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cDNA Cloning and Characterization of a High Affinity Aryl Hydrocarbon Receptor in a Cetacean, the Beluga, *Delphinapterus leucas*

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Some cetaceans bioaccumulate substantial concentrations of planar halogenated aromatic hydrocarbons (PHAHs) in their tissues, but little is known about the effects of such burdens on cetacean health. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related PHAHs cause toxicity via activation of the aryl hydrocarbon receptor (AHR), a member of the bHLH-PAS family of transcription factors. Differences in AHR structure and function are known to contribute to species-specific differences in susceptibility to PHAH toxicity. To ascertain the potential for PHAH effects in a cetacean, we characterized an AHR from the beluga whale, *Delphinapterus leucas*. The 3.2 kb cDNA encodes an 845-amino acid protein with a predicted size of 95.5 kDa. Overall, the beluga AHR shares 85% amino acid sequence identity with the human AHR and 75% identity with the mouse AHR Ah^{b-1} allele. Beluga AHR protein synthesized in a rabbit reticulocyte lysate system demonstrated specific, high-affinity [^{3}H]TCDD binding. Saturation binding analysis was used to compare the [^{3}H]TCDD binding affinity of the *in vitro*-expressed beluga AHR with affinities of *in vitro*-expressed AHRs from a dioxin-sensitive mouse strain (Ah^{b-1} allele) and humans. The beluga AHR bound [^{3}H]TCDD with an affinity (K_d = 0.43 ± 0.16 nM) that was at least as high as that of the mouse AHR (K_d = 0.68 ± 0.23 nM), and significantly greater than that of the human AHR (K_d = 1.63 ± 0.64 nM). In electrophoretic mobility shift assays, the beluga AHR exhibited sequence-specific, Arnt-dependent binding to a dioxin responsive enhancer (DRE). Upon transient transfection into mammalian cells, the beluga AHR activated transcription of a luciferase reporter under control of a DRE-containing fragment of the mouse *Cyp1a1* promoter. These results show that in an *in vitro* system, the beluga AHR possesses characteristics similar to those of AHRs from other mammals that are considered sensitive to toxic effects of PHAHs. Together, these results demonstrate that the use of *in vitro*-expressed proteins is a promising approach for addressing molecular and biochemical questions concerning PHAH toxicity in endangered or protected species.

Key Words: aryl hydrocarbon receptor; TCDD; *in vitro* expression; beluga; cetacean; species-specific; susceptibility; dissociation constant.

Persistent organic pollutants (POPs) are ubiquitous in the marine environment. These compounds are generally hydrophobic and resistant to metabolic and chemical degradation, and thus tend to partition to lipid-rich components of tissues and cellular compartments. Because many POPs tend to bioaccumulate, organisms at the highest trophic levels—including marine mammals such as odontocete cetaceans (toothed whales)—may be exposed to high concentrations of these compounds. For example, beluga whales (*Delphinapterus leucas*, family Delphinidae) that inhabit the St. Lawrence Estuary, Canada, accumulate polychlorinated biphenyl (PCB) concentrations as high as 500 μg/g wet weight in blubber (Martineau *et al.*, 1987; Muir *et al.*, 1990, 1996). Consequently, POP exposure has long been suspected of playing a role in the observed cancer and reduced rate of reproduction in this population (Beland *et al.*, 1993; De Guise *et al.*, 1995; Martineau *et al.*, 1994). However, evaluation of the actual effects of POPs in beluga and other cetacean species and populations is hindered by the possible interplay of multiple environmental variables, including habitat degradation and the occurrence of high levels of other types of anthropogenic pollutants. Furthermore, establishing definitive cause and effect relationships is difficult, because logistical, moral, or ethical concerns preclude direct toxicity testing in cetaceans.

Among the several classes of POPs, the *planar* halogenated aromatic hydrocarbons (PHAH) are known to be especially toxic to vertebrate animals. PHAHs include 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), other polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, and certain non- and mono-*ortho*-substituted PCBs. These compounds cause a number of toxic and biochemical effects in laboratory rodents, including immunosuppression, endocrine dysfunction, enzyme induction, cancer, and reproductive/developmental toxicity (Pohjanvirta and Tuomisto, 1994; Poland and Knutson, 1982;

This work was presented in part at the 1999 annual meeting of the Society of Toxicology (Jensen *et al.*, 1999) and at the Thirteenth Biennial Conference on the Biology of Marine Mammals (November, 1999). The nucleotide sequence has been deposited in the GenBank database under accession number AF332999.

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Schmidt and Bradfield, 1996). Although the mechanisms by which TCDD and other PHAHs cause toxicity are not completely understood, it is known that these compounds exert their toxic and gene-regulatory effects *via* the aryl hydrocarbon receptor (AHR) (Hahn, 1998a; Poland *et al.*, 1976; Schmidt and Bradfield, 1996), a soluble, ligand-activated transcription factor and a member of the basic helix-loop-helix family of transcription factors (Gu *et al.*, 2000). Studies in AHR-null mice have shown that this protein is required for TCDD toxicity (Fernandez-Salguero *et al.*, 1996; Hundeiker *et al.*, 1999; Mimura *et al.*, 1997; Peters *et al.*, 1999; Thurmond *et al.*, 2000). In addition, strain- and species-specific differences in sensitivity to the effects of TCDD have been shown to depend, at least in part, on the properties of the respective AHRs, including their ligand-binding affinities (Ema *et al.*, 1994b; Poland *et al.*, 1994; Sanderson and Bellward, 1995).

Several laboratories have provided evidence for a functional AHR signal transduction pathway in cetaceans. Cytochrome P450 (CYP) forms in the 1A and 1B subfamilies, which in other mammals are regulated by the AHR, have been identified by catalytic, immunochemical, and molecular assays in cetaceans (Godard *et al.*, 2000; Goksøyr *et al.*, 1986, 1988; Teramitsu *et al.*, 2000; White *et al.*, 1994). A strong correlation between hepatic levels of CYP1A1 and blubber concentrations of non-*ortho*-substituted PCBs provided indirect evidence for CYP1A1 induction in beluga from the Arctic (White *et al.*, 1994). The presence of a cetacean AHR that is capable of specific binding to dioxins was confirmed using dolphin kidney cell lysates (Carvan *et al.*, 1994) and beluga liver cytosol (Hahn *et al.*, 1994).

To assess the potential susceptibility of cetaceans to PHAHs, we are characterizing the AHR signaling pathway of the beluga (Hahn *et al.*, 1994; Jensen, 2000; White *et al.*, 1994, 2000). This species was chosen for several reasons: (1) the existence of distinct stocks that vary in their exposure to PHAHs (Muir *et al.*, 1990); (2) the postulated role of PHAH in the decline of the St. Lawrence beluga stock (De Guise *et al.*, 1995; Martineau *et al.*, 1994; Sargeant and Hoek, 1988); (3) the recent proposal of beluga as a "model odontocete cetacean species" by a working group of marine mammal toxicologists convened by the Marine Mammal Commission (Marine Mammal Commission, 1999); and (4) the potential for follow-up research on captive populations. In the present report, we describe the cloning, *in vitro* expression, and initial functional analysis of a beluga AHR. We compare this beluga AHR to a relatively high affinity AHR, the product of the mouse Ah^{b-1} allele (Burbach *et al.*, 1992; Ema *et al.*, 1992; Poland *et al.*, 1994), as well as to the human AHR (Dolwick *et al.*, 1993a). This is the first molecular characterization of an AHR in a marine mammal. The results of these analyses suggest that beluga may be sensitive to the effects of PHAHs. More generally, our results show that cDNA cloning with *in vitro* characterization of proteins involved in mechanisms of toxicity is a promising approach for gathering species-specific data that may contribute to an improved understanding of the contaminant sensitivity of protected species, for which *in vivo* dosing experiments are ethically, legally, and logistically impossible.

TABLE 1
Gene-Specific Primers Used for Amplification and Cloning of the Beluga AHR

Primer	Sequence
Dlb538	5′–TTC CTT TGG CAT CAC AAC CAG TAG–3′
Dlb467	5′–CGG ATT TCA AGT ATG GAT GGT GG–3′
Dlf152	5′–GGG CGT TAA ATC CTT CAC AGT GTC C–3′
Dlf258	5′–GAA CTC TTC GTC TAT GGA AAG GTG C–3′
Dlf467	5′–CCA CCA TCC ATA CTT GAA ATC CG–3′
Dlf538	5′–CCT ACT GGT TGT GAT GCC AAA GGA A–3′
DlExon9F	5′–TGC AGT CGA ATG CAC GCT TAG–3′
DlUTRrev	5′–AAC CAA GAT GAA AAA TGG GCT TG–3′
5nat-hind3	5′–CCC AAG CTT GGG CAC CAT GAA CAG CAG C–3′
5koz-hind3	5′–CCC AAG CTT GGG CAC CAT GGA CAG CAG C–3′
xba1-utr	5′–GCT CTA GAG CAA CCA AGA TGA AAA ATG GGC TTG–3′

MATERIALS AND METHODS

Materials. Expression vectors for the mouse AHR (pSPORTmoAHR; Ah^{b-1} allele; Burbach *et al.*, 1992), human AHR (pSPORThuAHR2; Dolwick *et al.*, 1993a), and human Arnt (pSPORTArnt) were graciously provided by Dr. C. Bradfield (McArdle Center for Cancer Research, Madison WI). The luciferase reporter construct pGudLuc6.1, derived from pGudLuc1.1 (Garrison *et al.*, 1996) as described elsewhere (Long *et al.*, 1998), was a gift from Dr. M. Denison (U.C. Davis). 2,3,7,8-Tetrachloro[1,6–^{3}H]dibenzo-*p*-dioxin (33 Ci/mmol) was obtained from Chemsyn Science Laboratories (Lenexa, KS) and purified to ~95% radiochemical purity by high performance liquid chromatography (Gasiewicz and Neal, 1979). 2,3,7,8-Tetrachlorodibenzofuran (TCDF) was obtained from Ultra Scientific (Hope, RI). Methylated [*methyl*-^{14}C]ovalbumin was obtained from NEN Life Science Products, Inc. (Boston, MA). Methylated [*methyl*-^{14}C]catalase was synthesized as described (Dottavio-Martin and Ravel, 1978). Charcoal (Norit A alkaline) was from Fisher Scientific. The C57BL/6 mouse was provided by Dr. D. S. Sherr (Boston University School of Public Health, Boston, MA).

Tissue collection. Beluga liver was collected from Mackenzie River Delta, NWT, Canada, as described earlier (White *et al.*, 1994). Liver tissue was also collected from a subsistence hunt of Chukchi Sea beluga in Alaska during the summer of 1997. Liver tissue was snap frozen in liquid nitrogen approximately 3–4 hours after death. Tissues were maintained at liquid nitrogen temperatures throughout transport, storage, and powdering with mortar and pestle. The C57BL/6J mouse was killed by cervical dislocation and the liver was extracted from the animal and snap frozen minutes after death.

RNA isolation. Total RNA was isolated from liver tissue using the guanidinium isothiocyanate method (Clemens, 1984). PolyA$^+$ RNA was isolated using oligo dT columns (Collaborative Research, Bedford MA) as described by Maniatis *et al.* (1982). The quality of the total and polyA$^+$ RNA was confirmed by visualization on ethidium bromide-stained agarose minigels and quantity was determined by UV absorbance.

Oligonucleotide primers. Primers were synthesized by National Biosciences, Life Technologies, or Integrated DNA Technologies. The degenerate primers AHR-A1, AHR-A2, AHR-B1, and AHR-B2 have been described previously (Hahn and Karchner, 1995; Karchner and Hahn, 1996). The gene-specific primers used are presented in Table 1.

Cloning and sequencing of beluga AHR cDNA. PolyA$^+$ RNA isolated from a beluga that stranded in the Mackenzie River Delta, Canada, was used for synthesis of cDNA using random hexamers. Degenerate primers AHR-A2 and AHR-B2 containing inosines were used to amplify an internal beluga AHR fragment from the cDNA with the following PCR conditions: 94°C/5:00 min (95°/0:15 s, 45°/0:30, 72°/1:00) for 45 cycles, followed by a 7-min final extension (72°C). The same primers were used in direct sequencing of the amplified fragment in both directions using the Sequenase Version 2 Kit (U.S. Biochemical) with modifications for direct sequencing as described by Bachmann *et al.* (1990). Sequencing revealed 480 base pairs (bp) that shared high sequence identity with mammalian AHRs. Subsequent efforts to obtain a full-length clone using Rapid Amplification of cDNA Ends (RACE) (Frohman *et al.*, 1988; Ohara *et al.*, 1989) were unsuccessful. Suspecting that sample quality may have been compromised, we turned our efforts to beluga samples obtained in 1997 from Alaska.

PolyA$^+$ mRNA isolated from the Alaskan beluga was used with a GeneAmp RT/PCR kit (Applied Biosystems), with the following conditions in the Perkin Elmer 2400 thermocycler: 94°/5:00 (95°/0:15, 60°/0:30, 72°/1:00) for 35 cycles. Degenerate primers AHR-A1 and AHR-B1 containing inosines amplified a 648-bp fragment that was sequenced with a Licor4000 automated sequencing system using an Excel II cycle sequencing kit (Epicentre Technology).

Gene-specific primers were then designed for 5′- and 3′-RACE. The 5′ gene-specific primers Dlb538 and Dlb467 and the 3′ gene-specific primers Dlf152, Dlf258, Dlf467, and Dlf538 were used with the Marathon gene amplification kit (Clontech). To maximize specificity, "touchdown" PCR (Don *et al.*, 1991) was used for all RACE reactions: 94°/0:30 min (94°/0:05, 72°/2:00) (5 cycles); (94°/0:05,70°/2:00) (5 cycles); and (94°/0:05, 68°/2:00) (25 cycles). Nested 5′-RACE was carried out on the Dlb538 product using Dlb467. The 3′-RACE products were too large to sequence outright, so an additional PCR was performed to obtain the remaining sequence of the 3′-RACE clone using DLExon9F and DlUTR1rev with conditions 95°/5:00 (95°/0:30,56°/0:30,72°/0:30) (35 cycles); 72°/7:00. All sequencing was carried out on the Licor4000 automated sequencing system using an Excel II cycle sequencing kit. RT-PCR and RACE products were assembled to determine the consensus beluga AHR sequence. This sequence was based on 44 individual fragments; 38 were obtained by cycle sequencing of 21 separate clones and 6 sequences were from direct sequencing of PCR products in forward and reverse directions. The minimum number of sequences that were used to form a consensus at each base was 4 within the coding region and 2 in the untranslated regions (UTRs).

Multiple alignment of beluga, mouse, and human AHR was done with the ClustalW program contained in the MacVector software, version 6.5.3 (Oxford Molecular).

Expression vector construction. Once sequencing revealed the start and stop codons, primers were designed to amplify the full-length cDNA. Two 5′ primers were designed, one with the native sequence (5nat-hind3) and the other with a Kozak sequence (5koz-hind3) (A→G switch at +4) (Kozak, 1987). Both contained HindIII restriction sites outside of the coding region. The full-length beluga AHR was amplified using Klentaq DNA polymerase (Clontech). Primer pairs 5nat-hind3/xba1-utr and 5koz-hind3-/xba1-utr were used in touchdown PCR: 94°/1:00 (94°/0:05, 70°/2:00) (5 cycles); (94°/0:05, 68°/2:00) (5 cycles); (94°/0:05, 66°/2:00) (25 cycles); and 68°/7:00.

The full-length AHR products derived from primer pairs 5nat-hind3/xba1-utr and 5koz-hind3-/xba1-utr were cloned into the HindIII and XbaI sites of pcDNA3.0 (Invitrogen) and pSP64 Poly(A) (Promega) vectors. This generated constructs containing native and Kozak 5′ ends, each under control of either the T7 or SP6 promoter. The 4 constructs were tested for expression efficiency. Proteins were synthesized using the TNT Quick Coupled Transcription and Translation system (Promega) for T7 or SP6 promoters in the presence of [^{35}S]-methionine. The pSP64belAHRnat construct under control of the SP6 promoter expressed 2- to 3-fold higher levels of protein compared to the pcDNA constructs. The pcDNAbelAHRkoz construct expressed at higher levels compared with the pcDNAbelAHRnat sequence. Thus, the pSP64belAHRnat was used for *in vitro* translation studies, and the pcDNA-belAHRkoz was used for transfection experiments. To ensure that the pSP64belAHRnat expression plasmid (hereafter referred to as pSP64belAHR) and the pcDNAbelAHRkoz (hereafter referred to as pcDNAbelAHR) were free of PCR errors, each was completely sequenced and confirmed to match the belAHR consensus sequence that had been determined by RT-PCR and RACE.

Cytosol preparation. Liver cytosol was prepared as described in Hahn *et al.* (1994). Cryo-preserved liver was first powdered while under liquid nitrogen, then homogenized in MEEDGM (25 mM MOPS, 1 mM EDTA, 5 mM EGTA, 0.02% NaN_3, 20 mM Na_2MoO_4, 10% (v:v) glycerol, 1 mM DTT, pH 7.5) containing protease inhibitors (100 u/ml aprotinin, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 20 μM tosyl-L-phenylalanine chloromethyl ketone, and 0.1 mM phenylmethylsulfonyl fluoride). After serial centrifugations of 750g, 12,000g, and 100,000g, the supernatants were frozen in liquid nitrogen until analysis.

In vitro protein synthesis. Beluga, mouse and human AHR proteins were synthesized using the TNT Quick coupled Reticulocyte Lysate Systems (Promega) in the presence or absence of [^{35}S]-labeled methionine. The sizes of the proteins were confirmed by SDS-PAGE with 2 μl of a 25-μl TNT reaction containing [^{35}S]-labeled AHR (approximately 25 μg total protein), followed by fluorography and autoradiography.

Western blotting. Two μl of *in vitro* (TNT) synthesized mouse, human, and beluga AHRs and 100 μg beluga liver cytosolic protein were loaded onto a Tris-acetate gel (Novex). The gel was blotted onto 0.22 μm PVDF membrane, and probed with SA-210 rabbit antimouse AHR polyclonal antibody (Biomol) and secondary goat antirabbit antibody (Schleicher & Schuell). Bands were visualized with CSPD chemiluminescent substrate (Tropix).

Velocity sedimentation. *In vitro*-expressed beluga, mouse, and human AHR were analyzed by velocity sedimentation on sucrose gradients in a vertical tube rotor (Tsui and Okey, 1981). For each AHR or unprogrammed lysate control, two identical TNT reactions (100 μl total) were combined and diluted 1:2 with MEEDMG buffer (described above). Each sample was split into 2 aliquots and incubated with [^{3}H]TCDD (2 nM) ± TCDF (400 nM) for 8 h at 4°C in glass tubes. Following incubation, the expressed proteins were treated with 1.5 mg/ml dextran-coated charcoal (DCC) (charcoal:dextran 10:1 w:w) in a polypropylene tube. Cytosols were diluted to 5 mg protein/ml in MEEDMG buffer and incubated under the same conditions as the expressed proteins except that they were not washed with DCC. The [^{3}H]TCDD concentration was verified by sampling each tube for total counts. Samples were analyzed on 10–30% sucrose gradients as described earlier (Karchner *et al.*, 1999). Specific binding was defined as the difference between total binding (incubations containing [^{3}H]TCDD) and nonspecific binding (incubations containing [^{3}H]TCDD plus a 200-fold excess of TCDF).

[^{3}H]TCDD saturation binding analysis. The specific binding of [^{3}H]TCDD to *in vitro*-expressed AHRs was measured using a modification of the DCC-based binding assay of Poland *et al.* (1976). The beluga, mouse, and human AHR proteins were synthesized by *in vitro* transcription and translation as for the velocity sedimentation assay. The reactions were then diluted 1:8 in MEEDGM with protease inhibitors. Diluted TNT-expressed proteins were incubated in 16 × 100-mm glass tubes for 7–8 h with each of 9 concentrations (0 to 8 nM nominal) of [^{3}H]TCDD in DMSO. Duplicate aliquots were taken at the beginning of the incubation to measure the actual concentrations of [^{3}H]TCDD in each tube. After the incubation, 30 μl were transferred in triplicate aliquots to standard, 1.5-ml polypropylene tubes containing 30 μl of 2.3 mg DCC/ml MEEDGM. Tubes were vortexed × 3 for 5 s, with a 5-min incubation on ice between each vortexing. After a short spin to sediment the DCC, 40 μl of the supernatant were counted to measure bound [^{3}H]TCDD. Total counts and total binding were measured on a Beckman 5000 scintillation counter.

A difference in the assay used here as compared to the original method (Poland *et al.*, 1976) is our use of "unprogrammed" TNT lysate (UPL; TNT lysate plus an empty expression vector) to determine nonspecific binding. In tissue-based AHR binding assays, specific binding is determined from the

difference between total [^{3}H]TCDD binding and the [^{3}H]TCDD binding measured when excess cold ligand (usually TCDF) is used to block specific binding sites. The use of *in vitro*-expressed proteins provides the opportunity to measure nonspecific binding directly, by using a blank reaction containing UPL. Sucrose gradient analysis showed that UPL lacks specific binding when assayed in the presence and absence of excess TCDF (see Results and Karchner *et al.*, 1999). Therefore, analysis of unprogrammed TNT lysate provides a convenient means for measuring nonspecific binding without the need for duplicate tubes for each species tested. This method also avoids potential artifacts associated with the use of high concentrations of TCDF, which is poorly soluble in aqueous solutions.

All curves were plotted as "free" [^{3}H]TCDD (nM) vs. bound [^{3}H]TCDD. The amount of bound [^{3}H]TCDD was determined directly from measured radioactivity after DCC treatment. Free [^{3}H]TCDD was determined by subtracting the bound [^{3}H]TCDD concentration from the total [^{3}H]TCDD concentration for each tube. The binding of [^{3}H]TCDD to UPL (nonspecific binding) was also plotted as a function of the free [^{3}H]TCDD concentration and fit to a linear model; this relationship was used to calculate the predicted nonspecific binding at each concentration of free [^{3}H]TCDD in incubations containing *in vitro*-synthesized AHRs. This calculated value was then subtracted from the total binding in each tube to obtain the specific binding. The specific binding points were fit by nonlinear regression to the equation for the Langmuir binding isotherm:

$$B = \frac{B_{max}[L]}{[L] + K_d},$$

where B is specifically bound [^{3}H]TCDD, B_{max} is maximum bound receptor, L is the concentration of free ligand, and K_d is the equilibrium dissociation constant. Nonlinear regression analysis is the method of choice for determination of K_d and B_{max}, because it avoids the statistical disadvantages of linear transformations such as the traditional Scatchard analysis (Kenakin, 1999). The K_d values from 4 experiments each for beluga, mouse, and human AHRs were compared using one way ANOVA and Tukey's multiple-comparisons test. Curve fits were done with SigmaPlot 5.0 for the Macintosh (Jandel Scientific) and statistics were done with Prism version 3 software for the Macintosh (GraphPad).

Electrophoretic mobility shift assays. Electrophoretic mobility shift assays were performed using wild type and mutant DRE-containing oligonucleotides (Yao and Denison, 1992). Oligonucleotides were: wild-type (5′-GAT CTG GCT CTT CTC **ACG CAA** CTC CG-3′ and 5′-CGG AGT **TGC GTG** AGA AGA GCC AGA TC- 3′) and mutant (5′- GAT CTG GCT CTT CTC **AC<u>A</u> CAA** CTC CGG ATC- 3′ and 5′- GAT CCG GAG **TTG <u>T</u>GT G**AG AAG AGC CAG ATC-3′) (core sequence in bold; mutations underlined). To generate labeled dioxin-responsive enhancer (DRE)-containing probes, one strand of the wild-type DRE oligonucleotide was radiolabeled with [γ^{32}P]-ATP using T4 kinase according to the manufacturer's recommendations (Promega). After the labeling reaction, the complementary strand was added and annealed to the labeled strand by heating the combined oligonucleotides to 90°C and allowing them to slowly cool to room temperature. Unincorporated radionucleotides were removed by spinning the product through a Centri-Spin 20 column (Princeton Separations). For competition experiments, a double-stranded, unlabeled wild-type DRE oligonucleotide and a mutant DRE oligonucleotide that contains a single base pair substitution within the DRE core consensus sequence were used.

Prior to initiation of the *in vitro* expression reaction, the TNT Master Mix (Promega) was incubated with 2.5 μg DCC per μl Master Mix for 10 min on ice (Karchner *et al.*, 1999; Powell *et al.*, 1999). The DCC was then pelleted, and the Master Mix was removed and used in the *in vitro* expression reactions with pSP64belAHR, pSPORTmoAHR, and pSPORTArnt. Three μl of either the beluga or mouse AHR reactions were mixed with 3 μl human Arnt reaction and MEEDG (10 mM MOPS buffer, pH 7.5 with 1 mM dithiothreitol, 1 mM EDTA, 5 mM EGTA, 0.02% NaN3, and 10% glycerol). Where AHR or Arnt was left out of the reaction, an equal volume of unprogrammed lysate was substituted. Acetone or 40 nM TCDD was then added, and the mixture incubated for 2 h. For the competition assays, 3 μl unlabeled DRE, 3 μl mutant DRE, 0.6 μg anti-moAHR antibody (Biomol), or 0.6 μg rabbit IgG was added for an additional 15-min incubation. Finally, a master mix containing NaCl (165 mM final), pdIdC (1.25 μg/μl), glycerol (10%), and labeled DRE probe (50,000 cpm/rxn) was added to all tubes, and the incubation was continued for an additional 30 min. All incubations were at room temperature. The samples were resolved on a 4.5% TBE gel, and the gel was dried and exposed to X-ray film overnight.

Transfection assays. COS-7 monkey kidney cells, which express no AHR and very little Arnt (Ema *et al.*, 1994a,b) were transfected with human Arnt and beluga or mouse AHR, together with pGudLuc6.1 and a renilla luciferase plasmid (pRL-TK, Promega), to serve as the transfection control. pGudLuc6.1 contains the firefly luciferase gene under control of an MMTV promoter regulated by 4 DREs within a 480-bp fragment derived from the murine *Cyp1A1* promoter (Long *et al.*, 1998). Cells were transfected with 0, 10, or 50 ng of pcDNAbelAHR or pSPORTmoAHR, 0 or 50 ng pSPORTArnt, 20 ng GudLuc6.1, and 3 ng pRL-TK. The empty vector pcDNA3.1 (100–290 ng) was used to bring the total amount of DNA transfected to approximately 300 ng.

The transfection protocol was as follows. First, COS-7 cells were plated at 40,000 cells/well of a 48-well plate. Twenty-four hours later, the medium was replaced with serum-free medium and the cells were transfected using the Lipofectamine 2000 reagent (Gibco) as described by the manufacturer. Five hours later, cells were dosed with DMSO or 10 nM TCDD. Eighteen hours after dosing, the cells were harvested and luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) on a Turner TD-20/20 luminometer (Turner Designs).

RESULTS

Beluga AHR cloning. A beluga AHR cDNA was isolated from liver of beluga whales collected from Canada and Alaska. The initial sequencing utilized liver tissue from a beluga that was stranded in the Mackenzie River Delta, Canada. This individual was among those from which PCB levels and CYP1A expression were measured (White *et al.*, 1994) and an AHR was identified by photo-affinity labeling (Hahn *et al.*, 1994). Using degenerate primers, whose design was based on previously published mammalian AHR sequences (Hahn and Karchner, 1995; Karchner and Hahn, 1996), we isolated the AHR-A2/AHR-B2 fragment after using low-stringency conditions. Efforts to isolate the full-length beluga AHR cDNA using the Mackenzie River Delta beluga sample were not successful, so subsequent efforts utilized a beluga liver sample collected in Alaska in 1997. From freshly prepared mRNA, a 648-bp AHR-A1/AHR-B1 fragment was isolated that encompassed the AHR-A2/AHR-B2 fragment and matched its sequence exactly. The remaining beluga AHR sequence was obtained with 5′- and 3′-RACE using the 1997 Alaskan sample. Together, the RACE fragments and original PCR products spanned a 3.2 kb cDNA that contained a 2535-bp open reading frame (ORF), 30 bp of 5′-UTR, and 592 bp of 3′-UTR (Fig. 1). No polyA$^+$ tail was detected. The ORF encoded an 845-amino acid protein with a predicted size of 95.5 kDa.

An alignment of the beluga AHR amino acid sequence with that of the mouse AHR^{b-1} allele (Burbach *et al.*, 1992) and the human AHR (Dolwick *et al.*, 1993a) is shown in Figure 2.

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-30   CCG GGG AGA AGC CGC CGC CAG CCG GGC ACC ATG AAC AGC AGC AGC GCC AGC ATC ACC TAC GCC AGT CGC AAG CGG CGG
                                          M   N   S   S   S   A   S   I   T   Y   A   S   R   K   R   R  16
 49   AAG CCG GTG CAG AAA ACT GTC AAG CCA GTC CCA GCT GAA GGA ATC AAG TCG AAT CCT TCA AAG CGG CAT AGA GAC CGA
       K   P   V   Q   K   T   V   K   P   V   P   A   E   G   I   K   S   N   P   S   K   R   H   R   D   R  42
127   CTT AAT ACG GAA TTG GAC CGT TTG GCC AGC CTG CTG CCT TTT CCA CAA GAT GTT GTT AAT AAG CTG GAC AAA CTT TCA
       L   N   T   E   L   D   R   L   A   S   L   L   P   F   P   Q   D   V   V   N   K   L   D   K   L   S  68
205   GTT CTT AGG CTC AGT GTC AGT TAT CTA AGA GCC AAG AGC TTC TTT GAT GTT GCA TTA AAG TCC ACC CCA GCT GAC AGA
       V   L   R   L   S   V   S   Y   L   R   A   K   S   F   F   D   V   A   L   K   S   T   P   A   D   R  94
283   AAT GGA GTC CAG GAC AAC TGT AGA ACA AAA TTC AGA GAA GGC CTG AAC TTG CAG GAA GGA GAA TTC TTA CTG CAG GCA
       N   G   V   Q   D   N   C   R   T   K   F   R   E   G   L   N   L   Q   E   G   E   F   L   L   Q   A 120
361   CTG AAT GGC TTT GTA CTG GTT GTC ACT ACA GAT GCT TTG GTC TTT TAT GCT TCT TCT ACT ATA CAA GAT TAC CTG GGG
       L   N   G   F   V   L   V   V   T   T   D   A   L   V   F   Y   A   S   S   T   I   Q   D   Y   L   G 146
439   TTT CAG CAG TCT GAT GTC ATC CAT CAG AGT GTG TAT GAA CTG ATC CAT ACT GAA GAC CGA GCT GAA TTT CAG CGC CAG
       F   Q   Q   S   D   V   I   H   Q   S   V   Y   E   L   I   H   T   E   D   R   A   E   F   Q   R   Q 172
517   CTG CAC TGG GCG TTA AAT CCT TCA CAG TGT CCA GAC TCT GGA CAA AAA ATG GAT GAA GCT AAT GGC CTC TCA CAG CCA
       L   H   W   A   L   N   P   S   Q   C   P   D   S   G   Q   K   M   D   E   A   N   G   L   S   Q   P 198
595   GCA GTC TAT TAT AAC CCA GAC CAG GTT CCT CCA GAG AAC TCT TCG TCT ATG GAA AGG TGC TTC GTT TGC CGA CTA AGG
       A   V   Y   Y   N   P   D   Q   V   P   P   E   N   S   S   S   M   E   R   C   F   V   C   R   L   R 224
673   TGT CTG CTG GAT AAT TCA TCT GGT TTT CTG GCA ATG AAT TTC CAA GGG AGG TTG AAG TAT CTT CAC GGA CAG AAC AAG
       C   L   L   D   N   S   S   G   F   L   A   M   N   F   Q   G   R   L   K   Y   L   H   G   Q   N   K 250
751   AAA GGG AAA GAT GGA TCA ATA CTT CCA CCT CAG TTG GCT TTG TTT GCA ATA GCT ACT CCA CTG CAG CCA CCA TCC ATA
       K   G   K   D   G   S   I   L   P   P   Q   L   A   L   F   A   I   A   T   P   L   Q   P   P   S   I 276
829   CTT GAA ATC CGA ACC AAA AAT TTC ATC TTT AGA ACC AAA CAC AAG TTA GAC TTT ACA CCT ACT GGT TGT GAT GCC AAA
       L   E   I   R   T   K   N   F   I   F   R   T   K   H   K   L   D   F   T   P   T   G   C   D   A   K 302
907   GGA AGA ATT GTT TTA GGC TAT ACT GAA GCA GAG CTA TGC ATG AGA GGA TCA GGA TAT CAA TTT ATT CAT GCT GCT GAT
       G   R   I   V   L   G   Y   T   E   A   E   L   C   M   R   G   S   G   Y   Q   F   I   H   A   A   D 328
985   ATG CTT TAC TGT GCT GAG TAC CAT ATC CGG ATG ATT AAG ACT GGA GAG AGT GGC CTG ATA GTG TTC AGG CTT CTT ACG
       M   L   Y   C   A   E   Y   H   I   R   M   I   K   T   G   E   S   G   L   I   V   F   R   L   L   T 354
1063  AAA GAC AAT CGA TGG ACT TGG GTG CAG TCG AAT GCA CGC TTA GTT TAT AAG AAT GGA AGA CCA GAT TAT ATC ATT GCA
       K   D   N   R   W   T   W   V   Q   S   N   A   R   L   V   Y   K   N   G   R   P   D   Y   I   I   A 380
1141  ACT CAG AGA CCT CTA ACA GAT GAA GAA GGA ACA GAG CAT TTA CGA AAA CGA AAT CTG AAG TTG CCT TTT ATG TTT ACC
       T   Q   R   P   L   T   D   E   E   G   T   E   H   L   R   K   R   N   L   K   L   P   F   M   F   T 406
1219  ACT GGA GAA GCT GTT TTA TAT GAG GTA ACC AAC CCT TTT CCT CCC ATA ATG GAT CCC TTA CCA ATA AGG ACT AAA AAT
       T   G   E   A   V   L   Y   E   V   T   N   P   F   P   P   I   M   D   P   L   P   I   R   T   K   N 432
1297  GGT GCT GGT GGA AAA GAT TCT GCT ACC AAG TCA ACT CTA AGT AAG GAT TTC CTC AAT CCC AGC TCC CTC CTG AAT GCC
       G   A   G   G   K   D   S   A   T   K   S   T   L   S   K   D   F   L   N   P   S   S   L   L   N   A 458
1375  ATG ATG CAA CAA GAT GAA TCT ATT TAT CTC TAT CCT GCT TCA AGT AGT ACA CCT TTT GAA AGA AAC TTT TTC AGT GAC
       M   M   Q   Q   D   E   S   I   Y   L   Y   P   A   S   S   S   T   P   F   E   R   N   F   F   S   D 484
1453  TCT CAG AAC GAG TGC AGT AAT TGG CAA AAC AAT GTC GCA CCA ATG GGA AGT GAC GAT ATC CTG AAA CAC GAG CAG ATT
       S   Q   N   E   C   S   N   W   Q   N   N   V   A   P   M   G   S   D   D   I   L   K   H   E   Q   I 510
1531  GGC CAG TCT CAG GAA ATG AAC CCA ACC CTC TCT GGA GAT CAC GCA GGG CTC TTT CCA GAT AAT AGA AAT AGT GAC TTG
       G   Q   S   Q   E   M   N   P   T   L   S   G   D   H   A   G   L   F   P   D   N   R   N   S   D   L 536
1609  TAC AGC ATT ATG AAA CAC CTG GGC ATC GAT TTT GAA GAT ATC AAA CAC ATG CAA CAG AAT GAG GAA TTT TTC AGA ACT
       Y   S   I   M   K   H   L   G   I   D   F   E   D   I   K   H   M   Q   Q   N   E   E   F   F   R   T 562
1687  GAC TTT TCT GGT GAA GAT GAC TTC AGA GAT ATT GAC TTA ACA GAT GAA ATT CTG ACC TAC GTC GAA GAC TCT TTA AAT
       D   F   S   G   E   D   D   F   R   D   I   D   L   T   D   E   I   L   T   Y   V   E   D   S   L   N 588
1765  AAG TCT GCC TTG GGG TGT TCA GGT TAC CAT CCG CAA CAG TCC ATG GCT CTG AAC CCA AGC TGC ATG GTA CAG GAG CAC
       K   S   A   L   G   C   S   G   Y   H   P   Q   Q   S   M   A   L   N   P   S   C   M   V   Q   E   H 614
1843  CTC CAG TTA GAA CAG CAA GAG CAG CGA CAG CAG CAT CAG AAG CAC AGA GCA GTG GAG CAG CAA CAG CTG TGT CAG AAA
       L   Q   L   E   Q   Q   E   Q   R   Q   Q   H   Q   K   H   R   A   V   E   Q   Q   Q   L   C   Q   K 640
1921  ATG CAG CAT ATG CAA GTT AAT GGC ATG TTC GCA AAC TGG AGC TCG AAC CAA TCC GGG CCT TTT AAT TGT CCT CAG CCA
       M   Q   H   M   Q   V   N   G   M   F   A   N   W   S   S   N   Q   S   G   P   F   N   C   P   Q   P 666
1999  GAC TTA CAG CAG TAC GAT GTC TTT TCA GAC GTA CCT GGG ACC AGT CAA GAG TTT CCC TAC AAA TCT GAG ATT GAT ACT
       D   L   Q   Q   Y   D   V   F   S   D   V   P   G   T   S   Q   E   F   P   Y   K   S   E   I   D   T 692
2077  ATG CCT TAC GCA CAG AAC TTT ATT CCC TGT AGT CAG TCT GTG TTG CCG CCA CAT TCT AAG GGT ACA CAG TTA GAC TTT
       M   P   Y   A   Q   N   F   I   P   C   S   Q   S   V   L   P   P   H   S   K   G   T   Q   L   D   F 718
2155  CCC ATT GGG GAT TTT GAA CCA GCC CCA TAC CCT ACA ACT TCT TCT AAT TTA GAA GAC TTT GTC ACA TGT TTA CAA GTT
       P   I   G   D   F   E   P   A   P   Y   P   T   T   S   S   N   L   E   D   F   V   T   C   L   Q   V 744
2233  CCT CAA AGC CAA AGG CAC GGA CTC AAT CCA CAG TCA GCC ATA GTA ACT CCT CAG ACG TGT TAC ACT GGG GCT GTG TCA
       P   Q   S   Q   R   H   G   L   N   P   Q   S   A   I   V   T   P   Q   T   C   Y   T   G   A   V   S 770
2311  ATG TAC CAG TGC CAG CCG GAA GCT CAG CAC AGC CAT GTG GCT CAG ATG CAG TAC AAC CCA ACA GTG CCA GGC CCG CAG
       M   Y   Q   C   Q   P   E   A   Q   H   S   H   V   A   Q   M   Q   Y   N   P   T   V   P   G   P   Q 796
2389  GCA TTT TTA AAC AAG TTT CAG AAC GGA GGA GTC TTA AAT GAA ACC TAT CCA GCT GAA TTA AAC AGC ATA AAT AAC ACT
       A   F   L   N   K   F   Q   N   G   G   V   L   N   E   T   Y   P   A   E   L   N   S   I   N   N   T 822
2467  CAG CCT ACC ACC CAT CTT CAC CCG TCA GAA GCC AGA CCT TTC TCT GAC TTG ACA TCC AGT GGA TTC CTG TAA TTC CAA
       Q   P   T   T   H   L   H   P   S   E   A   R   P   F   S   D   L   T   S   S   G   F   L   *     845
2545  GCCCATTTTTCATCTTGGTTTTTTGATTAAATTCGTTTGTGAAACATTATTAGGGAGTAATAAAACTGTCATTGTTGGATGTTAGCAAGTTCACGTGGAGGCA
      TTGATGTATGTAGTTCTGTTTTTCCAAACCAAATTTTAATGATTTTATAAGAGGAGTTAATTACATATATTACCATCATTAAAATAGAAAGTATGCTGCCACA
      GAGGAGTGAAATAATCTACACTTTATAGTATTCTGAGATACCACAGAATATACAAAACTTATTCAGGGGGACATTAGGGGGACATTATGATACTTTTAAATAA
      GAACTTTTTCTCTGTTGGAATTATTTCTGTCAGAATAAAATGAAATACTTCGAGTTTTGAACTTCAACATTCTTATTAGTGTACCAAATATGAACTATTTTCT
      TTTATTGTAGCTTCTGCTTTTATCTCAGATGTTAAAATGAGTGGTTTGGTGCTTTTATAAAAAGATAATCTCAGTGCTCTCCTCCTTCCTTGGTAATGTAAGT
      GCCTCACAATTTTTTCTACCTATAACACTGTAGGAGTATGTTTTATATAAAATATACTTTCTCTTTTTAAAATCAAT
3167
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FIG. 1. Translation of a beluga AHR cDNA. The nucleotide and deduced amino acid sequence for a beluga AHR cDNA is shown. Bases are numbered on the left, starting with 1 at the start codon ATG, and encoded amino acids are numbered on the right; the stop codon is denoted with an asterisk.

Among published mammalian AHR sequences, the beluga AHR shares the highest overall amino acid sequence identity (85%) with the human AHR. The beluga and mouse AHRs share 75% identity. The AHR has functional regions whose boundaries have been determined for the mouse AHR (Burbach *et al.*, 1992; Dolwick *et al.*, 1993b; Fukunaga *et al.*, 1995; Poland *et al.*, 1994; Whitelaw *et al.*, 1993). In brief, the bHLH motif is involved in DNA binding, the PAS domain is involved

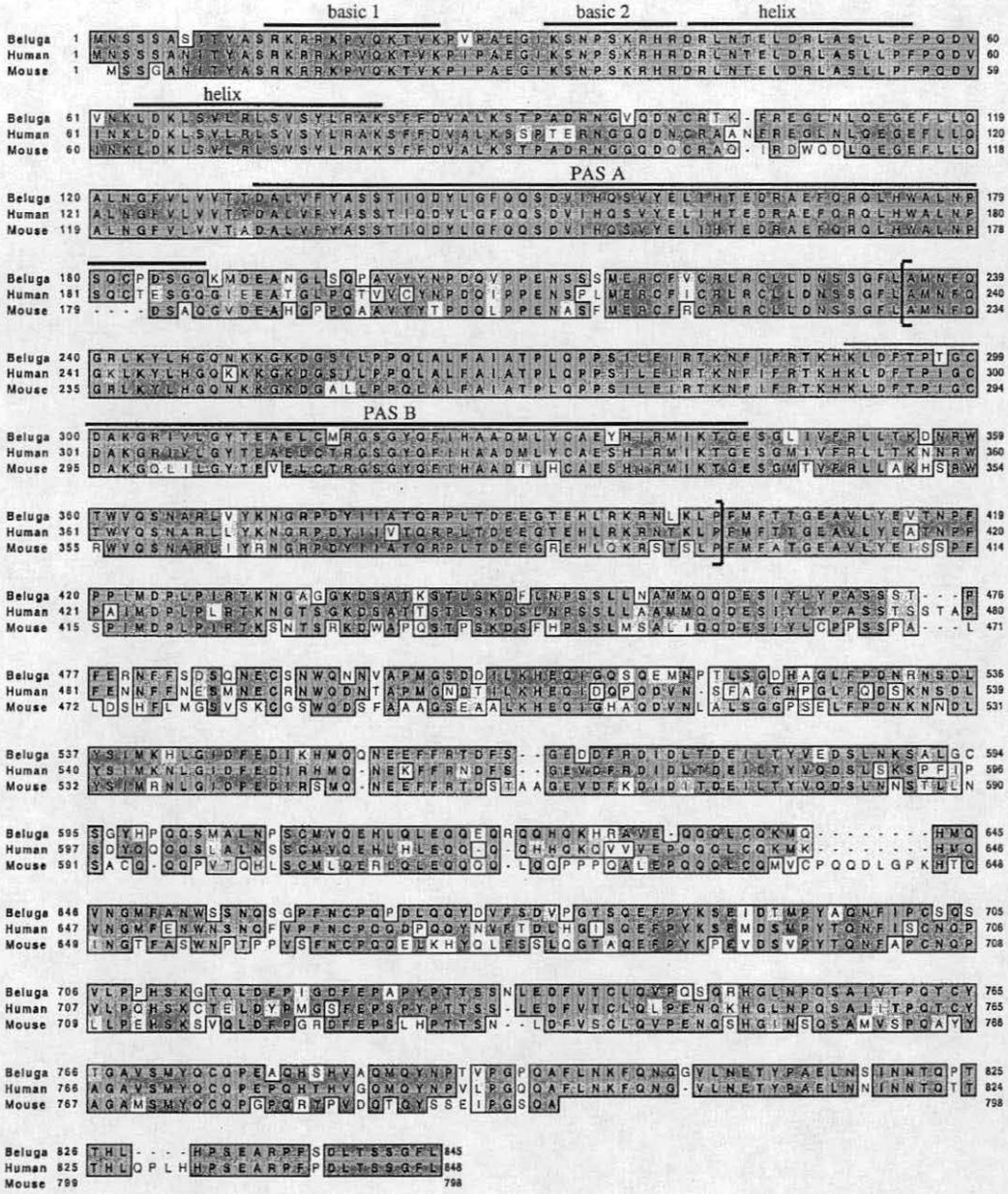

FIG. 2. Beluga, human, and mouse AHRs were aligned using the ClustalW alignment program within MacVector software, version 6.5.3 (Oxford Molecular). Human AHR (accession number L19872) and mouse AHR (accession number M94623) sequences were obtained from GenBank. The mouse AHR sequence reflects a Thr at residue 74 instead of a Ser, as noted by Sun *et al.*, (1997). The functional domains labeled were identified by homology to other mammalian AHRs (see text). The ligand-binding domain (Fukunaga *et al.*, 1995) is in brackets. Identical and similar amino acids are boxed, with identical amino acids shaded darkly, and similar amino acids shaded lightly. Gaps are indicated by dashes.

in ligand binding, dimerization with Arnt, and hsp90 binding, and the C-terminal half of the protein contains sequences that mediate transcriptional activation. When the homologous regions of the beluga AHR were compared to mouse and human AHR genes, the beluga AHR again shared the greatest sequence identity and similarity with the human AHR (Fig. 3). As in other bHLH/PAS proteins, the N-terminus is much more highly conserved among species as compared to the C-terminus. Clusters of glutamine residues appear among amino acids 598–675 of the beluga AHR, 20 in total (27%; Fig. 2). In the

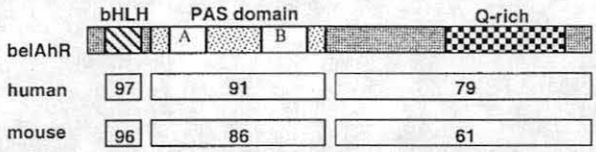

FIG. 3. Conservation within functional domains between beluga, mouse, and human AHRs. Pairwise alignments of the functional domains were generated using MacVector version 6.3. The numbers represent percent amino acid identity within the region spanned by the box.

same region, mouse AHR is 28% glutamine, and human AHR is 29% glutamine. This "Q-rich" domain, which has been shown to contribute to transcriptional activation in other transcription factors (Mitchell and Tjian, 1989), is suggested also to have a role in transcriptional activation by the AHR (Burbach *et al.*, 1992; Jain *et al.*, 1994; Rowlands *et al.*, 1996).

Certain amino acid changes in the AHR cause dramatic changes in its function. One of these is amino acid 375, of the mouse AHR; an alanine at this position in the Ah^{b-1} allele confers a 4- to 5-fold greater binding affinity when compared to the Ah^{d} allele, in which this residue is a valine (Ema *et al.*, 1994b; Poland *et al.*, 1994). Like the high-affinity mouse allele, the beluga AHR has an alanine at the homologous residue 380. Another notable mutation is found in the AHR of mouse Hepa-1 cells (mutant clone c35-3) at residue 216, between the PAS-A and PAS-B domains. A point mutation (C → Y) at this site caused a complete loss of DNA binding by the AHR-Arnt heterodimer (Sun *et al.*, 1997). As with the human AHR and the wild-type mouse Ah^{b-1} allele, the beluga AHR has the conserved cysteine at homologous residue 221 (222 in human AHR).

Characterization of beluga AHR synthesized in vitro. We analyzed the beluga AHR protein expressed in a rabbit reticulocyte lysate system under control of the SP6 and T7 promoters, with and without modification to create Kozak sequences near the start codon. An autoradiogram of a polyacrylamide gel containing the proteins made with [^{35}S]methionine revealed that the construct containing the native AHR sequence, under control of the SP6 promoter (pSP64belAHR), was the most efficiently expressed (not shown). The apparent molecular weight of the *in vitro*-expressed protein was ~110 kDa, consistent with its apparent size as observed by photo-affinity labeling of beluga hepatic cytosol (Hahn *et al.*, 1994). All functional assays of *in vitro* (TNT)-expressed protein were henceforth conducted using the pSP64belAHR plasmid.

The relative sizes of the expressed beluga, human, and mouse proteins synthesized in the presence of [^{35}S]methionine are consistent with their relative predicted molecular weights of 95.5, 90.6, and 96.0 kDa for pSP64belAHR, pSPORTmoAHR, and pSPORThuAHR products, respectively (Fig. 4A). Analysis by Western blot (Fig. 4B) confirmed the identity of the expressed proteins as AHRs, and showed that in each case the size of the expressed protein is the same as that derived from tissue cytosol. The relative intensities of the bands do not necessarily reflect quantitative differences between the beluga and mouse AHR expression in liver, because (1) the antibody we used was polyclonal and raised against the N-terminus of the mouse AHR, so equal cross-reactivity cannot be assumed, and (2) the beluga liver was sampled under suboptimal conditions.

Specific binding of [^{3}H]TCDD to the in vitro-expressed beluga AHR and beluga hepatic cytosol. We next determined the ability of the *in vitro*-expressed beluga AHR to bind [^{3}H]TCDD (2 nM). In a velocity sedimentation assay, the beluga, mouse, and human AHR TNT products all showed a clear peak of binding that was eliminated upon incubation with 200-fold excess TCDF, confirming that the [^{3}H]TCDD binding was specific (Fig. 5). The expressed beluga and human AHRs sedimented with peaks at 10.5 *S*, and the mouse AHR peak was at 10 *S*. In this experiment, the mouse AHR displayed the highest concentration of specific binding sites (111 fmol AHR/50 μl TNT reaction), followed by beluga (69 fmol/reaction) and human (24 fmol/reaction) AHRs. No specific binding was observed with the unprogrammed lysate (Fig. 5F), confirming that all detectable specific binding was from the expression products alone, and not from lysate proteins. Differences in the total number of specific binding sites among species in this experiment likely reflect variations in the efficiency of *in vitro* expression as well as species-specific differences in ligand binding properties.

To compare the ligand-binding properties of cloned (*in vitro*

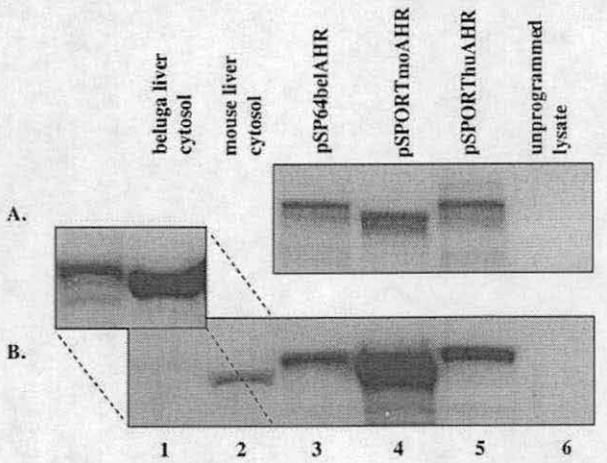

FIG. 4. Confirmation of *in vitro* expression of a beluga AHR. TNT reactions that were conducted in the presence of [^{35}S]-labeled methionine and 100 μg cytosolic protein isolated from beluga and mouse liver were analyzed by SDS-PAGE. (A) The gel was fixed, treated with Amplify fluorography reagent (Amersham), dried, and exposed to film overnight. (B) An identical set of samples was transferred to 0.2 μm PVDF membrane and blotted with antimouse AHR polyclonal Ab (SA-210; BioMol). Immunogenic proteins were visualized with chemiluminescence by exposure to film for 15 min (all lanes) or 30 min (inset lanes 1 and 2).

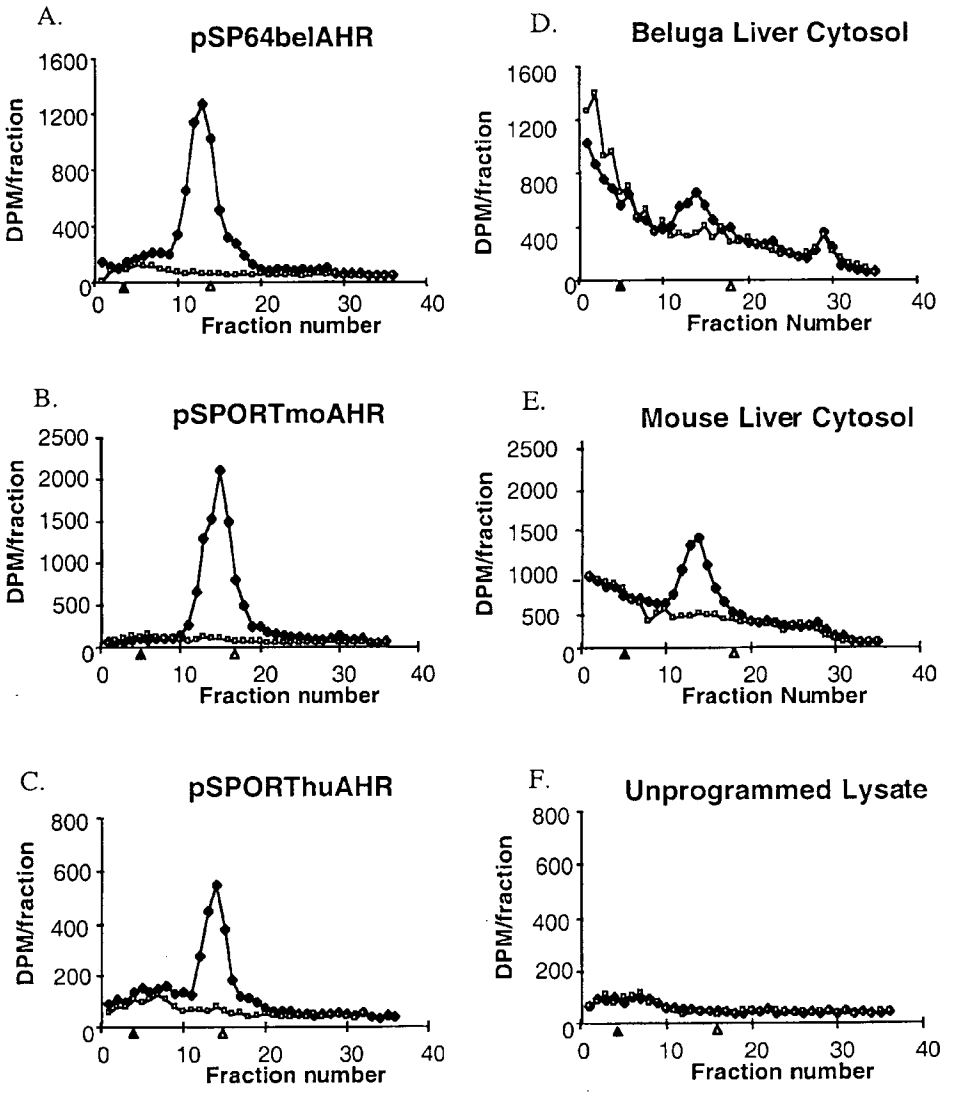

FIG. 5. Velocity sedimentation analysis of *in vitro*-expressed and cytosolic AHR. Beluga, mouse and human AHRs expressed *in vitro* and beluga and C57BL/6 liver cytosols were incubated with [^{3}H]TCDD (2 nM), with or without 200-fold excess cold TCDF. Total bound [^{3}H]TCDD (dpm) indicated by filled diamonds; nonspecific binding ([^{3}H]TCDD not displaced by 200-fold excess cold TCDF) indicated by open squares. The specific binding detected was as follows: (A) pSP64belAHR 115 fmol/mg, (B) pSPORTmoAHR 185 fmol/mg, (C) pSPORThuAHR 41 fmol/mg, (D) beluga liver cytosol 7.2 fmol/mg, and (E) C57BL/6 mouse liver cytosol 24 fmol/mg. (F) No specific binding was detected in UPL (TnT lysate programmed with empty pSP64polyA vector). [^{14}C]Ovalbumin (3.6 *S*; filled triangles on *x*-axis) and [^{14}C]catalase (11.3 *S*; open triangles on *x*-axis) were added to each tube as internal sedimentation markers.

expressed) and native (*in vivo* expressed) AHRs, we also performed velocity sedimentation analyses using cytosols prepared from beluga and C57BL/6 mouse livers, and both exhibited specific binding of [^{3}H]TCDD with peaks sedimenting at 9.5*S*, similar to the sedimentation coefficients of the *in vitro*-expressed AHRs. Specific binding in mouse liver cytosol was 24-fmol AHR/mg cytosol protein, and that in beluga liver cytosol was 7.2 fmol/mg. As with the Western-blotting results, the differences in specific binding of mouse and beluga cytosols do not necessarily reflect quantitative differences in AHR expression between these 2 species. When the beluga liver cytosol was treated with as little as 0.025–2.0 mg DCC/mg protein to adsorb free [^{3}H]TCDD, a significant amount of specific binding was lost (not shown). Sensitivity of cytosolic AHR to charcoal treatment has also been observed in human tissues (Manchester *et al.*, 1987; Nakai and Bunce, 1995), in monkey and dog tissues (Sandoz *et al.*, 1999), and in fish cells (Lorenzen and Okey, 1990). We did not observe this

sensitivity to charcoal with the TNT-expressed beluga AHR. Overall, these results show that the beluga AHR binds [^{3}H]TCDD in a highly specific manner, and that *in vitro* expression is a suitable way to generate large amounts of functional AHR protein, circumventing problems associated with obtaining, collecting, and processing cetacean tissue.

Relative binding affinities of beluga, mouse, and human AHRs expressed in vitro. Having established the ability of the beluga AHR to specifically bind [^{3}H]TCDD, we next sought to compare the TCDD-binding affinity (K_d) of the *in vitro*-expressed beluga AHR with the affinities of *in vitro*-expressed mouse and human AHRs. We used a saturation-binding assay modified from the DCC-based cytosolic-binding assay described by Poland *et al.* (1976). Critical to these experiments is the achievement of an abundance of "free" ligand relative to bound ligand, so that the [^{3}H]TCDD concentrations used to measure the dissociation constant are only minimally reduced by associations with lower-affinity binding sites. A satisfactory balance of detectable specific binding ($\geq$ 500 dpm) and fraction of "free" [^{3}H]TCDD ($\geq$ 80%) for the beluga AHR was achieved with an 8- to 10-fold dilution of the lysate (Jensen, 2000). After incubation with [^{3}H]TCDD, bound radioligand was separated from "free" radioligand with the use of DCC, and the amount of bound [^{3}H]TCDD was plotted as a function of the concentration of free [^{3}H]TCDD. Since the UPL does not bind [^{3}H]TCDD specifically (Fig. 5), it was used to determine the amount of nonspecific binding, as described in Materials and Methods. This eliminated the need for parallel incubations with [^{3}H]TCDD plus excess unlabeled competitor. Binding to UPL increased linearly with increasing amount of [^{3}H]TCDD in the concentration range used (Fig. 6), consistent with the theoretical properties of nonspecific binding sites (low affinity, high capacity, and not saturable).

Specific binding was calculated by subtracting nonspecific binding from total binding as described in Materials and Methods, and these values were fit to the Langmuir binding isotherm to determine the equilibrium dissociation constant (K_d) and the theoretical maximum binding (B_{max}). A representative set of experiments is shown in Figure 6, and data from 4 independent experiments are summarized in Table 2. The beluga AHR exhibited high affinity for [^{3}H]TCDD, with a K_d that was not significantly different from that of the mouse AHR; both the beluga and mouse AHRs had K_d values that were significantly lower than that of the human AHR, indicating greater binding affinities.

DRE binding and transactivation function of the beluga AHR. In order to characterize further the *in vitro*-expressed beluga AHR, we examined its ability to bind DRE sequences and activate transcription. Figure 7 shows the ability of the beluga and mouse AHRs to bind DNA in an electrophoretic mobility-shift assay (EMSA) using a double-stranded oligonucleotide that contains DRE3 from the mouse *Cyp1a1* promoter (Yao and Denison, 1992). The *in vitro* synthesized beluga AHR exhibited DNA binding, as demonstrated by the presence of a shifted band that coincided with a similar band produced by the mouse AHR (compare lanes 3 and 10). As expected, there was an absolute requirement for Arnt for DNA binding of both beluga and mouse AHRs (lanes 2–3 and 10–11). The ability of a wild-type but not a mutant DRE (containing a single base pair change within the consensus DRE sequence) to abolish binding demonstrates the specificity of the DNA binding by the beluga AHR-human Arnt and mouse AHR-human Arnt complexes (lanes 5–6 and 12–13). The ability of the anti-AHR antibody, but not nonspecific IgG, to eliminate the shifted band (lanes 7–8 and 14–15) demonstrates the presence of the beluga and mouse AHRs in the shifted complex. For both beluga and mouse AHRs, some DRE binding was observed in the absence of added ligand; TCDD caused a slight but reproducible increase in complex formation. Seemingly constitutive DRE binding by AHR-Arnt complexes in gel shift assays is often seen with *in vitro*-expressed AHRs, as noted previously (Dolwick *et al.*, 1993a; Hirose *et al.*, 1996; Karchner *et al.*, 1999; Numayama-Tsuruta *et al.*, 1997; Powell *et al.*, 1999). The causes of this apparent AHR activation are not clear, but could include the presence of natural ligands in the reticulocyte lysate (Phelan *et al.*, 1998; Sinal and Bend, 1997) or a deficiency of factors required to stabilize the AHR in the inactive configuration (Bell and Poland, 2000; Kazlauskas *et al.*, 2000; LaPres *et al.*, 2000; Petrulis *et al.*, 2000).

Figure 8 shows the ability of the beluga AHR to activate transcription of a luciferase reporter gene under control of murine *Cyp1a1* enhancer sequences in a transient cotransfection assay. Luciferase transcription was low to undetectable when the cells were transfected with pGudLuc6.1 alone or together with a human Arnt expression plasmid (lanes 1–4). Cotransfection of increasing concentrations of pcDNAbelAHR (0.5, 1.0, 5.0, 10, or 50 ng) resulted in a DNA concentration-dependent increase in transcription of the luciferase reporter (lanes 5–8 for 10 and 50 ng; lower DNA concentrations not shown). This occurred in the absence of cotransfected human Arnt expression plasmid, most likely because of the low levels of endogenous Arnt in these cells (lanes 9–10); however, luciferase transcription was enhanced in the presence of exogenous Arnt (compare lanes 7–8 to 9–10). The mouse AHR expression vector pSPORTmoAHR also stimulated transcription of the luciferase reporter (lanes 11–16). The greater degree of transactivation with the beluga AHR as compared to the mouse AHR could be because of the use of the pcDNA vector for beluga AHR expression; this vector contains the SV40 origin of replication, which leads to high plasmid copy number in COS-7 cells. In cells expressing beluga or mouse AHRs, luciferase activities in the absence of added TCDD were equal or nearly equal to those when TCDD (10 nM) was added. This "constitutive" (exogenous ligand-independent) reporter-gene expression and the resulting low TCDD inducibility in transient transfection assays have been noted by others. They are likely due to overexpression of the AHRs in the transfected

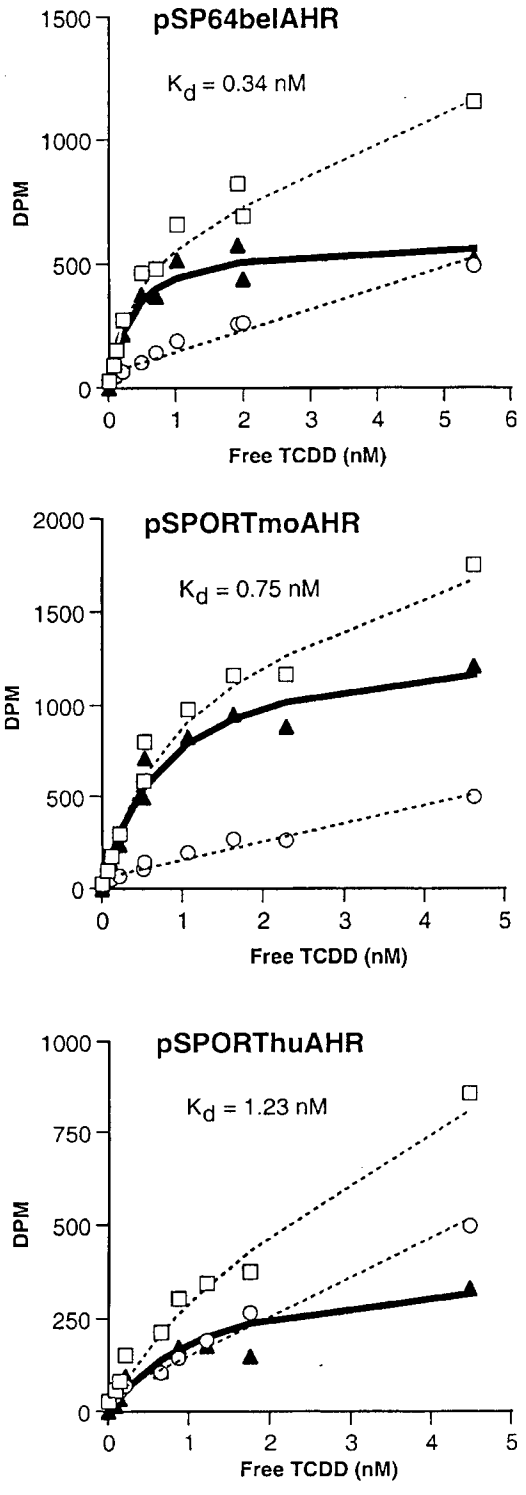

TABLE 2

Summary of Equilibrium Dissociation Constants (K_d) and Maximal Binding (B_{max}) Determined for *in Vitro*-Expressed AHRs

	n	Mean K_d ± SD (nM)	B_{max} ± SD (nM)	*p* vs. moAHR K_d	*p* vs. humAHR K_d
belAHR	4	0.43 ± 0.16	0.56 ± 0.16	>0.05	<0.01
moAHR	4	0.68 ± 0.23	0.86 ± 0.4	–	<0.05
huAHR	4	1.63 ± 0.64	0.32 ± 0.06	–	–

Note. K_ds of AHRs were analyzed using 1-way ANOVA (p = 0.0052, F = 9.9) and compared using the Tukey's multiple comparison test. For all 4 experiments, the range of r^2 values for fit of specific binding data to the Langmuir isotherm was 0.95–0.99 (beluga), 0.96–0.98 (mouse), 0.81–0.98 (human), and 0.95–1.0 (upl).

cells (Fukunaga *et al.*, 1995; Fukunaga and Hankinson, 1996; Mason *et al.*, 1994; Matsushita *et al.*, 1993; Whitelaw *et al.*, 1994;), possibly coupled with a deficiency of proteins required for cytoplasmic retention of unliganded receptor (Kazlauskas *et al.*, 2000; LaPres *et al.*, 2000; Petrulis *et al.*, 2000). Nevertheless, the results of these experiments clearly demonstrate the ability of the beluga AHR to bind to DRE sequences and subsequently to activate transcription.

DISCUSSION

The overall objective of these studies was to begin to assess the potential impact of a persistent and toxic class of environmental contaminants, the PHAHs, on the health of cetaceans. The toxicity of PHAHs has been clearly established in other mammalian species. For this reason, PHAHs, among other contaminants and factors related to environmental degradation, have been implicated in apparent increases in marine mammal disease and mortality. However, species-specific variability in sensitivity to PHAHs (Pohjanvirta and Tuomisto, 1994; Poland and Knutson, 1982) confounds and limits broad speculation on the toxicological significance of the high tissue burdens that are observed in marine mammals.

One approach for assessing the potential toxicological significance of PHAHs in a given species is to examine proteins involved in the mechanism of toxicity. We chose to examine

FIG. 6. Saturation binding curves for beluga, mouse, and human AHR. The panels show representative binding curves for the *in vitro*-expressed products derived from pSP64 belAHR, pSPORTmoAHR, and pSPORThuAHR. Nine concentrations of [^{3}H]TCDD were incubated with TNT reaction diluted in AHR buffer, as described in Materials and Methods. Open squares, total binding; open circles, nonspecific binding as determined using an empty pSP64 vector ("unprogrammed lysate"). Filled triangles, specific binding determined by subtracting the calculated nonspecific binding values from the total binding in each tube (100 dpm = 65 pM [^{3}H]TCDD). The curve through the specific binding points was derived by a nonlinear curve fit to the Langmuir binding isotherm using SigmaPlot software (Jandel Scientific). R^2 values (goodness of fit) for this experiment were 0.95 (beluga), 0.96 (mouse), 0.81 (human), and 0.97 (upl).

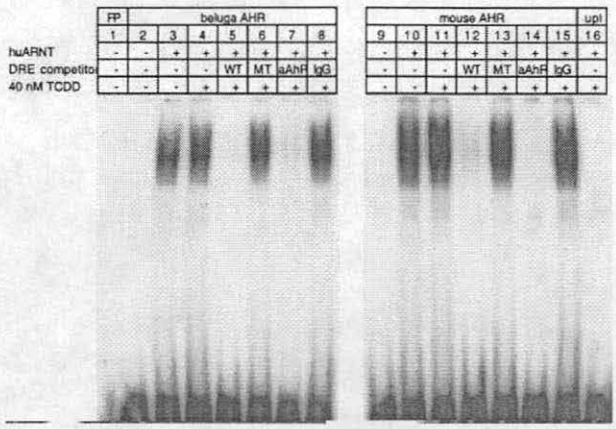

FIG. 7. DNA binding by the beluga AHR. *In vitro*-expressed beluga and mouse AHR were combined with unprogrammed lysate (lane 2) or human Arnt (lanes 3–8) and assessed for binding to a radiolabeled DRE-containing oligonucleotide as described in Materials and Methods. Competitors or treatments included excess unlabeled wild-type DRE (WT), excess unlabeled mutant DRE (MT), 0.6 μg SA-210 antimouse AHR polyclonal antibody (aAHR), and rabbit immunoglobulin (IgG). The results shown are representative of 3 experiments.

the AHR, a protein known to interact directly with PHAHs and to play an essential role in PHAH toxicity (Fernandez-Salguero *et al.*, 1996; Mimura *et al.*, 1997; Peters *et al.*, 1999). However, biochemical examination requires intact proteins from high quality tissue samples, which are difficult to collect from marine mammals because of the paucity of sampling opportunities, delays in excising tissues (leading to autolysis), and the *in vitro* lability and low abundance of the AHR. To circumvent these and other problems associated with the sampling and analysis of marine mammal tissues, we cloned a beluga AHR, expressed it *in vitro*, and characterized the TCDD-binding affinity and other functional properties of the expressed protein in comparison with AHRs from mouse and human. The results of our analyses provide suggestive evidence that beluga, and possibly other cetaceans, may be sensitive to AHR agonists, thus implying that PHAHs have the potential to affect cetacean health.

Beluga AHR Shares High Sequence Identity with Other Mammalian AHRs

Cloning and sequence analysis of a full-length cetacean AHR confirms and extends earlier observations of proteins in cytosols from beluga liver and a dolphin cell line that bind dioxin specifically (Carvan *et al.*, 1994; Hahn *et al.*, 1994). The beluga AHR possesses major functional domains that are characteristic of AHRs, including the bHLH, PAS A, PAS B, and glutamine-rich regions. As with other AHRs, the N-terminus of the beluga AHR is highly conserved, while the C-terminus is much less so, and might be termed "hypervariable," as noted by others (Dolwick *et al.*, 1993a). Phylogenetic analyses (not shown) demonstrate that the beluga AHR amino acid sequence groups with the "AHR1 clade" rather than the "AHR2 clade" recently identified in some vertebrates (Hahn *et al.*, 1997; Karchner *et al.*, 1999). Overall, the beluga AHR sequence is most closely related to the human AHR; these 2 proteins differ by only 3 residues in length and share 85% amino acid identity. The beluga AHR shares 75% identity with the mouse Ah^{b-1} allele (Burbach *et al.*, 1992), although within the bHLH and PAS domains this identity is much higher. The closer relationship of the beluga AHR to the human AHR, as compared to the mouse AHR, is unexpected in light of the phylogenetic relationships between cetacea, rodentia, and primates determined from other gene sequences (Madsen *et al.*, 2001; Murphy *et al.*, 2001). The basis and significance of this observation are not yet clear.

Despite the high degree of sequence identity among mammalian AHRs, subtle changes in the amino-acid sequence can cause remarkable changes in AHR function (Pohjanvirta *et al.*, 1998; Poland *et al.*, 1994; Sun *et al.*, 1997) that could be the basis for species-specific differences in sensitivity to AHR ligands. A much greater understanding of the AHR structure-function relationship is required before function may be deduced accurately from AHR sequence data. For this reason, we characterized the *in vitro* binding affinity and other properties of the beluga AHR.

Beluga Whales Possess a High Affinity AHR

In order to conduct a comparison of the beluga AHR with AHRs from terrestrial mammals that have been reasonably well characterized, we chose a relatively "high affinity" and a

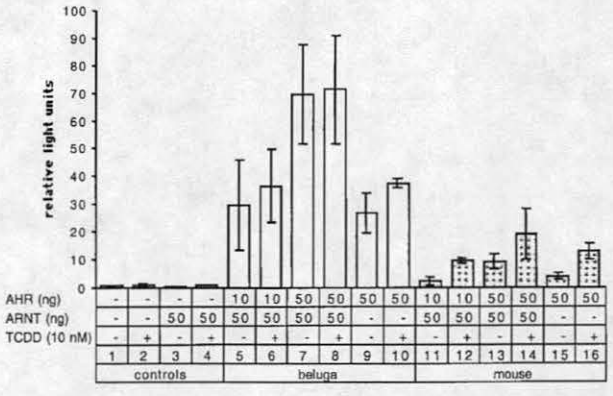

FIG. 8. Transcriptional activation by the beluga AHR. COS-7 cells were transfected with beluga and mouse AHR expression plasmids plus human Arnt where indicated. Each experimental and control well included 20ng luciferase reporter plasmid (pGudLuc6.1), and 3 ng renilla luciferase control plasmid (pRL-TK). Each combination of DNA was brought to ~300 ng total DNA with empty pcDNA3.1 vector. Five h after transfection, cells were dosed with DMSO or TCDD in DMSO, and cells were lysed and assayed 18 h later. Results shown represent means ± SD of 3 wells per group in one experiment. Transfection experiments using the beluga AHR were performed 3 times, with similar results.

TABLE 3
Dissociation Constants (K_d) of Mouse and Human AHRs Reported in the Literature

Protein source	Reference	K_d (nM)			
		Beluga	Mouse Ah^{b-1}	Mouse Ah^d	Human
In vitro transcription/translation	This study	0.43	0.68		1.63
In vitro transcription/translation	Poland *et al.*, 1994		0.010	0.037	
In vitro transcription/translation	Ema *et al.*, 1994b		0.27	1.66	1.58
Liver cytosol	Poland *et al.*, 1994		0.034	0.09–0.15	
Liver cytosol	Gasiewicz and Rucci, 1984		0.29	ND	
Liver cytosol	Manchester *et al.*, 1987; Okey *et al.*, 1989		1–3	16	
Placenta cytosol	Manchester *et al.*, 1987				6.8
Tonsil cytosol	Lorenzen and Okey, 1991				8.0
Hepa1c1c7 cytosol	Roberts *et al.*, 1990		1.0		
HepG2 cytosol	Roberts *et al.*, 1990				9.0
M2-Hep cytosol	Roberts *et al.*, 1991				4.4

Note. Data are grouped in columns by laboratory. K_ds reflect [^{3}H]-TCDD binding, except for the data from Poland *et al.*, 1994, which were derived using 2-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin. Standard deviations have been omitted for clarity. ND, not detected.

relatively "low affinity" AHR to use alongside the beluga AHR in our experimental system to determine comparative TCDD-binding affinities. The high affinity AHR was the mouse Ah^{b-1} allele (Burbach *et al.*, 1992). The representative low affinity AHR that we used was the human AHR that was cloned from the HepG2 hepatoma cell line (Dolwick *et al.*, 1993a). *In vitro*, the HepG2 AHR, like the mouse Ah^d allele, has a 4–5-fold lower affinity for TCDD than does the mouse Ah^{b-1} allele (Ema *et al.*, 1994b; Poland *et al.*, 1994).

Determination of the K_d for *in vitro*-expressed beluga, mouse, and human AHRs by saturation binding showed that the beluga AHR is a high affinity AHR. Absolute binding affinities are highly dependent on the protein concentrations used in the incubations, with apparent binding affinities decreasing with increasing protein concentrations (Bradfield *et al.*, 1988). Therefore, K_d values are best compared among those determined under similar experimental conditions and protein concentrations. Table 3 shows several intralaboratory data sets for which K_ds for mouse (Ah^{b-1} or Ah^d allele) and human AHRs were reported. When mouse and human AHR K_d values are compared within laboratories, the affinity of the mouse AHR (product of the Ah^{b-1} allele) is greater than that of the human AHR in each case. Regardless of whether the samples were tissue cytosols, cell-line cytosols, or *in vitro*-expressed proteins, the human AHR K_d values were 3- to 8-fold greater than those of the mouse. The robustness of this difference in binding affinities between experimental conditions and laboratories indicates a real functional difference between the mouse and human AHRs. Our findings show a 2.4-fold difference in binding affinity between mouse and human AHRs and a nearly 4-fold difference between beluga and human AHRs ($p < 0.05$, Table 2). Thus, the beluga AHR allele described here encodes a high affinity AHR.

These relative binding affinities may be used to infer the potential susceptibility of beluga to PHAH. The beluga AHR has a binding affinity that is similar to that of the mouse (Ah^{b-1}) AHR, but ~4-fold greater than the binding affinity of the human AHR (Table 2). In the case of inbred mice, a single alanine to valine change at residue number 375 is responsible for a 4–5 fold difference in the *in vitro* ligand binding affinities observed between the Ah^{b-1} allele and the Ah^d alleles (Ema *et al.*, 1994b; Poland *et al.*, 1994). At the homologous residues in the beluga and human AHRs, the beluga AHR possesses an alanine (Ala^{380}), as in the Ah^{b-1} allele, while the human AHR has a valine (Val^{381}), as in the Ah^d allele. This difference may contribute to the 4-fold difference in binding affinity between the beluga and human AHRs observed here. In *in vivo* studies using congenic mice, the homozygous Ah^{b-1} mouse strain shows 8- to 24-fold higher sensitivity to toxic effects compared to the homozygous Ah^d strain (Birnbaum *et al.*, 1990). Similarly, in a comparison among several bird species, the relative sensitivities to TCDD induction of CYP1A reflect the relative TCDD-binding affinities of their AHRs (Sanderson and Bellward, 1995). Thus, in the case of mouse strains and bird species, the relative AHR binding affinity can predict PHAH sensitivity. Of course, the AHR is not the only factor that can influence the susceptibility to PHAH effects. Altered expression or function of other components of the AHR-dependent signal transduction pathway, as well as other species-specific characteristics (e.g., biotransformation activities, pharmacokinetic differences) can also influence responsiveness (reviewed in Hahn, 1998b). Nevertheless, it is clear that the AHR plays an important—and possibly primary—role in determining susceptibility to PHAH toxicity. The presence of a high affinity AHR in beluga suggests that this species may be particularly sensitive to PHAH. Evidence for induction of CYP1A1 in beluga exposed to relatively low concentrations of PCBs in the Arctic (White *et al.*, 1994) is consistent with this hypothesis.

The Beluga AHR Binds DREs and Is Transcriptionally Active

To more completely assess the function of the beluga AHR, we examined its ability to participate in sequence-specific protein-DNA interactions with a murine DRE and to activate transcription of a reporter gene under control of a murine enhancer element containing multiple DRE sequences. These properties are characteristic of rodent and human AHRs (Burbach *et al.*, 1992; Dolwick *et al.*, 1993a,b; Fukunaga *et al.*, 1995; Whitelaw *et al.*, 1994) but had not yet been examined in a cetacean or other marine mammal. The results of these assays clearly demonstrated that the beluga AHR is capable of high affinity, sequence-specific, and Arnt-dependent interactions with mammalian DREs, and that this receptor is able to activate transcription of target genes, presumably through the glutamine-rich region or other putative transactivation domains in the C-terminal half of this protein. The demonstrated functionality of the beluga AHR *in vitro* is consistent with evidence for inducible *CYP1A1* expression *in vivo* (White *et al.*, 1994; 2000).

Significance of a High Affinity AHR in Cetaceans

Establishing a cause and effect relationship between contaminant burdens and disease in marine mammals is difficult. Because controlled dosing experiments in cetaceans are usually not feasible, a "weight of evidence" approach has been proposed for investigating the existence of contaminant-induced toxic effects in marine mammals (Ross, 2000). Currently, 4 lines of research are seen as contributing to this approach. These include (1) epidemiological and descriptive studies of wild populations, (2) mechanistic studies in laboratory rodents, (3) feeding studies in captive animals (possible only in limited cases), and (4) feeding studies involving rodents exposed to marine mammal diets or contaminated tissues.

Our results suggest a fifth type of data that can contribute to the weight-of-evidence approach: species-specific cloning and characterization of proteins involved in mechanisms of toxicity. This approach has been used recently to characterize the AHR and other receptors in nonmammalian species (Abnet *et al.*, 1999; Karchner *et al.*, 1999, 2000; Matthews and Zacharewski, 2000), but has not previously been applied to marine mammals. Such data can provide a link between mechanistic studies in laboratory rodents and epidemiological findings in wildlife. In this context, our study has demonstrated that belugas possess an AHR that shares a high degree of sequence identity with other mammalian AHRs, shares key functional properties with these AHRs, and binds TCDD with an affinity that is at least as high as a high affinity AHR from a dioxin-sensitive strain of mouse.

It may be informative to compare the binding affinity of the *in vitro*-expressed beluga AHR with the concentration of AHR ligands in beluga tissues, keeping in mind that such comparisons are complicated by the many differences that exist between *in vitro* and *in vivo* systems. The concentrations of "TCDD equivalents" (TEQs) in livers of St. Lawrence belugas are in the range of 0.01 to 0.13 nM (Gauthier *et al.*, 1998; Metcalfe *et al.*, 1999); Muir *et al.*, 1996).[3] Using the K_d of 0.43 nM for the *in vitro*-expressed beluga AHR (Table 2) and extrapolating directly to whole liver, these TEQ concentrations would be predicted to result in 2% to 23% receptor occupancy. At such levels of AHR occupancy, some effects might be expected, especially if belugas possess "spare" AHR capacity as shown in other systems (Hestermann *et al.*, 2000).

The presence of a high affinity AHR in a cetacean is consistent with a role for the AHR and PHAHs in the toxic effects observed in environmentally exposed cetaceans. Additional factors that will influence the sensitivity of cetaceans to PHAHs include the PHAH-AHR structure-binding relationships and the tissue- and cell-specific pattern of AHR expression. These questions are currently being addressed using the cloned beluga AHR cDNA and probes derived therefrom (Jensen, 2000). Together, these results demonstrate that the use of *in vitro*-expressed proteins is a promising approach for addressing molecular and biochemical questions concerning PHAH toxicity in endangered or protected species, in which logistical and ethical concerns preclude testing in live animals.

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[3] The value of 0.01 nM TEQ was calculated directly from the data provided by Gauthier *et al.* (1998) for a single neonatal beluga from the St. Lawrence estuary. The value of 0.13 nM TEQ was calculated for adult male beluga from the St. Lawrence estuary, using the data of Muir *et al.* (1996), as follows. These authors reported blubber TEQ values of 1400 ng/kg lipid. Assuming equilibration of PHAHs across tissues on a lipid basis, and assuming that the liver of these animals contained 3% lipid as shown for other St. Lawrence beluga (Metcalfe *et al.*, 1999), the hepatic concentration of TEQs in these beluga is estimated to be 42 ng/kg wet weight, which is equivalent to 0.13 nM TCDD.

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