THE OCEAN POUT (Macrozoarces americanus) ANTIFREEZE PROTEIN GENE PROMOTER DRIVES EXPRESSION OF ANTIFREEZE PROTEIN AND GROWTH HORMONE GENES IN TRANSGENIC ATLANTIC SALMON (Salmo salar)

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The ocean pout (*Macrozoarces americanus*) antifreeze protein gene promoter drives expression of antifreeze protein and growth hormone genes in transgenic Atlantic salmon (*Salmo salar*).

By

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Abstract

Expression of the type III antifreeze protein (AFP) gene from ocean pout (OP5a) was examined in ocean pout and Atlantic salmon transgenic for a truncated version this gene (t-OP5a). Also, Atlantic salmon transgenic for an "all-fish chimeric gene construct" composed of the ocean pout type III AFP gene 5' and 3' ends linked to the Chinook salmon growth hormone (GH) gene (EO-1α) was examined for GH mRNA. Northern blot analysis of ocean pout demonstrated that AFP mRNA was detected in most tissues with extremely high levels observed in the liver, and high levels in the stomach and gill. Low AFP mRNA levels were observed in skin, mouth skin, intestine, spleen, and kidney, while barely detectable levels were observed in heart, ovary, brain, and blood cells. No AFP mRNA was observed in muscle. For t-OP5a transgenic Atlantic salmon, AFP mRNA was detected in most tissues with high levels observed in heart, liver, stomach, and brain, low levels observed in mouth skin, intestine, spleen, gill, and muscle, and barely detectable levels observed in kidney, ovary and skin. No AFP mRNA was observed in blood cells. Northern blot analysis of EO-1α transgenic salmon demonstrated that expression was observed only in pituitary and spleen, with moderate expression detected in the pituitary and low expression in the spleen. Reverse transcription polymerase chain reaction (RT-PCR) demonstrated that the OP5a gene is expressed in all tissues of the ocean pout, and all tissues except for blood in both transgenic Atlantic salmon lines (t-OP5a and EO-1α). Blood plasma samples from the three strains of fish were tested for AFP

activity by determining the thermal hysteresis (TH) for each. TH measurements from winter acclimated ocean pout were high (approximately 1.36°C) with typical bipyrimidal ice crystal shaping indicative of a high amount of AFP, while t-OP5a transgenic fish showed little (< 0.01°C) TH activity, but yielded hexagonal shaped ice crystals indicative of the presence of AFP. On the other hand, EO-1α transgenics showed no (0°C) TH activity and no ice crystal shaping, as expected. The differences observed in the level of expression between ocean pout and the two transgenic fish lines may be attributed to gene copy number, where it is high in ocean pout and low in transgenic salmon, and promoter integrity, where the promoter is intact in ocean pout and truncated in both transgenic lines. In addition, differences in expression levels between the two transgenic lines may be a result of the presence or absence of intervening sequences (introns) in the DNA where the t-OP5a construct contains introns while the EO-1α construct does not. Finally, it is apparent by its ubiquitous expression in transgenic Atlantic salmon that, unlike the type I AFP from winter flounder, the introns within the ocean pout OP5a AFP gene do not contain tissue specific elements, or salmon lack the factors needed to recognize them.

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1. Introduction

1.1 General

Teleost fish living in ice-laden sea water protect themselves from freezing by producing antifreeze proteins or glycoproteins (collectively termed AF(G)P). To date, five distinct classes of AF(G)P have now been characterized in fish termed antifreeze protein (AFP) type I, II, III, and IV and antifreeze glycoprotein (AFGP) (Fletcher *et al.* 1999; Chan *et al.* 1993; Ewart *et al.* 1999; Marshall *et al.* 2004; Cheng, 1996). In summary, the type I AFP from righteye flounders (Pleuronectidae), shorthorn sculpins (*Myoxocephalus scorpius*) and snailfish (*Liparus sp.*) range in size from ca. 3.3 to 16,683 kDa, and are alanine rich amphipathic polypeptides with an α-helical secondary structure. Type II AFPs are found in sea raven (*Hemitripterus americanus*), smelts (Osmeridae) and herrings (Clupeidae), and are large (ca. 11-24 kDa), cystine rich molecules with a β-sheet secondary structure. Type III AFPs from ocean pout (*Macrozoarces americanus*) and wolfish (*Anarhichas lupus*) are small (ca. 6.5 kDa) with a β-sandwich secondary structure and type IV from longhorn sculpin (*Myoxocephalus octodecemspinosus*), are thought to be helix bundle proteins (ca. 12,296 kDa). Finally, the antifreeze glycoproteins (AFGP) contain Ala-Ala-Thr repeats attached to a disaccharide and are found in Antarctic nototheniods and Northern cods (*Gadus morhua, G. ogac*).

Despite the wide variety of fish species that produce AF(G)P, and that they are all biochemically distinct, they all function by lowering the

freezing point of aqueous solutions non-colligatively, while having virtually no effect on their melting point (Fletcher *et al.* 1992). This results in a temperature gap between the freezing and melting temperatures, known as thermal hysteresis, which is now used as a measure of antifreeze protein activity.

The mechanism of action involves binding to seed ice crystals thereby inhibiting their growth by preventing water from joining the ice lattice (Fletcher *et al.* 1992; Fletcher *et al.* 1999). As a result of this specific binding, these molecules are up to 500 times more effective at lowering the freezing temperature than any known solute molecule (Fletcher *et al.* 1999).

Most of the AF(G)P producing teleost fish inhabiting the potentially ice-laden waters off the northeastern coast of North America have distinct seasonal cycles in the expression levels of AF(G)P in the blood plasma that are inversely correlated with sea water temperatures. For example, winter flounder, shorthorn sculpin and Atlantic cod (Fletcher *et al.* 1985) produce negligible levels of AF(G)P during the summer and high levels during the winter. One exception to this generalization is the ocean pout where plasma AF(G)P levels remain relatively high during the summer, suggesting that AF(G)P genes in this species are expressed constitutively.

Other exceptions to the cyclic pattern of AF(G)P production can be found in species of fish that live in polar waters where the temperatures are near freezing year-round. For example, the Arctic sculpin (*Myoxocephalus scorpius*) and some Antarctic nototheniods (*Trematomus borchgrevinki* and

Rhigophilia dearborni) may be genetically predisposed to produce AF(G)P year round in response to the constant environmental threat of freezing (DeVries and Lin, 1977; Fletcher *et al.* 1982).

Research on the annual cycle of AF(G)P gene expression has concentrated on the winter flounder. There are three elements involved in AF(G)P gene expression at the level of transcription: tissue specificity, environmental cues and endocrine control (Davies *et al.* 1999). The liver was found to be the source of the plasma AF(G)P, and cDNA cloning has showed that the abundant preproproteins forms are encoded in the liver mRNA (Pickett *et al.* 1984). With the use of mRNA probes it was clearly demonstrated that the annual cycle of AF(G)P mRNA levels in the liver was closely associated with the annual cycle of plasma AF(G)P levels (Pickett *et al.* 1984).

The primary environmental factor involved in regulating the annual cycle of AF(G)P expression in winter flounder liver is photoperiod which, acting through the central nervous system, controls the production and secretion of growth hormone (GH) by the pituitary gland (Fletcher *et al.* 1989). The current hypothesis is that growth hormone inhibits the production of AF(G)P during the summer when the fish are feeding, and during the fall, shortening day lengths result in the decline of plasma GH levels below that needed to suppress AF(G)P mRNA transcription (Idler *et al.* 1989; Miao *et al.* 2002; Vaisius *et al.* 1989). Details of the proposed mechanism for GH

suppression of AF(G)P gene transcription in the winter flounder liver has been presented by Chan *et al.* (1997), Miao *et al.* (1998a) and Miao *et al.* (2002).

Until quite recently, the generally accepted idea was that AF(G)P were expressed and synthesized solely in the liver from where they are secreted into the blood for circulation throughout the body, providing extra-cellular freeze protection to the fish by lowering the freezing point of the body fluids to that of the surrounding sea water. This view changed, however, when Gong *et al.* (1992) discovered AF(G)P gene expression in a wide variety of tissues in the winter flounder and ocean pout; type I AFP and type III AFP producers, respectively. This study suggested that these AF(G)P genes had little or no tissue specificity. However, in a follow-up experiment, Gong *et al.* (1995) noted that the DNA sequences obtained from cDNA clones isolated from the skin of winter flounder were more similar to each other then to the clones isolated from the liver. Finally, upon detailed examination of a skin cDNA library from the winter flounder, they discovered that there were actually two distinct families of AFP genes responsible for the expression pattern previously observed. These were named "liver-type", for those expressed solely in the liver and secreted into the blood, and "skin-type" for those expressed in a variety of tissues, but mainly in external epithelia (Gong *et al.* 1995).

An interesting characteristic of these "skin-type" AFP is the absence of a signal sequence and a pro-region. The absence of a signal sequence suggested that the skin-type AFP remained intracellular. This was supported

by the fact that no skin-type AFP had been found in the blood plasma of winter flounder (Gong *et al.* 1995). However, recent immunohistochemical studies of winter flounder tissues suggest that this may not always be the case. In these studies, Murray *et al.* (2002, 2003) found that the skin-type AFP was indeed restricted to the cytoplasm of gill epithelial cells. However, in the skin these AFP could only be found located outside of the cells in the interstitial space. This signifies that despite the lack of a signal sequence skin-type AFP can be exported from the cells.

Since their discovery in winter flounder, other "skin-type" AFP have been discovered and characterized in the shorthorn sculpin (*Myoxocephalus scorpius*) (Low *et al.* 1988), longhorn sculpin (*Myoxocephalus octodecemspinosus*) (Low *et al.* 2001), and in the Atlantic and dusky snailfish (*Liparus atlanticus* and *L. gibbus*, respectively) (Evans and Fletcher, 2001) illustrating that the presence of "skin-type" AFP in external epithelia is a widespread biological phenomenon. It is hypothesized that these AFP prevent the propagation of ice crystals into and across the epidermis itself, thereby providing a first line of defense against ice formation and thus cell damage.

1.2 Ocean Pout

The ocean pout produces a family of at least 12 independently active AFP, which lack pro-sequences and are fully functional post-translationally (Li *et al.* 1985; Hew *et al.* 1988). In Newfoundland populations, these AFP occur at concentrations of 20-25 mg/ml in the blood plasma during the winter

and are maintained at approximately half of this level during the summer. In contrast, plasma AFP concentrations in New Brunswick populations of ocean pout are only a tenth of those found in Newfoundland (Fletcher *et al.* 1985). Genomic Southern analyses revealed that there are approximately 100-150 copies of the AFP genes in Newfoundland ocean pout whereas there are less than a quarter as many AFP genes in the New Brunswick ocean pout (Hew *et al.* 1988). This strongly suggests that the level of expression of AFP in ocean pout is dependent on gene copy number.

A study of antifreeze protein gene expression in ocean pout using northern blot analysis revealed that AFP was produced in most tissues, with the highest levels occurring in the liver (Gong *et al.* 1992). Given that there are 100-150 AFP genes in the ocean pout, it is unlikely that the cDNA probe [(corresponding to AFP SP1-C (HPLC-6) (Li *et al.* 1985, Hew *et al.* 1988)] used to detect the AFP mRNA was specific to any particular AFP gene. Therefore, it is unknown as to whether the ocean pout, like the winter flounder, possess AFP gene families which differ with regards to tissue specific expression.

Over the past 10-15 years, one of the ocean pout AFP genes (OP5a) (Figure 1) has been used with the goal of assisting the aquaculture industry by producing genetically superior transgenic Atlantic salmon brood stocks that are capable of being cultured in sea cages throughout Atlantic Canada.

One problem faced by the aquaculture industry in eastern Canada is the widespread prevalence of sub-zero water temperatures and ice during the

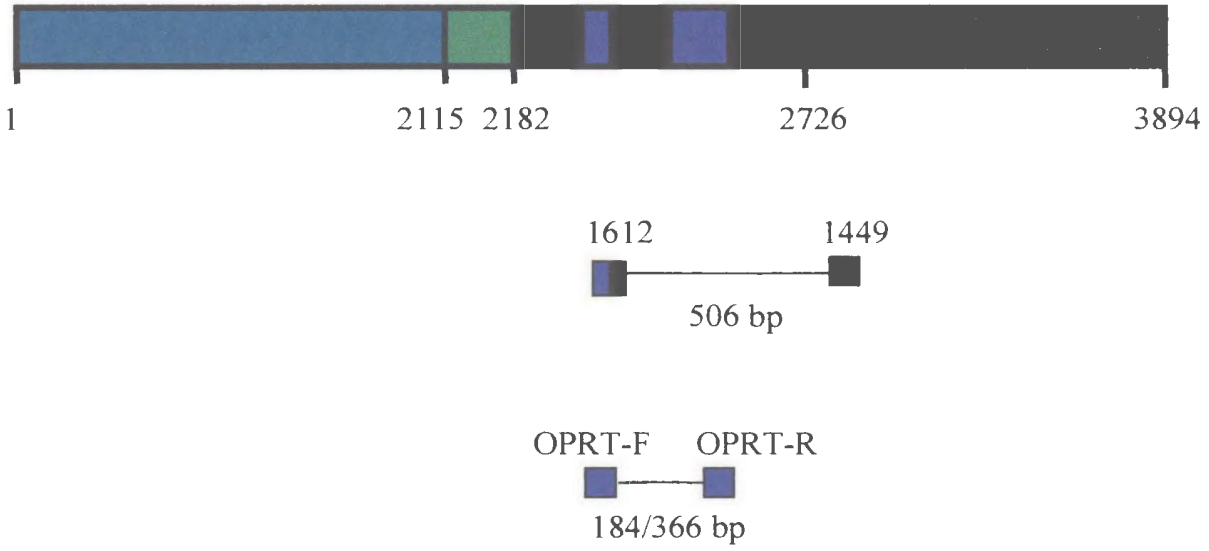

Figure 1: Structure of the ocean pout type III AFP gene (Op5a). PCR primers used in transgenic identification are indicated. An intron between primers OPRT-F and OPRT-R results in a 184 bp product in cDNA and a 366 bp product in genomic DNA. Figure modified from Wu (1994).

winter. The cold water conditions result in slow growth rates and, when combined with the presence of ice, they can be lethal. Therefore, sea cage culture of salmon is almost entirely restricted to a relatively small area in the most southerly part of the Atlantic region. The aim of the transgenic research was to produce salmon with enhanced growth rates and improved freeze resistance. A successful outcome from one or both of these tasks could help facilitate the expansion of aquaculture and thus economic development throughout the entire Atlantic coastal region (Hew *et al.* 1999; Hew *et al.* 1995; Du *et al.* 1992a; Fletcher *et al.* 2001).

Two lines of transgenic salmon have been produced utilizing an ocean pout antifreeze gene; one that was created from the transfer of a truncated version of the OP5a AFP gene (t-OP5a) (Wu 1994), and the other from the transfer of a chimeric growth hormone (GH) gene consisting of the promoter region from the OP5a AFP gene linked to a Chinook salmon GH cDNA (opAFP-GHc2) (Du *et al.* 1992a, 1992b).

Since the elements of a gene that direct tissue specific expression generally lie within the promoter region (von Hippel *et al.* 1982; de Freitas *et al.* 1994; Dynan and Tjian, 1985) the two transgenic salmon lines serve as excellent models that can be used to determine the tissue specificity of the ocean pout OP5a AFP gene promoter.

The objective of this study is to determine the tissue specific expression of the t-OP5a and EO-1α transgenes in transgenic Atlantic salmon

using RT-PCR and northern blotting procedures, and compare these expression patterns to that of the OP5a AFP gene in ocean pout.

2. Materials and Methods

2.1. Experimental Animals

Ocean pout (*Macrozoarces americanus*) were caught by SCUBA divers in Conception Bay, Newfoundland and Labrador, transported live to the Ocean Sciences Centre and maintained in 300 liter aquaria at seasonally ambient conditions of temperature and photoperiod.

Atlantic salmon were obtained from Newfoundland stocks cultured at the Ocean Sciences Centre. Three distinct strains were used in this study: one strain transgenic for an ocean pout antifreeze protein gene (t-OP5a), a second strain transgenic for a Chinook salmon growth hormone gene (EO-1α) and a third non-transgenic control strain (for details see Table 1). All salmon were fed a commercial salmon feed (Corey Feeds) to satiation once daily.

The transgenic strain (t-OP5a) of Atlantic salmon containing an ocean pout antifreeze protein gene (OP5a) was created in 1990 by injecting fertilized Atlantic salmon eggs with a truncated OP5a genomic clone (t-OP5a) (Figure 2) (Wu 1994). The specific salmon used in this study were F_2 offspring resulting from a cross between an F_1 generation male and a control (wild type) female. Approximately 50% of these F_2 offspring inherited the OP5a transgene, indicating that the transgene was integrated into a single chromosome. Salmon were identified as transgenic via polymerase chain reaction (PCR) prior to this study as previously described (Wu 1994).

The transgenic strain (EO-1α) of Atlantic salmon containing the Chinook salmon growth hormone gene was created in 1989 by injecting

Table 1. Characteristics of transgenic fish used in RT-PCR and Northern analyses.

Fish Name	Fish Line/Cross	Fish #	Weight (g) [c]	Age (yrs.)	Experiment
t-OP5a 1	ASC 4565 x Wild	BS 3009	1000	6	RT-PCR[a]
t-OP5a 2	ASC 4565 x Wild	BS 3033	1000	6	RT-PCR[a]
t-OP5a 3	ASC 4565 x Wild	BS 3073	1000	7	Northern[b]
t-OP5a 4	ASC 4565 x Wild	BS 3111	1000	7	Northern[b]
EO-1α 1	960048 x Wild	990456	400	2	RT-PCR[a]
EO-1α 2	960044 x Wild	990371	400	2	RT-PCR[a]
EO-1α 3	960044 x Wild	990247	800	3	Northern[b]
EO-1α 4	960044 x Wild	990233	800	3	Northern[b]

[a] Used for reverse transcription polymerase chain reaction experiments.

[b] Used for Northern blot analyses.

[c] Denotes approximate weight at time of death.

fertilized Atlantic salmon eggs with a chimeric gene construct consisting of the OP5a antifreeze protein gene promoter linked to a Chinook salmon growth hormone cDNA (opAFP-GHc2) (Figure 3) (Du *et al.* 1992a, 1992b). This construct has been fully characterized in the transgenic salmon revealing rearrangements resulting in truncation of the promoter region (Figure 4). The

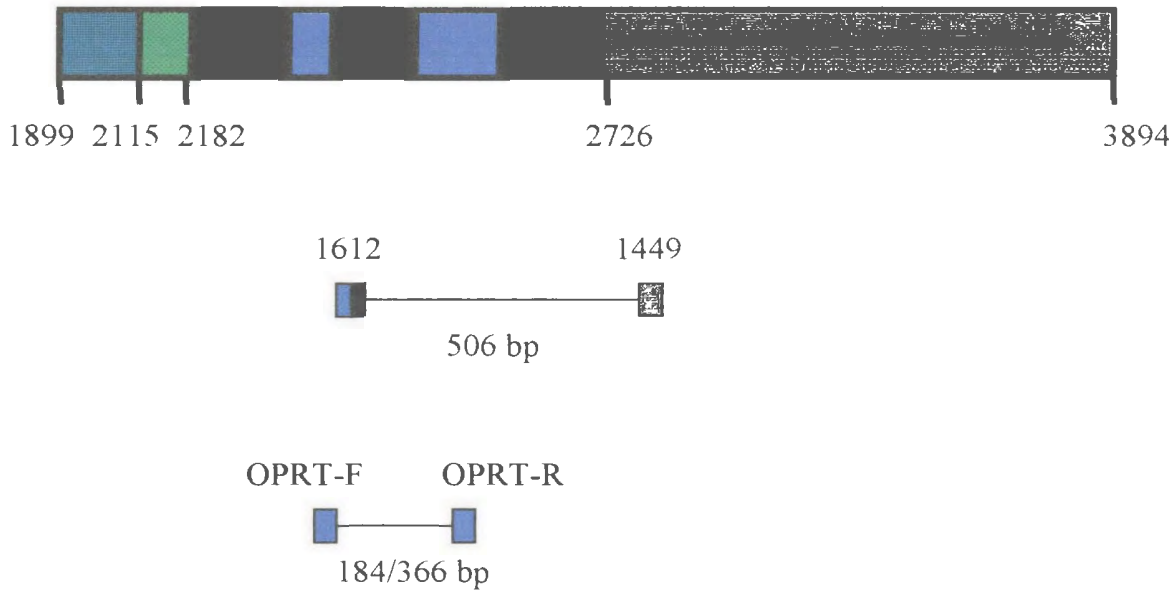

Figure 2: Structure of the truncated ocean pout AFP gene injected into Atlantic salmon eggs to produce t-Op5a transgenic Atlantic salmon. PCR primers used in transgenic identification are indicated. An intron between primers OPRT-F and OPRT-R results in a 184 bp product in cDNA and a 366 bp product in genomic DNA. Figure modified from Wu (1994).

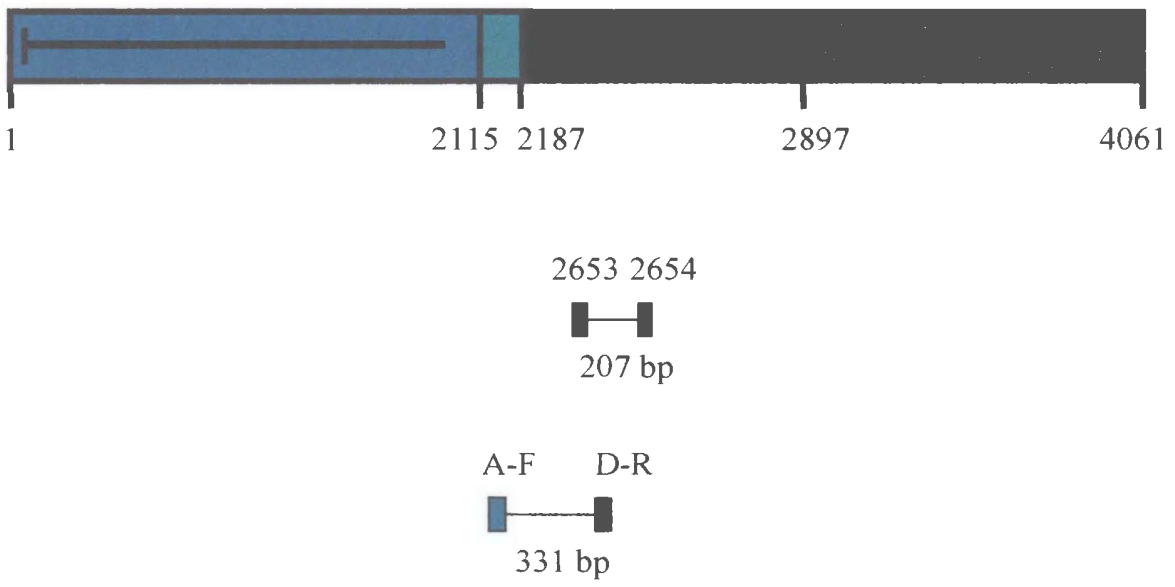

Figure 3: Structure of the "all fish chimeric gene construct" (opAFP-GHc2) injected into Atlantic salmon eggs to produce EO-1α transgenic Atlantic salmon. PCR and RT-PCR primers used as well as predicted band sizes are indicated. Figure modified from Du *et al.* 1992a.

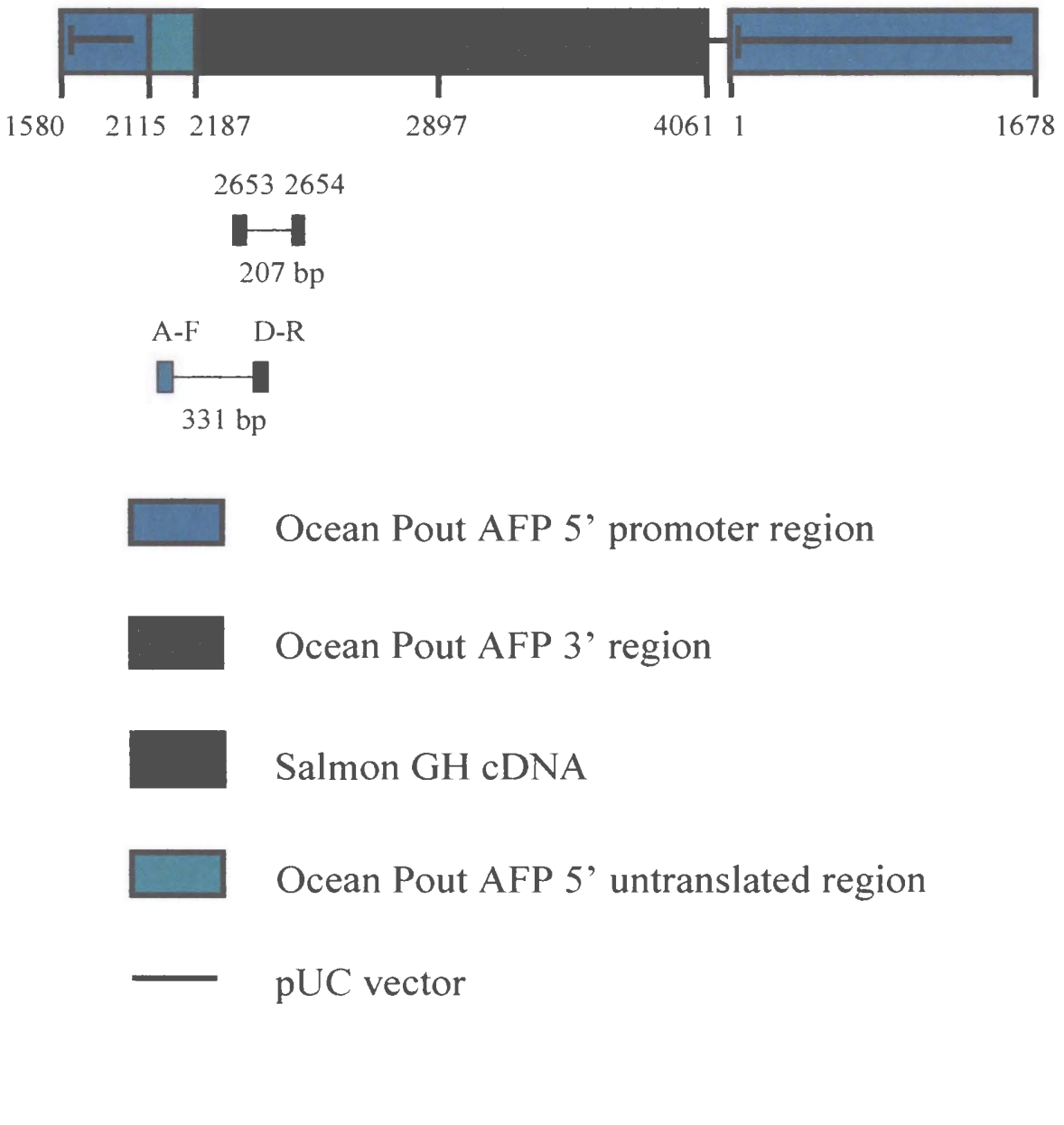

Figure 4. Schematic of the proposed structure of the EO-1α construct in transgenic Atlantic salmon. PCR primers used as well as predicted band sizes are indicated. Personal Communication, Ed Yaskowiak, AQUA Bounty Technologies, St. John's, Newfoundland and Labrador, Canada.

resulting transgene (EO-1α) and its rapid growth phenotype have exhibited stable Mendelian inheritance over 5 generations to date (unpublished data). The salmon used in the present study are from the F_4 generation. Approximately 50% of these F_4 offspring inherited the EO-1α gene construct, indicating that the transgene was integrated into a single chromosome. P_1 and F_1 salmon were identified as transgenic prior to this study as previously described (Du *et al.* 1992a, 1992b). Subsequent generations were identified using primers 2653/2654 as indicated in Figures 3 and 4. Three of the fish lines used in the present study (ocean pout, t-OP5a transgenic salmon and EO-1α transgenic salmon) contain the intact 3' region of the OP5a AFP gene.

2.2. Tissue sampling

Fish were anesthetized (3-aminobenzoic acid ethyl ester, 2 g/L, two minutes), blood sampled from a caudal blood vessel using a 5 cc syringe with a 21 gauge needle and then killed by anesthetic overdose (3-aminobenzoic acid ethyl ester, 2 g/L, 10 minutes). Tissues samples (stomach, intestine, spleen, liver, full kidney, heart, brain, pituitary, gonad, skin, gills, mouth skin, and muscle) were removed from the fish as rapidly as possible and immersed in RNA*later*™ RNA stabilization solution (Qiagen Inc., Chatsworth, CA) for 24 hours followed by storage at -70°C. Whole blood samples were divided into two portions, one portion was immediately frozen in liquid nitrogen and stored at -70 °C, and the other portion was centrifuged at 5000 x g for 15 minutes and the plasma stored at -70 °C until analyzed.

2.3. RNA Isolation

Total RNA was isolated using a commercially available RNA isolation method. (TRIzol® reagent; Invitrogen Life Technologies, Burlington, ON) (Chomczynski and Sacchi, 1987). Approximately 50 - 100 mg of tissue was homogenized in one ml of Trizol using disposable 1.5 ml pestles and tubes (Kontes glass company, Vineland, NJ). Samples were then centrifuged at 12,000 x g for 10 minutes at 6°C after which the clear homogenate was transferred to a fresh microcentrifuge tube. Samples were incubated at room temperature for five minutes and 0.2 ml chloroform was added. The samples were then shaken vigorously by hand for 15 seconds and incubated at room temperature for three minutes followed by centrifugation at 12,000 x g at 6°C for 15 minutes. This separated the sample into three phases, the lower phenol-chloroform phase, an interphase, and a clear upper phase. The upper aqueous RNA containing phase was transferred into a fresh microcentrifuge tube and 0.5 ml isopropyl alcohol was added. The samples were then incubated at room temperature for 10 minutes followed by centrifugation at 12,000 x g at 6°C for 10 minutes. The RNA pellet was washed once with one ml of 75% ethanol and centrifuged at 7,500 x g at 6°C for 5 minutes. The RNA pellet was then briefly air-dried and resuspended in Dnase/Rnase free water (Invitrogen™, Burlington, ON). Finally, the concentration was determined using the GeneQuant *pro* RNA/DNA Calculator (Amersham Pharmacia Biotech, Cambridge, ON).

2.4. Probe Production

2.4.1. Bacterial Preparation

E. coli bacteria (genotype: F-*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G) were grown in Luria-Bertani (LB) medium (10 g/L bacto-tryptone; five g/L bacto-yeast extract; 10 g/L NaCl, pH 7.0) which was supplemented with ampicillin (100 μg/ml) based on the resistance gene encoded by the plasmid used (pCR®II-TOPO®: Invitrogen Life Technologies Burlington, ON). The cultures were grown overnight with vigorous shaking at 37°C.

Transformations were carried out according to TOPO TA Cloning® (Invitrogen Life Technologies, Burlington, ON). Essentially, a cloning solution was mixed containing four μl of fresh PCR product of the gene sequence of interest (see following paragraph), one μl salt solution (1.2 M NaCl; 60 mM $MgCl_2$) and one μl pCR® II TOPO® vector and incubated at room temperature for 30 minutes. Next, two μl of this solution was added to a vial of chemically competent *E. coli*, mixed and incubated on ice for 30 minutes. The cells were then heat shocked for 30 seconds at 42°C and held on ice. 250 μl of room temperature SOC medium (2% Tryptone; 0.5% yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 mM glucose) was added to the solution and shaken horizontally (200 rpm) at 37°C for 1 hour. Approximately 50 μl of this solution was then added to pre-warmed, X-gal in dimethylformamide (DMF) treated (40 μl of 40 mg/ml solution/ plate) LB agar

(10 g/L bacto-tryptone; five g/L bacto-yeast extract; 10 g/L NaCl, pH 7.0; 15 g/L bacto-agar) plates supplemented with ampicillin (100 μg/ml) and incubated over night at 37°C. Individual colonies were then patch-plated for short-term maintenance. Bacteria were stored by picking cells from an individual colony or cells from a patch-plate and streaked on a fresh LB agar plate supplemented with ampicilin (100 μg/ml) periodically.

The gene sequence of interest for ocean pout and t-OP5a transgenic Atlantic salmon was obtained from RT-PCR using primer set OPRT-F/OPRT-R. The gene sequence of interest for EO-1α transgenic Atlantic salmon was obtained from RT-PCR using primer set A-F/D-R. The internal control (β-actin) gene sequence of interest was obtained from RT-PCR using primer set Actin-F/Actin-R (see Section 2.7).

2.4.2. Small-Scale Plasmid Purification

Small-scale plasmid purification was performed using a commercially available affinity resin in conjunction with a modified alkaline lysis protocol (Qiagen Inc., Chatsworth, CA) (Birnboim and Doly, 1979). A single colony was inoculated into two ml of LB media supplemented with ampicillin (100 μg/ml) and incubated with shaking overnight at 37°C. A 1.5 ml aliquot of the culture was transferred to a 1.5 ml microfuge tube and the cells were pelleted by centrifugation in a tabletop microfuge at 10,000 x g for 30 seconds. The supernatant was aspirated and the bacterial cell pellet was resuspended in 200 μl of P1 Resuspension Buffer (50 mM Tris-Cl, pH 8.0; 10 mM EDTA; 100

μg/ml RNase A) by vortexing. The cells were lysed by the addition of 200 μl of P2 Lysis Buffer [200 mM NaOH; 1% SDS (w/v)] and inverting the tubes six times to mix. The solution was then neutralized by the addition of 200 μl of N3 Neutralization Buffer (Proprietary composition; Qiagen Inc., Chatsworth, CA) and mixing the tubes immediately by inverting six times. Next, the samples were centrifuged at 10,000 x g for 10 minutes, after which the supernatant was removed and applied to a QIAprep Spin Column (Qiagen Inc., Chatsworth, CA) according to the manufacturer's protocol. The plasmid DNA was then eluted in 50 μl of 10 mM Tris-HCl (pH 8.5). The resulting DNA was analyzed using a diagnostic restriction enzyme (RE) digest as well as direct sequencing (See section 2.10 for details). For a diagnostic digest, four μl of the resulting DNA was digested with one μl EcoRI for 1 1/2 hours at 37°C, and the resulting DNA fragments analyzed by agarose gel electrophoresis.

2.4.3. Large-Scale Plasmid Purification

Large-scale plasmid purification was performed using a commercially available anion-exchange resin in conjunction with a modified alkaline lysis protocol (Qiagen Inc., Chatsworth, CA)(Birnboim and Doly, 1979). A single colony was inoculated into 150 ml of autoclave sterilized LB media supplemented with ampicillin (100 μg/ml) and incubated with shaking overnight at 37°C. The solution was poured into plastic centrifuge tubes and centrifuged at 6,300 rpm for 15 minutes at 4°C. The supernatant was discarded into bleach. Next, the pellet was resuspended in 10 ml of P1

Resuspension Buffer by vortexing and pipetting up and down using a 10 ml disposable pipette. The cells were then lysed by adding 10 ml of P2 Lysis Buffer, mixed by inverting the tubes six times and incubating at room temperature for five minutes. Next, 10 ml of chilled P3 Neutralization Buffer (3.0 M potassium acetate, pH 5.5) was added, immediately mixed by inverting the tubes six times and incubated on ice for 15 minutes. The tubes were then centrifuged at 13,300 rpm for 30 minutes at 4°C, after which the supernatant was filtered through cheesecloth. This solution was run through a Qiagen-tip 100 Anion-Exchange Resin (Qiagen Inc., Chatsworth, CA) according to the manufacturer's protocol to purify the plasmid DNA. The Qiagen-tip was then washed with two x 30 ml of QC Wash Buffer [1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)], and the DNA eluted in 15 ml of QF Elution Buffer [1.25 M NaCl; 50 mM Tris-Cl, pH 8.5; 15% isopropanol (v/v)]. Next, The DNA was precipitated by adding 10.5 ml of room temperature isopropanol to the eluted DNA, mixing and centrifuging at 5,000 x g for one hour at 4°C. After decanting the supernatant, the DNA pellet was washed with room temperature 70% ethanol followed by centrifugation at 5000 x g for one hour at 4°C. Finally, the pellet was air-dried until the ethanol completely evaporated (approximately 30 minutes) and then redissolved in 250 μl TE buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA). The concentration of the purified plasmid DNA was measured using the GeneQuant *pro* RNA/DNA Calculator (Amersham Pharmacia Biotech, Cambridge, ON). The plasmid was then linearized at a restriction site downstream of the cloned insert according to

standard RE protocol (Invitrogen Life Technologies, Burlington, ON). Specifically, 10 μg of plasmid DNA was cut using 10 μl of the appropriate RE and 10 μl of the appropriate RE buffer by incubating at 37°C for 1 ½ hours. The linearized plasmid was then purified using the QIAquick Gel Extraction Kit (Qiagen Inc., Chatsworth, CA) as per manufacturer's protocol. Essentially, the DNA sample was separated on a 1.5% agarose gel after which the DNA was excised and the agarose dissolved by incubating in three gel volumes of QG Buffer at 50°C. Next, one gel volume of isopropanol was added and the sample was pipetted into a QIAquick spin column. The sample was centrifuged at 13,300 x g for one minute and the flow through discarded. The DNA was washed by adding 0.75 ml of Buffer PE to the column and spinning at 13,300 x g for one minute. The flow through was discarded, and the column was spun again at 13,300 x g for one minute. Finally, the column was placed in a clean microcentrifuge tube, and the DNA was eluted by adding 50 μl of water to the center of the column and spinning at 13,300 x g for one minute. The concentration of the purified linearized plasmid DNA was measured using the GeneQuant *pro* RNA/DNA Calculator (Amersham Pharmacia Biotech, Cambridge, ON).

2.4.4. DIG-RNA Labelling

RNA was labelled for use as a probe for northern blot analyses in an *in vitro* transcription reaction with digoxigenin-11-UTP (DIG) according to manufacturers protocol (DIG Northern Starter Kit, Roche Applied Science,

Laval, QC). Specifically, one μg of linearized plasmid containing the gene sequence of interest in a total volume of 10 μl was added to four μl of 5 x Labelling mix, four μl of 5 x Transcriptional buffer and two μl of RNA polymerase (either SP6 or T7 depending on the orientation of the insert in the plasmid) on ice. The solution was then mixed and centrifuged briefly, followed by incubation at 42°C for one hour. Any remaining DNA template was then removed by adding two μl DNase I and incubating at 37°C for 15 minutes. Finally, the reaction was terminated by adding two μl of 0.2 M EDTA (pH 8.0).

2.5. Northern blot analysis

Formaldehyde agarose gel electrophoresis was performed as per methods outlined in DIG Northern Starter Kit (Roche Applied Science, Laval, QC) with modifications. A 1.2% formaldehyde agarose gel was prepared with 1 x MOPS buffer (20 mM MOPS; 5 mM NaAc; 1 mM EDTA; pH 7.0) and 0.22 M formaldehyde by adding 0.96 g agarose (Invitrogen Life Technologies, Burlington, ON) to 78.6 ml 1 x MOPS buffer and boiling in a microwave oven until the agarose had completely melted. After cooling to approximately 50 °C, 1.5 ml of a 37% formaldehyde solution (Fisher Scientific, Nepean, ON) was added and the solution was poured into a gel tray containing a gel comb and allowed to set. For ocean pout samples, two μg total RNA was used for each tissue, while 10 μg total RNA was used for t-OP5a transgenic salmon and 40 μg total RNA was used for EO-1α transgenic salmon. Samples were prepared in a final volume of 5-10 μl with water. 15-30 μl of freshly prepared

loading buffer (250 μl of 100% formamide; 83 μl of 37% formaldehyde solution; 50 μl 10 x MOPS; 50 μl 100% Rnase free glycerol; 10 μl 2.5% bromophenol blue; 57 μl water; six μl of 1.0 mg/ml ethidium bromide) (3:1 ratio with RNA) was added to each sample, mixed and heated at 70°C for 10 minutes and briefly chilled on ice. The addition of ethidium bromide enabled the RNA to be visualized directly without staining after electrophoresis. The samples were applied to the lanes of the gel and run at 130 V in 1 x MOPS until the bromophenol blue had run approximately 2/3 of the way down the gel. After electrophoresis the RNA was visualized via UV light and photographed using the MultiImage™ light cabinet and AlphaImager™ 1220 Documentation and Analysis System (Alpha Innotech Corporation, San Leandro, CA).

RNA was transferred to a positively charged nylon membrane (Roche Applied Science, Laval, QC) using the VacuGene™ XL Vacuum blotting system (Amersham Biosciences, Piscataway, NJ) as per manufacturers protocol. Essentially, the membrane was wetted with 10 x SSC (1.5 M NaCl; 150 mM sodium citrate, pH 7.0) and placed on a porous support screen which was pre-wetted with 2 x SSC. A plastic mask with a window cut slightly smaller than the membrane was then placed on the screen with the window over the membrane to create an airtight seal around the apparatus. The gel was positioned over the membrane avoiding air bubbles and the top frame was added and secured with clamps. The vacuum was then applied and set to approximately 50 mbar for two hours while ensuring that the gel was

constantly covered in 10 x SSC. After the transfer period, the RNA was cross-linked to the membrane twice via UV light using a UV Stratalinker® 2400 (Stratagene®, La Jolla, CA). Pre-hybridization and washing conditions were as per methods outlined in DIG Northern Starter Kit (Roche Applied Science, Laval, QC) with modifications. Initially, the membrane was soaked for one minute in Millipore (Millipore Corporation, Bedford, MA) filtered water, added to a hybridization tube containing 15 ml of DIG Easy Hyb solution (Roche Applied Science, Laval, QC) preheated to 68°C, and pre-hybridized with constant rotation for one-two hours in a hybridization oven/shaker (Amersham Biosciences, Piscataway, NJ). A DIG-labelled RNA probe was then denatured by boiling for five minutes followed by rapidly cooling on ice and was added to seven ml of DIG Easy Hyb solution preheated to 68°C. The pre-hybridization solution was poured out of the hybridization tube, and replaced with the probe-containing hybridization solution and hybridized with constant rotation overnight in a hybridization oven/shaker. After hybridization, the membrane was washed two x five minutes with stringency wash #1 (2 x SSC/ 0.1 % SDS) at room temperature, followed by two x 15 minute washing with stringency wash #2 (0.1 x SSC/ 0.1 % SDS) at 68°C. The remaining steps were performed at room temperature with gentle agitation unless otherwise instructed. The membrane was washed with 100 ml of Washing Buffer (DIG Wash and Block Buffer Set, Roche Applied Science, Laval, QC) by vigorous shaking for five minutes followed by incubation in Blocking Solution (DIG Wash and Block Buffer Set, Roche Applied Science, Laval, QC) for one hour.

This was followed by incubation in Antibody Solution (DIG Wash and Block Buffer Set, Roche Applied Science, Laval, QC) for 30 minutes. The membrane is then washed two x 15 minutes in Washing Buffer (DIG Wash and Block Buffer Set, Roche Applied Science, Laval, QC) with vigorous shaking followed by equilibration in Detection Buffer (1 M Tris-HCL (pH 9.5), 1 M NaCl, DIG Wash and Block Buffer Set, Roche Applied Science, Laval, QC) for five minutes. The membrane was then placed inside a development folder, covered with CDP-Star ready-to-use (Roche Applied Science, Laval, QC), and allowed to incubate for five minutes. Any excess fluid was then squeezed out and the folder sealed. Finally, the membrane was exposed to chemiluminescent film (Roche Applied Science, Laval, QC) for one - 60 minutes.

2.6. Reverse Transcription (RT)

Prior to reverse transcription, all RNA samples were treated with amplification grade Deoxyribonuclease (DNase) I (Invitrogen Life Technologies, Burlington, ON). Basically, one μg of RNA along with one μl of 10 x DNase I reaction buffer, one unit of DNase I, and water to 10 μl were added to a microcentrifuge tube and incubated at room temperature for 15 minutes. The reaction was inactivated by adding one μl of 25 mM EDTA and heating at 65°C for 10 minutes. RT reactions were carried out using the Superscript™ II RNase H⁻ Reverse Transcriptase kit (Invitrogen Life Technologies, Burlington, ON). In a 500 μl micro test tube, one μg of sample

RNA was added to one μl of 10 μM oligo-dT primer, one μl of dNTP Mix [containing 10 mM of dATP, dGTP, dCTP, and dTTP (Invitrogen Life Technologies, Burlington, ON)] and water to a final volume of 12 μl. This was incubated at 65°C for five minutes and then held on ice. A cocktail containing four μl of 5 x first strand buffer [250 mM Tris-HCL (pH 8.3), 375 mM KCL, 15 mM $MgCl_2$], two μl 0.1 M DTT, and one μl of RNaseOUT™ recombinant ribonuclease inhibitor (Invitrogen Life Technologies, Burlington, ON) was then added and incubated at 42°C for two minutes, followed by addition of 200 units of Superscript™ II reverse transcriptase. This was maintained at 42°C for 50 minutes followed by inactivating the reaction by heating at 70 °C for 15 minutes. Finally, the samples were treated with Ribonuclease H (Invitrogen Life Technologies, Burlington, ON). Essentially, two units of Ribonuclease H were added to the RT product and incubated at 37 °C for 20 minutes.

2.7. Polymerase Chain Reaction (PCR)

Table 2 lists the primer pairs used in the PCR reactions along with their annealing sites, while Figures 1 and 2 display the primer positioning with respect to the OP5a and t-OP5a gene constructs, and Figure 4 displays the primer positioning with respect to the EO-1α gene construct.

Primer set OPRT-F/OPRT-R was used to examine the tissue specificity of the OP5a gene in ocean pout (Figure 1). OPRT-F anneals to the OP5a coding sequence (cds) between bases 26 – 46 while OPRT-R anneals

Table 2. Primer pairs used for polymerase chain reaction and their annealing sites.

No.	Name	Sequence[a]	Annealing site[b]
1.	OPRT-F	CGG TTT GCT TTT CGT CCT CCT	OP5a CDS
	OPRT-R	TGG CGT GTT CAC TTG CTT CC	OP5a CDS
2.	1612	GTC CTC CTT TGT GTC GAC CAC ATG A	OP5a CDS
	1449	CCG GAC AGA CTT GGG TTT GTG ACA A	OP5a 3' UTR
3.	A-F	GTC AGA AGT CTC AGC TAC AGC	OP5a promoter
	D-R	ACA GAA GTC CAG CAG GAA TAT	Salmon GH CDS
4.	2653-F	GCT CTT CAA CAT CGC GGT CA	Salmon GH CDS
	2654-R	ATA TGG AGC AGC TTC AGG AC	Salmon GH CDS
5.	ACTIN-F[c]	CGC CGC ACT GGT TGT TGA CA	Atl. salmon β-actin
	ACTIN-R[c]	GCG GTG CCC ATC TCC TGC T	Atl. salmon β-actin

[a] Primer sequences are written in the 5'→3' direction. The forward primer is listed first and the reverse primer second. R and F denoted forward and reverse primers, respectively.

[b] Indicates site of primer annealing on gene of interest. See Figures 1 and 2 for the location of primer sets 1 and 2 and Figures 3 and 4 for the location of primer sets 3 and 4 relative to gene of interest.

[c] Internal control used in PCR experiments. Amplify 675 bp portion of Atlantic salmon β-actin gene. GenBank Accession # AF012125.

to the OP5a cds between bases 372 – 392 to yield a 184 base pair (bp) band in cDNA.

In t-OP5a transgenic Atlantic salmon, two sets of primers were used to examine the tissue specificity of the OP5a gene (Figure 2). The first set, 1612/1449 was used to identify the fish used as transgenics (Wu 1994). The second set, OPRT-F/OPRT-R was used to examine the tissue specificity of the OP5a gene in transgenic Atlantic salmon as it was in ocean pout.

In EO-1α transgenic Atlantic salmon, two sets of primers were used to examine the tissue specificity of the EO-1α gene (Figure 4). The first set, 2653/2654 was used to identify the fish used as transgenics (Du *et al.* 1992a). The second set, A-F/D-R was used to examine the tissue specificity of the EO-1α gene in transgenic Atlantic salmon. Primer A-F anneals to the OP5a 5' promoter region between bases 2080 – 2100 while D-R anneals to the salmon GH cds between bases 198 - 218 to yield a 331 bp band.

Finally, primer set Actin-F/Actin-R was used as an internal control to amplify a portion of the Atlantic salmon β-actin gene. Actin-F anneals to the β-actin cds between bases 15-34 while Actin-R anneals to the β-actin cds between 671 – 689 to give a 675 bp band (not shown).

The Atlantic salmon β-actin gene primers were included in the PCR reaction mixture along with the OP5a AFP gene primers (OPRT-F/OPRT-R) to determine AFP gene expression in ocean pout and t-OP5a AFP transgenic salmon tissues. Separate reactions with β-actin primers and EO-1α

transgene primers (A-F/D-R) were carried out on the tissues of salmon transgenic for the EO-1α transgene.

Polymerase chain reactions were performed in a Mastercycler® gradient thermal cycler (Eppendorf Scientific, Westbury, NY) with 35 cycles of amplification. PCR amplification was achieved using Taq DNA polymerase (Invitrogen Life Technologies, Burlington, ON). Primers were designed using the Gene Tool Version 1.0 from Biotools.com and purchased from Cortec DNA Service Laboratories Inc. A 50 μl reaction contained two units Taq polymerase, 1 x PCR buffer (200 mM Tris-HCL (pH 8.4), 500 mM KCL), 1.5 mM $MgCl_2$, four dNTP's [200 μM each of dATP, dGTP, dCTP, and dTTP (Invitrogen Life Technologies, Burlington, ON)], 100 ng template DNA, 0.2 μM of both upstream and downstream primers and was held on ice.

When all of the components were added to the reaction mixture, the samples were placed in the thermal cycler with the block preheated to 90 °C, and the lid preheated to 105 °C. The thermal cycling profile consisted of an initial denaturation at 94 °C for three minutes, followed by 35 cycles of denaturation at 94°C for 15 seconds, primer annealing at the appropriate temperature for 15 seconds and extension at 72 °C for 30 seconds. A final extension of four minutes at 72 °C was used to ensure complete extension. Samples were held at 4 °C until ready for agarose gel electrophoresis.

2.8. Agarose Gel Electrophoresis

100 ml gels (1.5 - 2%) were prepared by combining 1.5 - two g of agarose (Invitrogen Life Technologies, Burlington, ON) with 100 ml of 1 x TBE buffer (45 mM Tris-borate, 1 mM EDTA) and boiling in a microwave oven until the agarose was completely melted. The solution was allowed to cool to approximately 50 °C and poured into a gel tray containing a gel comb used for well formation and solidification of the gel. five μl of 6 x sample buffer (0.25% bromophenol blue; 0.25% xylene cyanol FF; 30% glycerol in water) was added to each sample and 12 μl per sample was added to the wells and electrophoresed in 1 x TBE running buffer at 100 – 130 V until the sample buffer had migrated approximately 2/3 of the way down the gel. To estimate the size of the DNA fragments, a molecular weight standard (100 base pair ladder or 1 KB Plus DNA ladder; Invitrogen Life Technologies, Burlington, ON) was run along side the DNA samples. The DNA was visualized by staining with 0.5 μg/μl solution of ethidium bromide (Sigma®, Oakville, ON) for approximately 20 minutes followed by exposure to UV light and photographed using the MultiImage™ light cabinet and AlphaImager™ 1220 Documentation and Analysis System (Alpha Innotech Corporation, San Leandro, CA).

2.9. DNA Sequencing

Samples were prepared via PCR and separated on agarose as previously described (sections 2.7 and 2.8). The band of interest was excised

and the DNA purified using the QIAquick® gel extraction kit (Qiagen Inc., Chatsworth, CA) and diluted with Dnase/Rnase free water (Invitrogen Life Technologies, Burlington, ON) to a final concentration of 10-20 ng/μl. DNA was sent to McMaster University's Mobix lab for sequencing to confirm sequence identity of products obtained from PCR. The ABI Big Dye terminator cycle sequencing chemistry was used to perform DNA sequencing. Conditions for the standard sequencing reaction were: annealing temperature 50°C, extension 60°C, 2.5 mM $MgCl_2$ and primer concentration is 0.2 mM. After the completion of the sequencing reaction, excess primer and unincorporated ddNTP's were removed using DyeEx columns (Qiagen Inc., Chatsworth, CA) and the DNA precipitated using sodium acetate and ethanol. The purified reaction was then resuspended in HiDi formamide (ABI) and the sample is placed in the 3100 DNA automated sequencer. Results were provided as alphabetic sequences as well as chromatograms. The chromatograms were proof read to confirm the alphabetic sequences.

2.10. Thermal Hysteresis Measurements

Thermal hysteresis, the difference between the freezing point and melting point of a solution, was used as a measure of antifreeze activity in plasma samples from all fish used in the study. The method used was based upon the methods used in Kao *et al.* (1986). Plasma samples were placed in cylindrical wells on a metal plate, which was placed on a cooling stage and viewed through a compound microscope. The microscope was connected to

a monitor to allow for straightforward viewing of samples. For each individual sample, triplicate measurements of the freezing and melting points of a single ice crystal were obtained using a Clifton nanoliter osmometer (Clifton Technical Physics, Hartford, NY)(1000 mOsmols = -1.86°C) and the average was used to calculate the thermal hysteresis of the sample. Control measurements were obtained by measuring the freezing and melting points of deionized water, and used to correct for background error of the osmometer.

3. Results

3.1. Expression of the OP5a AFP gene in ocean pout.

The relative level of expression of the ocean pout OP5a AFP gene was examined by northern analysis in ocean pout tissues obtained from two individuals. A blot obtained from a single individual is shown in figure 5. The results for these two individuals were identical, with AFP mRNA being detected in most tissues. The levels of AFP mRNA in the tissues could be ranked as follows: Liver > stomach and gill > skin, mouth skin, intestine, spleen, and kidney > ovary, heart > brain, and blood cells. No AFP mRNA was detected in muscle tissue, however see RT-PCR analysis below. These results are similar to those obtained by Gong *et al.* (1992). The control β-actin mRNA was readily detectable in all tissues with the exception of the liver where the band was barely detectable. This low level of β-actin mRNA in liver is likely due to the excess of AFP mRNA relative to the β-actin mRNA present in the two micrograms of total RNA loaded onto the gel (Figure 5). Although β-actin mRNA was detected in muscle, hybridization was visualized at a lower position on the gel with respect to all other tissues.

RT-PCR analysis indicated the presence of OP5a AFP and β-actin mRNA in all tissues including muscle as evidenced by the 184 bp and 675 bp bands, respectively (Figure 6). Both of the negative control (non-transgenic) Atlantic salmon testes were positive for the β-actin mRNA and negative for the AFP mRNA. An extra, higher molecular weight band (approximately 750

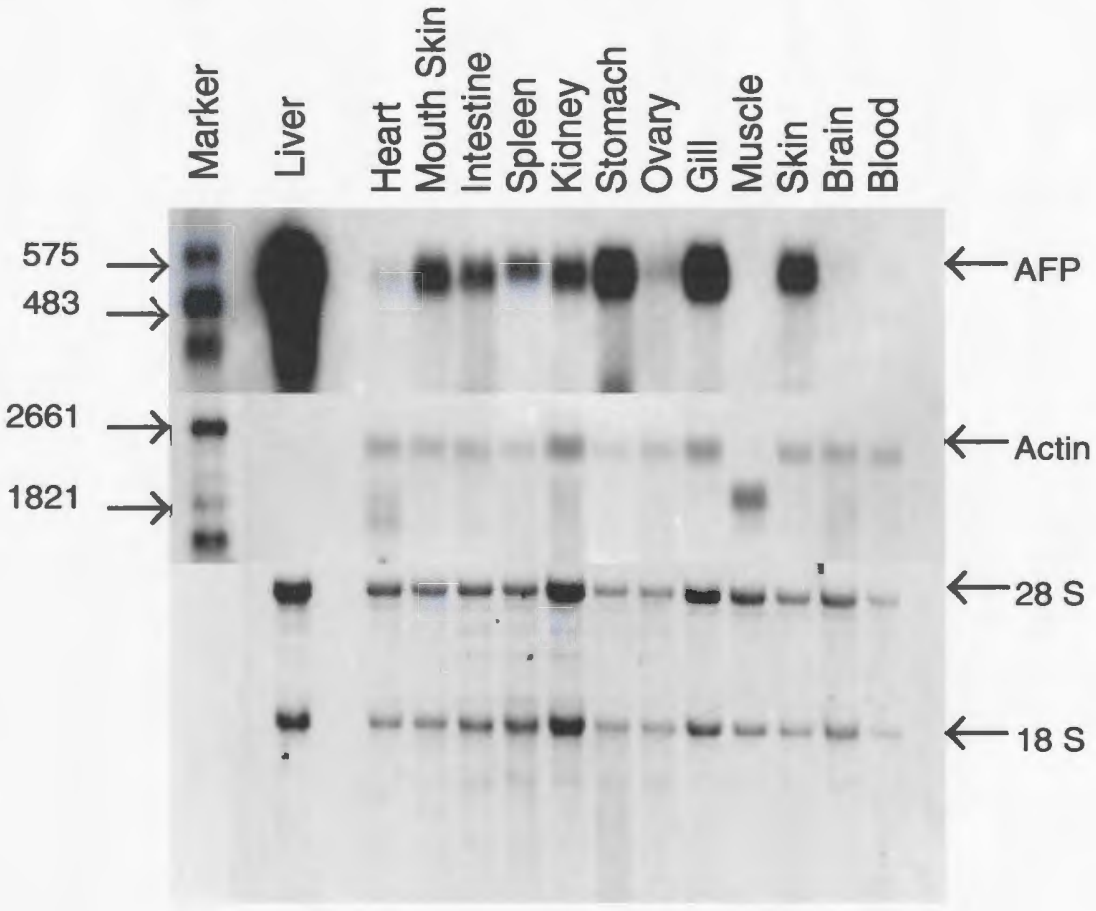

Figure 5. Northern blot analysis of ocean pout total RNA from various tissues as seen on a formaldehyde agarose gel. 2 μg total RNA was added for each sample. Top panel probed for ocean pout OP5a AFP RNA. Middle panel probed for Atlantic salmon β-actin RNA. Bottom panel is RNA after electrophoresis viewed under ultra violet light. Lane 1 is the DIG-labeled RNA Marker I from Roche Applied Science in bases (60 ng used).

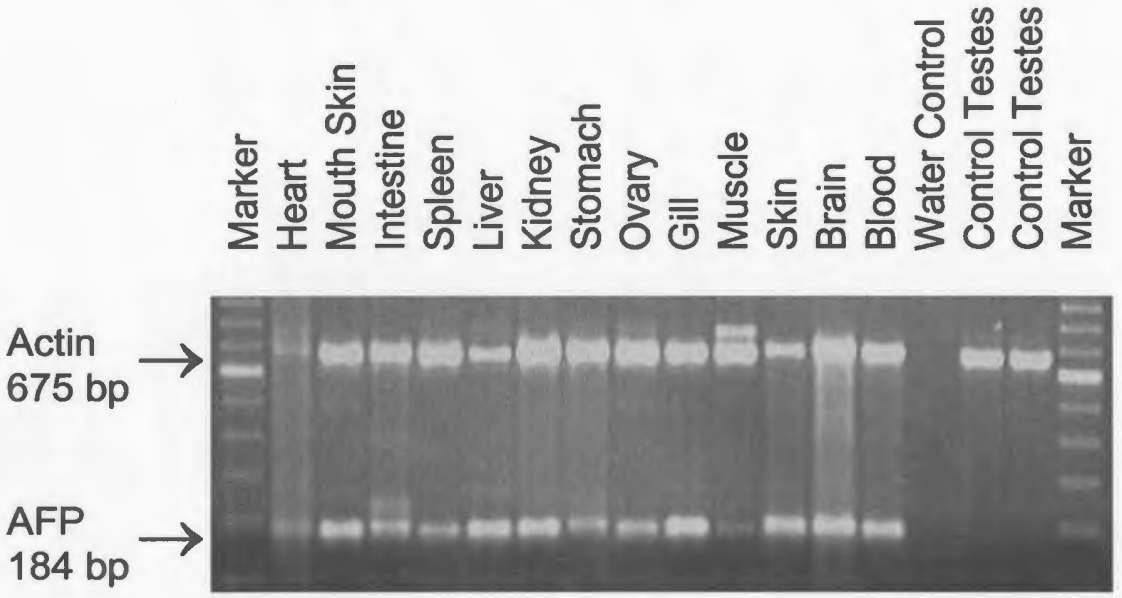

Figure 6. RT-PCR analysis of ocean pout total RNA from various tissues as seen on an agarose gel. Primers were designed from the Atlantic salmon β-actin gene sequence and the ocean pout Op5a antifreeze protein gene sequence to give a 675 base pair (bp) product and a 184 bp product, respectively. Wild Atlantic salmon testes RNA was used as a negative control for the Op5a antifreeze protein gene.

bp) was also observed in muscle when tested for β-actin. Similarly, intestine yielded a higher molecular weight band (approximately 220 bp) when tested for AFP. The absence of a 366 bp band that could have been generated by the primer set OPRT-F/OPRT-R if genomic DNA was present indicates that the RNA was not contaminated by genomic DNA (see Figure 2).

Sequence analysis of the AFP bands from brain and blood cells that had been amplified by RT-PCR indicated that they were indeed derived from a type III AFP gene (Figure 7). However, there are three possible base pair changes from the OP5a sequence in brain and blood samples located at bases 79, 99 and 145 (Figure 7) due to multiple peaks present on the sequencing chromatogram (data not shown). Also, sequencing of three liver samples from ocean pout identified combinations of these three base pair changes. In the Liver 1 sample, the base at location 79 is guanine in contrast to the adenine in the OP5a sequence while base 99 is cytosine in contrast to thymine in OP5a, corresponding to HPLC 9 in Hew *et al.* (1988). In addition, there is a base change from cytosine to thymine at position 46 (Figure 7). In the Liver 2 sample, once again, the base at location 79 is guanine in contrast to adenine in OP5a, while at position 99, the base matches that of OP5a, corresponding to HPLC 1 in Hew *et al.* (1988). Finally, in the Liver 3 sample, the base at position 79 matches that of the OP5a sequence while at position 99 is cytosine in contrast to thymine in OP5a, corresponding to HPLC 4, 6, and 11 in Hew *et al.* (1988). The base pair changes at positions 79 and 99

```
        1
OP5a    CGGTTTGCTT TTCGTCCTCC TTTGTGTCGA CCACATGACA
Brain                         TTGTGTCGA CCACATGACA
Blood                         TTGTGTCGA CCACATGACA
Liver1                        TTGTGTCGA CCACATGACA
Liver2                        TTGTGTCGA CCACATGACA
Liver3                        TTGTGTCGA CCACATGACA
        41
        GCCAGCCAGT CCGTGGTGGC CACCCAGCTG ATCCCCATAA
        GCCAGCCAGT CCGTGGTGGC CACCCAGCTG ATCCCCATXA
        GCCAGCCAGT CCGTGGTGGC CACCCAGCTG ATCCCCATXA
        GCCAGTCAGT CCGTGGTGGC CACCCAGCTG ATCCCCATGA
        GCCAGCCAGT CCGTGGTGGC CACCCAGCTG ATCCCCATGA
        GCCAGCCAGT CCGTGGTGGC CACCCAGCTG ATCCCCATAA
        81
        ATACTGCCCT GACTCCGGTG ATGATGGAGG GGAAGGTGAC
        ATACTGCCCT GACTCCGGYG ATGATGGAGG GGAAGGTGAC
        ATACTGCCCT GACTCCGGYG ATGATGGAGG GGAAGGTGAC
        ATACTGCCCT GACTCCGGCG ATGATGGAGG GGAAGGTGAC
        ATACTGCCCT GACTCCGGTG ATGATGGAGG GGAAGGTGAC
        ATACTGCCCT GACTCCGGCG ATGATGGAGG GGAAGGTGAC
        121
        CAACCCAATA GGCATCCCGT TCGCAGAGAT GTCCCAAATA
        CAACCCAATA GGCATCCCGT TCGCXGAGAT GTCCCAAATA
        CAACCCAATA GGCATCCCGT TCGCXGAGAT GTCCCAAATA
        CAACCCAATA GGCATCCCGT TCGCGGAGAT GTCCCAAATA
        CAACCCAATA GGCATCCCGT TCGCGGAGAT GTCCCAAATA
        CAACCCAATA GGCATCCCGT TCGCGGAGAT GTCCCAAATA
        161
        GTGGGGAAGC AAGTGAACAC GCCA
        GTGG
        GTGG
        GTGG
        GTGG
        GTGG
```

Figure 7. Sequence consensus between the Op5a gene sequence[a] between primers OPRT-F and OPRT-R and those obtained from sequencing of PCR products from ocean pout blood and brain. Location of primers OPRT-F and OPRT-R underlined. Note: *X* represents adenine or guanine and *Y* represents thymine or cytosine.

[a] Sequence of Op5a obtained from Hew *et al.* (1988).

lead to amino acid changes in the mature protein while changes at positions 46 and 145 do not.

3.2. Expression of the t-OP5a gene construct in transgenic Atlantic salmon.

Northern blot analysis of two t-OP5a AFP transgenic Atlantic salmon revealed the presence of AFP mRNA in most of the tissues with the approximate 500 bp band being the same as that observed for the positive control ocean pout liver. The relative levels of AFP mRNA expression in the various tissues differed from that observed for the ocean pout and could be ranked as follows: heart, liver, stomach and brain > mouth skin, intestine, spleen, gill, and muscle > kidney, ovary and skin. In contrast to the ocean pout, no AFP mRNA was detected in blood cells (Figure 8). The internal control β-actin mRNA was present in all of the salmon tissues. However despite the presence of AFP mRNA in the ocean pout liver, there was no detectable β-actin mRNA (Figure 8). This is because the total amount of ocean pout liver RNA loaded on to the gel (0.01 μg) was insufficient to detect its presence. No AFP mRNA was detected in any tissue sample from a control (non-transgenic) Atlantic salmon (data not shown). Once again, although β-actin mRNA was present in heart and muscle, hybridization was also visualized at a lower position on the gel with respect to other tissues.

RT-PCR analysis revealed that t-OP5a transgene mRNA was present in all tissues examined with the exception of the blood cells (Figure 9). Also

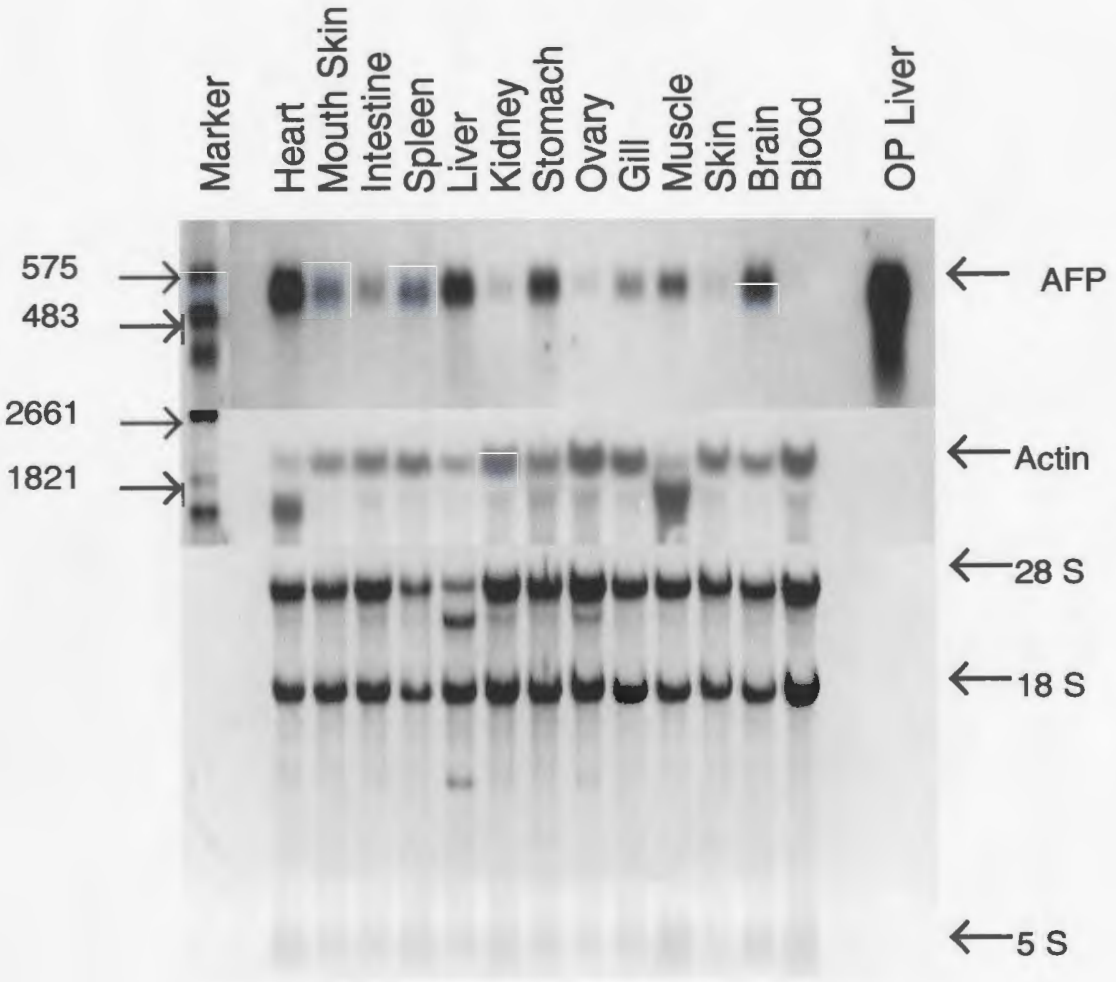

Figure 8. Northern blot analysis of t-Op5a transgenic Atlantic salmon total RNA from various tissues as seen on a formaldehyde agarose gel. 10 μg total RNA was added for each sample [0.01 μg for ocean pout liver (positive control)]. Top panel probed for ocean pout OP5a AFP RNA. Middle panel probed for Atlantic salmon β-actin RNA. Bottom panel is RNA after electrophoresis viewed under ultra violet light. Lane 1 is the DIG-labeled RNA Marker I from Roche Applied Science in bases (60 ng used) .

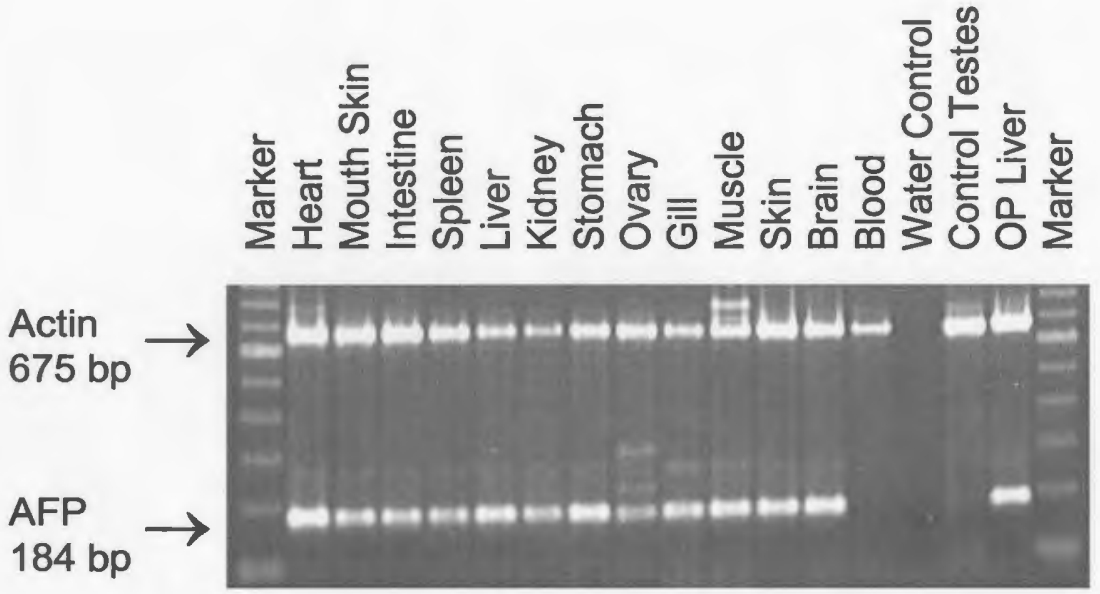

Figure 9. RT-PCR analysis of t-Op5a transgenic Atlantic salmon total RNA from various tissues as seen on an agarose gel. Primers were designed from the Atlantic salmon β-actin gene sequence and the ocean pout Op5a antifreeze protein gene sequence to give a 675 bp product and a 184 bp product, respectively. Ocean pout Liver cDNA was used as a positive control. Wild Atlantic salmon testes cDNA was used as a negative control for the Op5a antifreeze protein gene.

present were faint, higher molecular weight bands (approximately 280 bp for all tissues with the exception of blood), and two extra faint higher molecular weight bands (approximately 210 and 300 bp) in ovary. All of the tissues tested positive for the β-actin gene, with muscle yielding an extra, higher molecular weight band (approximately 800 bp). The control (non-transgenic) salmon testes was positive for the β-actin mRNA and negative for the transgene mRNA. The ocean pout liver, which was used as a positive control, tested positive for both t-OP5a and β-actin mRNA, while the water control tested negative for both.

Sequence analysis of the AFP band from brain tissue that had been amplified by RT-PCR indicate that it was indeed derived from the OP5a AFP transgene. (Figure 10).

```
          1
OP5a    CGGTTTGCTT TTCGTCCTCC TTTGTGTCGA CCACATGACA
Brain                         TTGTGTCGA CCACATGACA

        41
        GCCAGCCAGT CCGTGGTGGC CACCCAGCTG ATCCCCATAA
        GCCAGCCAGT CCGTGGTGGC CACCCAGCTG ATCCCCATAA

        81
        ATACTGCCCT GACTCCGGTG ATGATGGAGG GGAAGGTGAC
        ATACTGCCCT GACTCCGGYG ATGATGGAGG GGAAGGTGAC

        121
        CAACCCAATA GGCATCCCGT TCGCAGAGAT GTCCCAAATA
        CAACCCAATA GGCATCCCGT TCGCAGAGAT GTCCCAAATA

        161
        GTGGGGAAGC AAGTGAACAC GCCA
        GTGG
```

Figure 10. Sequence consensus between the OP5a gene sequence[a] between primers OPRT-F and OPRT-R and that obtained from sequencing of RT-PCR product from t-OP5a transgenic salmon brain. Location of primers OPRT-F and OPRT-R underlined.

[a] Sequence of OP5a obtained from Hew *et al.* (1988).

3.3. Expression of the EO-1α gene construct in transgenic Atlantic salmon.

Northern blot analysis of tissues obtained from two EO-1α transgenic Atlantic salmon demonstrated that expression of EO-1α mRNA was limited to the spleen and pituitary (Figure 11). The internal control β-actin mRNA was present in most of the salmon tissues. However despite the presence of GH mRNA in the GH transgenic Atlantic salmon pituitary, there was no detectable β-actin mRNA (Figure 11). This is because the total amount of pituitary RNA loaded on to the gel (0.6 μg) was insufficient to detect its presence. Once again, although β-actin mRNA was also present in heart and muscle, hybridization was visualized at a lower position on the gel with respect to other tissues. GH mRNA was not detected in any control (non-transgenic) Atlantic salmon tissue (data not shown) with the exception of pituitary (Personal communication, Madonna King, AQUA Bounty Technologies Inc., St. John's, Newfoundland and Labrador, Canada).

RT-PCR analysis indicated the presence of transgene mRNA (331 bp band) in all tissues except blood cells (Figure 12). All non-reverse transcribed samples (duplicates of the reversed transcribed samples), were negative for transgene mRNA as was the non-transgenic and water controls (Figure 12).

Since the EO-1α construct contained the GH cDNA, any tissue extract that was contaminated by genomic DNA would have shown the same 331 bp band as the RT-PCR product. The absence of a 331 bp band with the non-reverse transcribed controls eliminated this possibility. All tissues tested

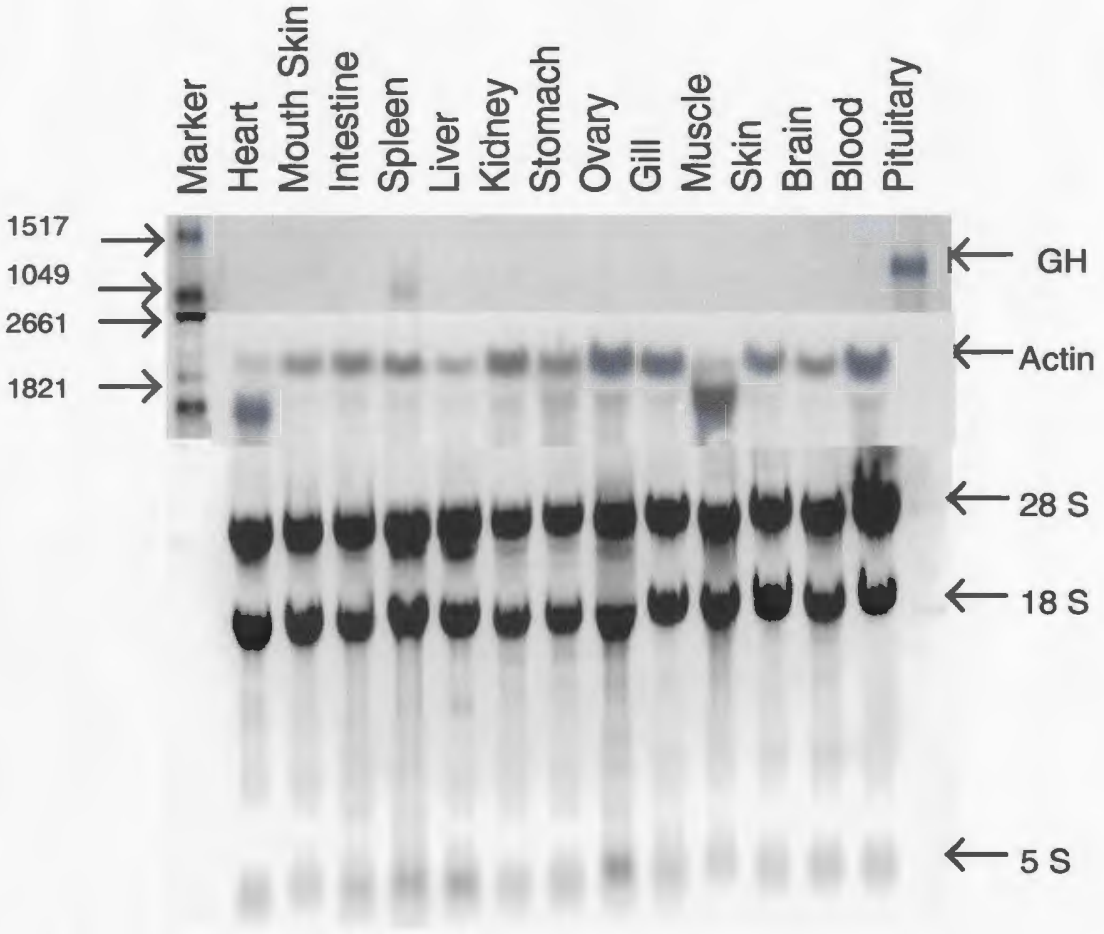

Figure 11. Northern blot analysis of EO-1α transgenic Atlantic salmon total RNA from various tissues as seen on a formaldehyde agarose gel. 40 μg of total RNA was added for each sample. Top panel probed for the EO-1α chimeric gene construct. Middle panel probed for Atlantic salmon β-actin gene. Bottom panel is RNA after electrophoresis viewed under ultra violet light. Lane 1 is the DIG-labeled RNA Marker I from Roche Applied Science in bases (60 ng used). Lane 15 is EO-1α transgenic Atlantic salmon pituitary total RNA [0.6 μg used (positive control)].

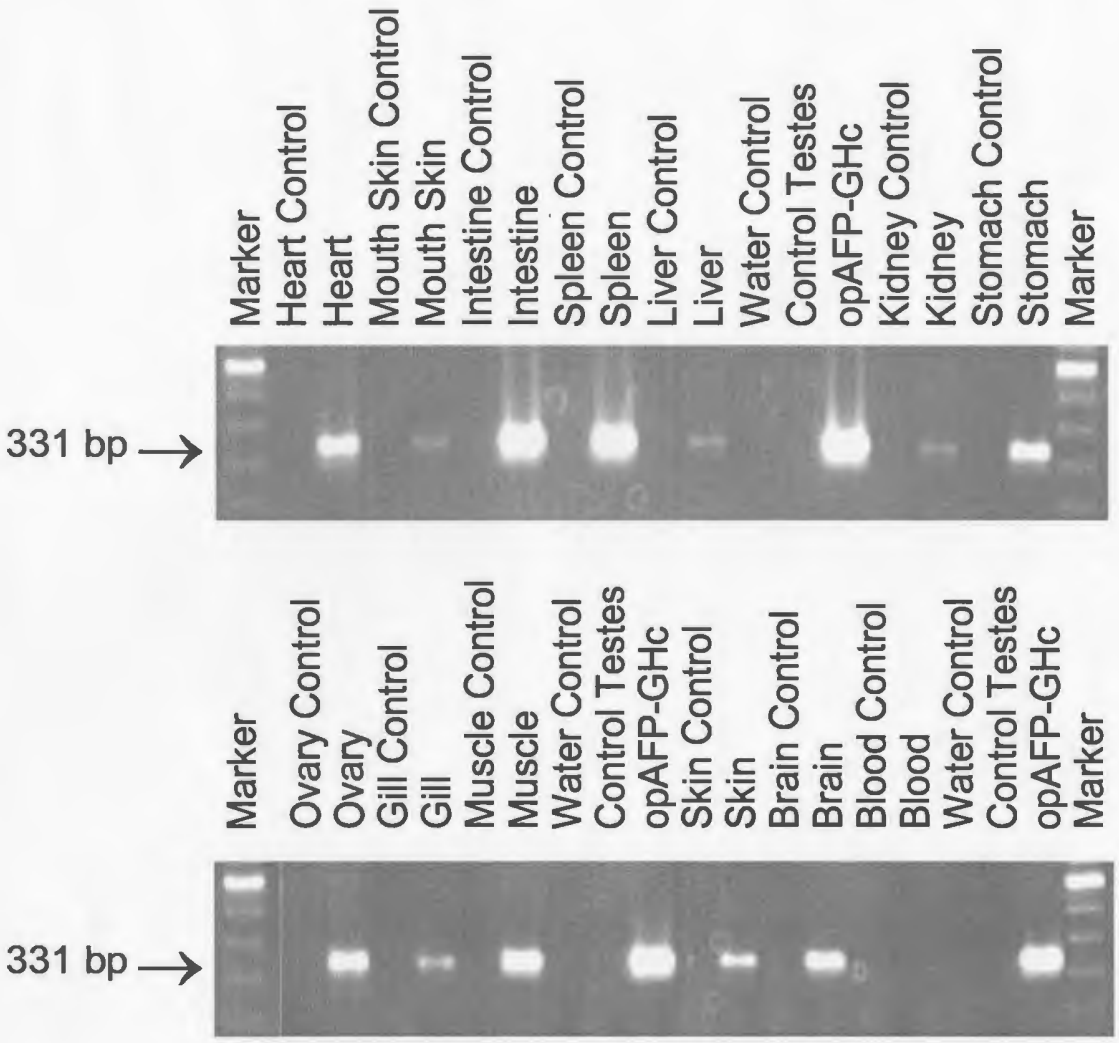

Figure 12. RT-PCR analysis of EO-1α transgenic Atlantic salmon total RNA from various tissues as seen on an agarose gel (GH specific). Primers were designed from the chimeric gene construct containing the Chinook salmon growth hormone gene (opAFP-GHc2) cDNA sequence to give a 331 bp product. All samples were treated with DNase and tissue controls were not reverse transcribed. Wild Atlantic salmon testes was used as a negative control while the opAFP-GHc2 construct in pUC 19 was used as a positive control.

positive for the salmon β-actin mRNA in both the transgenic and control salmon tissues with muscle yielding an extra, higher molecular weight band (approximately 800 bp). The water control tested negative (Figure 13).

Sequence

Analysis of the GH bands from muscle and spleen that had been amplified by RT-PCR indicated that they were derived from a type III AFP gene (Figure 14).

3.4. Thermal hysteresis

Thermal hysteresis (TH) measurements were taken on blood plasma samples from ocean pout and both lines of transgenic Atlantic salmon (t-OP5a and EO-1α) (Table 3). Samples from two ocean pout had an average TH of 1.355 °C and showed bipyrimidal ice crystal shaping typical of a high concentration of AFP (Figure 15). On the other hand, samples from six t-OP5a transgenic Atlantic salmon had an average TH of 0.019 °C and exhibited hexagonal ice crystal shaping indicative of the presence of a low concentration of AFP (Figure 16). Finally, samples from two EO-1α transgenic Atlantic salmon displayed no TH and no ice crystal shaping and therefore served as negative controls (Figure 17).

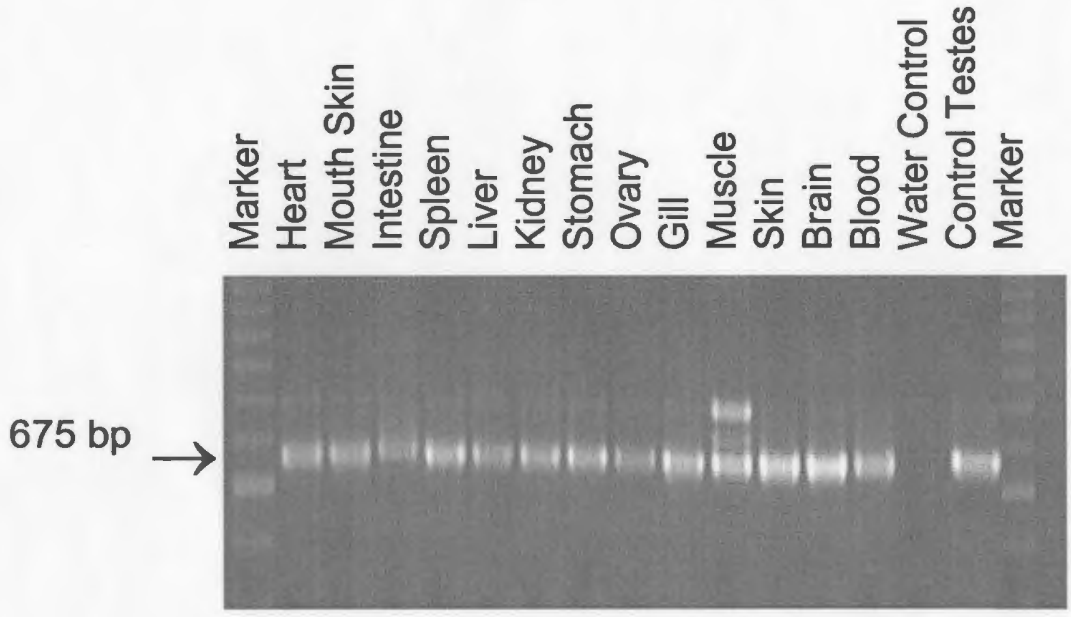

Figure 13. RT-PCR analysis of EO-1α transgenic Atlantic salmon total RNA from various tissues as seen on an agarose gel (Actin control). Primers were designed from the Atlantic salmon β-actin gene sequence to give a 675 bp product. Wild Atlantic salmon testes was used as a positive control.

```
            1
OpAFP-GHc2  GTCAGAAGTC TCAGCTACAG CTTTCACTTC GATCCAGATC
Muscle                             TTTCACTTC GATCCAGATC
Spleen                             TTTCACTTC GATCCAGATC
           41
           TTTTCACTTC GATCTCCGAT AATTAATTAA TTAATTAATT
           TTTTCACTTC GATCTCCGAT AATTAATTAA TTAATTAATT
           TTTTCACTTC GATCTCCGAT AATTAATTAA TTAATTAATT
           81
           ATTAATTAAT TAAGTCTCAG CCACTGCAGG TCGTAAAAAT
           ATTAATTAAT TAAGTCTCAG CCACTGCAGG TCGTAAAAAT
           ATTAATTAAT TAAGTCTCAG CCACTGCAGG TCGTAAAAAT
           121
           GGGACAAGTG TTTCTGCTGA TGCCAGTCTT ACTGGTCAGT
           GGGACAAGTG TTTCTGCTGA TGCCAGTCTT ACTGGTCAGT
           GGGACAAGTG TTTCTGCTGA TGCCAGTCTT ACTGGTCAGT
           161
           TGTTTCCTGA GTCAAGGGGC AGCGATAGAA AACCAACGGC
           TGTTTCCTGA GTCAAGGGGC AGCGATAGAA AACCAACGGC
           TGTTTCCTGA GTCAAGGGGC AGCGATAGAA AACCAACGGC
           201
           TCTTCAACAT CGCGGTCAGC CGGGTGCAAC ATCTCCACCT
           TCTTCAACAT CGCGGTCAGC CGGGTGCAAC ATCTCCACCT
           TCTTCAACAT CGCGGTCAGC CGGGTGCAAC ATCTCCACCT
           241
           ATTGGCTCAG AAAATGTTCA ATGACTTTGA CGGTACCCTG
           ATTGGCTCAG AAAATGTTCA ATGACTTTGA CGGTACCCTG
           ATTGGCTCAG AAAATGTTCA ATGACTTTGA CGGTACCCTG
           281
           TTGCCTGATG AACGCAGACA GCTGAACAAG ATATTCCTGC
           TTGCCTGATG AACGCAGACA GCTGAACAAG
           TTGCCTGATG AACGCAGACA GCTGAACAAG
           321
           TGGACTTCTG T
```

Figure 14. Sequence consensus between the opAFP-GHc2 gene construct sequence[a] between primers A-F and D-R and those obtained from sequencing of PCR products from EO-1α transgenic Atlantic salmon muscle and spleen. Locations of primers A-F and D-R underlined.

[a] Sequence of opAFP-GHc2 obtained from Hew *et al.* (1989); Du *et al.* (1992b); Personal communication, Madonna King, AQUA Bounty Technologies Inc., St. John's, Newfoundland and Labrador, Canada.

Table 3. Plasma thermal hysteresis measurements taken from ocean pout and transgenic Atlantic salmon.

Sample (Fish #)	Melting Point (mOsmols)	Freezing Point (mOsmols)	Average Melting Point (AvMP) (mOsmols)	Average Freezing Point (AvFP) (mOsmols)	Thermal Hysteresis (TH) (°C)
Ocean Pout #1	520 520 530	1280 1270 1280	523.3	1276.7	1.40
Ocean Pout #2	420 420 420	1120 1130 1130	420	1126.7	1.31
BS 3009 (t-OP5a salmon)	400 380 410	410 390 420	396.7	406.7	0.019
BS 3033 (t-OP5a salmon)	425 420 420	435 430 430	421.7	431.7	0.019
BS 3073 (t-OP5a salmon)	395 415 410	405 425 420	406.7	416.7	0.019
BS 3018 (t-OP5a salmon)	385 375 375	395 385 385	378.3	388.3	0.019
BS 3065 (t-OP5a salmon)	380 390 385	390 400 395	386.7	396.7	0.019
BS 2956 (t-OP5a salmon)	410 390 390	420 400 400	396.7	406.7	0.019
990456 t-(opAFP-GHc2 salmon)	360 390 400	360 390 400	383.3	383.3	0
990371 (t-opAFP-GHc2 salmon)	405 370 385	405 370 385	386.6	386.6	0

Thermal hysteresis was calculated using the following formula (Kao *et al.* 1986):

$$TH\ (^{\circ}C) = \frac{AvFP - AvMP \times 1.858}{1000}$$

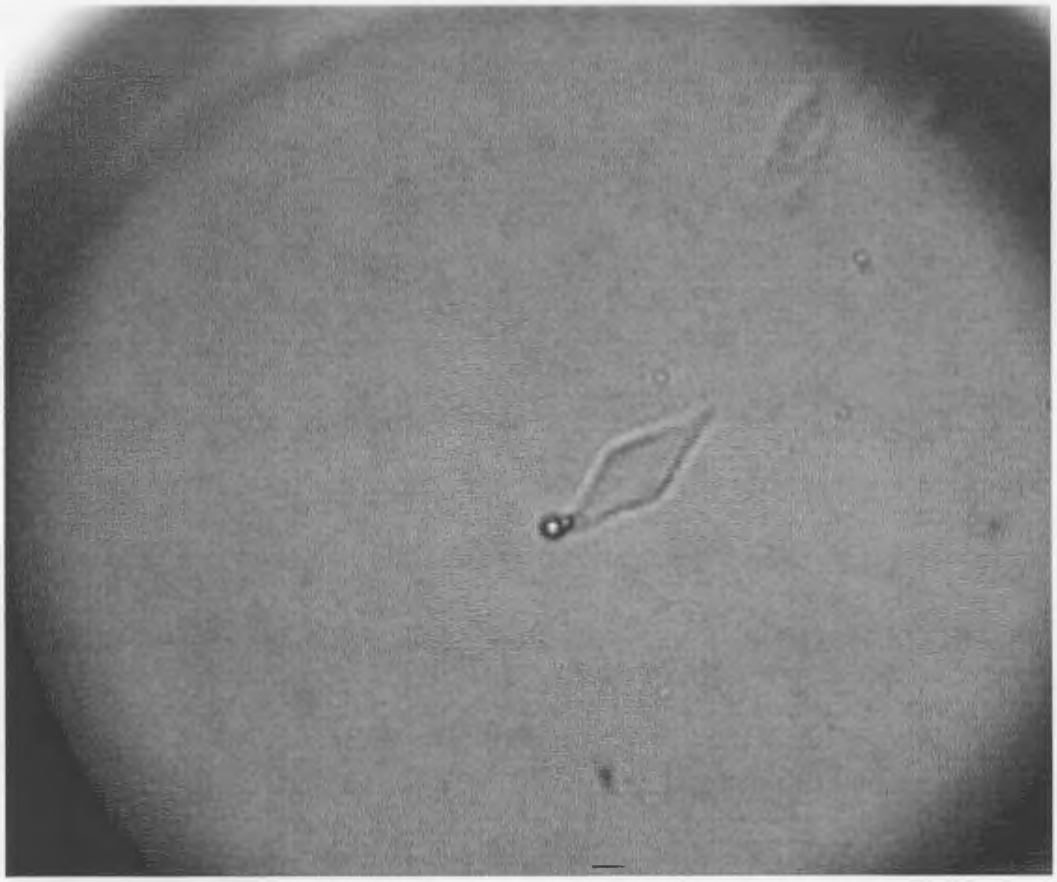

Figure 15. Ice crystal from ocean pout blood plasma illustrating bipyrimidal shaping due to the presence of a high concentration of AFP (320x magnification).

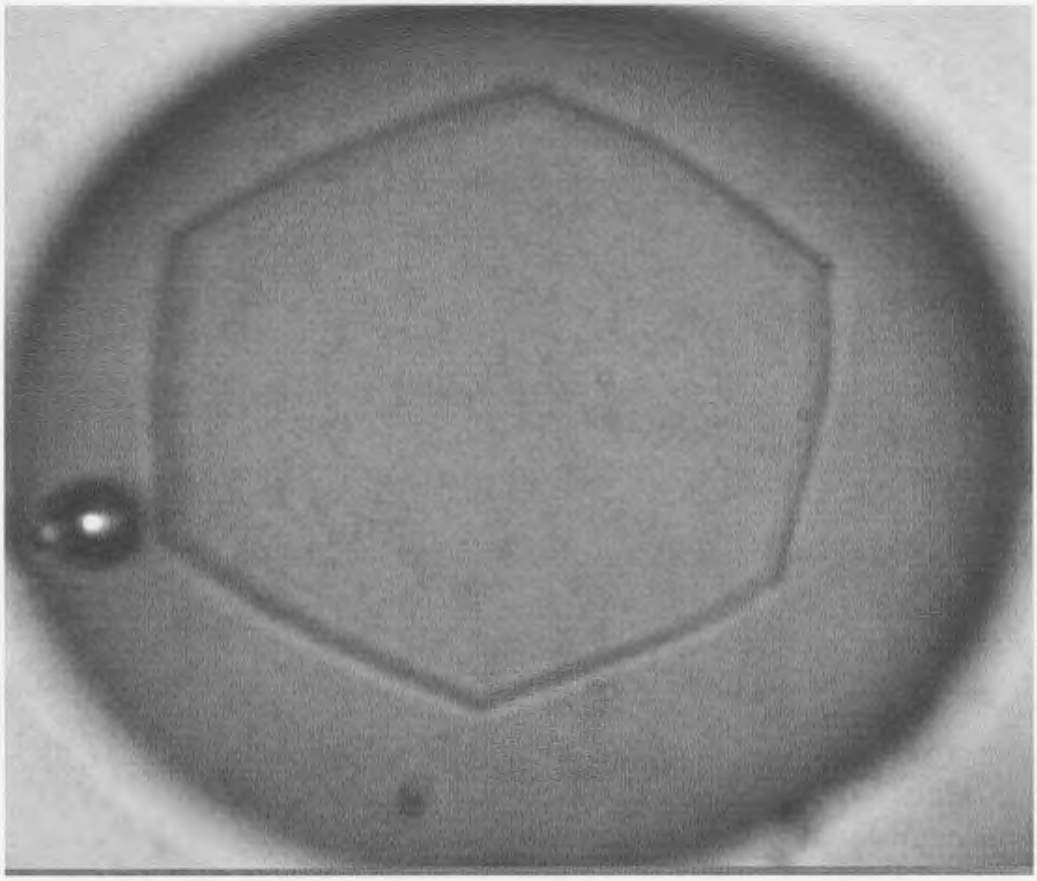

Figure 16. Ice crystal from t-Op5a transgenic Atlantic salmon BS 3009 blood plasma illustrating hexagonal shaping due to the presence of a low concentration of AFP (320 x magnification).

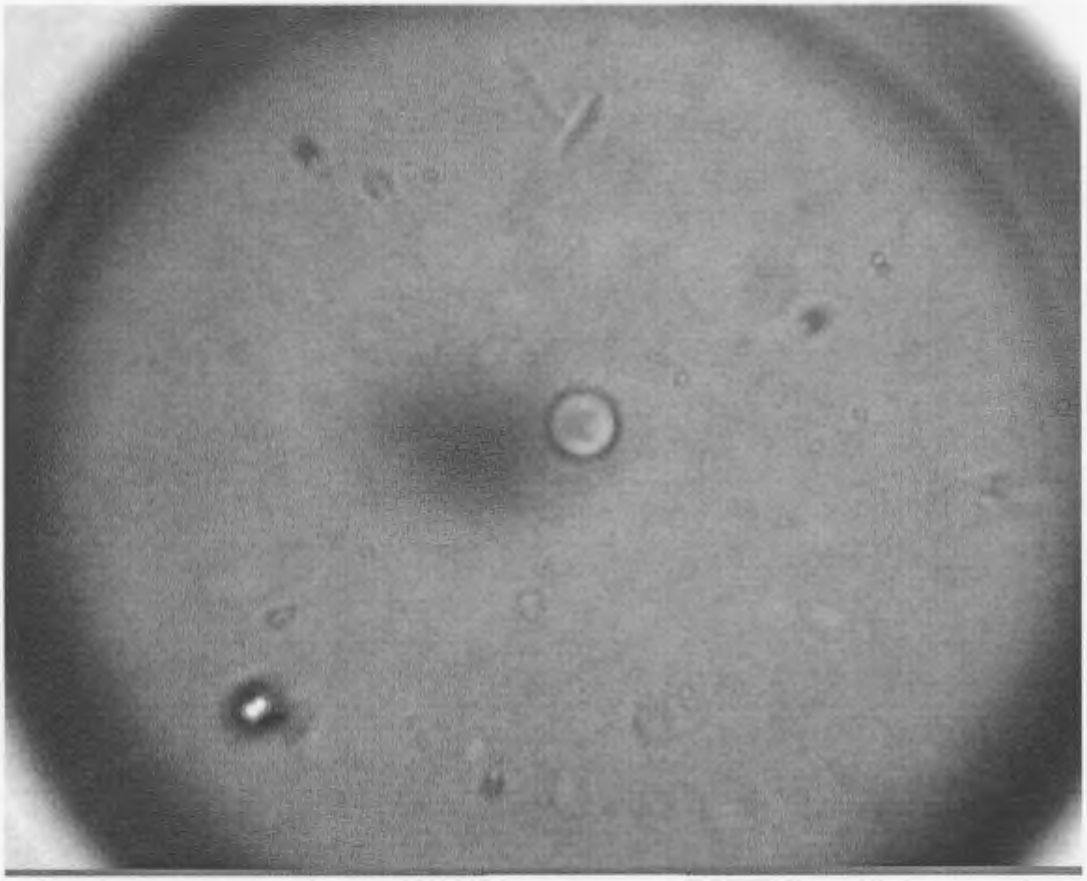

Figure 17. Ice crystal from EO-1α transgenic Atlantic salmon 990456 blood plasma illustrating no shaping due to the absence of AFP (320 x magnification).

4. Discussion

The OP5a AFP gene examined in this study was isolated from a Charon 30 genomic library of Newfoundland and Labrador ocean pout DNA. Although sequence analysis revealed that it did not code for any of the AFP purified from plasma it had all of the elements associated with a fully functional gene (Hew *et al.* 1988). A number of studies have demonstrated that the OP5a AFP promoter is indeed fully functional and capable of driving gene expression. Initial studies by Gong and Hew (1993) and more recently by Kirby (2005) have shown that the promoter was able to drive expression of chloramphenicol acetyltransferase (CAT) and luciferase reporter genes in a variety of fish and mammalian cell lines. These studies also demonstrated the presence of positive and negative regulatory elements within the promoter that would enable modulation of gene expression in a cell specific manner. The greatly enhanced growth rates of several fish species transgenic for chimeric growth hormone gene constructs driven by the OP5a AFP promoter also provides direct evidence for its ability to function *in vivo* (Du *et al.* 1992a, Devlin *et al.* 1995, Maclean *et al.* 2002).

In general the promoter region of a gene contains elements that direct tissue specific expression (von Hippel *et al.* 1982; de Freitas *et al.* 1994; Dynan and Tjian, 1985). Therefore, although the preceding studies demonstrate the functionality of the OP5a promoter *in vitro* they do not provide any information as to whether or not it contains tissue specific elements. The availability of stable lines of salmon transgenic for either

growth hormone or type III AFP both driven by modified OP5a AFP promoters provided a unique opportunity to examine the tissue specific expression of two different genes *in vivo* and compare their expression patterns with those observed in the ocean pout.

The results of northern blot analyses of ocean pout tissues confirmed conclusions by Gong *et al.* (1992) that the AFP genes in this species are expressed in most tissues, with levels ranging from the highest values in liver to barely detectable levels in brain and blood, and no expression in muscle (Figure 4). Further analysis using the more sensitive RT-PCR procedure (Foley *et al.* 1993; Mocharla *et al.* 1990) revealed that low levels of AFP mRNA transcripts were also present in the muscle tissue. Since the ocean pout has approximately 100-150 copies of homologous AFP genes, it was not possible to determine which genes were responsible for the observed expression patterns. It was also not possible to determine whether the OP5a AFP gene was being expressed in any or all of the tissues. Sequence analysis of the PCR amplicons from liver, blood and brain tissue (Figure 7) clearly indicates that they were the products of a number of different AFP genes whose nucleotide or amino acid sequences are identified in Hew *et al.* (1988).

The RT-PCR results showing transgene expression in all tissues except blood cells from the t-OP5a and EO-1α transgenics suggests that the OP5a AFP gene lacks tissue specific elements. Since the ocean pout expresses a large number of highly homologous AFP (Hew *et al.* 1988), it is

likely that the expression observed for the ocean pout blood cells is attributable the expression of an AFP gene other than OP5a. An alternative explanation is that Atlantic salmon blood cells lack the transcriptional factors needed for AFP expression that ocean pout blood cells express. These hypotheses can only be tested by further analysis of the ocean pout blood cell expression products.

Two aspects of AFP gene, and AFP promoter driven GH expression, as determined by northern analysis, differed between the ocean pout and the two lines of transgenic salmon; a) overall levels of expression and b) relative tissue specific expression patterns. The high levels of expression in the ocean pout tissues compared with those observed for the transgenic salmon is likely attributable to the large number of AFP genes expressed by the ocean pout. This argument is supported by sequence analysis of PCR amplicons from the ocean pout liver showing the presence of a number of different gene products (Figure 7). In addition, AFP gene dosage has been shown to be positively correlated with blood plasma AFP levels. For example ocean pout from Newfoundland have approximately ten times the plasma levels of AFP, and three to four times the number of AFP genes observed in New Brunswick ocean pout (Fletcher *et al.* 1985; Hew *et al.* 1988). Duncker *et al.* (1999) demonstrated with the use of a model transgenic host, the fruit fly *Drosophilia melanogaster*, that increased gene dosage can increase the production of type III AFP. The very low levels of expression observed in the EO-1α transgenic salmon which has only a single copy of the transgene also

lends support to the hypothesis that the level of expression can depend, at least in part, on the number of functional gene copies. Although the number of transgene copies present in the t-OP5a transgenic salmon is unknown it would appear from the trace amounts of AFP activity found in the plasma (Figure 16) that the gene copy number is low.

Another factor that could have played a role in the relatively low levels of expression in the transgenic salmon is the fact that in both cases the OP5a gene promoter was considerably truncated (Figures 2 and 4) (Wu 1994, Kirby 2005, Unpublished data, Ed Yaskowiak, AQUA Bounty Technologies Inc., St. John's, Newfoundland and Labrador, Canada). Promoter analysis has revealed that although these truncated promoters are equally functional, their ability to drive the expression of reporter genes in fish and mammalian cell lines was reduced to approximately a third the activity of the full length promoter (Gong and Hew 1993, Kirby 2005). These results indicated that the 5' end of the promoter that was removed contained enhancer elements. However in a recent promoter analysis study Kirby (2005) found that locating this 5' promoter section downstream of the 3' flanking region, as it is in the EO-1α GH transgenic salmon (Figure 17) restored little if any of the lost promoter strength, indicating possible directionality of the enhancer region.

Once again, the possibility exists that Atlantic salmon transcriptional factors are slightly different in sequence and therefore do not bind to ocean

pout promoters as well as ocean pout transcriptional factors, resulting in diminished expression levels.

The lower levels of expression in the EO-1α transgenic salmon as compared with those observed for the t-OP5a AFP transgenics could be attributable to the lack of introns in the EO-1α transgene. It is evident that introns can be essential if high levels of expression are desirable. Studies by Brinster *et al.* (1988) demonstrated, using the transgenic mouse model, that the presence of introns increased transcriptional efficiency. Palmiter *et al.* (1991) followed this study up by showing that the addition of heterologous introns between the metallothionein promoter and a growth hormone gene improved expression in transgenic mice. Similarly Duncker *et al.* (1997) demonstrated that introns boosted type III AFP gene expression in transgenic *Drosophilia*.

Despite the fact that the genes being examined were all driven by the same or a very similar AFP gene promoter the relative tissue specific expression patterns differed considerably between the three groups of fish. For example expression levels in ocean pout liver were at least 10 times greater than any of the other tissues examined (Figure 5, Gong *et al.* 1992). In contrast the level of expression in liver tissue obtained from t-OP5a AFP transgenic salmon did not differ appreciably from that observed for heart or brain; tissues that showed very low levels of expression in the ocean pout (Figures 5 and 8). In EO-1α GH transgenic salmon, the spleen was the only tissue that showed evidence for transgene expression using northern blot

analysis. Although the pituitary showed high levels of expression in these salmon, it is likely that the probe used for northern analysis was hybridizing to mRNA expressed by the endogenous GH genes as control non-transgenic salmon show a similar degree of hybridization (Personal communication, Madonna King, AQUA Bounty Technologies Inc., St. John's, Newfoundland and Labrador, Canada).

Some possible reasons for the observed differences in tissue specific expression between the fish groups studied could be related to species differences in their molecular capacity to transcribe AFP genes, the specific AFP gene under study (OP5a), and in the case of the transgenic fish, the intactness of the promoter and presence or absence of introns.

The relatively equitable expression levels of the AFP gene across a wide range of tissues in the t-OP5a AFP transgenic salmon clearly indicates that the OP5a AFP gene with its truncated promoter (Figure 2) has little or no tissue specificity. Since AFP are predominantly expressed in the ocean pout liver, the question arises as to whether the 5' region that was removed from the OP5a promoter contained liver specific elements. Although this is a possibility, there are several reasons to think that this may not be the case. The fact that the OP5a AFP gene does not code for any of the known plasma AFP suggests that at best it plays a minor role in AFP production by the liver. In addition the sequences obtained for the PCR amplicons from the ocean pout liver tissue did not correspond to the OP5a AFP gene but rather to several of the major AFP found in blood plasma (Figure 7). Furthermore the

presence of the 5' region of the OP5a AFP promoter at the 3' end of the EO-1α GH transgene (Figure 4) in the EO-1α line of transgenic salmon did not result in any significant liver expression (Figure 11). Based on the foregoing discussion, it seems that the predominance of liver AFP gene expression in the ocean pout must be attributable to AFP genes other than OP5a that have a high degree of liver specificity.

Studies by Miao *et al.* (1998a, 1998b, 2000) demonstrated that introns can play an important role in determining the tissue specificity of gene expression. These authors found that the intron in the winter flounder type I AFP genes not only possessed enhancer elements, it also dictated whether expression was relatively ubiquitous, as is the case for the skin type I AFP, or strictly liver specific. Since expression of the AFP gene in the t-OP5a transgenic salmon showed no significant tissue specificity it seems reasonable to conclude that unlike the winter flounder liver type AFP, the introns within the OP5a AFP gene do not contain tissue specific elements, or that salmon lack the factors needed to recognize them. This would suggest that the high degree of liver specific expression in the ocean pout must be encoded within one or more of the AFP genes that code for the AFP found in abundance in the blood plasma of this species.

The reason for the difference in tissue specific expression patterns between the t-OP5a AFP and the EO-1α transgenics is unknown. The low levels of expression in the EO-1α transgenics compared with those observed for the t-OP5a transgenics could be accounted for by the lack of introns.

However the considerable reduction in expression levels observed in heart, liver stomach and brain, etc., relative to the spleen suggests that the introns within the OP5a AFP gene may in some way be responsible for these differences between the two transgenic lines.

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