerization partners that function in a dominant negative manner.

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