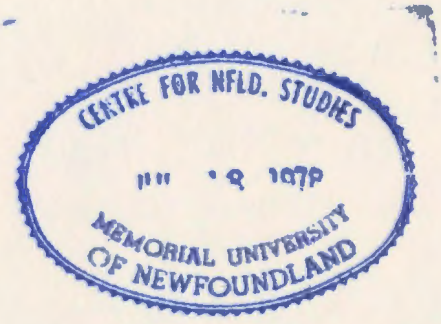
THE ONTOGENY OF TYPE I ANTIFREEZE PROTEIN EXPRESSION IN WINTER FLOUNDER, PLEURONECTES AMERICANUS

HARRY MICHAEL MURRAY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THE ONTOGENY OF TYPE I ANTIFREEZE PROTEIN EXPRESSION IN WINTER FLOUNDER, *PLEURONECTES AMERICANUS*

By

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ABSTRACT

Winter flounder (*Pleuronectes americanus*) produce Type I antifreeze proteins (AFPs) which are characterized as alanine rich and demonstrate an alpha-helical secondary structure. These proteins work by noncolligatively depressing the freezing point of tissues and tissue fluids. Two isotypes of Type I AFP, the liver-type and the skin-type, have been described in this species. The liver-type AFP is produced on a seasonal basis and secreted from hepatocytes into the blood and tissue fluids. The skin-type has just begun to be characterized and appears to have a wide tissue distribution and is expressed all year round. The absence of a signal sequence also suggests that this isotype has an intracellular site of activity. The aim of the present series of studies was to improve our understanding of the ontogeny of Type I AFP expression in the winter flounder, the cell types involved in skin-type AFP expression, their tissue distribution, seasonal expression characteristics, and relationship to liver AFP producing cells.

Adult winter flounder were captured from wild populations during the summer, fall and winter seasons. Winter flounder larvae were hatched and reared in the laboratory under seasonal temperatures and photoperiod. AFP gene expression was examined in adult winter flounder seasonally.

In situ hybridization (ISH) using AFP gene specific RNA probes and immuno-histochemistry (IH) using polyclonal and monoclonal antisera to AFP were used to identify cells responsible for AFP expression in both larval, juvenile and adult tissues. ISH and IH on whole-mounted larvae stained specifically cells running laterally along the fish. These were similar or closely associated in position with superficial neuromast cells when compared to similar species. The staining declined following yolk-sac absorption suggesting that expression in these cells was only important during the early larval stages. Larval integumental mucous cells also gave a specific IH reaction but no ISH reaction suggesting a possible variation in the genes involved. IH product in these cells continued through the larval stage but was shown to disappear around the time of metamorphosis.

ISH and IH on juvenile and adult skin sections showed a distribution of mRNA and skin-type AFP specific for the epidermis and epidermal pavement cells. Evidence of AFP mRNA and associated protein became detectable in the epidermis from early to mid fall of the fishes first year. The AFP appeared extracellular and was distributed throughout the extracellular or interstitial space usually in close association with the epidermal cells, suggesting that it may be important in slowing ice crystal formation in these interstitial regions and thus reducing cellular damage due to osmotic imbalance.

Adult and juvenile/larval gut (stomach and intestine) did show a positive immuno reaction for skin-type AFP antisera in cells throughout the stomach and intestinal mucosa. These cells also reacted with Alcian blue suggesting that they were mucous producing cells. This reaction indicated a co-secretion with mucous similar to that suggested for the integumental mucous cells of the larvae. Adult intestine did not show the IH reaction in mucous cells, but rather in-association with another population of cells. ISH with skin and liver RNA probes did not show any staining reaction in comparable regions.

During winter, adult liver cells were positive for liver AFP mRNA and the liver type protein following ISH and IH (polyclonal and monoclonal antibodies). Neither was

found present during the summer. Immuno-staining reactions for liver type AFP were localized to distinct regions of the hepatocyte cytoplasm . Skin AFP RNA probes indicated that skin AFP RNA was present throughout the year in liver but interestingly the skin type protein was never detected using polyclonal antibodies. This suggested that while the skin AFP mRNA is present constitutively in liver the actual protein is undetectable in hepatocytes using IH regardless of the time of the year. Ontogenetically, expression of AFP (skin and liver types) was first detected in the livers of juvenile flounder in November of the fishes first year. Distinct ISH staining reactions were obvious in liver at this time using both liver specific and skin specific AFP probes. As with the adult liver, IH staining was only evident with liver type AFP antisera.

Adult gill filaments showed a skin-type AFP mRNA distribution associated with cells throughout the lamellae and to some extent in the filament. The association with lamellae suggested that respiratory cells or pavement cells were likely involved. IH with antibodies for skin AFP identified specific cells corresponding to those detected using ISH. Parallel experiments with antibodies specific for chloride cells showed that these cells were not involved in AFP expression. Similarly, goblet cells did not show reactivity with the AFP antibodies. Based on these two observations the cells expressing the genes are likely gill pavement cells. Ontogenetically the gills arise from extensions of the ventral pharyngeal cartilage and continue to develop over time through increasing complexity of the filament and lamellar structures. Evidence of AFP expression in gill does not become obvious until beyond metamorphosis (October). Cellular distribution of immuno-positive product was similar to that of the adult gill. Interestingly, AFP mRNA was not detected in the gill of juvenile flounders. This may have been due to the delicate nature of the tissue resulting in less stability under the conditions of *in situ* hybridization.

Verification of IH and ISH results using Reverse Transcription Polymerase Chain Reaction (RT PCR) and gene specific primers showed as expected that liver AFP expression only occurred in liver tissue and only during the winter. Skin AFP expression was detectable in all adult tissues examined (skin, gill, liver, stomach and intestine) with no observed seasonal effect indicating that the constitutive nature of these genes was continuous across tissue types. Sequencing of each of the skin AFP PCR products indicated that they were all of similar nature. Isolated AFP clones from the stomach and intestine showed the most divergence from the original skin AFP clone (WFP9). Sequencing of PCR products obtained using primers specific for the pre-pro sequence of the liver AFP clone CT5 returned sequence identical to this clone, thus verifying that these primers and RT PCR can be used to examine the development of liver type AFP expression. RT PCR analysis of total RNA from larval flounder ranging in age from hatch to metamorphosis indicated that skin type AFP expression is present as early as hatch and continues through metamorphosis. Liver AFP expression however was not detectable until shortly after metamorphosis corresponding with changes in environmental conditions consistent with the onset of colder water and shortened photoperiod in the fall. This observation suggested that cold water naïve juvenile fish expressed the liver AFP gene in response to environmental stimuli known to initiate expression of the same genes in adults.

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DEDICATION

To Sharon, Timothy and Thomas…it was all for you!

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TABLE OF ABBREVIATIONS

AB	Alcian Blue
AFGP	Antifreeze Glycoprotein
AFP	Antifreeze Protein
BCIP	5-Bromo-4-chloro-3-indolyl-phosphate
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
DAB	Diaminobenzadine
DTT	1,4-Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis-(B-amino ethyl ether) N,N,N′,N′,-tetraacetic acid
FBT	FS + 2mg/ml BSA + 0.1% Triton-X-100
FS	Fish Saline
FTW	1XFS + 0.1 % Tween-20
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
LiCl	Lithium Chloride
MEMFA	0.1 M MOPS, 2mM EGTA, 1mM $MgSO_4$, and 3.7% Formaldehyde
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium Sulphate
MOPS	3-[N-Morpholino]propanesulfonic acid
NaCl	Sodium Chloride
NBT	Nitro Blue Tetrazolium
PAS	Periodic Acid-Schiff Reagent
PBS	Phosphate Buffered Saline
PES	Post-esophageal swelling
PBT	1X PBS, 2mg/ml Bovine Serum Albumin, 0.1% Triton-X-100
RT- PCR	Reverse Transcription Polymerase Chain Reaction
SCUBA	Self-Contained Underwater Breathing Apparatus
SSC	5X Sodium Chloride, Sodium Citrate
UV	Ultraviolet

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CHAPTER 1. GENERAL INTRODUCTION

1.1 FREEZING AND FISHES

Over the past three decades research investigating cold and freezing resistance in biological systems has revealed a tremendous diversity in the number of organisms producing antifreeze proteins (AFPs) or glycoproteins (AFGPs) (abbreviated collectively to AF(G)Ps) (Cheng 1998; Fletcher et al. 1998; Ewart et al. 1999). The vast majority of this work originated from early studies on freezing and freezing resistance in marine fish (Scholander et al. 1957), and as a result the resistance of some Arctic and Antarctic fish to freezing has been well documented (See reviews by Hew and Fletcher 1985; Hew et al. 1986, 1987; Scott et al. 1986; Davies et al. 1988; Fletcher et al. 1998; Ewart et al. 1999). Many of these species can tolerate temperatures to -1.8 °C, an entire degree below the normal freezing point of fish plasma (Holmes and Donaldson 1969). In many cases, this resistance has been shown to be correlated with the presence of small molecular weight (3.3 - 14 kDa) serum proteins which are of liver origin (DeVries et al. 1970; Duman and DeVries 1974, 1976; Fletcher 1976; Hew et al. 1980; Fletcher et al. 1998). These serum proteins have been found to be wide spread in many cold water species (Scott et al. 1986; Ewart et al. 1999). Currently there are five described types of AF(G)Ps (AFP Types I-IV and AFGP) all of which differ significantly in basic protein structure (primary and secondary) (Davies et al. 1982; Ng et al. 1986; Li et al. 1985; Ewart and Fletcher 1990; Ewart et al. 1992; Ewart and Fletcher 1993). Interestingly, regardless of peptide structure all types exhibit the one common characteristic of binding ice crystals, modifying their structure and growth characteristics and thus noncolligatively decreasing the freezing

point of a solution (thermal hysteresis) (see reviews by Davies and Sykes 1997; Cheng 1998; Deng and Laursen 1998; Zhao et al. 1998; Baardsnes et al. 1999; Ewart et al. 1999; Harding et al. 1999; Loewen et al. 1999).

While the ice modification characteristics of AF(G)Ps have been an important focus, parallel studies examining the physiological importance of these proteins have also raised numerous questions regarding their function and mode of regulation *in vivo* (see reviews by Chan et al. 1993; Fletcher et al. 1998).

1.2 AFP AND WINTER FLOUNDER

The winter flounder (*Pleuronectes americanus*) has provided an excellent working model for developing an understanding of antifreeze protein function and gene regulation in cold water marine fishes (Chan et al. 1993). This species produces large amounts of AFP Type I in the liver from November through to April, the majority of which are secreted into the blood and distributed throughout extracellular spaces (approximately 10-15 mg/ml of blood serum) (Fletcher 1976; Lin 1979; Lin and Long 1980). More recent studies on winter flounder have shown antifreeze protein gene expression and associated antifreeze activity in nonliver tissues such as gills and skin (Gong et al. 1992, 1995; Valerio et al. 1992), indicating that the liver is not the only source of this protein.

1.3 GENOMIC ORGANISATION AND TYPE I AFP PEPTIDE STRUCTURE

Winter flounder AFP is derived from a set of at least 40-50 gene copies. The genes coding for the two major AFP components [6 and 8 (High Performance Liquid Chromatography: HPLC); liver-specific] are clustered in tandem repeats, whereas the less

abundant components (skin-type) show linkage but are dispersed throughout the genome (Scott et al. 1985; Davies 1992; Davies et al. 1984; Davies and Gauthier 1992; Gong et al. 1992). Other Pleuronectids, like the yellowtail flounder do not possess the same extensive tandem repeat as observed in the winter flounder. This is thought to reflect differences in environmental selective pressure (Scott et al. 1987; 1988).

1.3.1 Liver-type AFP

The liver type AFP is produced as a preproprotein, consisting of 82 amino acids (Davies et al. 1984). The pre-component is known to be associated with the secretion signal sequence and is cleaved prior to secretion, whereas the pro-protein is thought to be secreted into the blood and processed later into the mature AFP within 24-48 hours of its appearance (Davies et al. 1984; Hew and Fletcher 1985; Hew et al. 1986b).

The primary protein structure is a 37 amino acid sequence organised into three 11 amino acid repeats, each consisting of Thr-x-x-polar aa-xxxxxxx, where the x represents alanyl residues (Hew et al. 1980; Hew and Fletcher 1985). The peptide itself is quite small (3000 to 5000 daltons) and contains about 60 % alanine, hence it is described as alanine rich (Hew et al. 1987). The secondary structure is principally an amphiphilic alpha helix (Hew et al. 1987), with the hydrophilic residues clustered on one side of the helix (Hew and Fletcher 1985).

1.3.2 Skin-type AFP

The skin-type AFPs from winter flounder are encoded by a separate subset of genes from the liver AFP (Gong et al. 1996). As with the serum protein, there are multiple isoforms of the skin AFP, all of which are distinct from that produced by the

liver. Generally, the protein contains the same 11 amino acid repeats found in the secretory liver type AFPs, however are produced as mature polypeptides lacking both the signal and prosequences (Gong et al. 1996). This indicates that the skin-type protein could be either acting intracellularly or is released from the cell through some pathway independent of the endoplasmic reticulum and Golgi apparatus.

1.4 WINTER FLOUNDER TYPE I AFP PHYSIOLOGY AND REGULATION

Expression of liver–type AFP genes in winter flounder follow a seasonal cycle controlled primarily by photoperiod (see reviews by Fletcher et al. 1989; Chan et al. 1993). The basic trend shows AFP production in the fall (October) well before the danger of ice crystals and cold water and continuing until spring (March/April) when the water temperatures begin to rise above zero (Petzel et al. 1980; Fletcher et al. 1989). The loss of long daylength in the fall is important in the initiation of hepatocyte synthesis of AFP mRNA and the subsequent production and secretion of the protein (Fletcher 1981; Fourney et al. 1984b). Further studies suggest that growth hormone (GH) is the regulator of AFP gene transcription in winter flounder (Vaisus et al. 1988; Fletcher et al. 1989). Extensive work examining the effects of hypophysectomy (surgical removal of the pituitary) on AFP synthesis showed that production of the AFP could be initiated even during summer, whereas the injection of pituitary extracts or purified GH inhibited production (Fletcher et al. 1984; Fletcher et al. 1978; Fourney et al. 1984c). This work provides strong evidence implicating GH as an inhibitor of hepatic AFP gene expression. Further investigations by Chan et al. (1997) described a liver specific transcription factor AEP which bound to an AP-1 site in the AFP intron element B. AP-1 binding proteins are

known to heterodimerize with transcription factors related to the Fos and Jun families. Fos and Jun like transcription factors are induced by somatotrophin (GH). Chan et al. (1997) further speculated that if the AEP is an AP-1 site binding protein then during the summer when somatotrophin is likely to be at its highest concentration, Fos and Jun will also be present with the potential for binding AEP and inhibiting transcription of the liver specific AFP gene. This proposed molecular interaction could explain the GH regulated seasonality of liver specific AFP expression.

Water temperature does not appear to be important in the initiation of AFP mRNA or AFP synthesis in the fall, nor does it appear to be important in terminating AFP synthesis in the spring. Studies by Fletcher (1981) and later by Price et al. (1986, 1990) showed that cold temperature was important for the effective accumulation of AFP mRNA and the synthesis of sufficient levels of plasma AFP in the fall. Vaisius et al. (1989) also found that temperature does not have any effect on AFP gene transcriptional initiation rate, however warm temperatures did appear to reduce the amount of antifreeze protein in the blood and the overall stability of the AFP mRNA. Interestingly, Pickett et al. (1983b) showed that alanyl tRNA synthetase is most active at temperatures of 0 °C to 5 °C and is less active at higher temperatures. Similarly it was also shown that there is an increase in alanine associated tRNA in the winter months, suggesting a selection for alanine rich proteins such as Type I.

Far less is known regarding the regulatory mechanisms behind the expression of skin-type AFPs. These AFPs from winter flounder do not appear to be regulated through the same mechanisms as the liver AFP, thus there is a significant level of expression of

these AFPs throughout the year (Gong et al. 1992; 1995). Gong et al. (1995) showed that hypophysectomy had no apparent effect on the accumulation of skin-type AFP mRNA, suggesting that photoperiod is not a major regulatory factor. However, it is possible that temperature will play a role in this case. It is well known that water temperature is not important in the initiation of liver type AFP expression in the fall but is important in the accumulation of the mRNA and the stability of the protein (Price et al. 1986). These data suggests post-transcriptional control. It is clear that further study is necessary to dissect the various control mechanisms involved in this case.

1.5 A THEORY OF ANTIFREEZE FUNCTION

Many of the functional features of AFPs have been derived from structural studies on AFGP and winter flounder liver type AFP. Early studies determined that the mode of AFP activity was non-colligative, that is the presence of the protein, even at the high concentrations found under winter conditions, did not have a significant effect on the osmolarity of the serum (Davies et al. 1988). DeVries and Lin (1977) and Yang et al. (1988) both proposed that the amphiphilic alpha-helical structure of type I AFP was of primary importance to its action. They surmised that the amphiphilic structure allowed for a separation of hydrophobic and hydrophilic groups on the molecule resulting in a specific orientation allowing the hydrophilic residues to bind to the growing front of ice crystals preventing further advancement by the exclusion of water molecules on the hydrophobic side.

The importance of AFP type I structure has been further defined (Hew et al. 1987; Wen and Laursen 1992). Hew et al. (1987) outlined three absolute requirements for AFP

activity. These include the presence of a ridged helical structure important in aligning the protein with the ice crystal lattice, the amphiphilicity of the protein causing differential exclusion of water and ice, and finally the flexibility of the hydrophilic side chains allowing for hydrogen bonding with the ice lattice. A recent review by Harding et al. (1999) further defined the importance of the hydrophobicity in ice growth inhibition.

Another line of research has suggested a second mode of action for AF(G)Ps as a general group. Rubinsky et al. (1990) carried out a series of studies examining the effects of Antarctic nototheniid AFGPs on the survival of pig oocytes at hypothermic temperatures. It was discovered that mixtures of these AFGPs could protect the structural integrity of the oolemma and thus inhibit ion leakage across the membrane at hypothermic temperatures. Similar work utilising AFPs from winter flounder (Type I), sea raven (Type II) and ocean pout (Type III) resulted in significant increases in survival, maturation, and fertilization success of bovine oolemma exposed to hypothermic temperatures (Rubinsky et al. 1991). Later studies using rabbit parietal cells and pig granulosa cells indicated that AFPs appeared to be blocking ion channels, specifically those associated with Ca^{2+} and K^{+} thus preventing leakage (Negulescu et al. 1992; Rubinsky et al. 1992). While these latter studies were carried out at room temperature, the results do indicate a mechanism by which AFPs could maintain the integrity of cell membranes. These observations become important in the light of many of the current theories of how low temperature damage actually occurs. Hochachka (1986; 1988a,b) and Steponkus (1984) proposed that a major cause of cell damage at low temperatures is due to a decoupling between metabolic activities and membrane function. This would

result in a disruption of various ion gradients within the membrane and thus an increase in intracellular Ca^{2+} concentration, membrane hydrolysis and cell death. Taken generally, these data suggest that AF(G)Ps may not only have the ability to control freezing and ice crystal growth in fish but may also protect cell membranes under hypothermic conditions.

1.6 REGIONAL AND CELLULAR FEATURES OF AFP EXPRESSION

The observation of a significant freezing point depression in skin associated tissues from sculpin (Schneppenheim and Theede 1982), cunner (Valerio et al. 1990), and flounder (Valerio et al. 1992) suggested that either high levels of AFP of liver origin was diffusing through to these regions or the tissue itself had the potential for AFP production.

Subsequent studies by Gong et al. (1992, 1995) showed significant levels of AFP gene expression, as well as potential differential regulation in many non-liver tissues e.g., skin, scales, and gills from winter flounder, thus strongly supporting the idea that non-liver cells also have the capacity to produce AFPs in this species. To date the AFP producing cells in non-liver tissue have not been identified. Therefore, little is known about the cell types, their developmental features and characteristics that provide them with the potential to enhance freeze resistance in fish. In consideration of recent observations of AFPs exhibiting membrane protective properties and the fact that many of the external tissues (i.e. gills) in Arctic fishes like the winter flounder are constantly exposed to extreme cold water and ice conditions further work is warranted to improve the current understanding of how antifreezes are distributed through these tissues and the

characteristics of the cells involved in their synthesis.

1.7 RESEARCH OBJECTIVES

The discovery of the ability of AF(G)Ps to affect the stability of cell membranes under cold conditions and the subsequent description of antifreeze proteins from a variety of epithelial tissues in fishes has resulted in a need for a re-evaluation of how these proteins work to protect cells in intimate contact with cold water and ice crystals. Further, the constitutive expression characteristics of the skin type genes from winter flounder raise many questions as to when these proteins become important during the development of the fish. In consideration of these discoveries the present series of studies were designed to improve our understanding of the cell types involved in skin-type AFP expression, their tissue distribution, seasonal expression characteristics, relationship to liver AFP producing cells and their importance during the ontogeny of the winter flounder.

In order to achieve the objectives I proposed to:

1) Determine the cell types involved in **Skin-type** AFP production during post-embryonic development of larval winter flounder and describe their spatial distributions and morphological characteristics.

2) Describe the cell types involved in tissue specific expression of **Skin-type** and **Liver-type** AFP in adult winter flounder and compare these to cells expressing the genes in larval/juvenile stages.

CHAPTER 2. ANTIFREEZE PROTEIN EXPRESSION IN INTEGUMENTAL CELLS OF LARVAL WINTER FLOUNDER

2.1 INTRODUCTION

The winter flounder (*Pleuronectes americanus)* is commonly found in near shore locations off the East Coast of Canada. It is usually found in depths ranging from 1.8 - 36.6 m, and while some seasonal migration has been observed, it is common to find this species, in inshore areas during the winter (Scott and Scott 1988). Seawater temperatures during mid winter (January/February) in Newfoundland have been recorded as low -1.5^{0} C with the frequent occurrence of ice crystals in the water column (Fletcher 1977). This environment would be deadly to most species however, winter flounder survive in these conditions by producing AFPs that lower the freezing point of their body fluids to levels similar to that of seawater. Typically, winter flounder produce Type I AFPs, which are small alanine-rich proteins (≈ 3300- 4500 Daltons) that characteristically have an alpha helical secondary structure (Hew et al. 1986a).

Current knowledge indicates that the winter flounder produce two different Type I AFPs, the liver-type and the skin-type. The liver-type is synthesized in the hepatocytes and secreted into the blood circulation based on seasonal control and photoperiod regulation (Davies et al. 1988; Fletcher et al. 1998). In contrast to the liver AFP, the skin-type is expressed in most tissues, is not environmentally regulated and thus is present all year (Gong et al. 1992; 1995). While skin-type AFP expression appears to have a wide tissue distribution, it occurs more abundantly in epithelia including skin, gills

and digestive tract. These AFPs differ further from the liver type in that they lack a signal sequence suggesting that they remain and function intracellularly (Gong et al. 1996). Recent studies however indicate that this may not always be the case, for they provide evidence that these proteins may occur in extracellular or interstitial spaces (see Chapter 3) or are released in association with other cellular secretions such as mucus (see Chapter 4).

Winter flounder are known to spawn from late April to June in waters around Newfoundland (Scott and Scott 1988). During this period, temperatures can range from 0.5 ^{0}C to 6 ^{0}C depending on region and wind conditions (Fletcher 1977; Fletcher et al. 1985b; Fletcher et al. 1987). Eggs hatch in 11 or 12 days at between 5 and 7 ^{0}C but can take as long as 23 days or more at 2 ^{0}C and below (Buckley 1982). Similarly, time to yolk-sac absorption is about 7 to 8 days at between 5 and 7 ^{0}C or as long as 13 days or more at 2 ^{0}C and below (Buckley 1982). Following yolk-sac absorption, larvae continue to develop reaching metamorphosis in 2.5 to 3 months. Chambers and Leggett (1987) observed an average time to metamorphosis for winter flounder cultured at 6 ^{0}C to be 59.5 days.

Little is known of skin-type AFP gene expression, its onset and the tissues involved in post-embryonic larvae of winter flounder. Recent studies have shown that it is prevalent in the skin of adult winter flounder throughout the year (Gong et al. 1992; 1995). The present study was designed to examine fish ranging in age from hatch to metamorphosis in order to determine the developmental timing of AFP expression and specifically the cell types involved in skin-type AFP expression in the post-embryonic

larval integument. The occurrence of total AFP transcripts will be examined using Reverse Transcription Polymerase Chain Reaction (RT PCR) and gene specific primers. Identification of specific cell types will be accomplished by using whole-mount immuno-histochemistry to detect the cellular presence of the protein and by the detection of skin AFP mRNA using whole-mount *in situ* hybridization and gene specific probes.

2.2 MATERIALS AND METHODS

2.2.1 Adult Maintenance

Winter flounder were captured by SCUBA (November, 1996; January, 1997) and transported to the Ocean Sciences Centre where they were held in tanks maintained overwinter with running seawater under ambient photo-period and temperature.

In early spring, animals were checked daily for signs of gonadal swelling and the presence of eggs or milt. When fish of appropriate condition were found they were immediately isolated from the main tank in preparation for spawning.

2.2.2 Spawning and Fertilization

2.2.2.1 Spawning

Eggs were collected by first carefully drying the genital pore area and then applying gentle pressure, allowing the eggs to pour easily into a clean dry container. The container with eggs was then stored in a cool place until fertilization. Sperm was collected by placing a syringe near the genital pore and gently drawing up the milt. Following gamete collection all further procedures were carried out at 5 °C.

2.2.2.2 Fertilization

500 to 1000 µl of eggs in ovarian fluid were placed in a dry sterile plastic Petrie dish and mixed gently with 100 µl of milt. The sperm were activated by adding a few drops of UV treated and filtered seawater (0.2 µm Millipore filter system). The eggs were then allowed to sit for 5 minutes to fertilize and were subsequently flooded with fresh filtered seawater and allowed to water harden for an additional 20 minutes.

Following water hardening the eggs were flushed once more to remove any surplus milt and allowed to develop normally. Water was changed every other day by decanting off old and adding new fresh filtered seawater. Following fertilization, eggs were incubated at 5 ^{0}C and hatched within 14 days.

2.2.3 Hatching and Transfer to Rearing Containers

Following hatch, 1000 to 2000 larvae were transferred to 4 litre plastic buckets lined with black plastic bags to enhance contrast for prey visibility. Buckets were set in flowing seawater baths at ambient temperature/photoperiod. Temperatures ranged from 5 to 7 ^{0}C in May/June to as high as 14^{0}C in August. The buckets contained 3 litres of filtered (0.2 µm) and UV sterilized seawater plus 500 mls of mixed algae (*Isochrysis* sp. and *Tetraselmis* sp.). Water was part changed (50%) and replaced every 2 to 3 days with mixed algae. Animals were fed daily on a ration of 5000 rotifers (variety: *Sap)* per litre and supplemented with a further 300 millilitres of algae until metamorphosis (about 60 days). Fish reaching metamorphosis and thus settlement were transferred to an aquarium with flowing ambient seawater and aeration and weaned onto a ration of 100 000 *Artemia*

per day. Subsequently, fish were further weaned onto a dry diet i.e. Biokyowa.

All animals were maintained and killed according to the guidelines set by the Canadian Council of Animal Care (Olfert et al. 1993).

2.2.4 Tissue Sampling

2.2.4.1 RNA Analysis

Larval and metamorphosed winter flounder were taken for total RNA extraction. These samples were flash frozen in liquid nitrogen by two methods. For larvae ranging in age from newly hatched to premetamorphosis, 10 to 50 fish were anesthesized in MS-222 and transferred to a 1.5-ml Eppendorf tube and subsequently exposed to brief centrifugation at 10,000 rpm for 30 to 90s to form a pellet. Water was carefully decanted without disturbing the pellet, and the tube was immersed directly in liquid nitrogen. Metamorphs were frozen by first anesthesizing them and then immersing them directly into a bath of liquid nitrogen. All samples were stored at -70 °C until analysis.

2.2.4.2 Whole-mount In *situ* Hybridization and Immuno-histochemistry

Thirty larvae were sampled immediately on hatch and then at 5-10 day intervals until metamorphosis. Animals were fixed in 4 % paraformaldehyde in PBS for 2 hours at room temperature and subsequently post-fixed and stored in methanol at -20 °C. During early stages, approximately twenty larvae were chosen randomly for each experiment. These were divided so that even numbers were used for experimental and control groups (ten larvae per group). In later stages with larger fish, numbers were reduced to as little as two to four fish per group.

2.2.5 Total RNA Extraction and RT PCR

2.2.5.1 Total RNA Extraction

Total RNA was extracted using either the Qiagen®, Rneasy® Mini RNA prep kit following disruption and homogenisation using the QIAshredder ® and the included homogenisation buffer or alternatively with Trizol ® reagent, according to the manufacturer's suggested protocol. Following extraction, RNA was treated with Life Technologies amplification grade DNAse I according to the manufacturer's recommendations, aliquoted and frozen at -70 °C until use.

2.2.5.2 PCR Primers

PCR primers (WFP9A and WFP9B) were designed to amplify a 250 base pair (bp) fragment analogous to an AFP cDNA clone (WFP9) originally isolated from a winter flounder skin cDNA library (Gong et al., 1996). The sequence of the primers corresponded to 5'-GTCGAACACTCAGAATCACTG-3' and 5'-AGCTTCTCAGGTTTATTCAGC-3', respectively.

2.2.5.3 RT PCR

One microgram of total RNA, primers, and the required components of the Life Technologies SuperScript ™ One-Step ™ RT-PCR System were mixed according to the manufacturer's recommendations. The mixture was incubated at 50 °C for 30 minutes for the reverse transcription reaction and then carried through PCR on a Perkin-Elmer Model 480 thermal cycler. The amplification consisted of 35 cycles at 94 °C, (30s), 60 °C (30s), and 68 ° C (45s).

2.2.5.4 Sequencing of PCR Products

At least five consecutive RT PCR reactions were run and the reactions pooled, DNA was ethanol precipitated and then resuspended in 50 ul of sterile water. This volume was run on a 2% agarose gel for at least 1 hour at 100 V and stained with ethidium bromide. The gel fragment was excised and the DNA purified using the QIAquick Gel Extraction Kit (Qiagen).

Purified PCR products and appropriate primers were subsequently sent to the DNA Sequencing Facility/The Centre for Applied Genomics at the Hospital for Sick Children, Toronto Ontario. For comparison, obtained sequences were checked against known AFP sequences to verify the result.

2.2.6 Detection of AFP using Whole-Mount Immuno-Histochemistry

Winter flounder skin AFP was secretory expressed in *E. coli* JM105 and purified to homogeneity by Lin et al. (1999). Polyclonal antisera to winter flounder skin AFP were produced in rabbits following conjugation to keyhole limpet hemocyanin (Lin et al. 1999). Antiserum to sea raven Type II antifreeze protein was also produced in rabbits but without conjugation (Ng et al. 1986). Specificity of antisera for the associated protein was shown through Western Blotting (Lin et al. 1999; Ng et al. 1986). Immunohistochemical negative controls included normal rabbit serum, the use of unrelated antisera (i.e. sea raven Type II AFP) and the absence of the primary antibody.

Samples chosen for immuno-histochemistry were re-hydrated through methanol /Phosphate buffer saline (PBS) series for 5 minutes and finally washed in fish saline for 10 minutes, after which they were transferred to PBT (1xPBS, 2mg/ml Bovine Serum

Albumin (BSA), 0.01 % Triton-X-100) for 10 minutes. Non-specific sites were blocked in a solution of PBT and 10 % normal goat serum for 60 minutes. Larvae were incubated with primary antibody at a dilution of 1/1000 in PBS and 5% normal goat serum overnight at 4 ^{0}C. Following primary incubation, samples were rinsed once in PBT and then three further washes for 30 minutes each to remove any excess antibody.

The secondary antibody was anti-rabbit conjugated to horseradish peroxidase (Sigma). Fish were exposed to a 1/500 dilution in PBT and 5 % Goat serum for 60 minutes at room temperature. Excess antibody was removed as for the primary antibody. Immuno-reactivity was detected by exposing the tissue to a solution of ImmunoPure® Metal Enhanced Diaminobenzadine (DAB) substrate (Pierce). Following development, samples were dehydrated, cleared in xylene and mounted on glass slides for photo-microscopy.

2.2.7 Detection of AFP mRNA using Whole-Mount *In situ* Hybridization

2.2.7.1 Probe Production

Winter flounder skin-type antifreeze protein cDNA WFP9 was ligated to an EcoRI/XhoI site in $_{p}$Bluescript SK (+/-) plasmid vector (Gong et al. 1996). Winter flounder liver antifreeze protein cDNA pKenc-17 (Pickett et al. 1984) was subcloned from plasmid vector PUC-18 and ligated to a BamHI/XBAI site in $_{p}$Bluescript SK (+/-). In order to make antisense or sense RNA probes, plasmids containing WFP9 were linearized with either EcoRI or XhoI respectively whereas those containing pKenc-17 were linearized with either BamHI or XBAI respectively. Probes were produced during *in vitro* transcription (Melton et al. 1984; Jowcltt and Lettice (1994). The reaction mixture

contained 5 µl linearized plasmid (1 µg/ul), 5 µl 10x buffer, 2 µl 250 mM 1, 4-Dithiothreitol (DTT)(Sigma), 0.5 µl RNAsin (Sigma), 10 µl 2.5 mM NTP mix containing digoxigenin tagged UTP (Boehringer Mannheim # 1209) and 1.5 µl of the relevant RNA polymerase, i.e. T7 or T3. The mixture was incubated at 37 ^{0} C for 2 hours, digested with DNase I for 15 minutes at the same temperature and then precipitated with 5.0 µl of 0.2 M Ethylenediaminetetraacetic acid (EDTA) , pH 8.0, 6.25 µl 4 M lithium chloride (LiCl), and 200 µl cold ethanol overnight at -20 ^{0} C. Pellets were resuspended in 1 ml of hybridization buffer (50 % deionized formamide, 5 x sodium chloride, sodium citrate (SSC), 1 mg/ml Torula RNA, 100 µg/ml Heparin, 1 x Denharts, 0.1 % Tween-20, and 0.1 % 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and stored at -20 ^{0} C.

2.2.7.2 Hybridization Protocol

This protocol was modified from Hemmati-Brivanlou et al. (1990). Larvae were rehydrated in single 5 minute washes of 100 % methanol, 75/25 methanol/water and finally 25/75 methanol/FTW (1 x Fish Saline (FS) (Valerio et al. 1992) + 0.1 % Tween-20), followed by four 5 minute washes in 100 % FTW. Tissue was then rinsed in 0.1 M Triethanolamine (pH 7-8) for 2 times 5 minutes followed by 2 washes for 5 minutes each in the same buffer with acetic anhydride. Tissues were subsequently incubated in 1 ml hybridization buffer for 10 minutes at 63 ^{0} C and then prehybridized for a further 6 hours in fresh buffer. Following prehybridization, buffer was replaced with the probe solution and hybridization was allowed to occur overnight at

63 ^{0} C.

Following hybridization, tissue was treated for 10 minutes at 63 ^{0} C with a series of increasing stringency washes containing 100 % hybridization buffer, 50/50 hybridization buffer/2 x SSC in 0.3 % CHAPS, 25/75 hybridization buffer/2 x SSC in 0.3 % CHAPS. These washes were followed by two further washes (20 minutes) in 2 x SSC in 0.3 % CHAPS at 37 ^{0}C. Tissues were then subsequently treated with RNAase at 37 ^{0} C for 30 minutes and washed once in 2 x SSC with 0.3 % CHAPS at room temperature for 10 minutes and then twice more in 0.2 x SSC with 0.3 % CHAPS at 63 ^{0}C for 30 minutes.

2.2.7.3 Immuno-histochemical Detection of Hybridization Products

Following stringency washes the tissue was washed twice in Ftw with 0.3 % CHAPS for 10 minutes at 63 ^{0} C, then once without CHAPS. The final volume of FTW was replaced by FBT (FS + 2 mg/ml BSA + 0.1 % Triton-X-100) and the tissue was washed at room temperature for 15 minutes.

Tissue was blocked with fresh FBT + 20 % heat treated lamb serum for 1 hour at room temperature, after which the solution was replaced with a duplicate solution containing a 1/1000 dilution of the affinity purified sheep anti-digoxigenin coupled to alkaline phosphatase antibody (Boehringer Mannheim) and rocked overnight at 4 ^{0} C.

Excess antibody was removed through five 1 hour washes with FBT, followed by two washes with chromogenic buffer (100 mM Tris, pH 9.5, 50 mM magnesium chloride ($MgCl_2$), 100 mM sodium chloride (NaCl), 0.1 % Tween-20, 1mM Levamisol).

The last chromogenic wash was replaced with 2 mls of the same solution

containing 4.5 ul NBT/ml (Nitro blue tetrazolium in 70 % Dimethyl formamide) and 3.5 ul/ml BCIP (5-bromo-4-chloro-3-indolyl-phosphate). The reaction was stopped by washing tissue in 4% paraformaldehyde. Tissue was dehydrated through ethanol series, cleared in benzoate/benzyl alcohol and mounted on glass slides for observation and photomicroscopy.

2.2.7.4 Controls

Sense RNA probes were used in identical hybridization conditions as the anti-sense probes on larvae of the same age and stage of development.

2.3 RESULTS

2.3.1 RT PCR

2.3.1.1 Testing Primers

The skin AFP sequence was amplified from purified plasmid DNA WFP9 using the relevant primers to test for specificity. Primers showed good amplification when reacted with their respective purified DNA (Figure 2.1).

2.3.1.2 RT PCR on Total RNA from Larvae and Juveniles

RT-PCR with skin primers and total RNA isolated from winter flounder larvae and juveniles consistently gave a strong 250-bp band corresponding to the original skin cDNA clone. (Figure 2.2). This band was apparent in newly hatched larvae and continued to be present throughout the larval stages and beyond metamorphosis to the juveniles.

2.3.1.3 Sequence Comparison

All PCR products showed high levels of identity with the original skin AFP clone WFP9. The amino acid sequences translated from RT PCR products of newly hatched

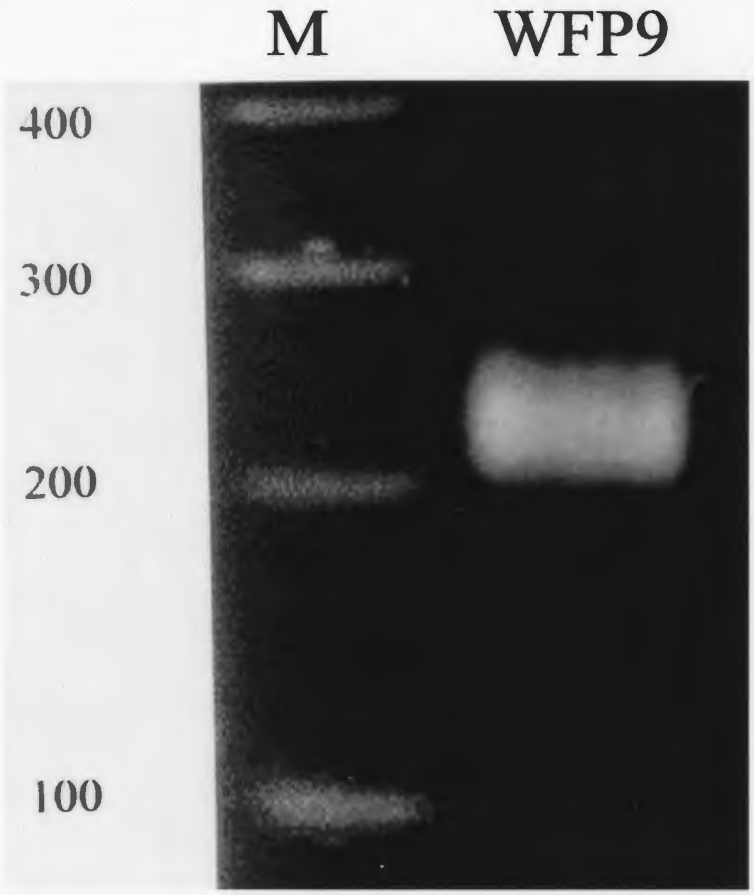


Figure 2.1 PCR verification of the specificity of primers for skin AFP clone WFP9 with corresponding plasmid DNA. Samples were run on a 2% agarose gel at 100V for one hour and stained in ethidium bromide

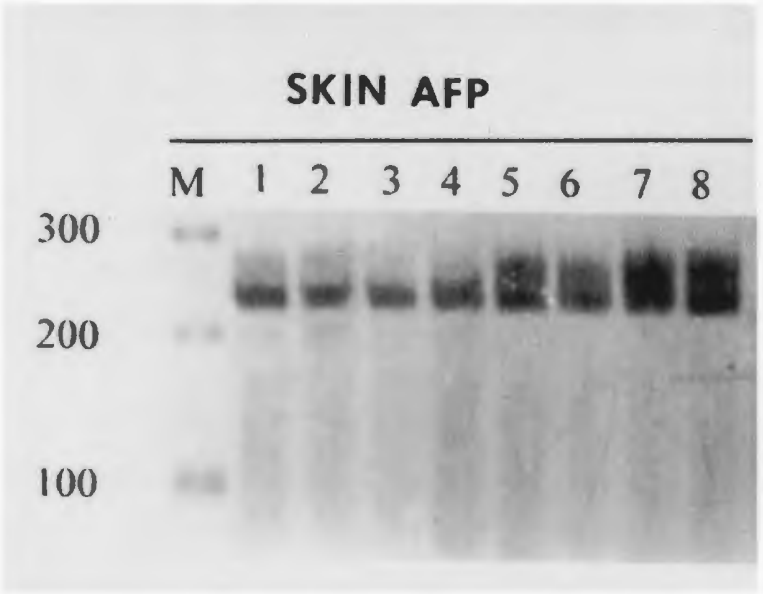


Figure 2.2 RT PCR results showing the onset of skin AFP expression over the postembryonic development of winter flounder. New hatch (lane 1), 4 days (lane 2), 10 days (lane 3), 20 days (lane 4), premetamorph (lane 5), metamorph (lane 6), first fall juvenile (lane 7) and first winter juvenile (lane 8). Samples were run on a 2% agarose gel at 100V and stained in ethidium bromide.

(A)

Source

```
WFP9(skin)  1 GTCGAACACTCAGAATCACTGACATCAACATGGAC
Larvae      1 -----------------------------------

WFP9(skin) 36 GCACCAGCCAAAGCCGCCGCAGCCACCGCCGCCG
Larvae     36 -----------------------------------

WFP9(skin) 70 CCGCCAAGGCCGCCGCAGAAGCCACCGCCGCCGC
Larvae     70 -----------------------------------

WFP9(skin)104 AGCCGCCAAAGCAGCAGCCGACACCAAAGCCAAA
Larvae    104 T----------------------------------

WFP9(skin)138 GCAGCCCGTTAAGGATCGTGGTCGTCTTGATGTGG
Larvae    138 -----------------------------------

WFP9(skin)173 GATCATGTGAACATCTGAGCAGCGAGATGTTACCA
Larvae    173 -----------------------------------

WFP9(skin)208 ATCTGCTGAATAAACCTGAGAAGCTG ttt
Larvae    208 -----------------------A--  ---
```

(B)

Source

```
          1         10        20        30
WFP9      MDAPAKAAAATAAAAKAAAEATAAAAAKAAADTKAKAAR
Larval    -----------------------------------------
```

Figure 2.3 A) Nucleotide sequences of skin AFP cDNA amplified from total RNA isolated from newly hatched winter flounder larva using primers specific for the known sequence of skin AFP clone WFP9. Dashes represent regions of identity. B) Translated amino acid sequences from the above cDNA showing regions of identity.

larvae show 100 % identity to the original clone from which the primers were designed (Figure 2.3).

2.3.2 Immuno-histochemistry

Immuno-histochemistry using antisera to winter flounder skin-type AFP revealed staining that was specific for two distinct groups of epidermal cells, the "lateral spindle-shaped cells" and a more randomly distributed population of cells (2.4ab). At hatch, the spindle cells had spindle shaped bodies that were characterized by the presence of numerous small regularly shaped vesicles (Figure 2.4b). The apical ends of the spindles appeared to extend outward. As the fish neared the end of yolk-sac stage staining intensity gradually diminished (Figure 2.5; 2.6a; 2.6b) eventually showing a complete absence of staining in corresponding areas of older larvae (Figure 2.7). The gradual loss of staining was also accompanied by a loss of the spindle shape.

The more randomly distributed cells were observed throughout the larval epidermis. They were irregular in shape, exhibiting a lightly staining cytoplasm with an obvious darkly staining "plug" of material. These cells continued to give a strong staining reaction beyond yolk-sac absorption, continuing to metamorphosis where the cells began to redistribute toward the lateral fins eventually becoming difficult to detect (Figure 2.8ab). Parallel experiments staining whole-mounted animals with Alcian Blue (pH, 2.5) following immuno-histochemistry showed a staining pattern indicative of a co-localization of both Alcian blue positive mucopolysaccharide material and immuno-positive AFP in the scattered integumental cells (Figure 2.9). The spindle shaped cells showed no similar reaction.

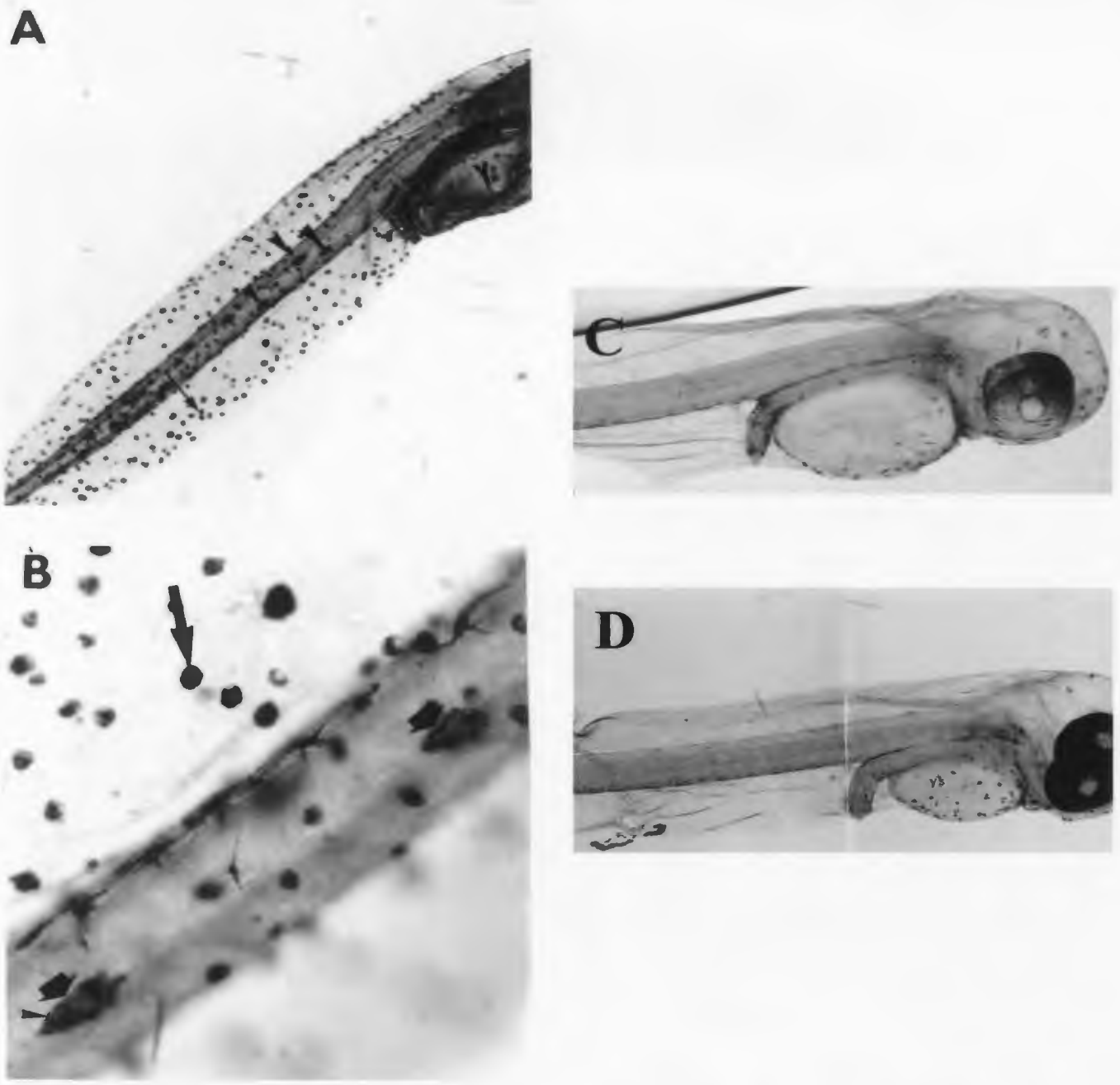


Figure 2.4 (A) Newly hatched winter flounder larva following incubation with antisera for skin-type AFP. Note spindle shaped cells situated along the lateral trunk region (arrowheads) and randomly distributed cells through the epidermis (arrow). (X165). Ys, yolk sac. (B) Higher magnification of above fish showing further detail of the cell types. Note that the lateral spindle shaped cells are composed of numerous small spherical bodies (arrowheads) (X825). (C) Normal rabbit serum control (X330). (D) Incubation with antiserum for sea raven type II AFP (X 330).

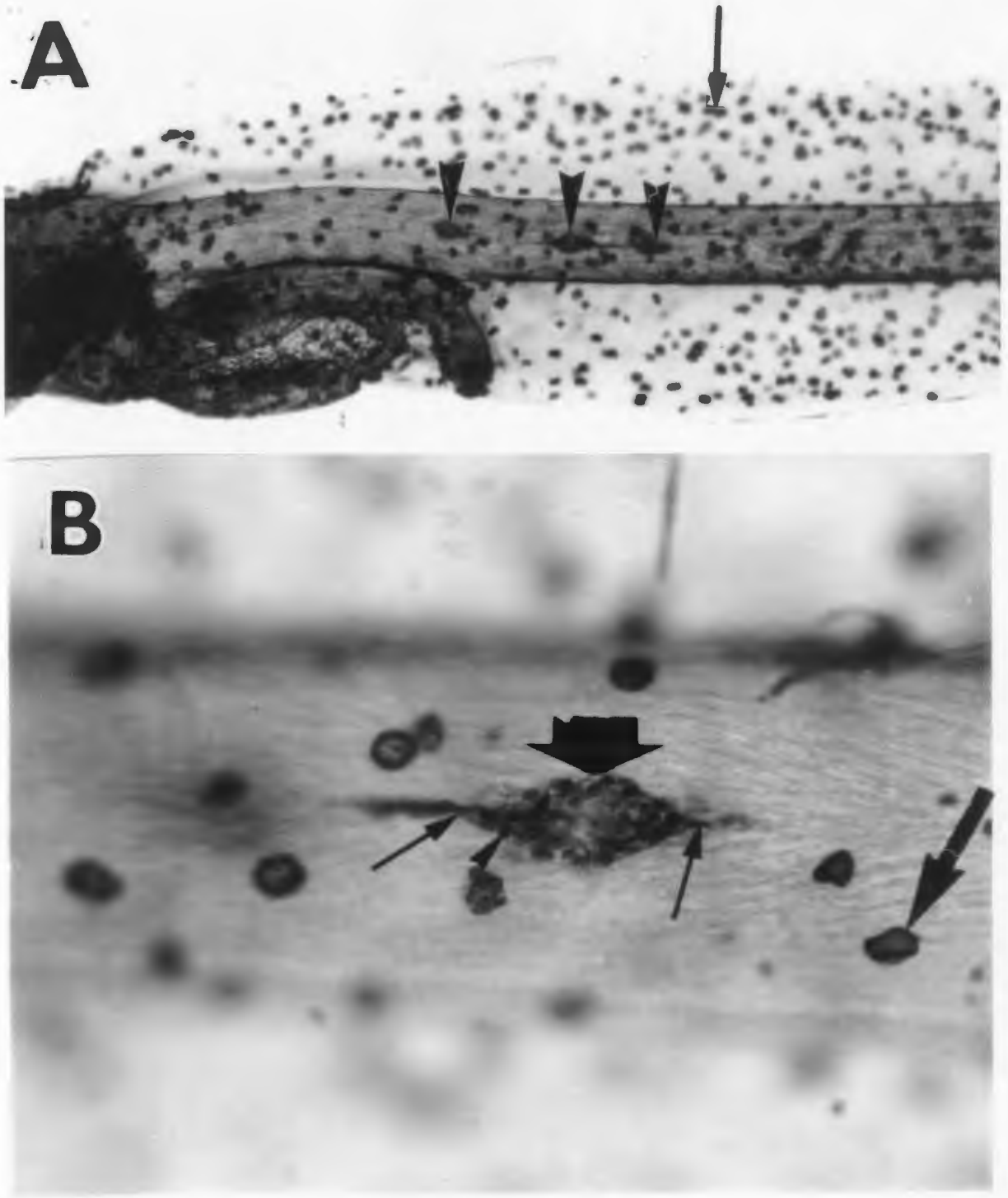


Figure 2.5 (A) Winter flounder larva at about 50% yolk sac absorption following incubation with antisera for skin-type AFP showing further detail of the distribution of the immuno-stain in the spindle cells (arrowheads) compared to the more randomly distributed epidermal cells (arrow). (X165). (B) High magnification of (A) showing detail of a lateral spindle shaped cell (big arrowhead). Note cytoplasmic extensions (small arrows) and granules (arrowhead). (X825).

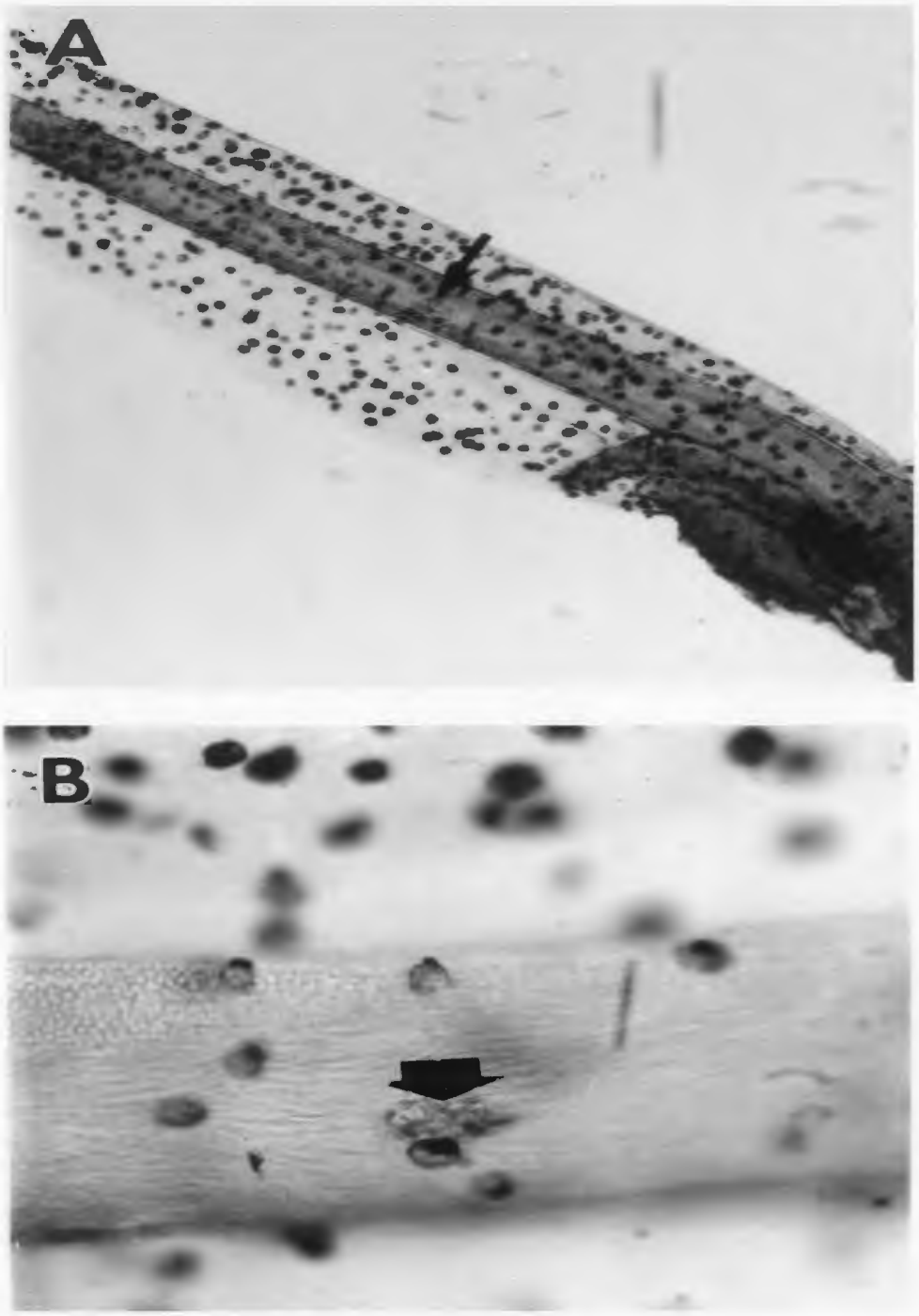


Figure 2.6 (A) Winter flounder larva at about 75% yolk sac absorption following incubation with antisera for skin-type AFP. Note the dramatic decrease in staining intensity in association with lateral spindle shaped cells (arrow) with little effect on the randomly distributed epidermal cells (X165). (B) Higher magnification of the above fish showing detail of an individual spindle shaped cell indicating a redistribution of the cytoplasm and loss of obvious granules (arrowhead) (X825).

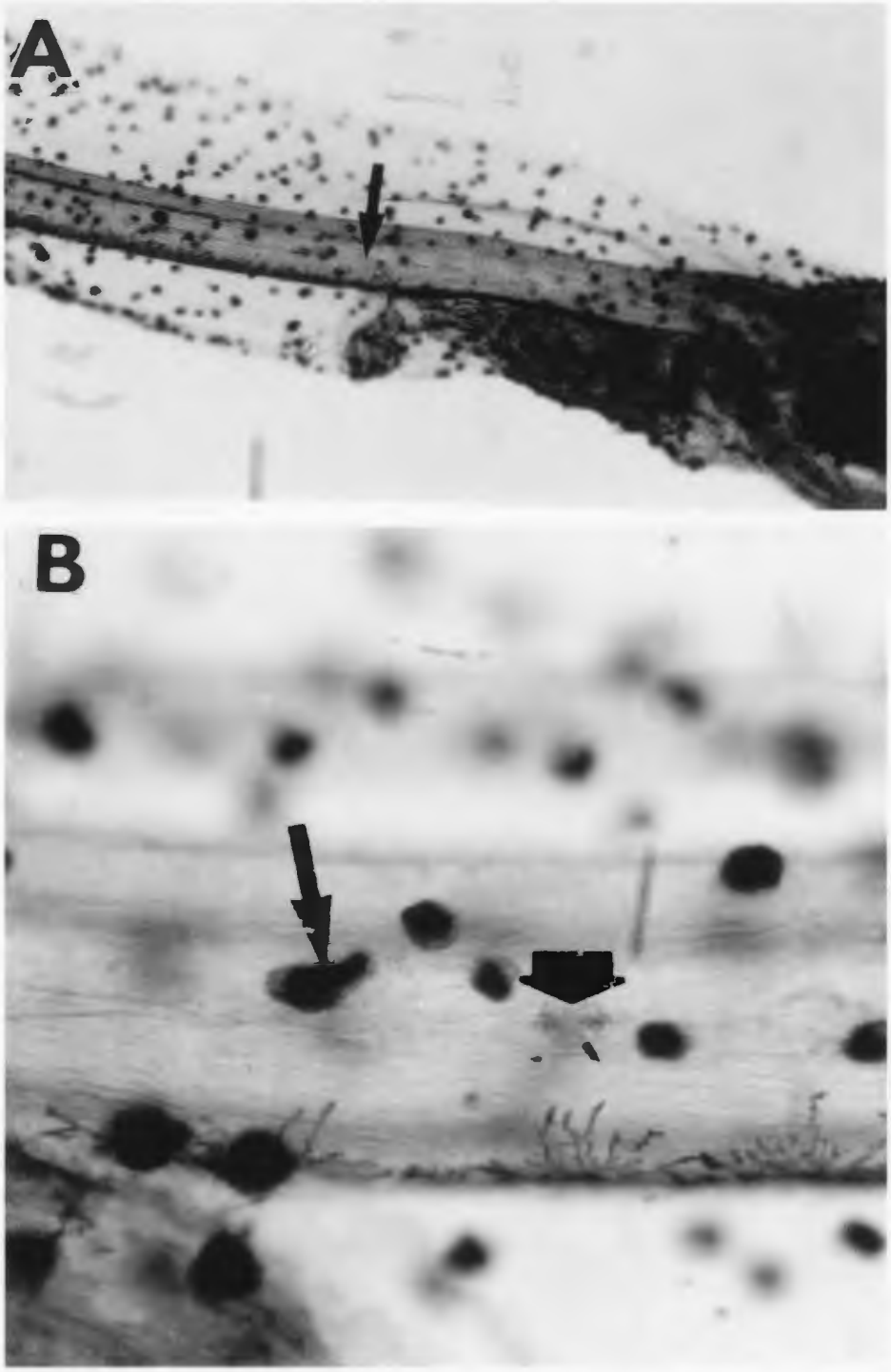


Figure 2.7 (A) Winter flounder larva following incubation with antisera for skin-type AFP, showing the significant reduction in staining associated with spindle cells at the end of yolk-sac stage (arrowhead). Note the lack of a corresponding loss of staining intensity in the more randomly distributed epidermal cells (arrow). (X165). (B) Higher magnification of the above fish showing significant loss of immuno-stain in spindle cell region (arrowhead). Note randomly distributed cells (arrow). (X825).

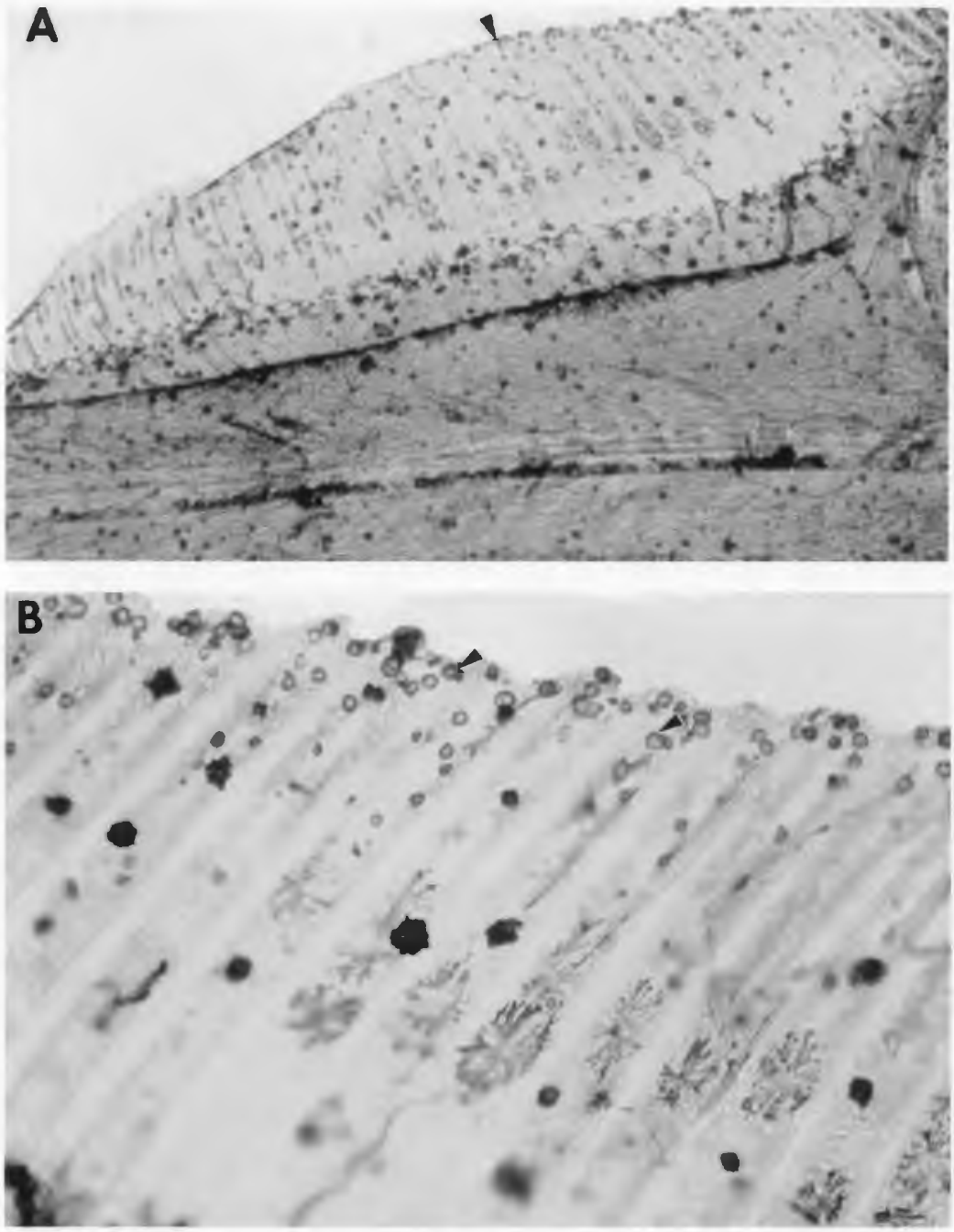


Figure 2.8 (A) Whole mount winter flounder metamorph following incubation with antisera for skin-type AFP, showing distribution of AFP positive integumental cell(arrowhead) (X165). (B) Lateral fin from above showing further detail of AFP positive cell distribution (arrowheads) (X320).

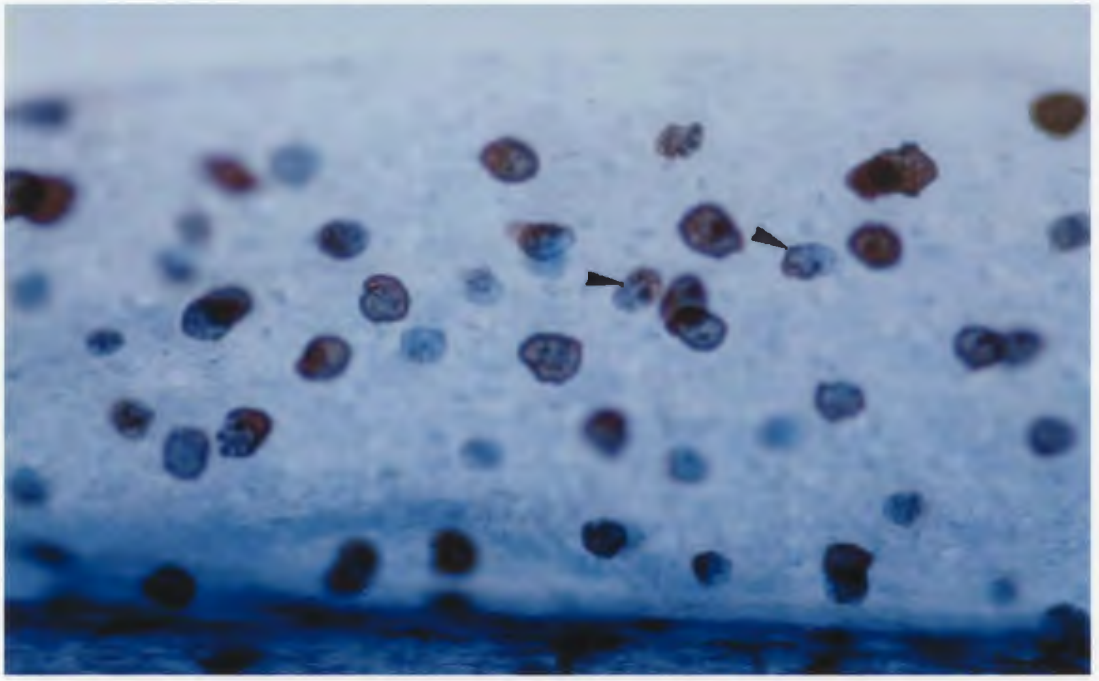


Figure 2.9 Integument of newly hatched winter flounder larva demonstrating co-localization of acid mucopolysaccharides and AFP in randomly distributed epidermal cells (arrowheads). (Alcian blue, pH 2.5) (X1320)

Western Blotting and immuno-blotting experiments have previously shown good specificity of the antisera for skin -type AFP (Unpublished results). Experiments to show equivalent specificity in tissue were run with antisera, which were pre-absorbed with a 100-fold excess of purified winter flounder skin-type AFP or serum winter flounder Type I AFP (Data not shown). In all cases, the addition of AFP eliminated the staining reactions. Further controls involved using normal rabbit serum (Figure 2.4c) or using unrelated antibody i.e.-polyclonal antisera to Type II AFP from sea raven (Figure 2.4d). These reactions were consistently shown to give negative staining results.

2.3.3 *In situ* Hybridization

Hybridization products were only observed when the antisense skin-type AFP ribo-probe was used. The liver-type AFP ribo-probe did not reveal the presence of liver-type AFP mRNA despite repeated attempts. This probe was shown to react with liver-type AFP mRNA in liver sections (see Chapter 3 and 5).

When hybridized with the skin-type AFP probe, newly hatched larvae showed a distinct staining reaction corresponding to a series of spindle shaped cell clusters running laterally from anterior to posterior (Figure 2.10a). Each cluster was paired with a companion cluster situated on the opposite side of the fish (Figure 2.10a). Initially, the staining appeared to be distributed randomly throughout cells in the cluster (Figure 2.10b). As the fish neared yolk-sac absorption, the clusters appeared to become more elongate (Figure 2.11a; 2.11b) with the reaction gradually showing a redistribution toward the apical ends of the spindle (2.12a; 2.12b). Nearing the end of the visible yolk-sac stage, the stain became highly concentrated at the spindle ends with a complete disappearance following shortly (Figure 2.13a; 2.13b). Sense probes made from the same cDNA for skin AFP showed no staining reaction corresponding to the cell clusters at any developmental stage when hybridized to identical tissue under the same conditions (Figure 2.10c). Hybridization products were at no time associated with the former scattered epidermal immuno-reactive cells.

2.4 DISCUSSION

The present chapter describes two distinctly different populations of integumental

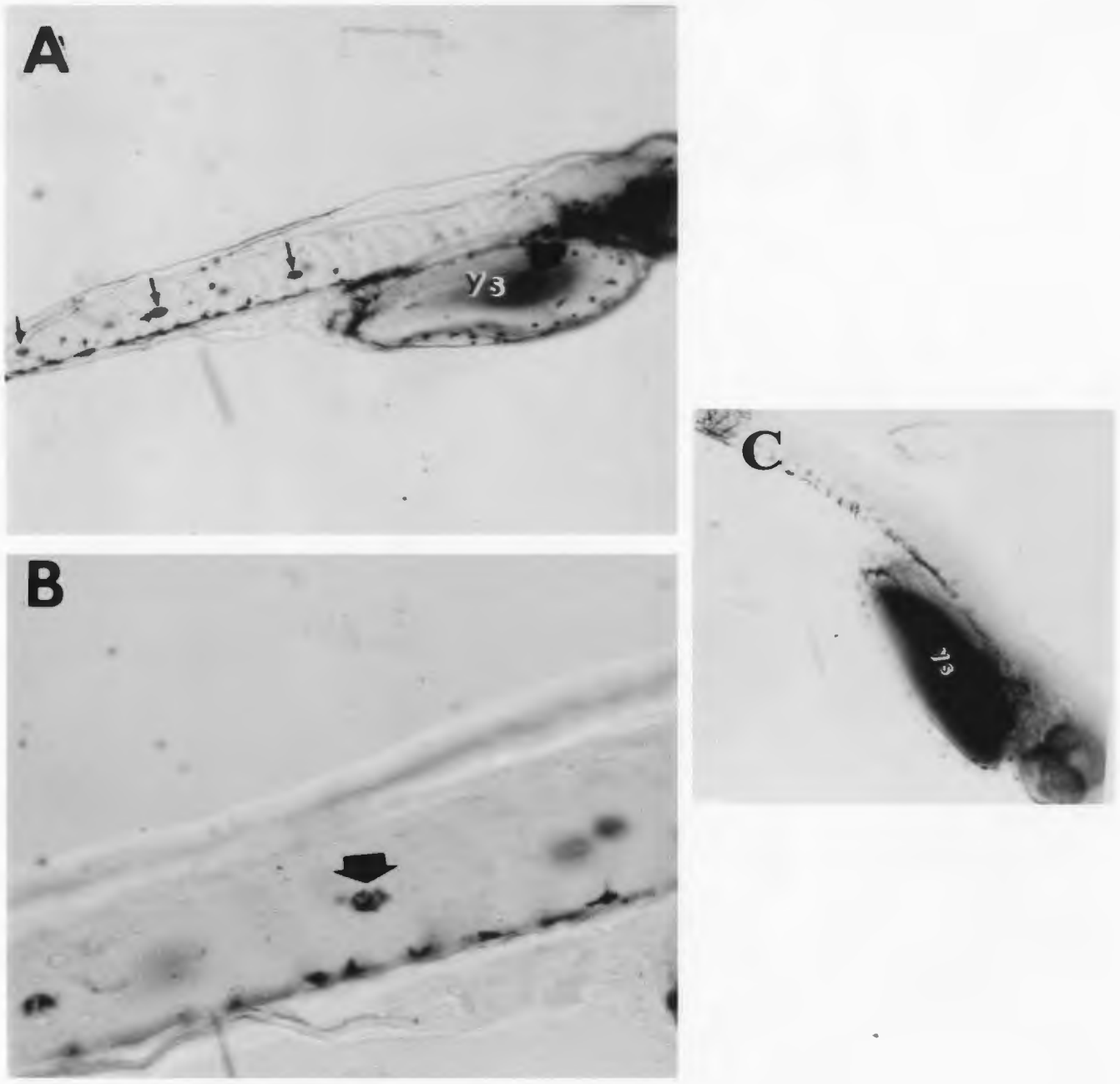


Figure 2.10 (A) Newly hatched winter flounder larva following whole-mount *in situ* hybridization with skin-type AFP ribo-probe showing staining associated with paired spindle shaped cells (arrows). (X320) ys, yolk sac. (B) Higher magnification of same larva showing detail of cell (arrow) (X825). (C) Sense probe control hybridization on newly hatched winter flounder. Ys, yolk sac (X320).

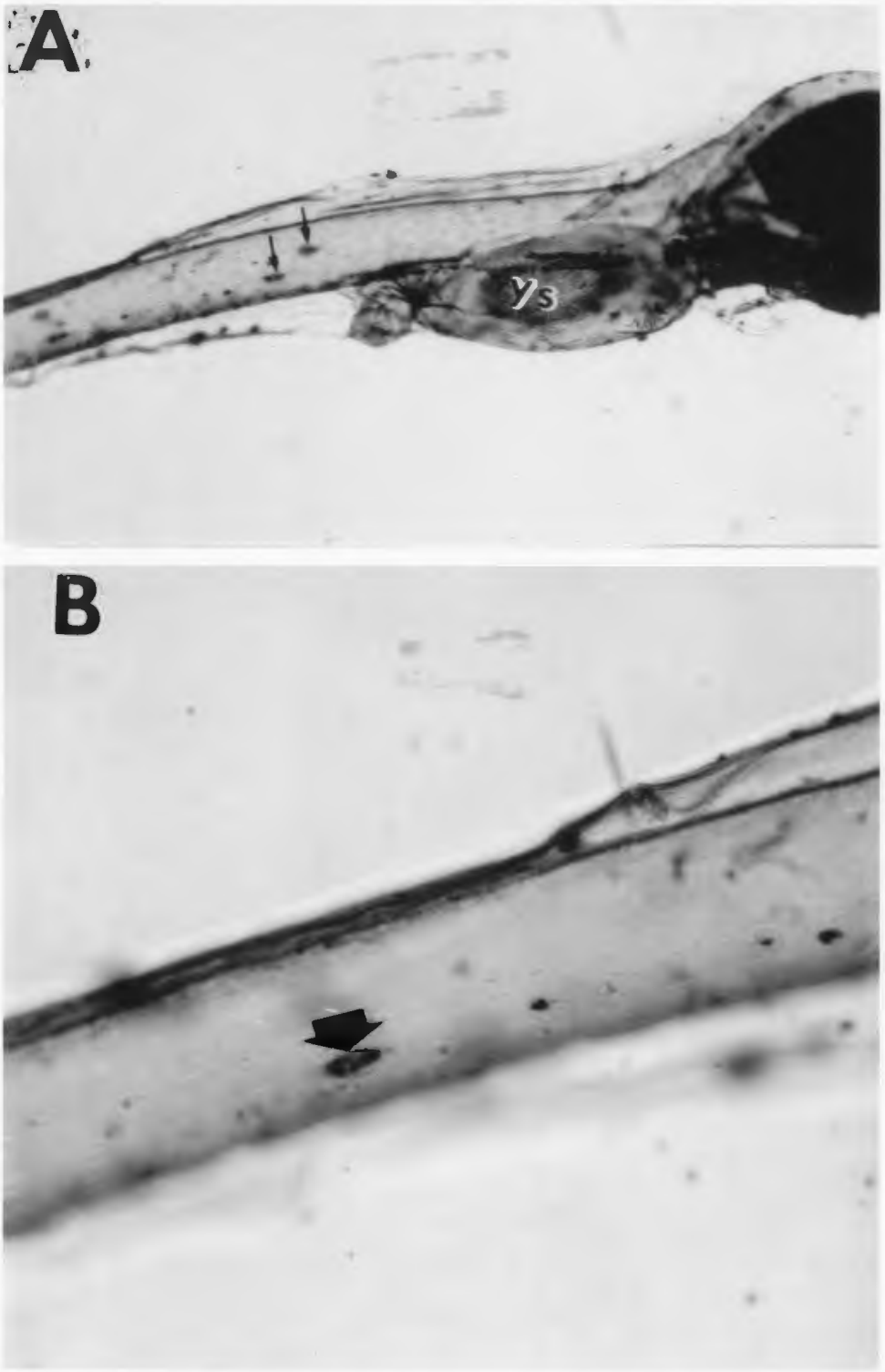


Figure 2.11 (A) Winter flounder larva shown at about 50% yolk sac absorption and following whole-mount *in situ* hybridization. Notice paired cells with a slight elongate structure (arrows). (X330). (B) Higher magnification of the same fish showing further detail of the lateral cell. (arrow) (X825). Note slight redistribution of staining reaction toward the ends of the spindle.

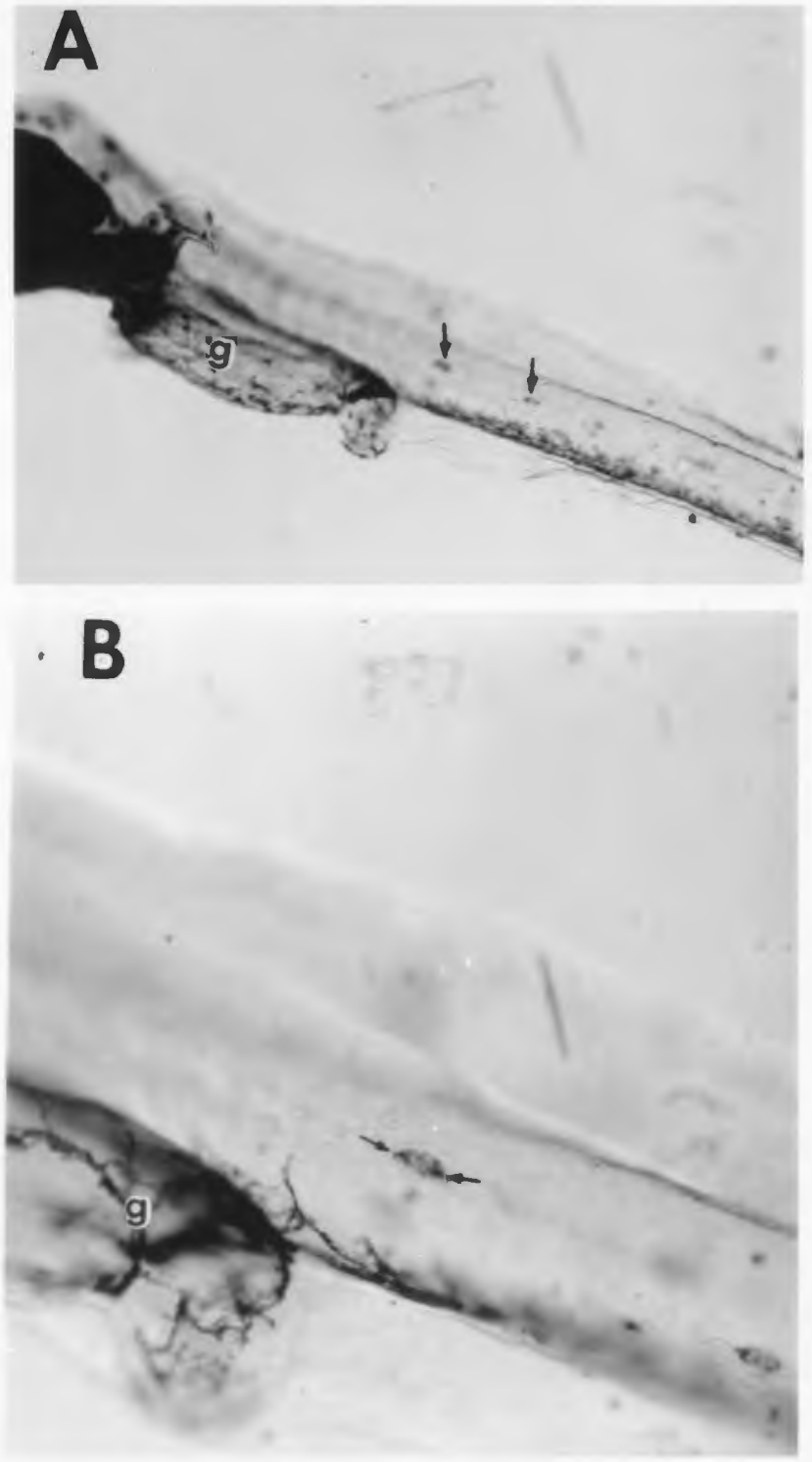


Figure 2.12 (A) Winter flounder larva near the end of yolk-sac absorption and following *in situ* hybridization. Note the further elongation of cell shape and the peripheral distribution of the signal (arrows). (X330) g, gut. (B) High magnification of same fish showing detail of the cell (arrows). (X825). g, gut.

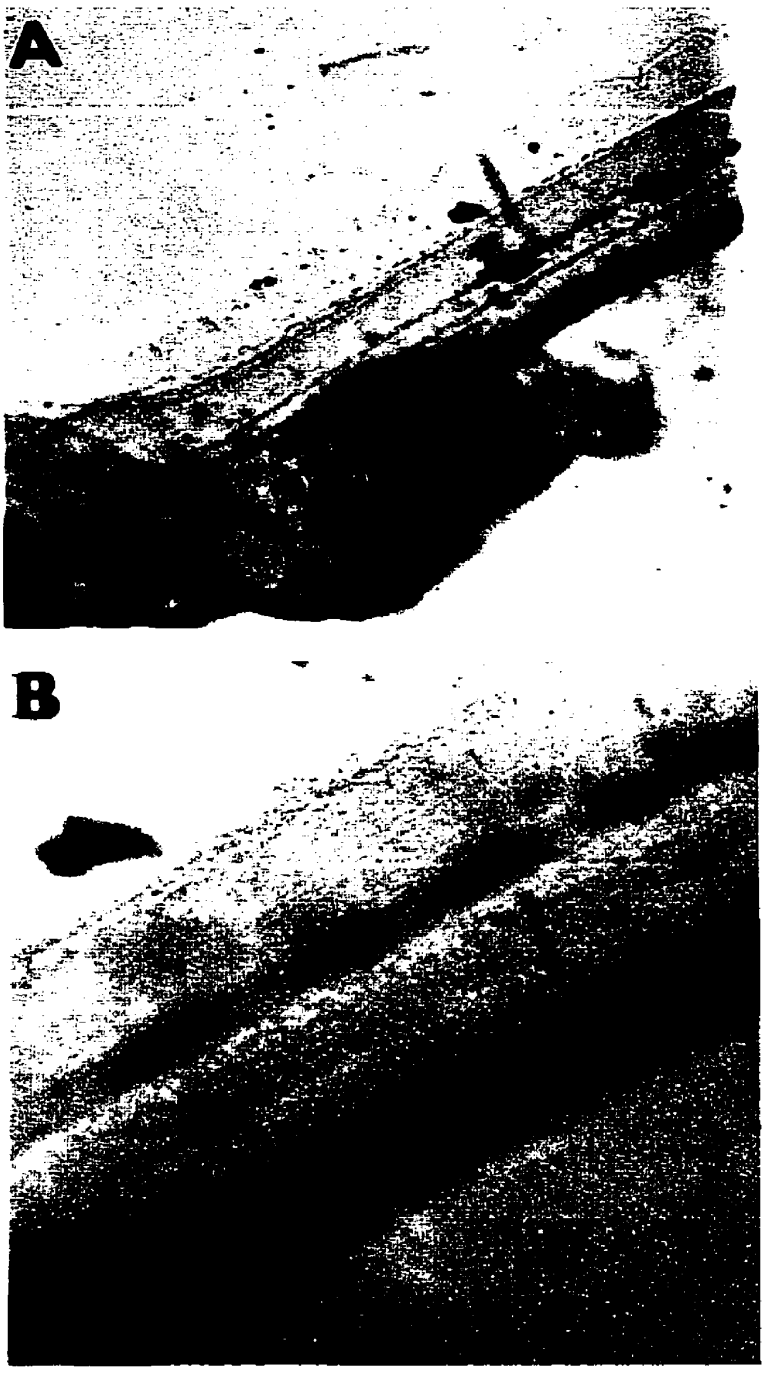


Figure 2.13 (A) Larvae at the end of yolk sac stage showing the near disappearance of stain in the cells (arrows). (X330)g, gut. (B) High magnification of lateral cells from the same fish showing extreme peripheral localization of the staining reaction (arrows). (X825).

cells that appear to be important in skin-type AFP production in post-embryonic larval winter flounder, 1. Laterally distributed spindle shaped cells, 2. Randomly distributed irregular integumental cells.

The spindle shaped cells were AFP immuno-positive and laterally paired, extending along the mid-lateral region during early post-embryonic larval development. Over the period of yolk sac absorption these cells showed a gradual redistribution and loss of staining reaction associated with reactivity with antisera for skin-type AFP. *In situ* hybridization products corresponding to skin-type AFP mRNA further verified that skin-type AFP gene expression was occurring in these cells. As with the immuno-staining, the hybridization products became undetectable following yolk-sac absorption.

A comparison with literature descriptions suggested that the spindle shaped cells were consistent in number and position with known locations of free neuromast cells or closely associated cells appearing early in the post-hatch development of other species (Blaxter and Fuiman 1989). Free neuromast cells are common in fish and while the numbers vary with species, they proliferate over development (Roper 1981; Noakes and Godin 1988; Metcalfe 1989, Webb 1989). Typically, these are the first important mechano-receptors to arise before true lateral line development and while numbers and position are known to vary, they are apparent on the sides of the head and trunk of newly hatched fish of a wide range of species (Noakes and Godin 1988; Blaxter and Fuiman 1989).

RT PCR data from this study showed the presence of skin AFP transcripts throughout post-embryonic ontogeny, however the appearance and gradual loss of AFP

expression in the spindle shaped cells of winter flounder larvae, suggests that AFP expression is only important early on in development in these cells, implying an increased need to protect them from low temperatures during the first few days post-hatch. The continued RT PCR results suggest that expression is occurring in another region of the fish i.e. gut (Chapter 4). Regardless, AFP expression here indicates that these cells likely have some importance early in the post-hatch development of the larvae.

Little is known regarding the function of free neuromast cells early in development. Blaxter and Fuiman (1989) suggested that the presence and size of these cells even at hatching, indicate that they must play a role in the sensory awareness of larvae. Blaxter and Fuiman (1989) also point out that it is unlikely that they play a role in feeding and that most likely the function is in the perception of physical stimuli from predators or conspecifics, thus it was determined that they are possibly important mechano-receptors and an early component of the developing lateral line.

In the northern limits of their range winter flounder are known to spawn in the early spring when water temperatures can be extremely variable (Petrie et al. 1988; Scott and Scott 1988). Larvae begin to occur in the wild during May and June (Chambers and Leggett 1987). During this time period, while temperatures have been known to dip as low as 0 ^{0}C, typically they range from 2 to 5 ^{0}C (Fletcher 1977; Fletcher et al. 1985b; Fletcher et al. 1987). Based on these data the chances of exposure of larvae to ice crystals and extreme cold water would be low. This raises many questions as to the actual function of AFPs in the integumental cells of post-embryonic flounder. If the chances of ice crystal exposure are minimal then what could their function be? Interestingly, while

the majority of work on antifreeze proteins in fish has concentrated on their direct affect on ice crystal morphology and the subsequent reduction in tissue fluid and blood freezing point (Davies et al. 1988), some studies have suggested that antifreeze proteins may also affect membrane ion channel function during exposure to above freezing but hypothermic temperatures (Rubinsky et al. 1990; 1991; 1992). Larvae are certainly exposed to hypothermic temperatures during their early development, thus one potential function of AFPs in early larvae could be to protect important sensory cells like neuromasts.

Before complete yolk-sac absorption the larvae show very little activity and have been shown to exhibit slow escape times for non-tactile predators (Williams and Brown 1992). The initial detection of predators, is likely dependent on the effective function of the trunk neuromasts (Blaxter and Fuiman 1989). The delicate structure and function of the neuromast hair cell/cupulae interaction (Kroese and van Netten 1988), suggests that cold water exposure could affect the efficient transfer of sensory information to the associated nerves.

The discovery of an antifreeze protein of epithelial cell origin and potential intracellular function further supports the theory that some types of antifreeze proteins could be important in enhancing or protecting physiologically significant epithelial cells (Gong et al. 1996). The sensory system association of the neuromast cells or closely associated cells in the developing lateral line and the production of antifreeze protein in alliance with them, suggests that the AFPs could be important in protecting the physiological function of these cells in the early spring when water temperatures are low enough to cause physiological problems. AFPs could be working to improve the survival

of the larval fish during the period of yolk-sac absorption.

While exhibiting immuno-reactivity with skin-type AFP antisera the more randomly distributed integumental cells also stained positively for Alcian blue at pH 2.5 suggesting that they were acid mucopolysaccharide secreting cells. Surprisingly, these cells did not however show hybridization with RNA probes for skin-type AFP genes. Also the cells did not decrease in immuno-reactivity following yolk-sac absorption but continued to show strong staining reactions until metamorphosis when the distribution of these cells changed and eventually became difficult to detect. Throughout the larval period, control competition experiments with purified AFP could consistently out compete the immuno-reaction in these cells. Related studies have shown that these cells do not correspond to those important in skin AFP production in juvenile and adult flounder (see Chapter 3). As well, mucous producing cells from other tissues (with the exception of juvenile gut (Chapter 4)) do not react with the skin AFP antibody (See Chapter 3 and 6). This indicates that skin AFP antibody only reacts with proteins from specific types of mucous secreting cells from specific tissues. The potential association of AFPs with secreted mucous here, indicates an alternative mode of secretion allowing AFP "spiked" mucous to overlay the larval epidermis acting as a barrier to ice crystal propagation, in this way forming an external epithelial layer fortified with AFP.

The importance of mucous layers on epithelial surfaces in fish, have been examined frequently (Murray et al. 1994a;b; 1996; Shephard 1994; Ottesen and Olafsen 1997; Scocco et al. 1998). Many of these studies have looked at changes in the composition of the mucous layer on exposure to adverse environmental changes, bacterial

pathogens or noxious compounds (Cole et al. 1997; Brokken et al. 1998). Recently, workers have shown that a variety of proteins are secreted in conjunction with mucous from epidermal mucous cells. Cole et al. (1997) showed that an antimicrobial peptide (pleurocidin) was present in the mucous secretions of adult winter flounder skin and that it was produced and secreted from goblet cells. The secondary protein structure of pleurocidin was described as amphipathic, alpha-helical and thus very similar to the secondary structure of Type I AFPs (Cole et al. 1997). This similarity in protein structure might allow for cross-reactivity between the antibody to Skin Type I AFP and a protein like pleurocidin thus explaining the absence of hybridization with mRNA probes. Interestingly, the absence of a skin-AFP immuno-staining reaction in goblet cells of juvenile and adult skin from winter flounder (Chapter 3) where pleurocidin should be present, would suggest that this might not be the case in older fish. One cannot however rule out the possibility that a similar but distinct protein with antimicrobial function could be important in the integumental mucous cells of larval flounder. Typically, the antimicrobial peptides have been shown to adopt amphipathic, alpha-helical conformations in hydrophobic media, suggesting that this structural type of peptide binds anionic phospholipid-rich membranes similar to bacterial membranes and emulsifies them like detergents. While the primary amino acid content of skin-AFP is different from that described for pleurocidin (alanine-rich), the secondary structure could be significant enough to suggest antimicrobial activity. It is noteworthy that AFP genes are expressed in tissues that do not ever get exposed to ice i.e. brain (Woon-Kai et al. 1998). These observations raise further questions regarding tissue specific function of AFPs.

CHAPTER 3. ONTOGENY AND SPATIAL EXPRESSION PATTERNS OF EPITHELIAL ANTIFREEZE PROTEIN IN THE SKIN OF JUVENILE AND ADULT WINTER FLOUNDER

3.1 INTRODUCTION

The expression of antifreeze proteins (AFPs) in external tissues of the winter flounder (*Pleuronectes americanus*) was originally described by Gong et al. (1992). The skin was shown to have one of the more significant levels of detectable expression when compared to other exterior tissues such as gills (Gong et al. 1992). The proteins produced from this distinct set of genes displayed unique characteristics, making them different from the liver associated AFPs (Gong et al. 1995; 1996). Some of these characteristics included constitutive expression, and a less pronounced seasonality, thus providing the potential for being differentially regulated from the liver expressed genes (Gong et al. 1995). Similarly, the absence of both secretory and prosequences indicate the possibility of an intracellularly active protein or one secreted through some atypical pathway (Mignatti et al. 1992; Gong et al. 1996).

While Gong et al. (1992; 1995; 1996) showed that expression of AFPs did occur in the skin of winter flounder, little is known regarding when these genes become active during development, what cells are involved and what their mode of action is. In Chapter 2 it was shown that expression of these skin type AFP genes can be detected in two distinct types of epidermal cells of the flounder larval integument. However expression in association with these cells was shown to decline as the fish neared the end of the larval stage. This change in expression pattern suggested the likelihood of a change in the cell

type involved in AFP expression in the flounder integument possibly as a consequence of metamorphosis.

The anatomical changes that occur at metamorphosis characterize the flounders as a group. Laroche (1981) provided a detailed description of the development of winter flounder larvae to metamorphosis and the changes associated with this transition. The key features indicating the completion of the transformation from a larva to a benthic juvenile include the complete migration of the eye, the formation of pectoral and pelvic fin rays and a thickening and a cellular diversification of the skin epidermis (Laroche 1981; Ottesen and Olafsen 1997).

The general structure and cellular dynamics of fish skin have been examined in detail (Hawkes 1974; Whitear 1986). More specifically, the histology of the adult winter flounder skin epidermis was described by Burton and Fletcher (1983). Later work concentrated on seasonal changes in the flounder epidermis associated with the reproductive cycle and or following exposure to environmental contaminants (Burton and Fletcher 1983; Burton et al. 1984; Burton and Burton 1989a,b; Burton and Everard 1991a,b).

The discovery of skin type AFPs in winter flounder raises many questions as to their mode of action. The purpose of this chapter is to describe when developmentally these proteins become detectable in " true" skin as opposed to larval epidermis and secondly to identify and describe the distribution of cell types involved in the expression of these genes. It is hoped that this work will provide fuel for the development of hypotheses regarding the biological significance and mode of action of these proteins in

winter flounder skin.

3.2 MATERIALS AND METHODS

3.2.1 Adult Maintenance

Winter flounder were captured by SCUBA (November, 1996; January, 1997) and transported to the Ocean Sciences Centre where they were held in tanks maintained overwinter with running seawater under ambient photo-period and temperature.

In early spring, animals were checked daily for signs of gonadal swelling and the presence of eggs or milt. When fish of appropriate condition were found they were immediately isolated from the main tank in preparation for spawning.

3.2.2 Spawning and Fertilization

3.2.2.1 Spawning

Eggs were collected by first carefully drying the genital pore area and then applying gentle pressure, allowing the eggs to pour easily into a clean dry container. The container with eggs was then stored in a cool place until fertilization. Sperm was collected by placing a syringe near the genital pore and gently drawing up the milt. Following gamete collection all further procedures were carried out at 5 °C.

3.2.2.2 Fertilization

500 to 1000 ul of eggs in ovarian fluid were placed in a dry sterile plastic Petrie dish and mixed gently with 100 µl of milt. The sperm were activated by adding a few drops of UV treated and filtered seawater (0.2 µm Millipore filter system). The eggs were then allowed to sit for 5 minutes to fertilize and were subsequently flooded with fresh

filtered seawater and allowed to water harden for an additional 20 minutes.

Following water hardening the eggs were flushed once more to remove any surplus milt and allowed to develop normally. Water was changed every other day by decanting off old and adding new fresh filtered seawater. Following fertilization eggs were incubated at 5 ^{0}C and hatched within 14 days.

3.2.3 Hatching and Transfer to Rearing Containers

Following hatch, 1000 to 2000 larvae were transferred to 4 litre plastic buckets lined with black plastic bags to enhance contrast for prey visibility. Buckets were set in flowing seawater baths at ambient temperature/photoperiod. Temperatures ranged from 5 to 7 ^{0}C in May/June to as high as 14^{0}C in August. The buckets contained 3 litres of filtered (0.2 μm) and UV sterilized seawater plus 500 mls of mixed algae (*Isochrysis* sp. and *Tetraselmis* sp.). Water was part changed (50%) and replaced every 2 to 3 days with mixed algae. Animals were fed daily on a ration of 5000 rotifers (variety: *Sap)* per litre and supplemented with a further 300 millilitres of algae until metamorphosis (about 60 days). Once they had reached metamorphosis the fish were transferred to aquaria with flowing seawater and aeration. Temperatures were allowed to remain ambient showing a maximum of 14 ^{0} C in August and dropping gradually to approximately 5 ^{0} C in November and then decreasing further to near 0 ^{0} C in December, January and February. Temperature began to rise again in April and May and continued to do so through the summer. All animals were maintained and killed according to the guidelines set by the Canadian Council of Animal Care (Olfert et al. 1993).

3.2.4 Tissue Sampling

3.2.4.1 RNA Analysis

Three adult winter flounder were sampled in either December (winter) or July (summer). All fish were sacrificed by a blow to the head followed by cervical severance. Scale (skin) tissues were removed and immediately flash frozen in liquid nitrogen. Tissues were stored at -70° C until analysis.

3.2.4.2 *In situ* Hybridization and Immuno-histochemistry

Five to ten fish were sampled monthly from the time of metamorphosis to the middle of the first winter (January/February 1998). Samples were also taken during the following summer (July 1998) and during December 1998. In addition, 1-cm^2 blocks of skin were excised from the pigmented and nonpigmented side of adult winter flounder in either winter (February) or summer seasons (June or July). Tissues were either fixed in 4 % paraformaldehyde in PBS for 30 minutes for *in situ* hybridization or fixed for 24-48 hours in Bouins fluid for mucous histochemistry and immuno-histochemistry. All fixations were carried out at room temperature. Following fixation tissue was processed for embedding in paraffin.

3.2.4.3 Mucous Histochemistry

The examination of skin structure, mucous content and chemistry was performed using Alcian blue (AB) pH 2.5/periodic acid-Schiffs reagent (PAS) (Bancroft and Cook 1984). Histochemical reactions showed blue for acid mucins, red for neutral mucins and reddish purple for a combination of neutral and acid mucins.

3.2.5 Total RNA Extraction and RT PCR

3.2.5.1 Total RNA Extraction

Total RNA was extracted from whole scales using either the Qiagen®, Rneasy® Mini RNA prep kit following disruption and homogenisation using the QIAshredder ® and the included homogenisation buffer or alternatively with Trizol ® reagent, according to the manufacturer's suggested protocol. Following extraction, RNA was treated with Life Technologies amplification grade DNAse I according to the manufacturer's recommendations, aliquoted and frozen at -70 ° C until use.

3.2.5.2 PCR Primers

PCR primers (WFP9A and WFP9B) were designed to amplify a 250 base pair (bp) fragment analogous to an AFP cDNA clone (WFP9) originally isolated from a winter flounder skin cDNA library (Gong et al. 1996). The sequence of the primers corresponded to 5'-GTCGAACACTCAGAATCACTG-3' and 5'-AGCTTCTCAGGTTTATTCAGC-3', respectively.

3.2.5.3 RT PCR

One microgram of total RNA, primers, and the required components of the Life Technologies SuperScript ™ One-Step ™ RT-PCR System were mixed according to the manufacturer's recommendations. The mixture was incubated at 50 ° C for 30 minutes for the reverse transcription reaction and then carried through PCR on a Perkin-Elmer Model 480 thermal cycler. The amplification consisted of 35 cycles at 94 ° C, (30s), 60 ° C (30s), and 68 ° C (45s).

3.2.5.4 Sequencing of PCR Products

At least five consecutive RT PCR reactions were run and the reactions pooled, DNA ethanol precipitated and then resuspended in 50 ul of sterile water. This volume was run on a 2% agarose gel for at least 1 hour at 100 V and stained with ethidium bromide. The gel fragment was excised and the DNA purified using the QIAquick Gel Extraction Kit (Qiagen).

Purified PCR products and appropriate primers were subsequently sent to the DNA Sequencing Facility/The Centre for Applied Genomics at the Hospital for Sick Children, Toronto Ontario. For comparison, obtained sequences were checked against known AFP sequences to verify the result.

3.2.6 *In Situ* Hybridization

3.2.6.1 Probe Production

Winter flounder skin-type antifreeze protein cDNA WFP9 was ligated to an EcoRI/XhoI site in $_p$Bluescript SK (+/-) plasmid vector (Gong et al. 1996). Winter flounder liver antifreeze protein cDNA pKenc-17 (Pickett et al. 1984) was subcloned from plasmid vector PUC-18 and ligated to a BamHI/XBAI site in $_p$Bluescript SK (+/-). In order to make antisense or sense RNA probes, plasmids containing WFP9 were linearized with either EcoRI or XhoI respectively whereas those containing pKenc-17 were linearized with either BamHI or XBAI respectively. Probes were produced during *in vitro* transcription (Melton et al. 1984; Jowett and Lettice 1994). The reaction mixture contained 5 µl linearized plasmid (1 µg/ul), 5 ul 10x buffer, 2 µl 250 mM 1, 4-

Dithiothreitol (DTT)(Sigma), 0.5 µl RNAsin (Sigma), 10 µl 2.5 mM NTP mix containing digoxigenin tagged UTP (Boehringer Mannheim # 1209) and 1.5 µl of the relevant RNA polymerase, i.e. T7 or T3. The mixture was incubated at 37 ^{0} C for 15 minutes and then precipitated with 5.0 µl of 0.2 M Ethylenediaminetetraacetic acid (EDTA) , pH 8.0, 6.25 µl 4 M lithium chloride (LiCl), and 200 µl cold ethanol overnight at -20 ^{0} C. Pellets were resuspended in 1 ml of hybridization buffer (50 % deionized formamide, 5 x sodium chloride, sodium citrate (SSC), 1 mg/ml Torula RNA, 100 ug/ml Heparin, 1 x Denharts, 0.1 % Tween-20, and 0.1 % 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and stored at -20 ^{0} C.

3.2.6.2 Section Pre-treatment

Protocol was modified from Uehara et al. (1993). Seven-micrometer wax sections were deparaffinized in two washes of xylene for 10 minutes each and subsequently rehydrated through an ethanol series (2 minutes in each of 100 %, 95 %, 70 %, and 50 %). Tissue was then digested in proteinase K (20 µg/ml) for 15 minutes at 37 ^{0}C, rinsed in 1 x fish saline, refixed in 4 % paraformaldehyde in 1 x fish saline for 2 minutes and washed in 1 x fish saline with 2 mg/ml glycine for 2 minutes. Nonspecific binding sites caused by electrostatic interaction between the probe and basic protein groups were blocked by equilibrating specimens for 5 minutes in 0.1 M triethanolamine buffer pH 7.5 and rinsing in the same buffer with 125 ul acetic anhydride for 10 minutes at room temperature. Tissue was further rinsed in 2 x saline sodium citrate (2 x SSC) before being dehydrated in a graded series of ethanol and air dried for 10 minutes

3.2.6.3 Hybridization

Sixty micro-litres of probe solution were added to each slide and cover slipped. Slides were placed in a moist chamber with 50 % formamide in 2 x SSC, covered and sealed with parafilm. Hybridization was allowed to occur for up to 24 hrs at 45 °C.

3.2.6.4 RNAase Treatment and Post-hybridization Stringency Washes

Cover-slips were removed by washing gently in 2 x SSC and slides were washed in 50% formamide in 2 x SSC for one hour at 45 °C and subsequently rinsed in 10 mM Tris, pH 8.0, 500 mM NaCl for two minutes. Tissues were then incubated with RNAase T_1 (10 units/ml) and of RNAase A (20 μg/ml) in the same buffer at 37 ° C for 30 minutes. Following RNAase treatment, slides were washed once more in the same buffer for 30 minutes at 37° C. Slides were next washed at 50 °C for 30 minutes in each of 2 x SSC, 0.5 x SSC and 0.1 x SSC.

3.2.6.5 Immunological Detection of Hybridization Products

Slides were rinsed briefly in 0.1M Tris, pH 7.5, 150 mM NaCl and then incubated in the same buffer for 30 minutes with 1% BSA at room temperature to block nonspecific sites. Sections were then treated with a 1/250 dilution of anti-digoxigenin antibody conjugated to alkaline phosphatase in the above buffer with 1% BSA and incubated overnight at 4 ° C. Following antibody treatment slides were washed for 30 minutes in 0.1M Tris, pH 7.5, 150 mM NaCl with 1% BSA at room temperature to remove any unbound antibody. The chromogenic reaction was as outlined for the whole-mount *in situ* hybridization protocol in Chapter 2 (2.2.7.3). Approximately, 500 ul of BCIP + NBT

in chromogenic buffer was added to each slide and the reaction allowed to proceed in the dark until the desired background was achieved (usually within 20 minutes). The reaction was stopped by washing slides in 1 X FS and then dehydrated rapidly through ethanol series, clearing briefly in xylene and mounted in Cytoseal (Stephens Scientific) for observation and photo-microscopy.

3.2.7 Immuno-histochemistry

3.2.7.1 Tissue Preparation and Blocking of Non-specific Sites

Tissue sections were deparaffinized by washing twice in xylene for 10 minutes, hydrated through ethanol series and washed three times 5 minutes in PBT (1x Phosphate Buffered Saline, pH 7.4, 0.1% Triton-X-100, 2mg/ml BSA). Non-specific sites were blocked by incubating tissue in 20% goat serum in buffer for 15 minutes at room temperature.

3.2.7.2 Antibody Treatment

Winter flounder skin AFP was secretory expressed in *E. coli* JM105 and purified to homogeneity by Lin et al. (1999). Polyclonal antisera to winter flounder skin AFP was produced in rabbits following conjugation to keyhole limpet hemocyanin (Lin et al. 1999).

Antiserum was diluted in PBT with 5 % goat serum and applied to sections for 30 minutes at room temperature, dilutions ranged from 1/500 to 1/1000. Following antibody application, slides were washed four times in PBT for five minutes each.

The secondary antibody (goat-anti-rabbit conjugated to horseradish peroxidase (HRP) (Sigma)) was diluted in PBT with 5 % goat serum, applied to sections and

incubated in an identical manner to the above. Dilutions ranged from 1/300 to 1/500. Slides were again washed four times in buffer for five minutes each.

3.2.7.3 Stain Development and Mounting

Following the final wash, sections were covered with 500 ul of Immuno-Pure Metal Enhanced DAB substrate (Pierce) and incubated at room temperature for 5 minutes or until the chromogenic reaction was of favourable intensity. Reaction was then stopped by washing in PBT, dehydrated in ethanol series, cleared in xylene and mounted in Cytoseal (Stephens Scientific) for observation and photography.

3.2.7.4 Controls

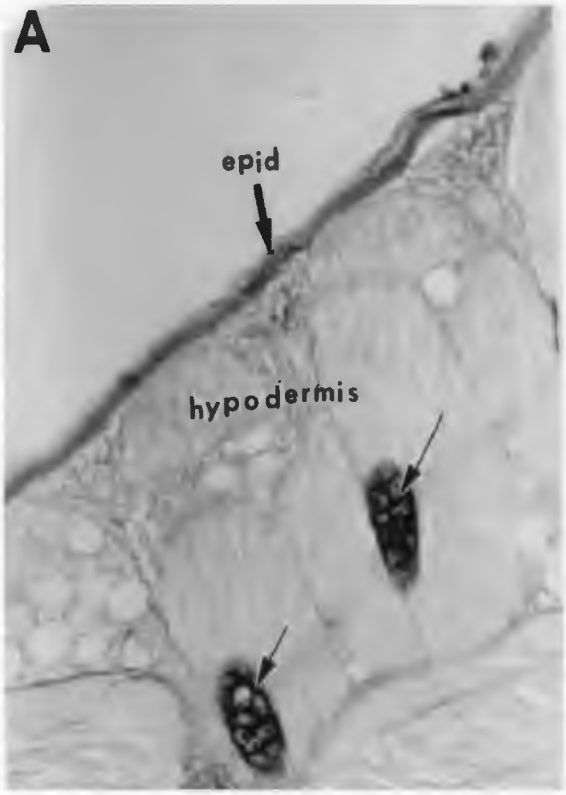
In parallel sets of experiments, negative controls involved the replacement of the primary antibody with normal rabbit serum or antisera for Type II AFP from sea raven. Further experiments showed that the cell specific staining could also be out competed with a 100-fold excess of purified AFP Type I. Immuno-blotting and Western blotting have previously shown antisera/protein specificity (C. Hew, personal communication).

3.3 RESULTS

3.3.1 Histology and Mucous Histochemistry

Generally first metamorphs were observed in early to mid August when water temperatures were in the range of 13 to 14 ^{0}C. At this time winter flounder larvae show a distinct broadening of the body as the fish orients itself for a laterally compressed lifestyle. Histologically the integument at this stage showed a thickened dermis and hypodermis with deeper layers of subcutaneous muscle (Figure 3.1a.). The epidermis however was very thin lying upon a prominent basement membrane that stained strongly

Figure 3.1 A. The integument of a newly metamorphosed juvenile winter flounder (August), showing thin epidermis (epid) with prominent basal lamina and thick dermal region with subcutaneous muscle and developing cartilaginous fin rays (arrows). (X660) B. Skin of a winter flounder juvenile in November of its first year. Note significant epidermal thickening compared to (A) and the proliferation of goblet cells (arrow). The superficial layer of cells (arrowhead) consistently stained positive for acid glycoproteins. (X660). C. Skin of a winter flounder juvenile in February. Note proliferation of goblet cells (asterix) and a further thickening of the epidermis (epid). As with the November sample the superficial layer of cells stained with Alcian blue (arrowhead). Melanophore are also obvious in the dermal region (thick arrowhead).(X660). D. Skin of a winter flounder juvenile in July of its second summer. Note decrease in the number of epidermal cell layers compared to the winter fish and the conspicuous lack of goblet cells (X660) hypo, hypodermis.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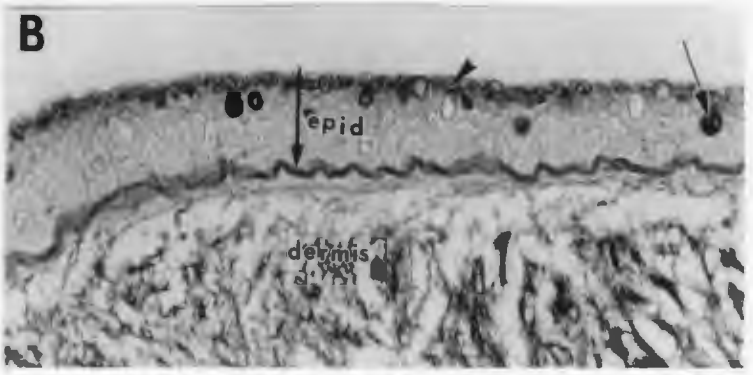
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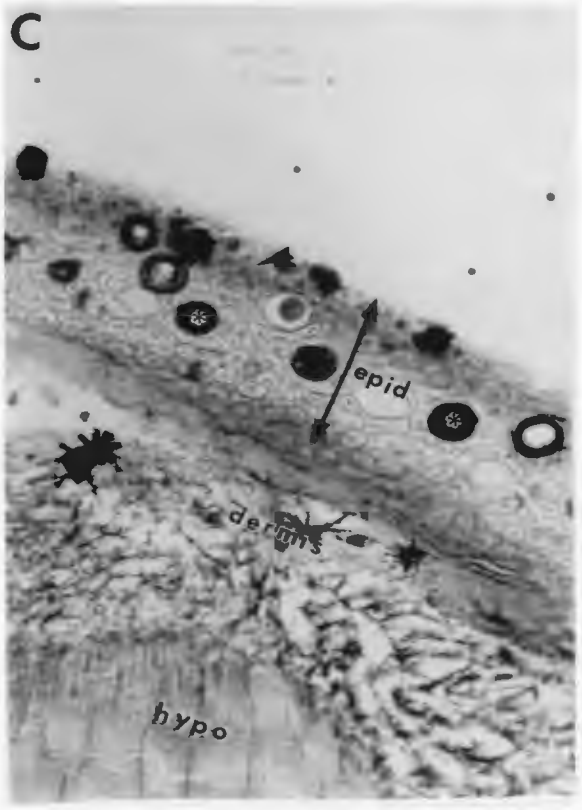
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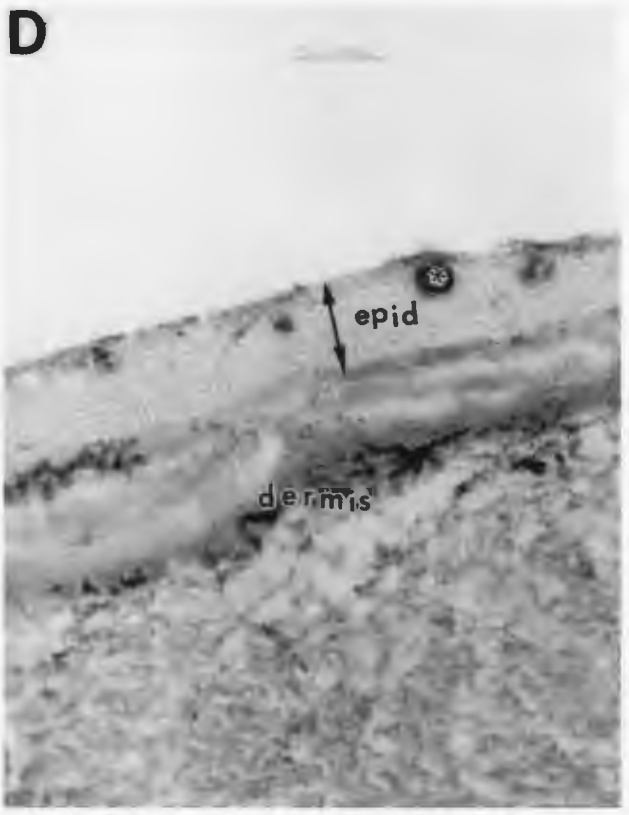
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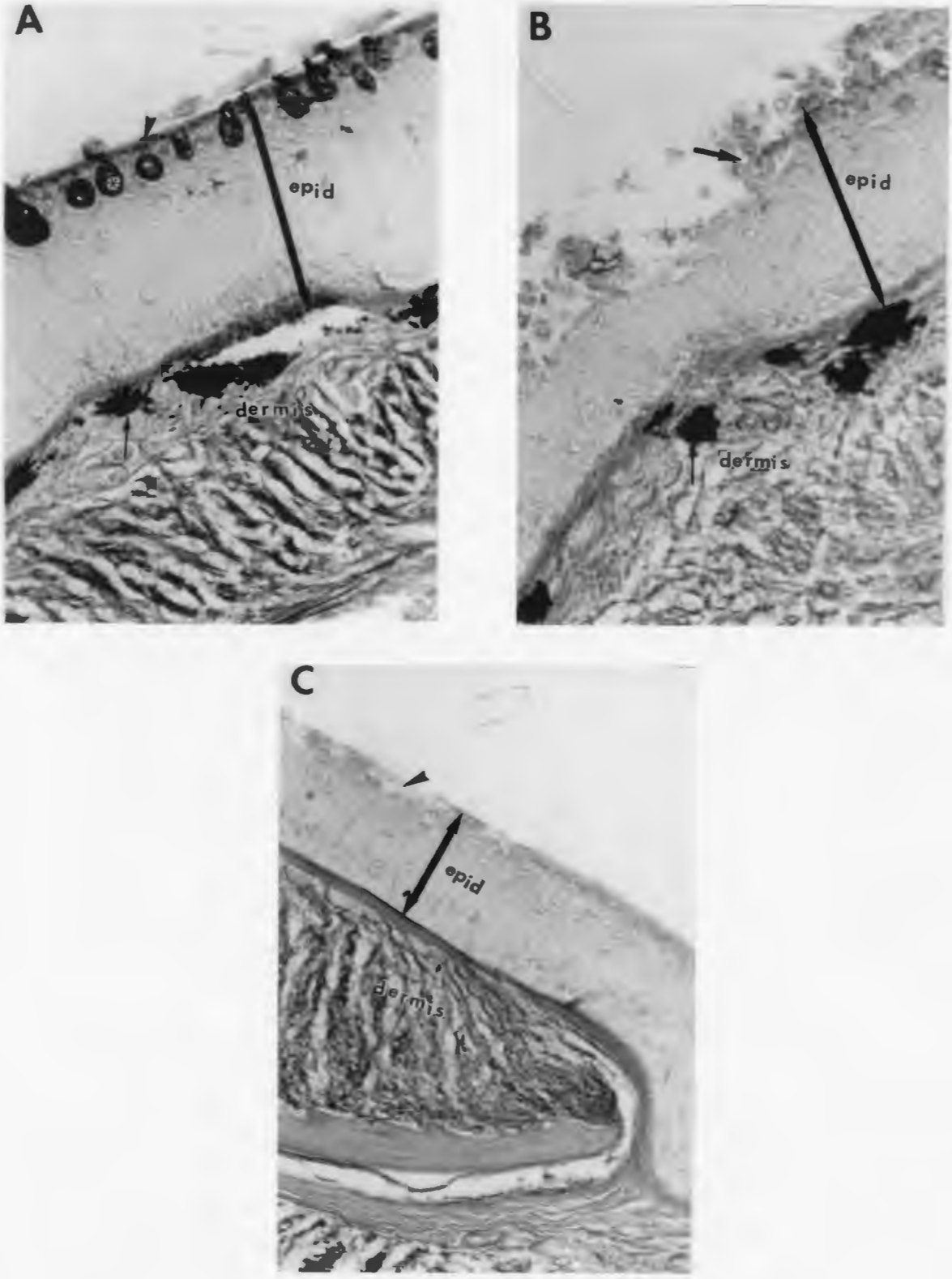
for PAS (Figure 3.1a). The epidermis changed dramatically over the subsequent fall and into the winter. The majority of this change is associated with a dramatic epidermal thickening (Figure 3.1bc). Epidermal mucous cells also become prominent at this time. Histochemically, these cells appear to contain mixtures of acid and neutral mucins (Figure 3.1bc). The outermost layers of epidermal cells, also known as the superficial layer, consistently stained strongly with Alcian blue, indicating the presence of acid glycoproteins. Sections through skin of juveniles in their second summer showed decreases in cellular diversity and evidence of epidermal thinning.

Few goblet cells were present at this stage and they only stained for acid mucins. Once more the outermost later of epidermal cells continued to show a strong reaction with Alcian blue, indicating that within this layer of cells are numerous acid glycoprotein producers (Figure 3c). The epidermis began to show evidence of an increase in mucous cell diversification as the fish moved into its second fall. The goblet cells began to show mixtures of neutral and acid mucins, as well as just neutral mucins. This trend continued into the winter season.

Adult fish sampled in the winter (January) when water temperatures are near 0 ^{0}C also had a distinctly thickened ocular-side epidermis, resulting from numerous layers of cells (Figure 3.2). As was the case in the juvenile fishes, the epidermal layer rests upon a PAS positive basement membrane. Histochemically, with the exception of goblet cells and the outermost layer of cells, the majority of the epidermis did not stain with Alcian blue/PAS during the winter season. The staining reactions in these cells once more indicate a predominance of acid glycoproteins (Figure 3.2).

Figure 3.2 A. Section through the ocular-side skin of an adult winter flounder during winter showing thick epidermal layer (epid) with numerous goblet cells secreting mucous onto the surface (asterix). As with the juveniles, the superficial layer of skin continues to react with Alcian blue (arrowhead). Note the presence of dermal melanocytes (arrow). (X660) B. Section through the ocular-side skin of adult winter flounder in July. Note the extensive necrosis at the epidermal (epid) surface. Note the presence of dermal melanophores (arrow). (X660). C. Section through the blind-side skin of an adult winter flounder in July. Note slight necrosis at the superficial layer and minimal reaction with Alcian blue (arrowhead) (X660) epid, epidermis.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Ocular side skin from summer fish demonstrated an apparent decrease in overall epidermal thickness. The epidermis also showed a slight cellular necrosis at the outer surface. Blind side epidermis from both adult males and females appeared slightly thicker than the ocular surface and the mucous histochemical staining appeared slightly less intense although the distribution was similar (Figure 3.2).

3.3.2 RT PCR

3.3.2.1 RT PCR on Total RNA from Adult Flounder Scale

RT-PCR with skin primers and total RNA isolated from winter flounder scale (ocular side) consistently gave a strong 250-bp band corresponding to the original skin cDNA clone (Figure 3.3). No difference was observed in expression between the scales taken from the ocular side and the blind side.

3.3.2.2 Sequence Comparison

All PCR products showed high levels of identity with the original skin AFP clone WFP9. The amino acid sequences translated from RT PCR products of adult scale showed 100 % identity to the original clone from which the primers were designed (Figure 3.4ab).

3.3.3 *In situ* Hybridization

Whole sections of juvenile epidermis were examined from September through February. Early fall tissue showed no indication of a hybridization signal in any histological region using skin AFP specific probes (Figure 3.5a). November juveniles however, showed a light but distinct reaction in association with epidermal cells (Figure 3.5b). The dermis and other underlying tissues did not show any reaction. February

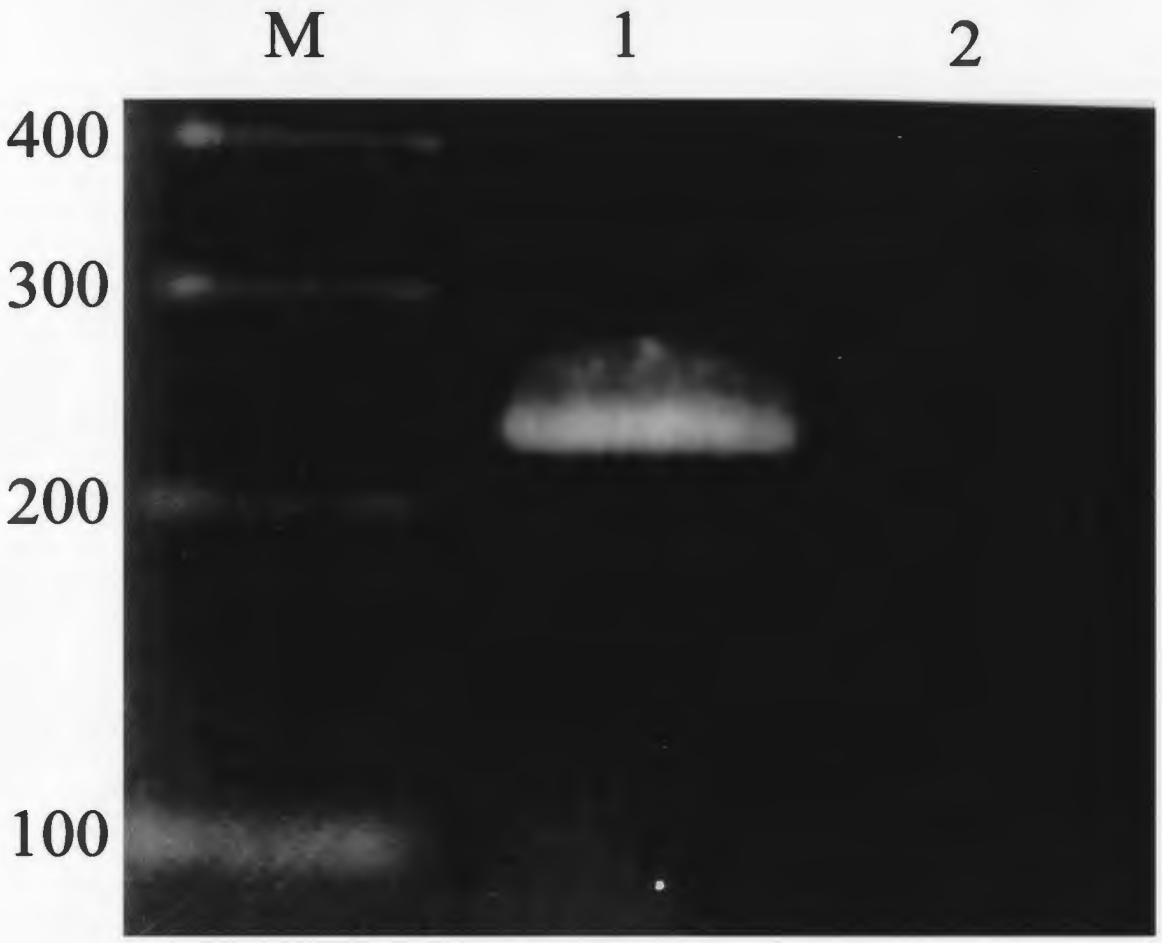

Figure 3.3 RT-PCR using primers for skin AFP WFP9, showing products amplified from total RNA isolated from winter flounder scale (lane 1). Lane 2 shows the results from a similar experiment in the absence of reverse transcription. Samples were run on a 2% agarose gel at 100V for one hour and stained in ethidium bromide.

(A)

Source

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WFP9(skin) 1 GTCGAACACTCAGAATCACTGACATCAACATGGACG
Scale      1 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

WFP9(skin)37 CACCAGCCAAAGCCGCCGCAGCCACCGCCGCCGCC
Scale      37- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

WFP9(skin)72 GCCAAGGCCGCCGCAGAAGCCACCGCCGCCGCAGC
Scale      72- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

WFP9(skin)107 CGCCAAAGCAGCAGCCGACACCAAAGCCAAAGCAG
Scale      107 T- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

WFP9(skin)142 CCCGTTAAGGATCGTGGTCGTCTTGATGTGGGATC
Scale      142- - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

WFP9(skin)177 TGTGAACATCTGAGCAGCGAGATGTTACCAATCTG
Scale      177 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

WFP9(skin)212 CTGAATAAACCTGAGAAG CTG ttt
Scale      212 - - - - - - - - - - - - - - - - - - - - - - - -A ---
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(B)

Source

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           1              10              20              30
WFP9       MDAPAKAAAATAAAAKAAAEATAAAAAKAAADTKAKAAR
Scale      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
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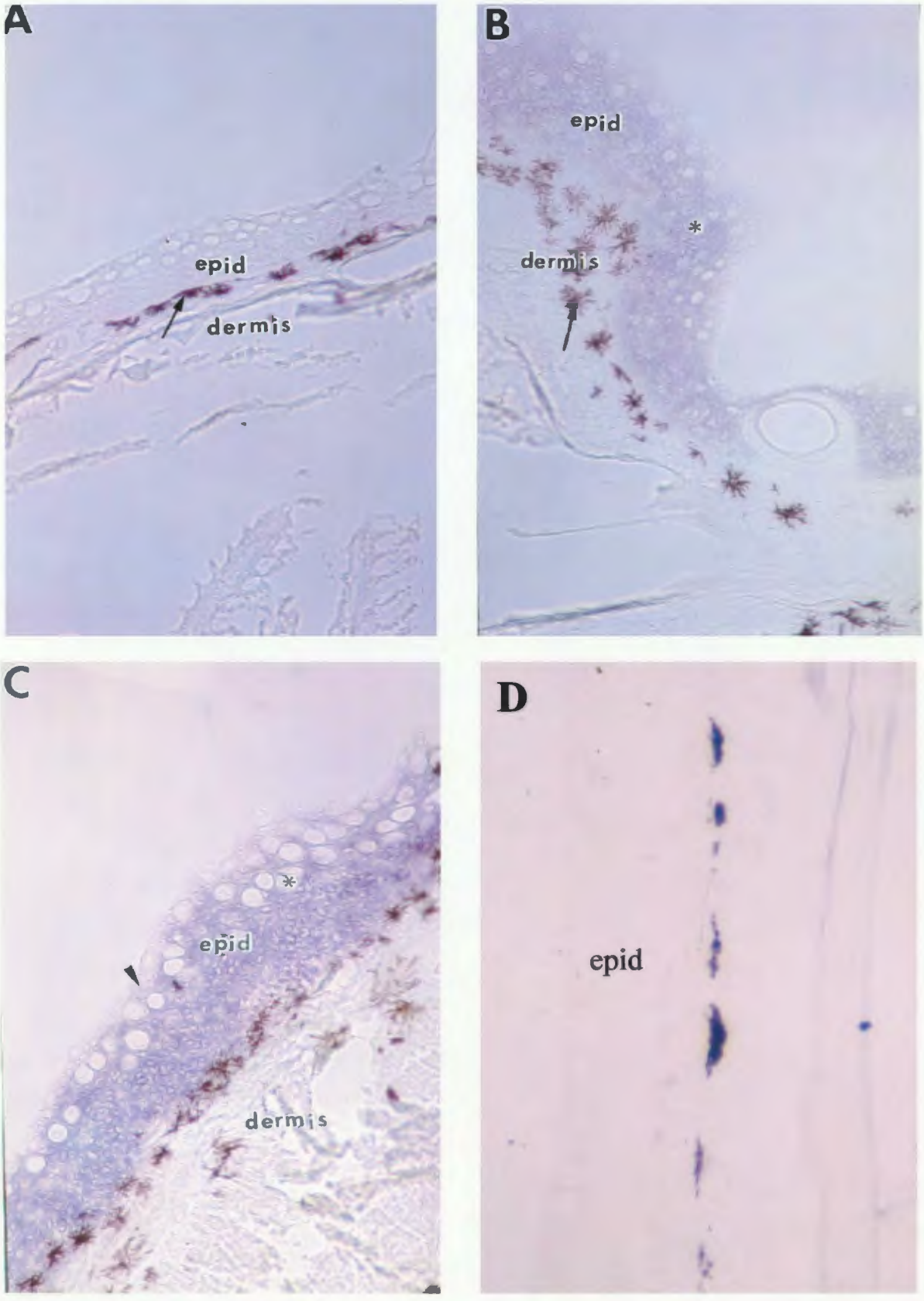
Figure 3.4 A) Nucleotide sequences of skin AFP cDNA amplified from total RNA isolated from adult winter flounder scale using primers specific for the known sequence of skin AFP clone WFP9. Dashes represent regions of identity. B) Translated protein sequences from the above cDNA showing regions of identity.

Figure 3.5 Juvenile flounder *in situ* hybridization with a riboprobe for skin AFP-juvenile flounder. A. Skin epidermis of a juvenile winter flounder sampled in October of its first year. Note lack of any staining reaction in epidermal layer (epid). Dark areas correspond to melanocytes (arrow). (X330). B. Skin epidermis of a juvenile winter flounder in November of its first season. Note light staining reaction in epidermal layer. Goblet cells are free of stain (asterix). Dermal layer is also clear of stain. Note the dermal melanphores (arrow). (X330). C. Skin epidermis of a juvenile flounder in February. in the epidermal layer show a strong signal, whereas none is detected in the dermis. Goblet cells and those of the superficial layer also do not show significant staining reaction (X330). D. Sense control (epid) (X330).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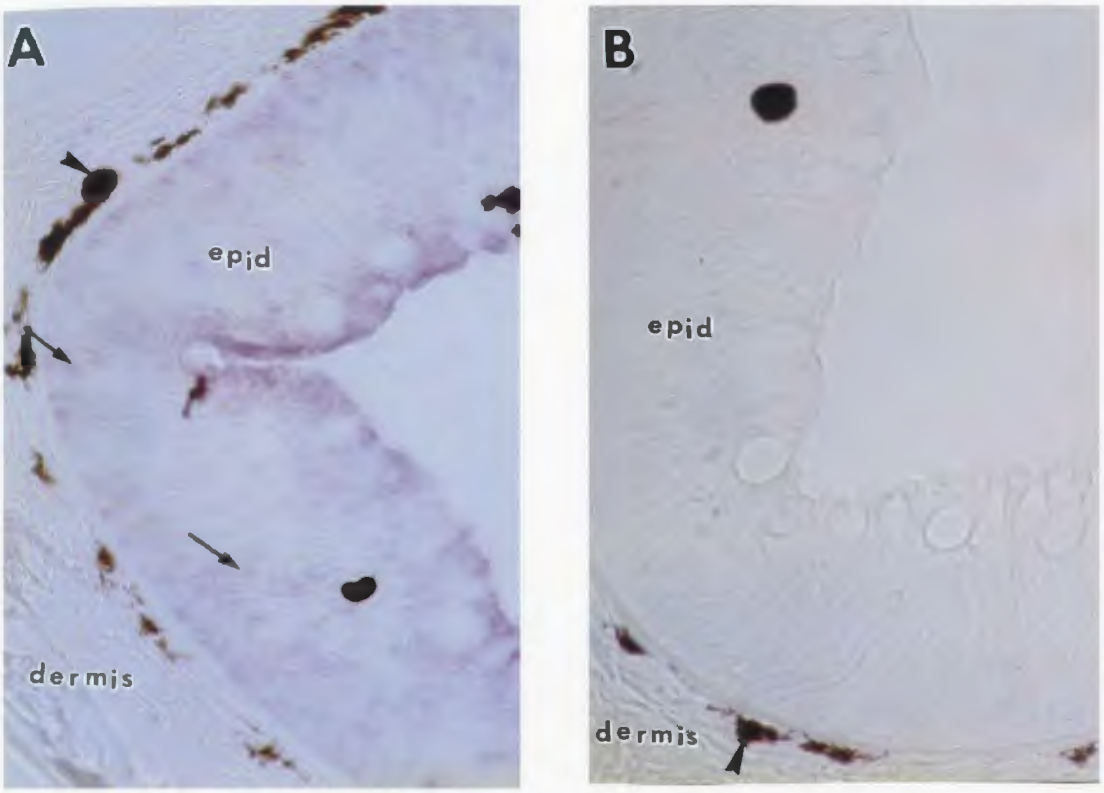


Figure 3.6 *In situ* hybridization with riboprobe for skin AFP-adult flounder. (X330). (A) Skin epidermis of an adult winter flounder in December showing *in situ* hybridization signal in association with epidermal cells (epid)(arrows). Note the absence of reaction in the underlying dermis (X330). (B) Skin epidermis (epid) of adult winter flounder in December following hybridization with sense probe made from skin AFP clone WFP9 (X330). Note dermal melanophore (arrowhead).

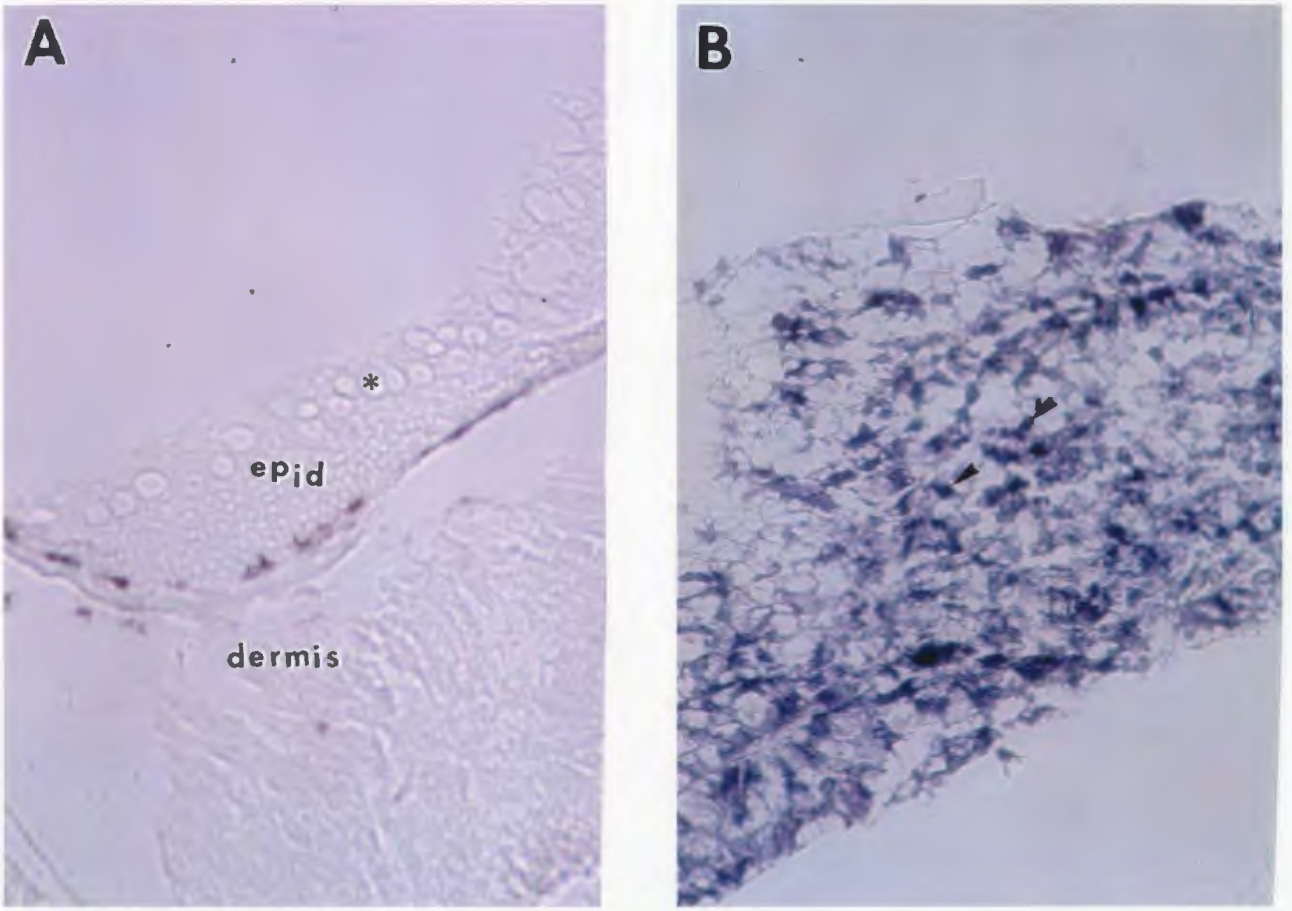


Figure 3.7 *In situ* hybridization with riboprobe for liver AFP (pKenc-17)-juvenile flounder. (A) Skin epidermis of a juvenile winter flounder in February of its first winter showing complete absence of signal in the tissue section. (X330). (B) Section through the liver of a juvenile flounder in February of its first season. Note the strong staining reaction association with the hepatocytes (arrowheads). (X330).

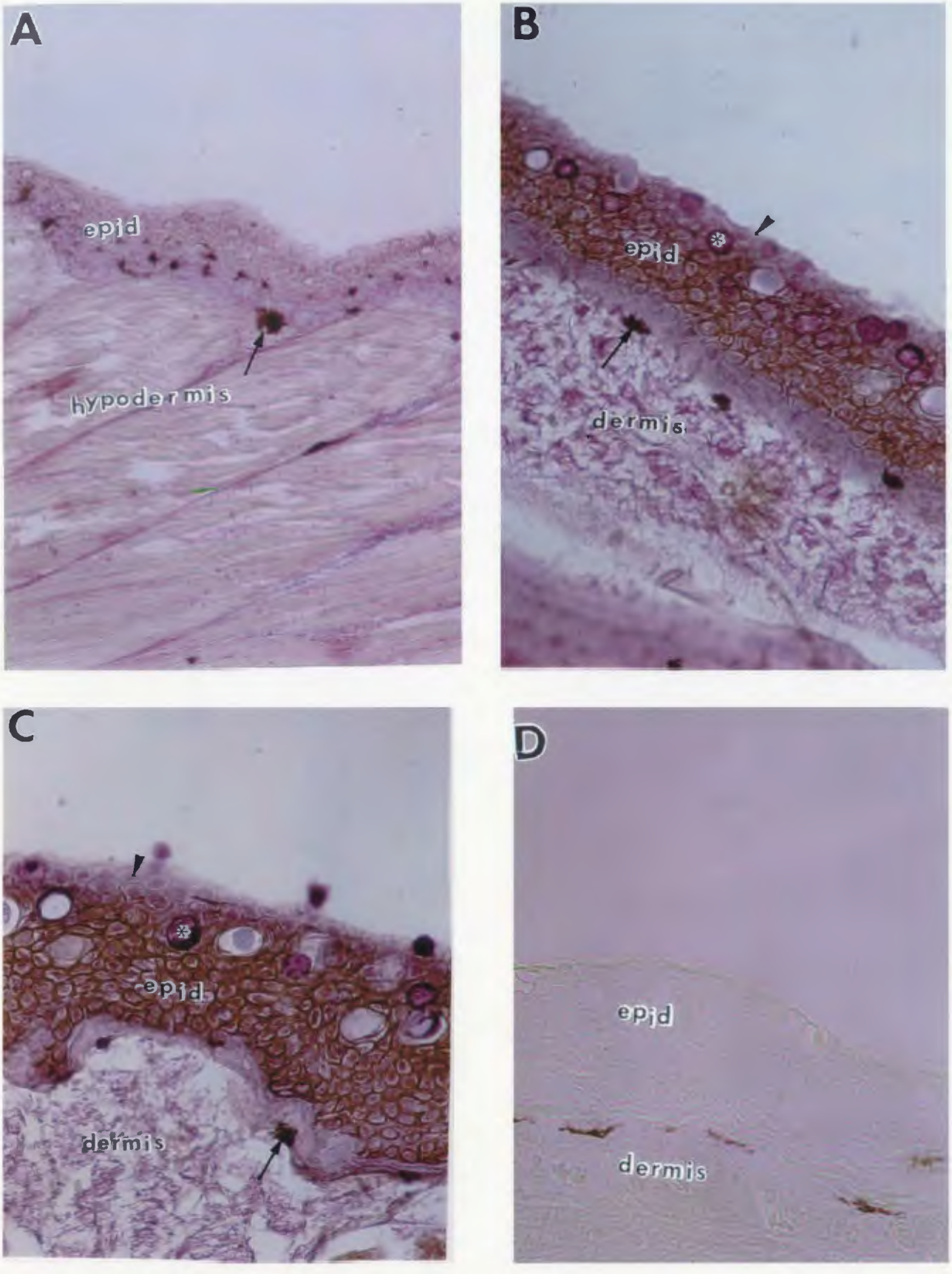
juveniles showed a dramatic increase in stain reaction associated again with cells in the epidermal region (Figure 3.5c). Sections of adult skin tissue from winter showed hybridization to comparable regions of the epidermis (Figure 3.6ab). Mucous cells did not show any hybridization products.

In order to test the specificity of the skin riboprobe, the liver specific AFP riboprobe was used in hybridization experiments on skin tissue from adult and winter juveniles (Figure 3.7b). No hybridization product was detected in any tissue of skin origin using this probe (Figure 3.7). However, the liver AFP probe did react with liver tissue from February juveniles and adult fish sampled in January (chapter 5).

3.3.4 Immuno-histochemistry

As newly metamorphosed winter flounder entered into the fall (September) of their first year, immuno-histochemical results showed minimal or no discernable skin AFP immuno-reaction in the epidermis (Figure 3.8a). The subsequent thickening of the epidermis through the fall (November) and into the first winter (February) showed a significant increase in immuno-detectable AFP (Figure 3.8bc). The reaction product was observed to be consistently associated with the epidermal pavement cells or filament containing cells. These cells exhibited a wide distribution through the tissue. Mucous cells did not contain any obvious reaction product (Figure 3.8c). Consistently, a thin layer of cells at the epidermal surface also showed a distinct absence of immuno-detectable AFP. Similarly, no immuno-reactivity was associated with the dermis and hypodermis of the skin. A closer examination of epidermal tissue using oil immersion revealed that the AFP appeared outside of the epithelial cells in intimate contact with the plasma

Figure 3.8 Juvenile flounder immuno-histochemistry with antisera for skin-type AFP-Juvenile flounder. A. Skin epidermis (epid) of a juvenile winter flounder in September of its first year showing absence of AFP immuno-stain in the epidermal layer. Note dermal melanocyte (arrow) (X330). B. Skin epidermis (epid) of a juvenile winter flounder in November of its first season showing strong immuno-stain throughout the epidermal layer with the exception of the superficial layer (arrowhead). Note dermal melanocyte (arrow) (X660). C. Skin epidermis (epid) of a juvenile winter flounder in February showing extensive immuno-stain throughout the epidermal layer with the exception of the superficial region. Note dermal melanophore (arrow) (X660). D. Normal rabbit serum control for February juvenile skin (X660).


A
epid
hypodermis
B
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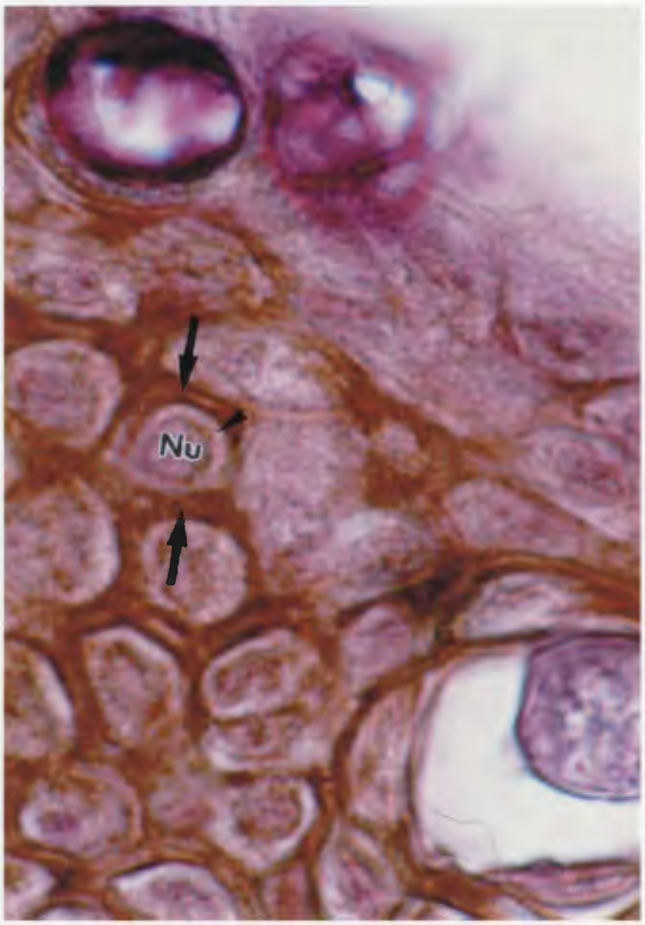
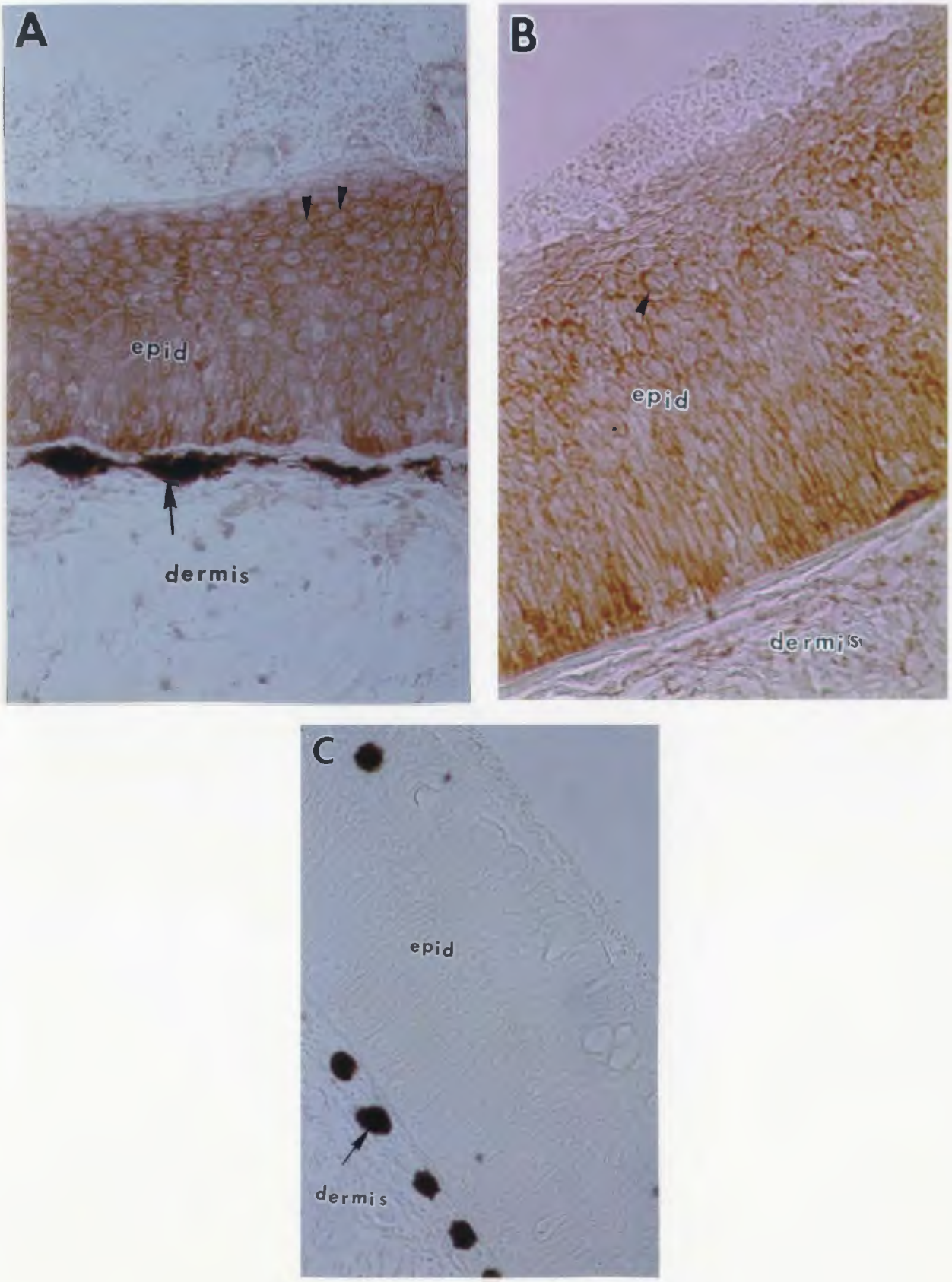


Figure 3.9 High-resolution micrograph of epidermal cells from a February juvenile showing the extracellular distribution of immuno-reaction product following immuno-histochemistry with anti-serum for skin-type AFP. Note the relatively clear cytoplasm (arrowhead) and the interstitial localization of the AFP (arrows). Nu, nucleus. (X3300).

Figure 3.10 Adult winter flounder immuno-histochemistry with antisera for skin-type AFP-Adult winter flounder. A. Immuno-reaction on adult winter skin showing specificity for the epidermal region (epid). Note the extracellular distribution of the immuno-reaction product (arrowheads) (X660). B. Immuno-reaction on adult summer skin taken from the blind side. Note similar stain distribution to that of winter skin (arrowhead) (X660). C. Normal rabbit serum control for winter skin from ocular side (X660). Note also dermal melanocytes in association with ocular side skin (arrows).


A
epid
dermis
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C
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membrane (Figure 3.9). With the exception of occasional small cytoplasmic granules, the cytoplasm and nucleus had no immuno-staining product.

Adult skin tissue from winter fish showed a distribution of AFP immuno-positive cells identical to that observed in the juvenile fish in November and February (Figure 3.10a). Once more the signal was observed to be associated with only one cell type but with an extensive distribution throughout the epidermis with the exception of a thin layer of cells at the surface (Figure 3.10a). As with the juvenile epidermal cells, these AFP immuno-positive cells also gave a distribution of reaction product closely associated with the external plasma membrane and extracellular matrix (Figure 3.10).

The immuno-reaction to the skin type AFP antisera exhibited only minimal detectable background when compared to normal rabbit serum controls (Figure 3.10c). Summer fish showed a distribution of AFP immuno-positive cells identical to that in winter fish, however the intensity of the stain on individual cells appeared to be less (Figure 3.10b). In some cases slightly more intense stain was observed in cells at the base of the epidermis. Comparisons of ocular and blind side epidermis showed comparable immuno-reactions, although the ocular side epidermis was decidedly thinner and exhibited some evidence of cellular damage.

3.4 DISCUSSION

Liver synthesized antifreeze proteins secreted into the blood were originally thought to protect outlying regions like skin by diffusing from the blood plasma through to these areas and subsequently bathing the tissues in AFP. Valerio et al. (1992) provided good evidence suggesting that the skin itself could act as an effective barrier to ice crystal

propagation especially in conjunction with AFPs. It was further indicated that in winter flounder this protection might be a combination of skin structure, extracellular solute concentration and AFPs. Gong et al. (1992) showed that the skin of winter flounder was in fact acting as its own source of AFP. They later showed that the genes responsible for expression of AFPs in skin were not seasonally regulated but constitutively expressed thus inherently different from the expression of liver specific AFPs (Gong et al. 1995). Further work showed that the skin-type AFP appeared to lack a secretion signal sequence and thus had potential for acting as an intracellular AFP (Gong et al. 1996).

Chapter 2 showed that epithelial AFP gene expression in winter flounder occurs very early in post-embryonic development. RT PCR indicated that skin AFP transcripts were present from hatch. *In situ* hybridization and immuno-histochemistry further illustrated that this expression occurred in association with two types of larval integumental cells. A band of paired spindle shaped cells was found to run along the lateral surface of the larva. Temporally these cells were observed to lose detectable expression at the end of yolk sac absorption suggesting that they were only important during this early period of development. Concurrently, immuno-histochemistry and mucous histochemistry showed that integumental mucous cells also reacted strongly with the "skin AFP" antiserum suggesting that these cells could be secreting AFPs together with acid glycoproteins. These cells continued to show expression until the time of metamorphosis at which point the numbers of cells showing immuno-reaction product appeared to diminish (Chapter 2). In the present study juvenile skin samples were not obtained for RT PCR however adult scale slips did show the presence of AFP transcripts.

Regardless, the cells localized as AFP producers in both juveniles and adults did not appear to be related to either of the larval integumental AFP producers previously described. In this case the cells are likely epidermal pavement cells or filament cells (Malpighian cells) similar to those described in plaice by Roberts et al. (1973). These cells are the most common cell type found in teleost epidermal tissue (Whitear 1986).

The onset of AFP expression in juvenile flounder skin appears to correspond to changes occurring in the histology of the epidermis, during the development of juveniles following metamorphosis. These histological changes reflect the life history divergence from a pelagic to a primarily benthic dwelling animal. Roberts et al. (1973) suggested that similar changes in the skin histology of the plaice during metamorphosis were probably a reflection of these lifestyle changes which allow for the skin's protective function in the adult. Newly metamorphosed animals from the present study (August and September juveniles) showed a gradual increase in epidermal thickness through the fall and into winter with a corresponding increase in detectable skin-AFP mRNA and antifreeze protein, thus the onset of AFP expression in the epidermis of juvenile flounder entering their first winter was concomitant with the changes in epidermal histology that occurred during and following metamorphosis. This suggests that the initiation of epidermal "skin-type" AFP gene expression may be developmentally rather then environmentally regulated. During this period it cannot be ignored that environmental features like decreasing water temperature and shortening photoperiod are occurring as well and these factors are well known to be important in the initiation and the quantity of liver type AFP expression in winter flounder (Price et al. 1990). It thus seems probable

that the simultaneous occurrence of epidermal thickening and skin-type AFP expression may be developmental preparation for exposure to cold water and potential ice.

The sub-cellular distribution of the AFP in this study raises some very interesting questions. As previously noted, Gong et al. (1996) described the skin-type AFP as lacking a pro-sequence and a secretion signal suggesting that the protein site of activity was intracellular. If this is the case then what is the intracellular function? Mazur (1984) noted that under normal circumstances ice will form intracellularly only at rapid rates of freezing, thus the occurrence would be unlikely at the relatively slow ice propagation temperatures in normal biological systems. Hochachka (1988ab) related that the majority of cellular damage is more likely to occur under these conditions due to a loss of osmotic integrity and cellular dehydration. These events probably occur during ice formation extracellularly. Hochachka (1986, 1988ab) further proposed that cell damage at low temperatures primarily occurs due to a decoupling between metabolic activity and membrane function, resulting in an increase in intracellular calcium. Considering these factors, an AFP physically or chemically protecting the osmotic integrity of the membrane or one localized to the extracellular matrix would biologically make a lot of sense. This region is exactly where one would predict the AFP should be, considering the current theories of how cells respond to ice propagation and freezing. Based upon experimental data examining ice propagation across winter flounder skin, Valerio et al. (1992) proposed that solute concentration (including that of AFP) in the interstitial space of the epidermis could be responsible for an observed lower ice propagation temperature. This suggests that the greater danger of ice propagation is between cells rather then

through them. If this were the case and to be consistent with the observation of Gong et al. (1996), then the skin AFP would likely be released from the cell through some pathway independent of the endoplasmic reticulum (ER) and the Golgi apparatus. Bereiter-Hahn et al. (1980) showed that glycoprotein secretions of "flame cells" in seahorse epidermis were synthesized in the ER but by-passed the Golgi for secretion. Mignatti et al. (1992) observed that basic fibroblast growth factor is secreted from cells in a way that completely bypasses the protein packaging machinery. These descriptions provide evidence that proteins can be released from cells in ways other then the typical secretion pathway, thus supporting the idea that the winter flounder skin AFP could be released from the cell into the extracellular matrix. Data from the present study suggests that the protein is distributed not only in close proximity to the cell membrane but also extends into the extracellular space. While one must certainly consider the spatial dynamics of the immuno-reaction products within the tissue section, the lack of excessive stain product in the cytoplasm strongly suggests extracellular localization and thus activity. In this way the AFP could protect the cell by slowing or preventing ice crystal proliferation and hence decreasing the osmotic shock and membrane damage caused by extracellular ice formation.

Teleost skin is known to be a very dynamic organ and unlike the integument of many higher vertebrates it is vital throughout (Roberts et al. 1972; Roberts and Bullock 1980; Whitear 1986). Because of its direct exposure to the environment, it frequently becomes the first line of defence against many biological and physical threats. Whitear (1986) reviewed the wide range of skin secretions from fishes, including both true

mucous cell glycoprotein secretions and secretions from non-mucous epithelial cells including the Malpighian cells. Some of these secretions may be pheromones but generally the majority of mucous secretion is associated with protection from abrasion (Mittal and Banerjee 1980; Whitear 1986). Other researchers have found that the co-production of two or more active proteins with mucous can add further functional benefits to these secretions. Murray and Fletcher (1976) found lysozyme activity in plaice integumental tissue suggesting that epidermal cells were producing this biologically active protein. Similarly, Cole et al. (1997) showed that winter flounder skin epidermal goblet cells were a source of a protein (pleurocidin) which exhibited distinct antimicrobial activity. Burkhardt-Holm et al. (1998) further showed expression of hsp (heat shock protein) 70 in the epidermal filament cells of brown trout skin after sudden temperature rise. The discovery of a skin produced protein important in freezing protection can now be added to this list. All of these studies substantiate the built in protective qualities of the fish epidermis.

The histological changes in juvenile flounder skin observed in this study were consistent with those previously reported for other juvenile flatfish and adult winter flounder. The gradual epidermal change from a thin structure of about 2 to 3 cell layers during summer to at least 4 or 5 layers through the fall and winter is comparable to the epidermal modifications observed in juvenile plaice (*Pleuronectes platessa*) (Roberts et al. 1973) and Atlantic halibut (*Hippoglossus hippoglossus*) of the same age (Ottesen and Olafsen 1997). The pattern of epidermal change observed in winter flounder juveniles is also reminiscent of the seasonal cycle in skin thickness that occurs in adult winter

flounder (Burton and Fletcher 1983; Burton and Burton 1989ab; Burton and Everard 1991b). Further studies on adult winter flounder related some of the seasonal changes in epidermal structure to serum levels of hormones associated with sexual maturation (Burton and Everard 1991a; Burton et al. 1984). However, Newfoundland winter flounder do not normally mature until 5 years of age (Burton and Idler 1984) and there was no evidence of sexual maturation in any of the juveniles used in the present study. Therefore the epidermal thickening that occurs during the fall in juvenile flounder may be related to a need for greater protection during the winter months.

The glycoprotein or mucous histochemistry of the epidermal layer in juvenile winter flounder was similar to that observed in juvenile and adult plaice (Roberts et al. 1972; 1973) and the Atlantic halibut (Ottesen and Olafsen 1997). In both cases, skin mucous cells consistently contained mixtures of neutral and sulphated acid mucins. Fletcher et al. (1976) found that adult plaice epidermal mucous cells contained only sulphated acid mucins. This observation is very similar to that seen in goblet cells from summer season adult and juvenile winter flounder in the present study. The consistent observation of Alcian blue positive cells in the superficial layer of all life history stages of fish examined in this study was comparable to that described in Burton et al. (1984). It has been suggested that a proliferation of such acid glycoprotein production in these cells could be a response to stress or handling but more generally these cells are probably involved in the secretion of the mucoid cuticle which likely adds a further protective barrier against damage (Burton et al. 1984; Whitear 1986).

CHAPTER 4. THE ONTOGENY OF AFP EXPRESSION IN THE DIGESTIVE TRACT OF WINTER FLOUNDER

4.1 INTRODUCTION

O'Grady et al. (1982; 1983) reported the presence of antifreeze protein (AFP) activity (thermal hysteresis) in the intestinal fluids of a variety of Antarctic and Arctic fishes, and suggested that they functioned to protect intestinal fluids from freezing when the fish ingested seawater laden with ice crystals. Since similar activity was also detected in the bile they speculated that the AFPs were produced in the liver, and reached the digestive tract via the bile duct. Although significant thermal hysteresis activity can be found in the bile of winter flounder, no evidence for the presence of AFP in intestinal fluids has been found (O'Grady et al. 1982; Fletcher et al. unpublished data).

Subsequent studies, using Northern blotting techniques, discovered significant expression of AFP mRNA in stomach and intestinal mucosa of adult winter flounder, suggesting that the cells lining the digestive tract were protected from freezing by producing AFP (Gong et al. 1992). Moreover, unlike the liver where expression of AFP mRNA is distinctly seasonal, the mucosal cell AFP mRNA, in common with other non-liver tissues, was expressed throughout the year (Gong et al. 1992; 1996).

Recently, Gong et al. (1996) discovered that winter flounder possessed two distinct types of AFP genes; one type expressed almost exclusively in the liver (liver-type AFP) and a second type that were predominately expressed in epithelial tissues (skin-type AFP), and expressed to a lesser extent in most other body tissues. Gong et al. (1996) also discovered that the skin-type AFP lacked a secretory signal sequence, and thus unlike the

liver-type AFP, which were secreted into the bloodstream, the skin-type AFP were likely to remain, and function intracellularly. Contrary to this, data from the present series of studies suggests that in adult and juvenile epidermis, AFPs may actually occur in the extracellular spaces (Chapter 3).

The present study was carried out to determine when, and by what cell types, AFP expression takes place in the digestive tract of the winter flounder during post-embryonic development and in the adult.

4.2 MATERIALS AND METHODS

4.2.1 Adult Maintenance

Winter flounder were captured by SCUBA (November, 1996; January, 1997) and transported to the Ocean Sciences Centre where they were held in tanks maintained overwinter with running seawater under ambient photo-period and temperature.

In early spring, animals were checked daily for signs of gonadal swelling and the presence of eggs or milt. When fish of appropriate condition were found they were immediately isolated from the main tank in preparation for spawning.

4.2.2 Spawning and Fertilization

4.2.2.1 Spawning

Eggs were collected by first carefully drying the genital pore area and then applying gentle pressure, allowing the eggs to pour easily into a clean dry container. The container with eggs was then stored at 5 °C until fertilization. Sperm was collected by placing a syringe near the genital pore and gently drawing up the milt. Following gamete collection all further procedures were carried out at 5 °C.

4.2.2.2 Fertilization

Five hundred to one thousand microliters of eggs in ovarian fluid were placed in a dry sterile plastic Petrie dish and mixed gently with 100 µl of milt. The sperm were activated by adding a few drops of UV treated and filtered seawater (0.2 µm Millipore filter system). The eggs were then allowed to sit for 5 minutes to fertilize and were subsequently flooded with fresh filtered seawater (0.2 µm Millipore filter system) and allowed to water harden for an additional 20 minutes.

Following water hardening the eggs were flushed once more to remove any surplus milt and allowed to develop. Water was changed every other day by decanting off old and adding new filtered seawater. Following fertilization eggs were incubated at 5 ^{0}C and hatched within 14 days.

4.2.3 Hatching and Transfer to Rearing Containers

Following hatch, 1000 to 2000 larvae were transferred to 4 l plastic buckets (8 ¼ inches X 6 inches) lined with black plastic to enhance contrast for prey visibility. Buckets were set in flowing seawater baths at ambient temperature/photoperiod. Temperatures ranged from 5 to 7 ^{0}C in May/June to as high as 14^{0}C in August. The buckets contained 3 litres of filtered (0.2 µm) and UV sterilized seawater plus 500 ml of mixed algae ie *Isochrysis* sp. and *Tetraselmis* sp. Water was changed (50%) and replaced every 2 to 3 days with mixed algae. Animals were fed daily on a ration of 5000 rotifers (variety: *Sap)* per litre and supplemented with a further 300 ml of algae until metamorphosis (about 60 days). Once they had reached metamorphosis the fish were transferred to aquaria with

flowing seawater and aeration. Temperatures were allowed to remain ambient showing a maximum of 14 ^{0} C in August and dropping gradually to approximately 5 ^{0} C in November and then decreasing further to near 0 ^{0} C in December, January and February. Temperature began to rise above zero in April and May and continued to rise through the summer. All animals were maintained and killed according to the guidelines set by the Canadian Council of Animal Care (Olfert et al. 1993).

4.2.4 Tissue Sampling

4.2.4.1 RNA Analysis

Three adult winter flounder were sampled in either December (winter) or July (summer). All fish were killed by a blow to the head followed by cervical severance. Portions of stomach and intestine were dissected from a zone midway along each region of gut and immediately flash frozen in liquid nitrogen. Tissues were stored at -70 °C until analysis.

4.2.4.2 Immuno-histochemistry

Winter flounder larvae were sampled at 10-day intervals from hatch to metamorphosis. Groups of five animals were anaesthetized in tricane methanesulfonate and fixed *in toto* in Bouin's fluid at room temperature for 24 - 48 hours. Following metamorphosis, 5-10 juveniles were sampled on monthly intervals beginning in August until the middle of the first winter (February 1998). Samples were also taken during the following summer (July 1998). Five animals were fixed *in toto* in Bouin's fluid and subsequently processed for paraffin embedding. Serial saggital sections, 7 um thick, were cut for all tissues. In addition, six adult winter flounder were captured by SCUBA

divers in either winter (February) or summer seasons (June or July) and sacrificed by a blow to the head, followed by cervical severance. One-centimetre pieces of tissue were removed from mid-stomach and intestine. The tissues were fixed in Bouins fluid for 24 to 48 hours at room temperature and processed for paraffin embedding.

4.2.4.3 Mucous Histochemistry

Positive reactions for mucous histochemistry using Alcian blue (AB) pH 2.5/periodic acid-Schiffs reagent (PAS) (Bancroft and Cook 1984) were blue for acid mucins, red for neutral mucins and reddish purple for a combination of neutral and acid mucin.

4.2.5 Total RNA Extraction and RT PCR

4.2.5.1 Total RNA Extraction

Total RNA was extracted from adult winter flounder stomach and intestine using either the Qiagen®, Rneasy® Mini RNA prep kit following disruption and homogenisation using the QIAshredder ® and the included homogenisation buffer or alternatively with Trizol ® reagent, according to the manufacturer's suggested protocol. Following extraction, RNA was treated with Life Technologies amplification grade DNAse I according to the manufacturer's recommendations, aliquoted and frozen at -70° C until use.

4.2.5.2 PCR Primers

PCR primers (WFP9A and WFP9B) were designed to amplify a 250 base pair (bp) fragment analogous to an AFP cDNA clone (WFP9) originally isolated from a winter flounder skin cDNA library (Gong et al. 1996). The sequence of the primers

corresponded to 5'-GTCGAACACTCAGAATCACTG-3' and 5'-AGCTTCTCAGGTTTATTCAGC-3', respectively.

4.2.5.3 RT PCR

One microgram of total RNA, primers, and the required components of the Life Technologies SuperScript ™ One-Step ™ RT-PCR System were mixed according to the manufacturer's recommendations. The mixture was incubated at 50 °C for 30 minutes for the reverse transcription reaction and then carried through PCR on a Perkin-Elmer Model 480 thermal cycler. The amplification consisted of 35 cycles at 94 °C, (30s), 60 °C (30s), and 68 ° C (45s).

4.2.5.4 Sequencing of PCR Products

At least five consecutive RT PCR reactions were run and the reactions pooled, DNA ethanol precipitated and then resuspended in 50 µl of sterile water. This volume was run on a 2% agarose gel for at least 1 hour at 100 V and stained with ethidium bromide. The gel fragment was excised and the DNA purified using the QIAquick Gel Extraction Kit (Qiagen).

Purified PCR products and appropriate primers were subsequently sent to the DNA Sequencing Facility/The Centre for Applied Genomics at the Hospital for Sick Children, Toronto Ontario. For comparison, obtained sequences were checked against known AFP sequences to verify the result.

4.2.6 Immuno-histochemistry

4.2.6.1 Tissue Preparation and Blocking of Non-specific Sites

Tissue sections were deparaffinized by washing twice in xylene for 10 minutes, hydrated through ethanol series and washed three times 5 minutes in PBT (1x Phosphate Buffered Saline, pH 7.4, 0.1% Triton-X-100, 2mg/ml BSA). Non-specific sites were blocked by incubating tissue in 20% goat serum in buffer for 15 minutes at room temperature.

4.2.6.2 Antibody Treatment

Winter flounder skin AFP was secretory expressed in *E. coli* JM105 and purified to homogeneity by Lin et al., (1999). Polyclonal antisera to winter flounder skin AFP was produced in rabbits following conjugation to keyhole limpet hemocyanin (Lin et al. 1999).

Antiserum was diluted in PBT with 5 % goat serum and applied to sections for 30 minutes at room temperature, dilutions ranged from 1/500 to 1/1000. Following antibody application, slides were washed four times in PBT for five minutes each.

The secondary antibody (goat-anti-rabbit conjugated to horseradish peroxidase (HRP) (Sigma)) was diluted in PBT with 5 % goat serum, applied to sections and incubated in an identical manner to the above. Dilutions ranged from 1/300 to 1/500. Slides were again washed four times in buffer for five minutes each.

4.2.6.3 Stain Development and Mounting

Following the final wash, sections were covered with 500 ul of Immuno-Pure Metal Enhanced DAB substrate (Pierce) and incubated at room temperature for 5 minutes

or until the chromogenic reaction was of favourable intensity. Reaction was then stopped by washing in PBT, dehydrated in ethanol series, cleared in xylene and mounted in Cytoseal (Stephens Scientific) for observation and photography.

4.2.6.4 Controls

In parallel sets of experiments, negative controls involved the replacement of the primary antibody with normal rabbit serum or antisera for sea raven Type II AFP. Further experiments showed that the cell specific staining could also be out competed with a 100-fold excess of purified AFP Type I. Immuno-blotting and Western blotting have previously shown antisera/protein specificity (C. Hew personal communication).

4.3 RESULTS

4.3.1 Histology and Mucous Histochemistry

4.3.1.1 Buccal Cavity, Pharynx and Esophagus

Immediately on hatch the gut is represented by a simple tube lined with a simple columnar epithelium (Figure 4.1a). The most anterior region (esophagus) and pharyngeal-buccal cavity were lined with low cuboidal cells that appear stratified (Figure 4.1b). The buccal cavity was observed to open to the exterior well before gut differentiation (Figure 4.1b). A thin layer of simple squamous epithelium was observed to line the most anterior buccal cavity (Figure 4.1b).

At four days post-hatch, digestive system differentiation became obvious (Figure 4.2). At this point the anterior gut was characterized by a distinct pharyngeal cavity lined with a thin epithelium, which gives way to a thickened stratified region of cuboidal cells identifying an esophageal/post-esophageal junction. The luminal surface of this region

stains lightly with Alcian blue.

At 10 days post-hatch a low simple squamous or cuboidal epithelium showing some evidence of stratification characterizes the anterior buccal cavity/pharynx (Figure 4.3). The pharyngeal gill arches at this stage begin to show a distinct epithelial thickening, indicating filament formation (Figure 4.3). The posterior pharynx gives way to the esophagus, characterized by a well-defined transition to a stratified cuboidal epithelium interspersed with numerous goblet cells, all of which are strongly positive for Alcian blue (Figure 4.3). Posteriorly, near the post-esophageal swelling (PES) the esophageal goblet cells end abruptly and the epithelium becomes simple columnar.

At 24 days post-hatch the pharynx region indicates further epithelial growth of the developing gill filament structure (Figure 4.4). The structure of the buccal cavity and the pharynx are identical to the previous stage, however the esophagus shows a dramatic proliferation of goblet cells (Figure 4.4). The goblet cells are limited to the most anterior region of the esophagus and stain identically to those in the previous stage. The posterior region is characterized by a folded but stratified cuboidal epithelium with the most distal area of the posterior esophagus giving way to an obvious transitional junction resulting in a change in cell type signifying the beginning of the PES (Figure 4.4).

At 30 days post-hatch, fish are beginning to show signs of an increase in body width. At this stage the branchio-pharynx begins to show significant gill filament proliferation with some lamellar development (Figure 4.5). The epithelium lining this area is thin and composed of simple squamous or low cuboidal cells which exhibit an Alcian blue reaction in the apical cytoplasm (Figure 4.5). Occasional goblet cells, similar to the

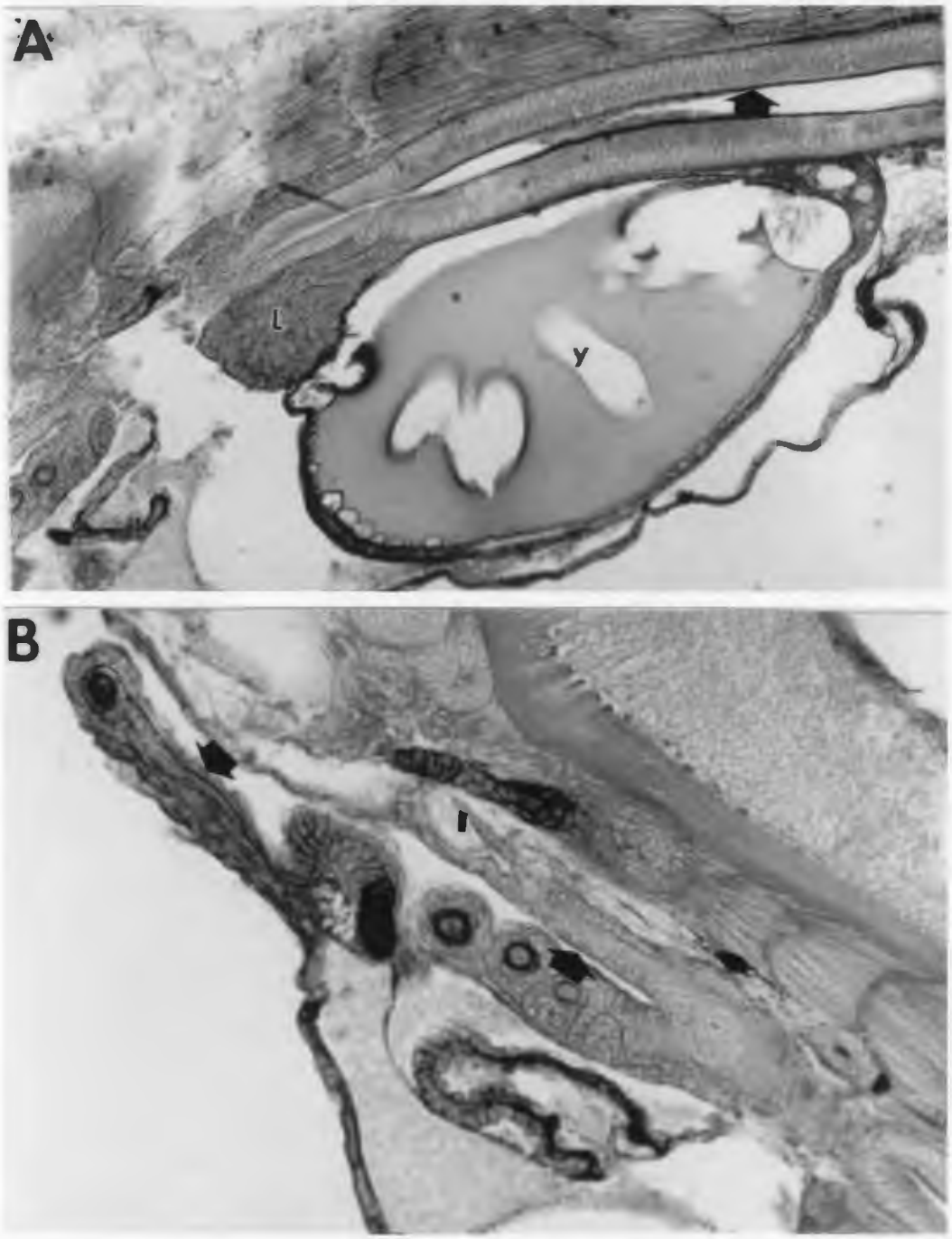


Figure 4.1 (A) Posterior portion of digestive tract of newly hatched winter flounder showing the characteristic columnar epithelial lining in this region (arrowhead). (X825). (B) The structure of the most anterior pharynx and buccal cavity shows the transition from a slightly stratified region to the low squamous epithelium of the mouth (arrowheads). (X825). L, liver; y, yolksac.

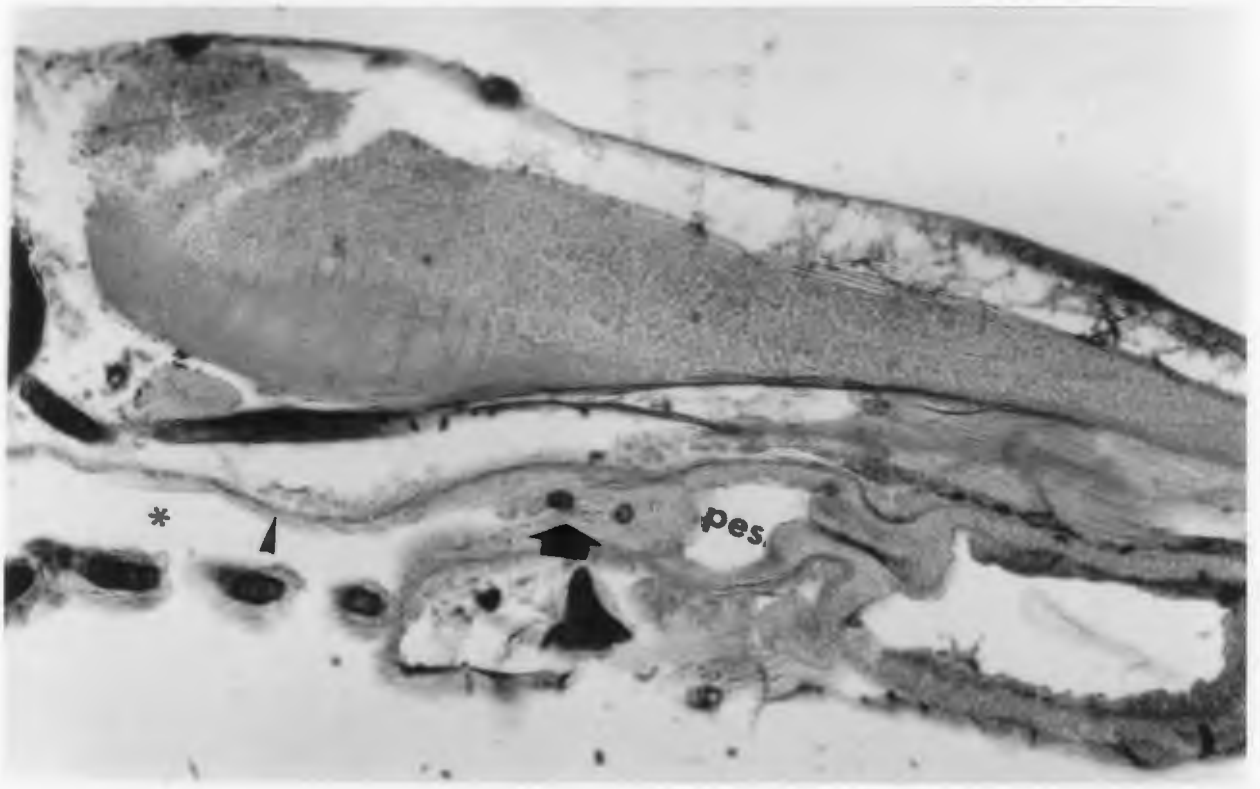


Figure 4.2 Digestive tract of a four-day-old larva showing regional differentiation of the foregut. The pharynx (asterix) is still lined by a thin squamous epithelium (small arrowhead). Note the thickening of the posterior portion of the pharynx/esophagus (arrowhead) leading to the post-esophageal swelling (PES). (X660).

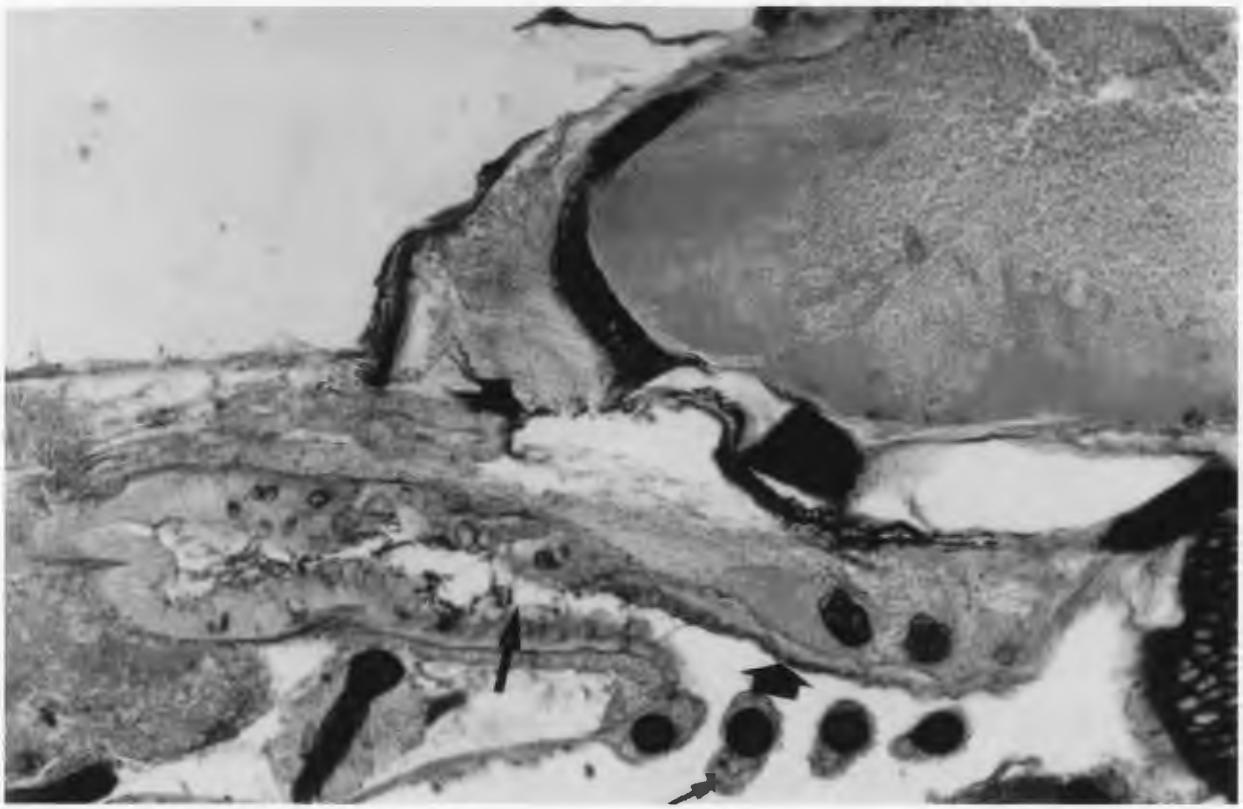


Figure 4.3 Anterior digestive tract of a 10 day old larva showing further development of this region including a thickening of the pharyngeal lining (arrowhead), the occurrence of esophageal goblet cells (thick arrow) and a thickening of the gill filaments (thin arrow). (X660).

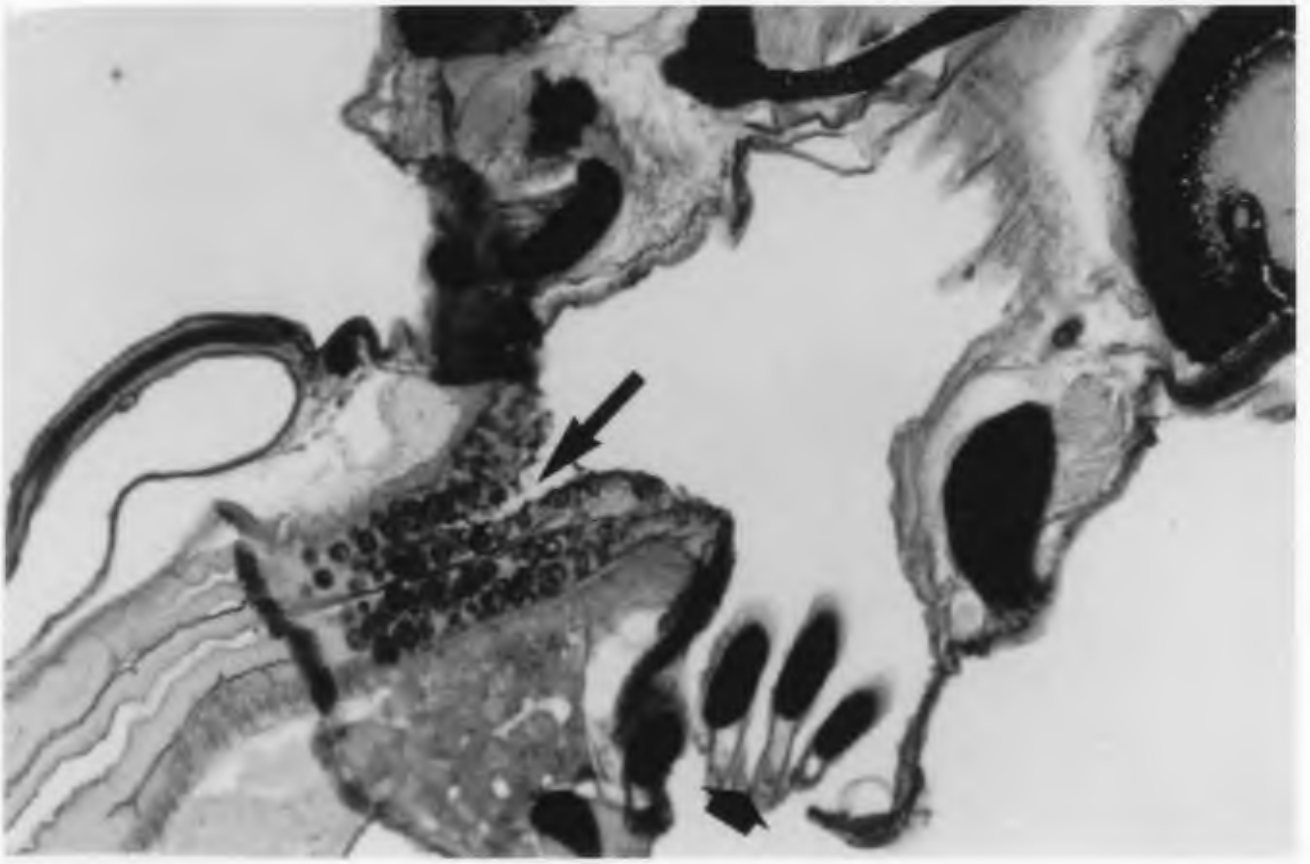


Figure 4.4 Pharyngeal/esophageal region of a twenty-four day old larva showing the various tissue and cell types present. Note the proliferation of goblet cells in the esophagus (thickened arrow) and the growth of gill filaments in the pharynx (arrowhead). (X330).

esophageal types in both structure and staining reaction, were scattered randomly throughout the region. The epithelium of the dorsal side of the gill arches also exhibit a similar Alcian blue staining reaction but has no goblet cells. The esophagus shows a further proliferation of goblet cells, all of which stain blue-green with Alcian blue and extend all the way to the stomach/esophagus transition (cardiac sphincter) (Figure 4.5).

At 40-45 days post hatch, winter flounder started to settle (early metamorphosis). The buccal/pharyngeal cavity region at this stage is lined by a stratified cuboidal epithelium (Figure 4.6a). The cells in the superficial layer of the stratified epithelium stain positive for Alcian blue (blue/green), very similar to esophageal epithelial cells. The epithelium also contained numerous spherical goblet cells that were positive for Alcian blue (dark blue). The underlying connective tissue in this region also contains evidence of pharyngeal tooth development (Figure 4.6a).

Further into the esophagus, the entire epithelium becomes dominated by goblet cells, all staining blue/green with Alcian blue (Figure 4.6b). A distinct inner layer of circular and an outer layer of longitudinal striated muscle are also part of the wall of the esophagus (muscularis externa).

As juveniles enter their first winter their digestive system is similar in structure to that observed in the early metamorph stage. As with the early metamorph the pharynx/buccal cavity is still lined by a stratified cuboidal epithelium. The luminal layer of cells is characterized by a blue/green Alcian blue reaction similar to that found in the esophageal epidermal cells. Occasional spherical mucous cells are also evident, continuing into the esophagus where they increase significantly in number toward the

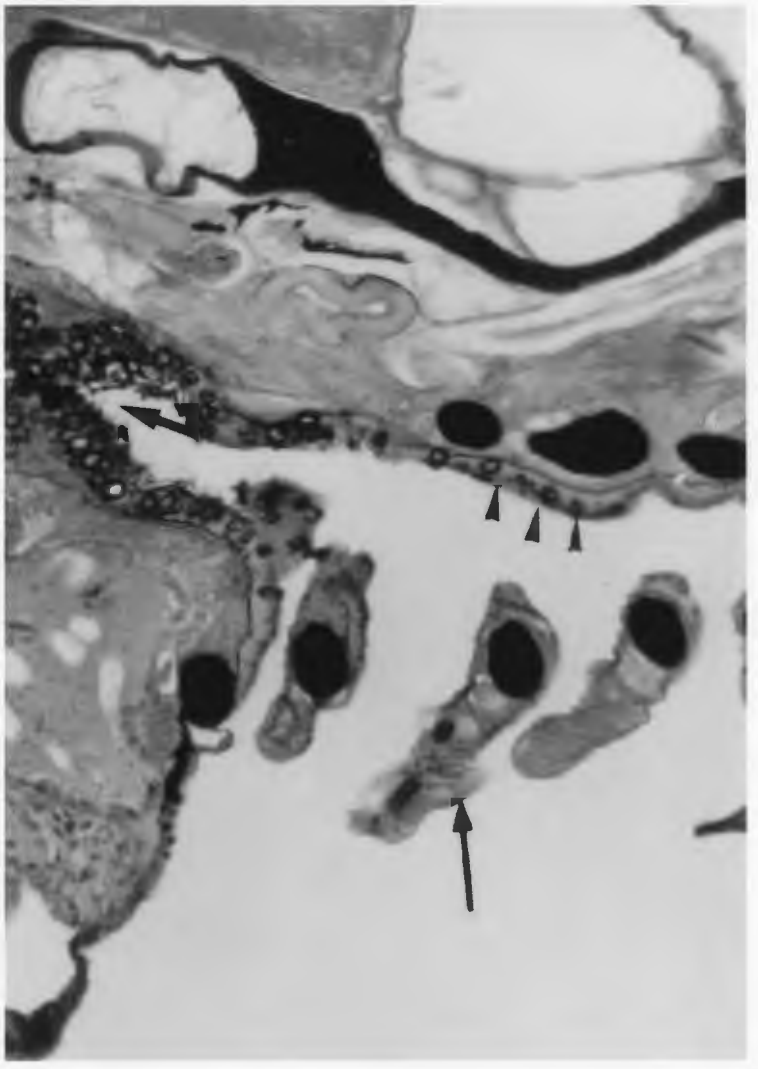


Figure 4.5 Pharyngeal/esophageal region of a thirty-day-old larva showing further proliferation of goblet cells in the esophagus and posterior pharynx (thick arrow and arrowheads) and the formation of gill lamellae in association with developing filaments (thin arrow). (X330).

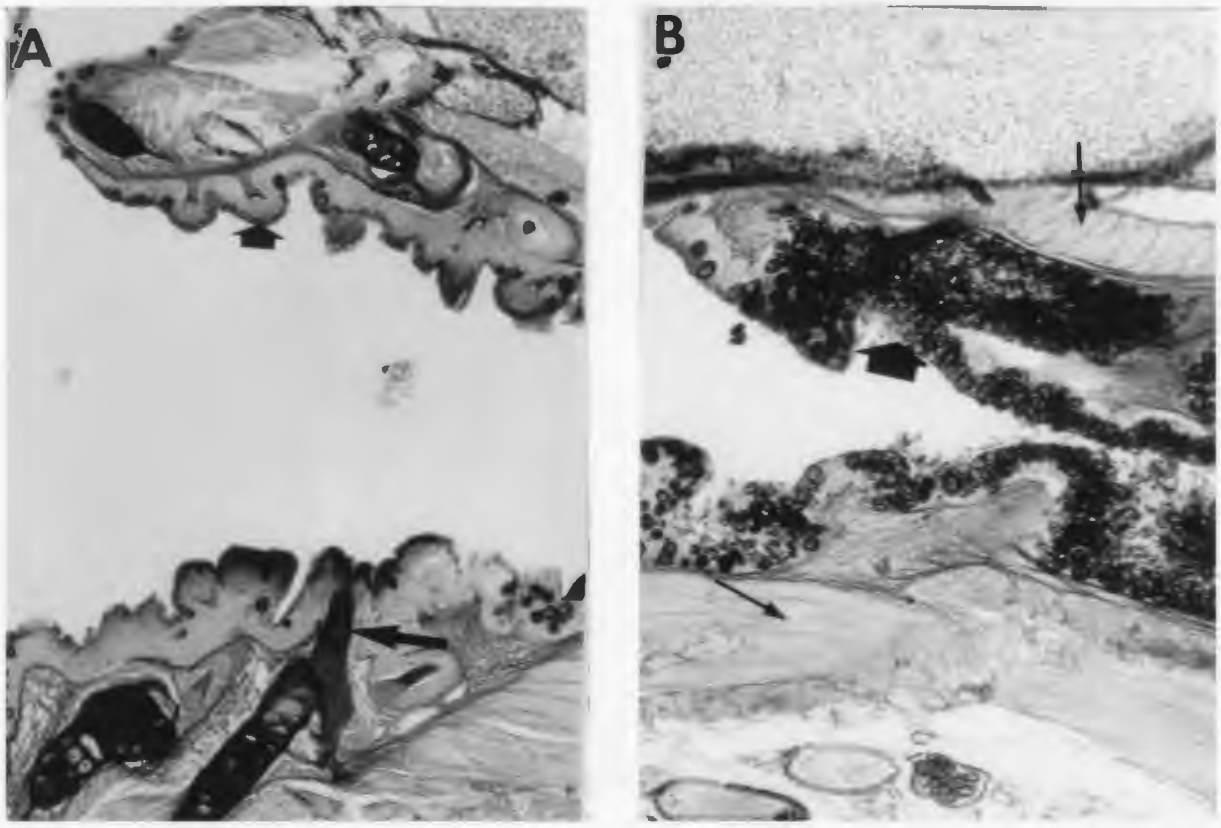


Figure 4.6 Esophageal/pharyngeal region of a newly metamorphosed juvenile. (A) Posterior pharyngeal region showing a thickening of the epithelium (arrowhead) and the occurrence of pharyngeal teeth (thick arrow). (X330). (B) Entrance to the esophagus illustrating the large population of goblet cells characteristic of this region (large arrowhead). Note also the surrounding layers of muscularis externa (thin arrows). (X330).

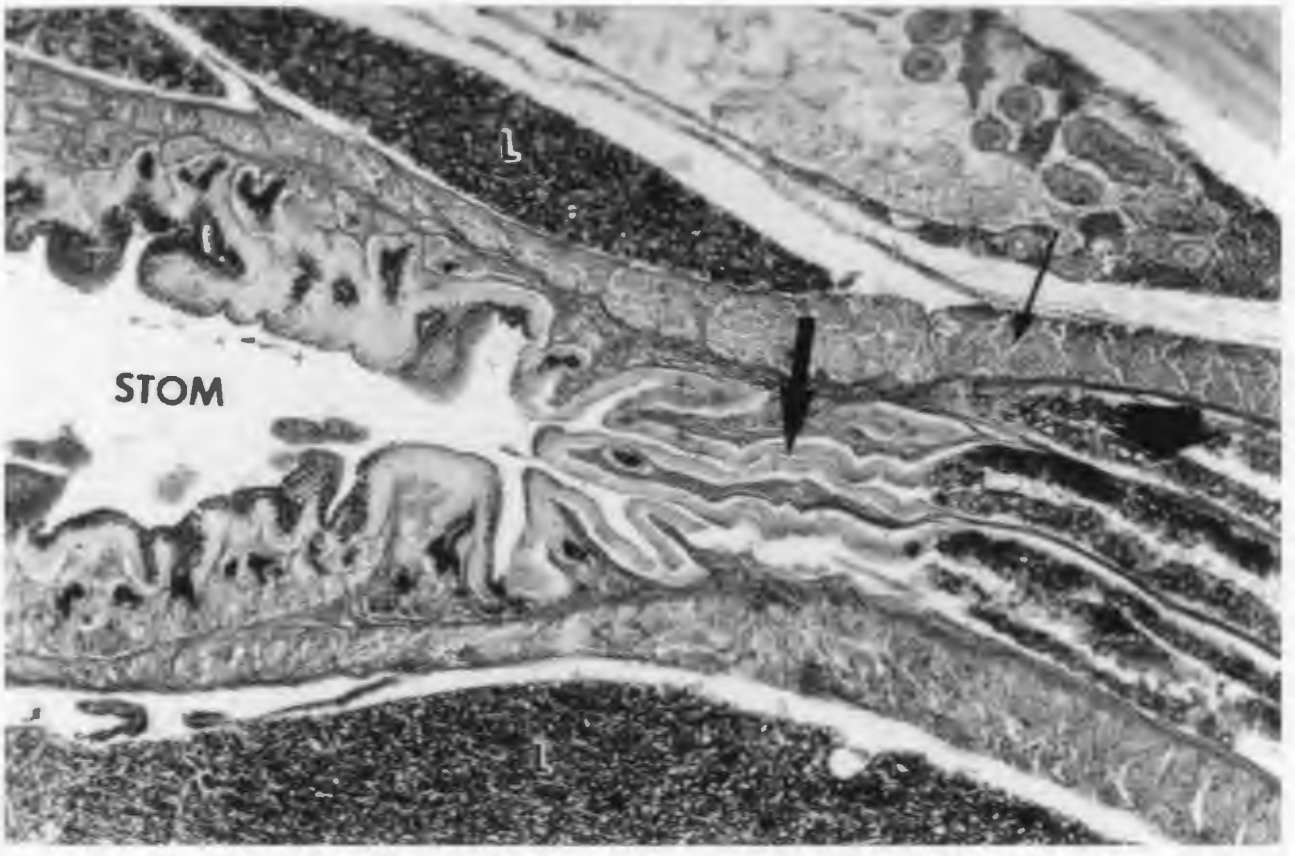


Figure 4.7 Posterior esophagus of a first winter juvenile showing the transition from esophagus to cardiac stomach. Note the extensive folding at the esophageal/gastric junction (thick arrow) and the thickened muscle forming the junction itself (thin arrow). (X330). STOM, stomach.

stomach (Figure 4.7). Epithelial folding becomes more pronounced near the esophageal/cardiac stomach junction and gives way to a region of deep multicellular glands and columnar gastric mucous cells (Figure 4.7). A thickening of the muscularis externa and underlying connective tissue characterize the junction (Figure 4.7).

4.3.1.2 Post-Esophageal Swelling (PES)/Stomach

At about four to 10 days post-hatch, a low simple cuboidal epithelium defines the PES. Multicellular gland formation is not obvious at this stage. The posterior end of this swelling is further characterized by a second thickening of the underlying connective tissue creating a junction and a transition to the hindgut (Figure 4.8).

At 24 days post-hatch, moderate epithelial folding becomes obvious at this stage in the PES, indicating the beginnings of gland formation (Figure 4.9a). At the posterior end of the PES a sphincter acts as a further transition to the upper intestine. It is defined by a connective tissue thickening that produces an obvious constriction (Figure 4.9b). Muscularis externa development is sparse even at this stage.

At 30 days post-hatch, the cells are simple columnar and the epithelium is heavily folded (Figure 4.10). The apical or luminal surface of the cells is strongly PAS positive. At this stage multicellular glands are obvious indicating the transition to a true stomach. The pyloric stomach is aglandular, however the epithelium is still columnar with a distinct PAS positive glycocalyx.

Following age 40-45 days, the stomach can be histologically divided into two distinct regions, the anterior cardiac and the posterior pyloric. The cardiac region is characterized by the presence of deep multicellular glands (Figure 4.11a). The luminal

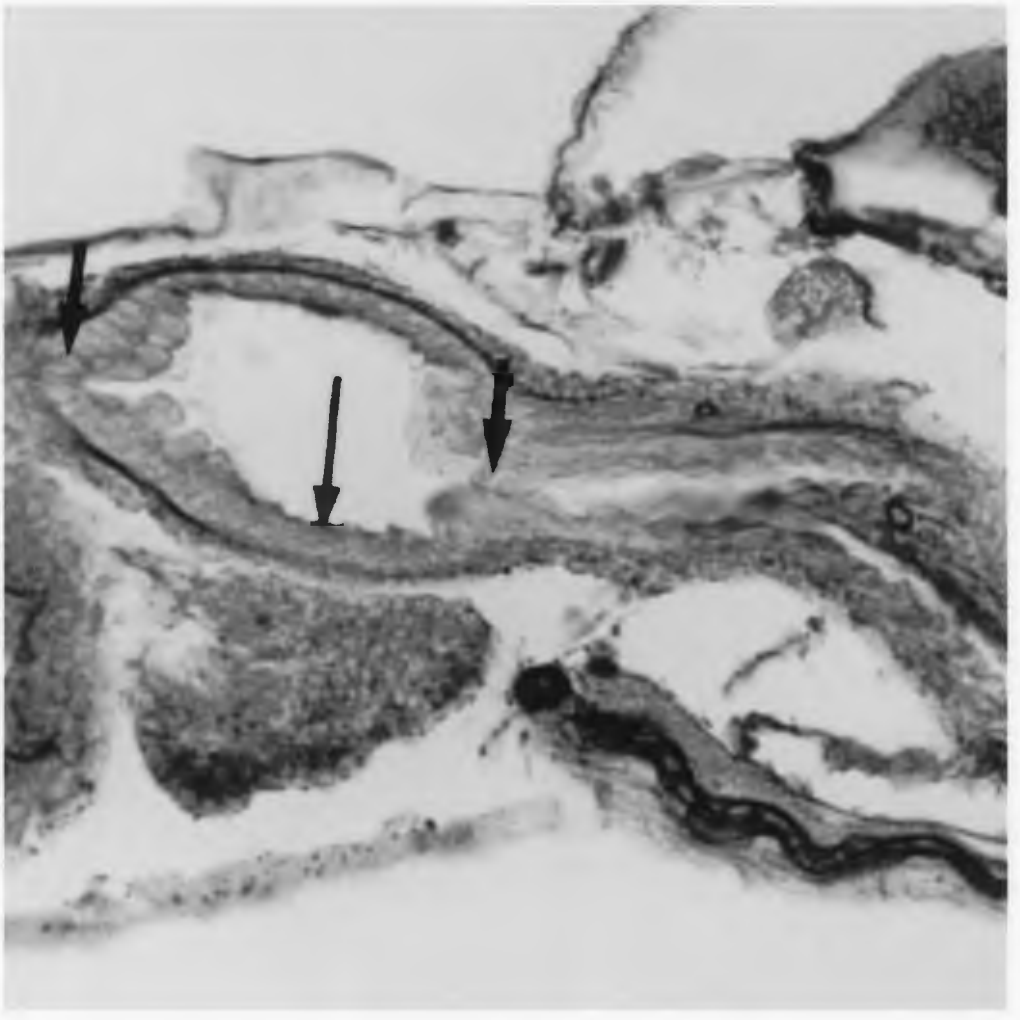


Figure 4.8 Post-esophageal swelling of a four day old larva. Note the relatively thin mucosal lining (thin arrow) and the junctions at both the cardiac and pyloric ends (thick arrows). (X660).

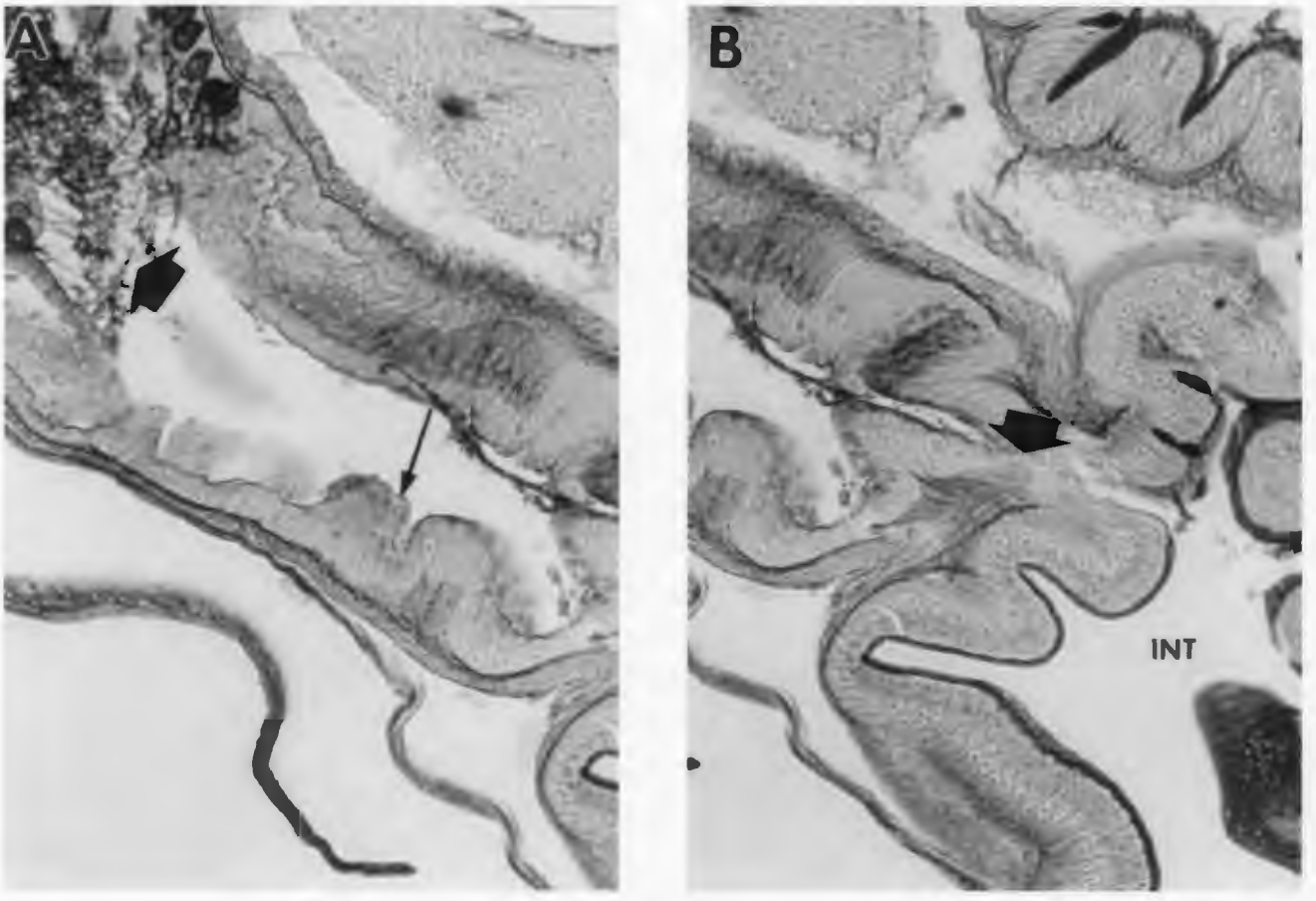


Figure 4.9 Post-esophageal swelling of a twenty-four day old larva. (A) Anterior region showing transition from the esophagus (arrohead) and the moderate folding of epithelium (arrow). (X660). (B) Posterior region showing transition to the upper intestine (arrowhead). (X660). INT, intestine.

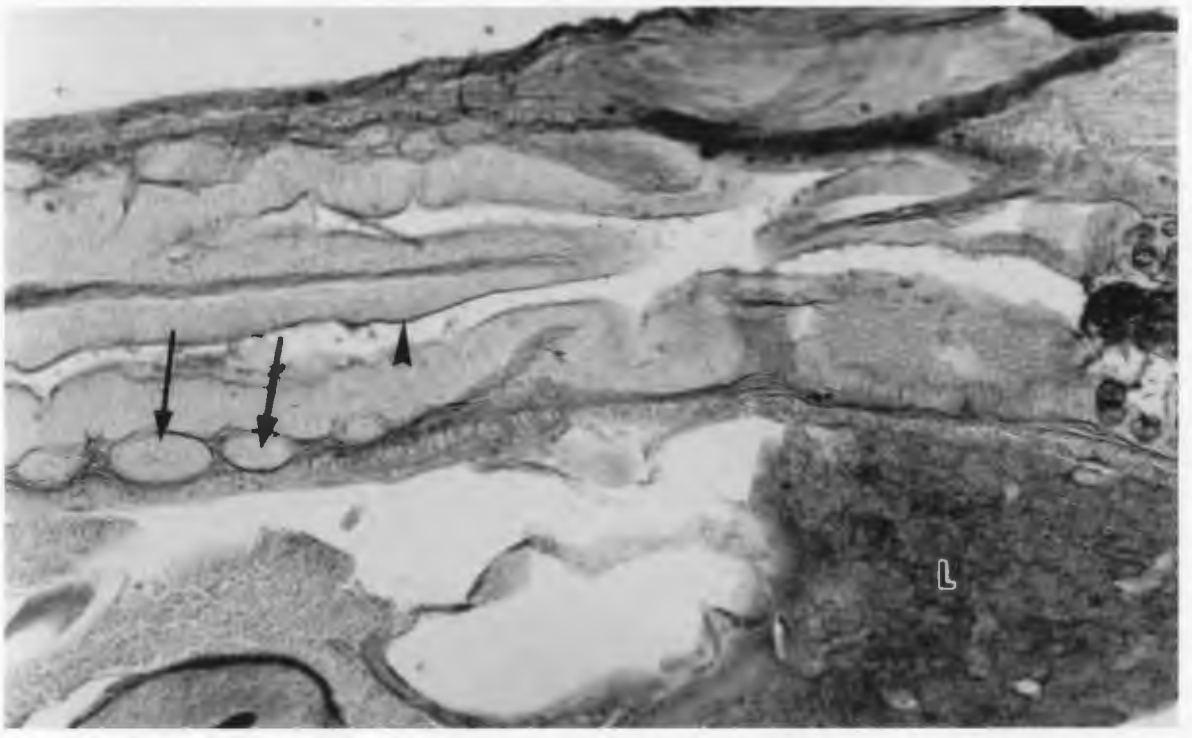


Figure 4.10 Evidence for the formation of a true stomach in thirty-day-old winter flounder larvae. Note the presence of multicellular glands (arrows). The luminal surface is PAS positive indicating a distinct brush border. (X330). L, liver.

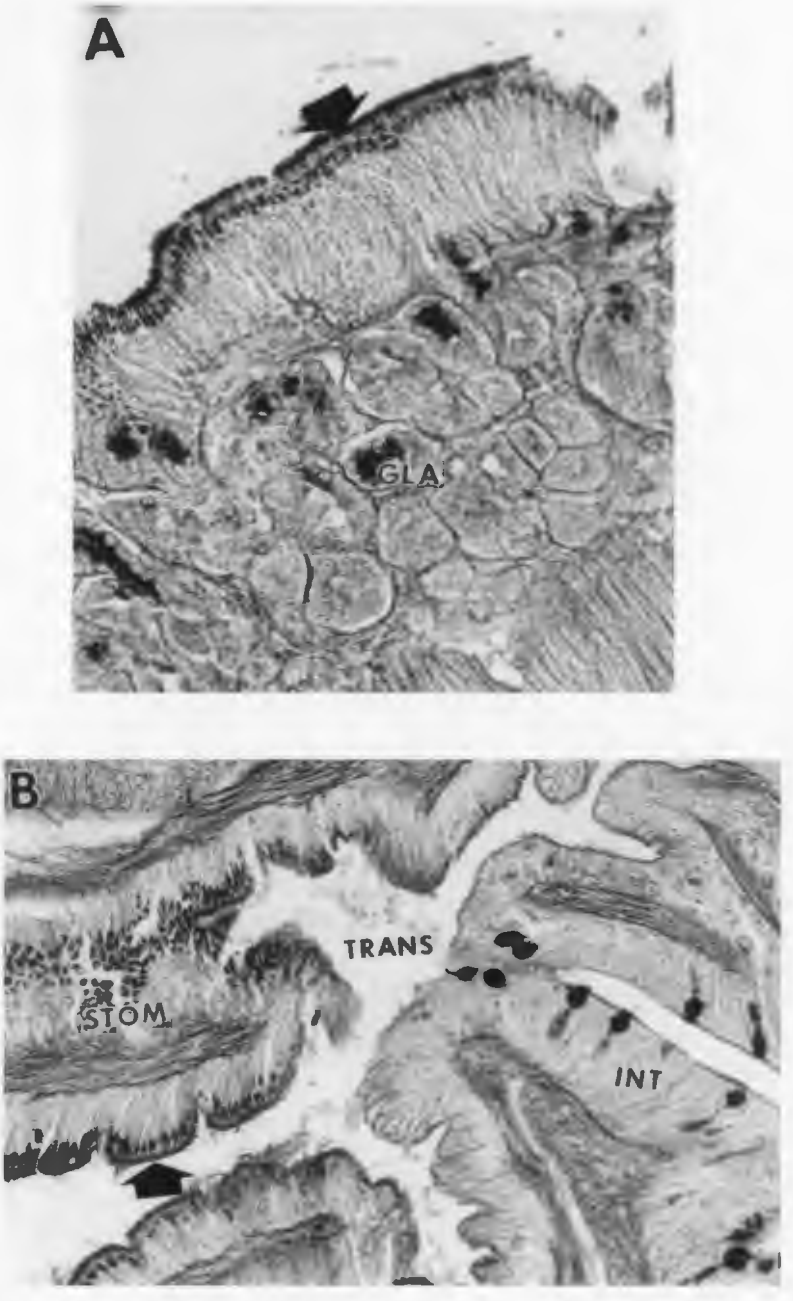


Figure 4.11 Stomach structure of a metamorphosed juvenile winter flounder. (PAS) (A) Cardiac stomach showing surface mucous cells (arrowhead) and deep glands. (X660). (B) Transitional zone (TRANS) of the pyloric stomach and intestine showing the change in cell type from pyloric stomach surface mucous cells and the enterocytes. (X660). GLA, stomach glands; STOM, pyloric stomach; INT, intestine.

epithelium is composed of columnar gastric mucous cells with PAS positive mucous granules in the apical cytoplasm and brush border. The pyloric region lacks deep glands, but the epithelium is composed of gastric mucous cells similar to those in the cardiac region (Figure 4.11b). Posteriorly, the pyloric region gives way to a transitional zone leading to the anterior intestine and is characterized by a thickening of both the underlying submucosal tissue and the muscularis externa. The pyloric stomach gastric mucus cells give way to a columnar epithelium with a strong PAS positive glycocalyx similar to what has been observed previously in younger stages (Figure 4.11b).

4.3.1.3 Intestine/Rectum/Anus

At 10 days post-hatch the intestinal simple epithelium becomes distinct and is characterized by columnar shaped cells with a distinct PAS positive brush border and basal nuclei (Figure 4.12). At 24 days post-hatch, the upper intestine is similar to the previous stage and is characterized by a columnar epithelium with basal nuclei (Figure 4.13). A distinct PAS positive brush border is also obvious. The apical cytoplasm is defined by numerous small PAS positive cytoplasmic inclusions. This area gives way to a more posterior hindgut region that is characterized by a lower simple columnar epithelium. These cells are very similar to those in the more anterior region however, the apical cytoplasmic inclusions are very large and prominent (Figure 4.13). They are also distinctly PAS positive. Overall the cytoplasm of these cells is darker then cells of the previous region, however a brush border is still very prominent.

At 30 days, the upper intestine is again characterized by a distinct simple columnar epithelium with a PAS/Alcian blue positive brush border (Figure 4.14a). Intestinal goblet cells become more numerous at this stage and give a strong Alcian blue

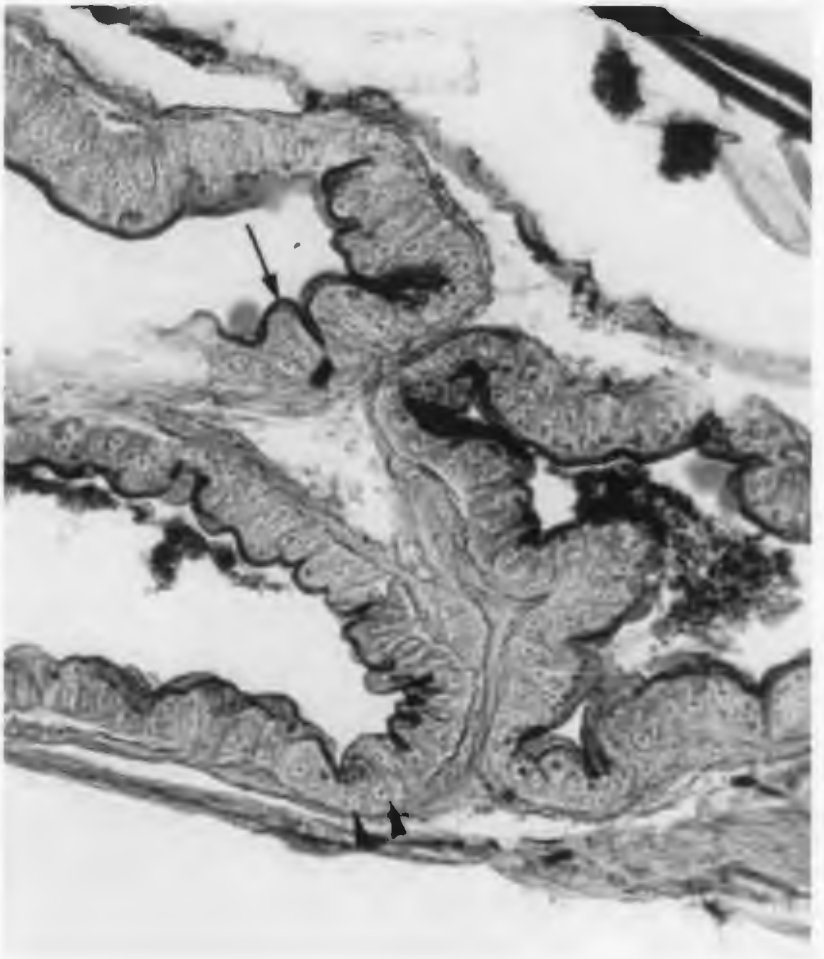


Figure 4.12 Hindgut structure in a 10-day-old winter flounder larva depicting the typical columnar cell with basal nuclei (arrowheads). Note strong PAS positive brush borders (arrow). (X660).

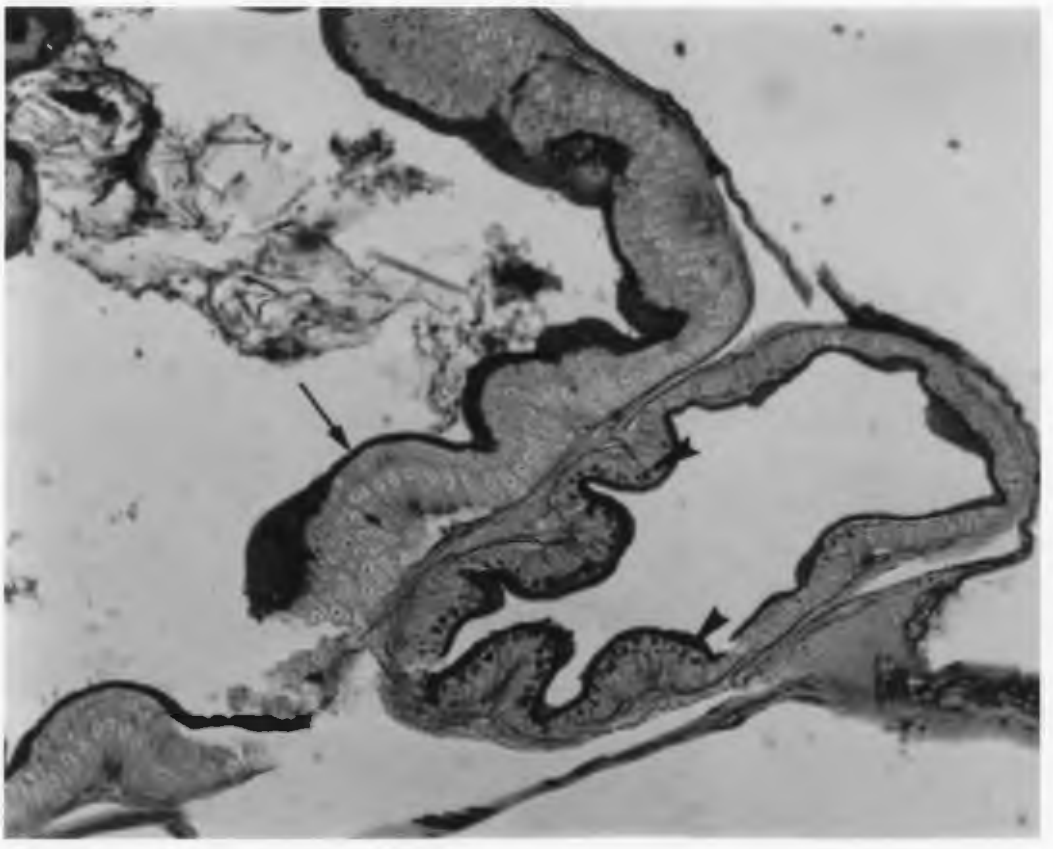


Figure 4.13 Comparison of intestine and rectal regions from a 24-day-old winter flounder larva. Note the large PAS positive granules in the apical cytoplasm of the rectal cells (arrowhead) and the strongly PAS positive brush border (arrow). (X660)

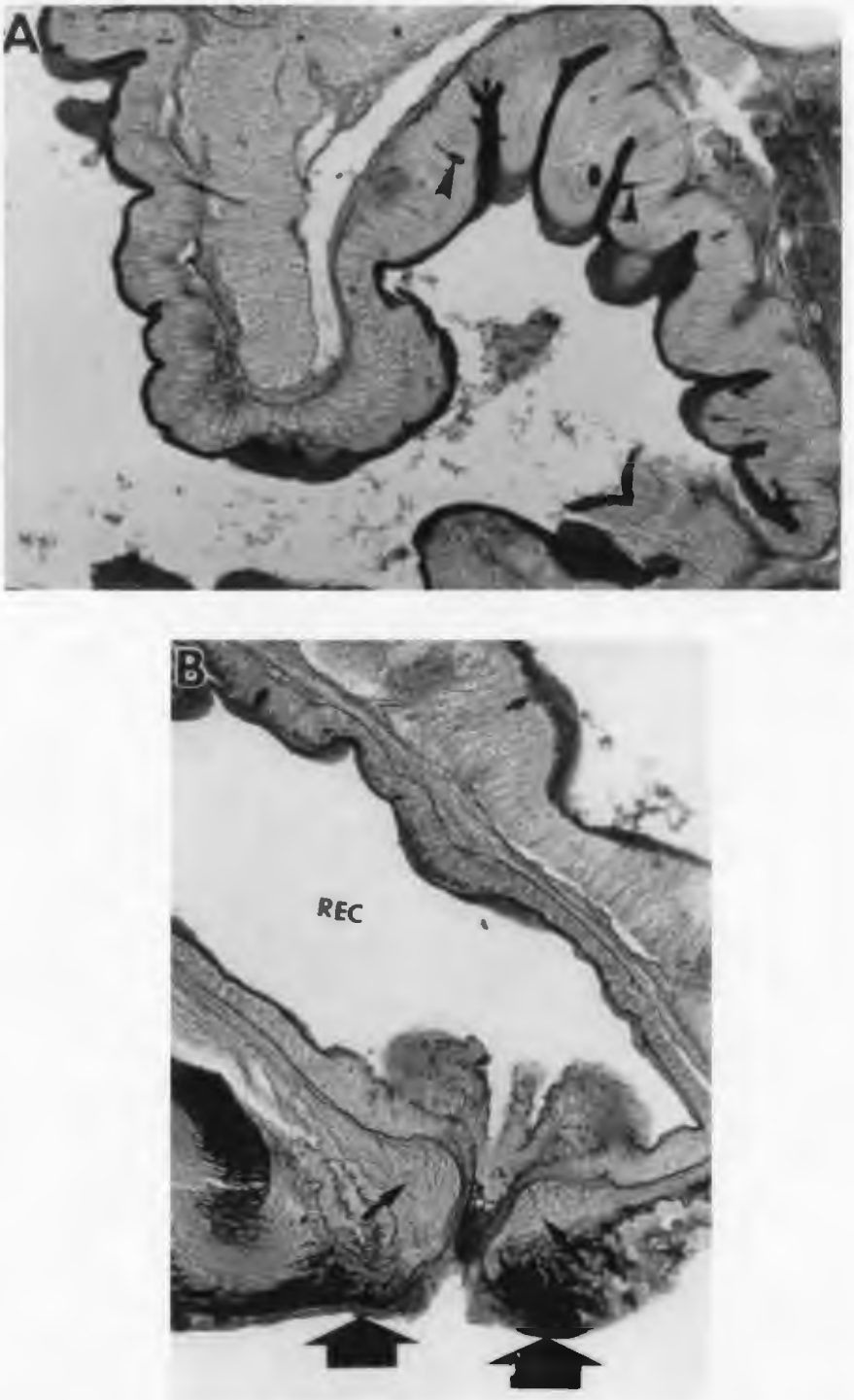


Figure 4.14 Intestinal and rectal region of a 30-day-old winter flounder larva. (A) Detail of intestine showing appearance of goblet cells (arrowheads). (X825) (B) Detail of hindgut/rectum showing the rectal anal junction and transition to the exterior (large arrowheads). (X825). REC, rectum.

reaction (Figure 4.14a). The apical cytoplasm of these cells still possess some small PAS positive granules. Typically, the connective tissue of the intestine is very thin with poor muscularis externa development. The hindgut or rectum is distinguished from the upper intestine by the presence of a folded lower simple columnar epithelium that exhibits an intense PAS reaction in both the brush border and the apical cytoplasm. Some cytoplasmic granules continue to be present here and also exhibit some PAS reaction. Alcian blue positive goblet cells similar to those in the upper intestine are also evident. The rectum undergoes a transition to the anus indicated by a thickening of the submucosal connective tissue (Figure 4.14b). The epithelium becomes stratified squamous with folding and leads to a smooth transition to the outlying skin epidermis and dermis.

Following metamorphosis, intestinal goblet cells become more common in this region and give an intense Alcian blue reaction (Figure 4.15). Small PAS positive granules continue to be obvious in the apical cytoplasm. The hindgut or rectum is very similar in structure to the previous stage, characterized by a low simple columnar epithelium with more extensive surface folding then the intestinal epithelium. The brush border exhibits an intense PAS reaction whereas goblet cells again stain intensely with Alcian blue. The posterior rectum gives way to the anus, which is once more characterized by a transition to a folded stratified squamous epithelium, which gradually leads into the skin epidermis and dermis of the outlying surface.

4.3.2 RT PCR

4.3.2.1 RT PCR on Total RNA from Adult Flounder Intestine and Stomach

Total RNA extracted from adult stomach and intestine and amplified with primers for Skin AFP produced the expected 250-bp band, however occasionally a doublet was observed for reactions using RNA from stomach (Figure 4.16). No seasonal differences in expression were observed.

4.3.2.2 Sequence Comparison

RT-PCR products showed high levels of identity with the original skin AFP clone WFP9, however some differences were noted. Nucleotide substitutions occurred at position 84 in stomach (G to A), and in both intestine and stomach, position 124 (A to C), positions 135 and 136 (A to G; A to C) and at position 150 (G to T) (Figure 4.17a). Intestine also showed a C to T substitution at position 107.

Translated amino acid sequence comparison showed expected results for intestine and stomach associated AFP sequences based upon the previous nucleotide sequence (Figure 4.17b). Here an alanine was replaced by a threonine at position 19 in stomach and alanines replaced aspartic acid and lysine at positions 32 and 36 respectively in stomach and intestine.

4.3.2 Immuno-histochemistry

During the first 10 days following hatch the gut of winter flounder larvae showed immuno-reactivity to skin-type AFP antisera in epithelial cells lining regions of the foregut and anterior midgut (Figure 4.18ab). The immuno-product was concentrated in the apical cytoplasm of the epithelial cells and also on their luminal surface, suggesting

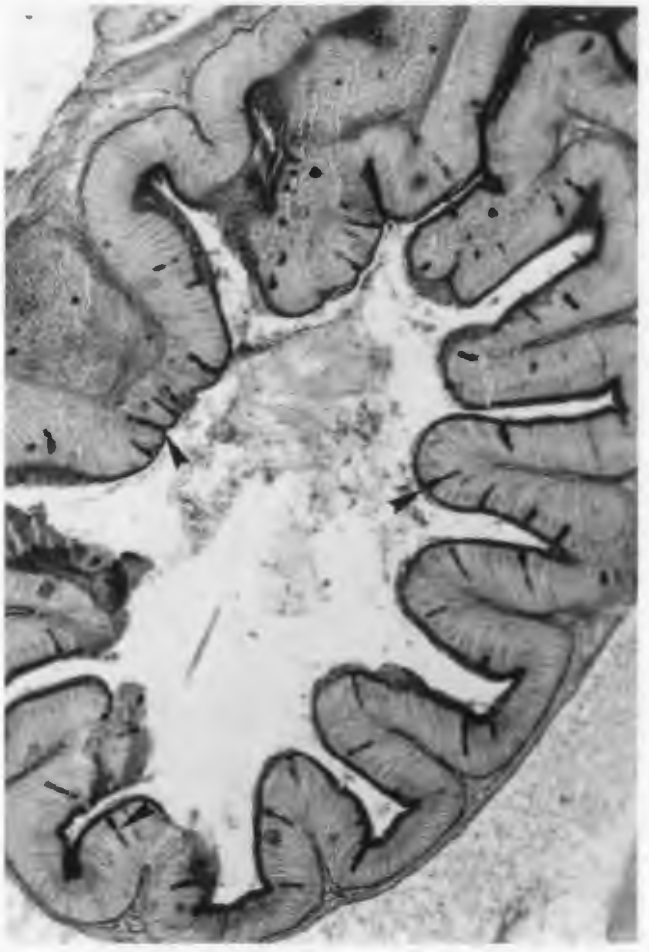


Figure 4.15 Intestinal region of winter flounder juvenile showing further proliferation of goblet cells and mucosal folding (arrowheads). (X825).

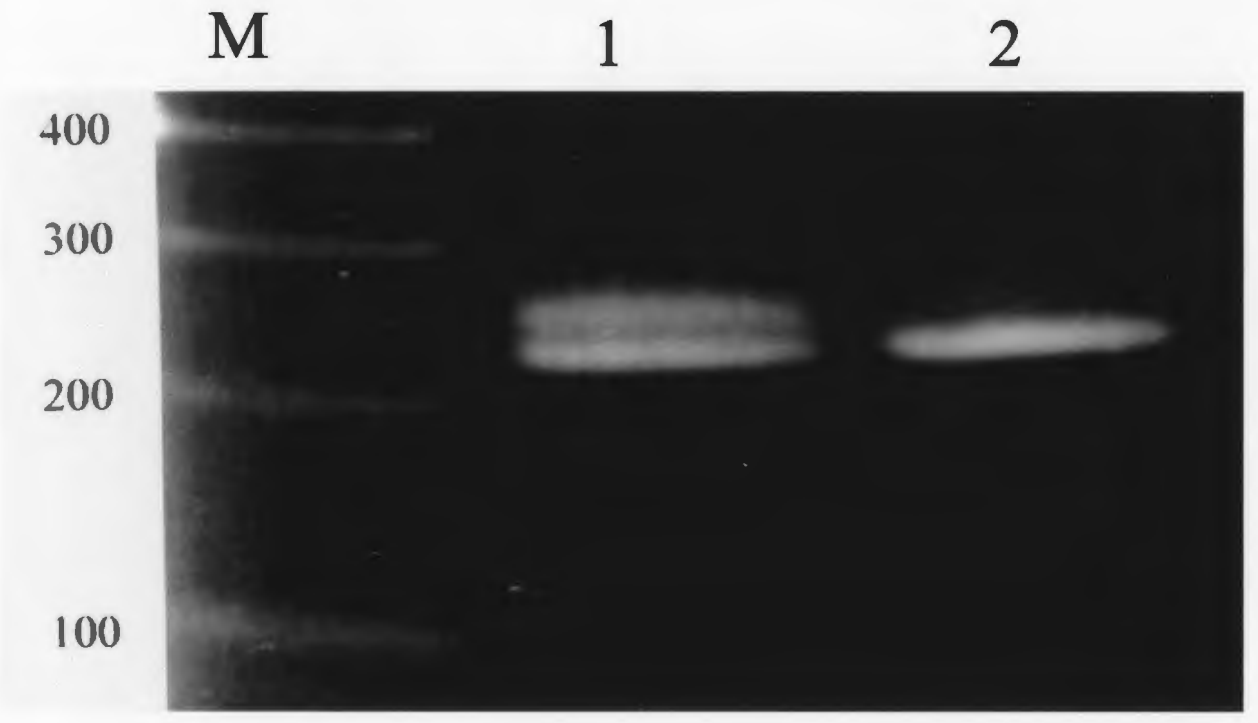


Figure 4.16 RT-PCR using primers for skin AFP WFP9, showing products amplified from total RNA isolated from winter flounder stomach (lane 1) and intestine (lane 2). Samples were run on a 2% agarose gel at 100V and stained in ethidium bromide.

Figure 4.17. (A) Nucleotide sequences of skin AFP cDNA amplified from total RNA isolated from adult winter flounder stomach and intestine using primers specific for the known sequence of skin AFP clone WFP9. Dashes represent identity. (B) Translated protein sequences from the above cDNA showing regions of identity.

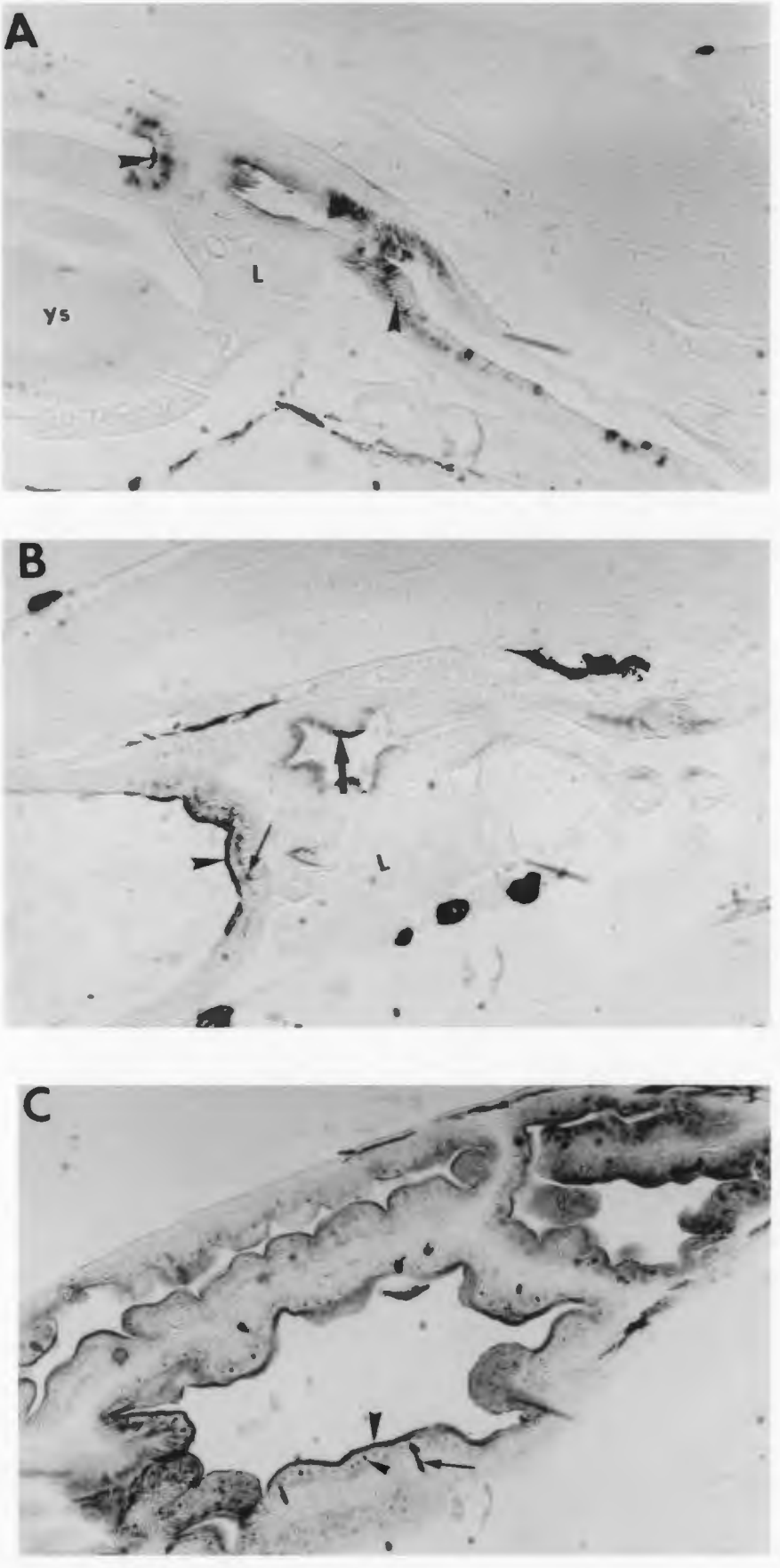
(A)

```
Source
WFP9(skin)   1 GTCGAACACTCAGAATCACTGACATCAACATGGAC
Intestine    1 ---------------------------------
Stomach      1 ---------------------------------

WFP9(skin)  36 GCACCAGCCAAAGCCGCCGCAGCCACCGCCGCCGC

Intestine   36 ---------------------------------
Stomach     36 ---------------------------------

WFP9(skin)  71 CGCCAAGGCCGCCGCAGAAGCCACCGCCGCCGCAG
Intestine   71 ---------------------------------
Stomach     71 ----------A----------------------

WFP9(skin) 106 CCGCCAAAGCAGCAGCCGACACCAAAGCCAAAGCA
Intestine  106 -T----------------C----------GC----
Stomach    106 ------------------C----------GC---

WFP9(skin) 141 GCCCGTTAAGGATCGTGGTCGTCTTGATGTGGGATC
Intestine  141 ---------T---------------------------
Stomach    141 ---------T---------------------------

WFP9(skin) 177 ATGTGAACATCTGAGCAGCGAGATGTTACCAATCT
Intestine  177 ---------------------------------
Stomach    177 ---------------------------------

WFP9(skin) 212 GCTGAATAAACCTGAGAAGCTGttt
Intestine  212 ------------------A--
Stomach    212 ------------------A---
```

(B)

```
Source
           1         10        20        30
WFP9       MDAPAKAAAATAAAAKAAAEATAAAAAKAAADTKAKAAR
Intestine  ---------------------------A---A-----
Stomach    ----------------T----------A---A-----
```

Figure 4.18 (A) Anterior gut of a newly hatched winter flounder larva (May 1998) following immuno-histochemistry with antiserum to skin-type AFP. Note that the immuno-reaction is only associated with cells in the epithelial layer (arrowheads). (X660). (B) Anterior gut of 10 day old larva following immuno-histochemistry (May 1998). Note the appearance of significant reaction product in the apical cytoplasm of the enterocytes (small arrow) and at the luminal surface in the posterior gut (arrowhead). Some lighter immuno-reaction is also present in the apical cytoplasm of the post-esophageal swelling (large arrow). (X660). (C) Gut of an approximately 25-day-old larva (July 1998). Note similar immuno-reaction as in previous stages (small and big arrowheads), however also note Immuno-reactivity in newly emerging gut mucous cells (arrow). (X660) Ys. yolk sac, L. liver.


A
L
ys
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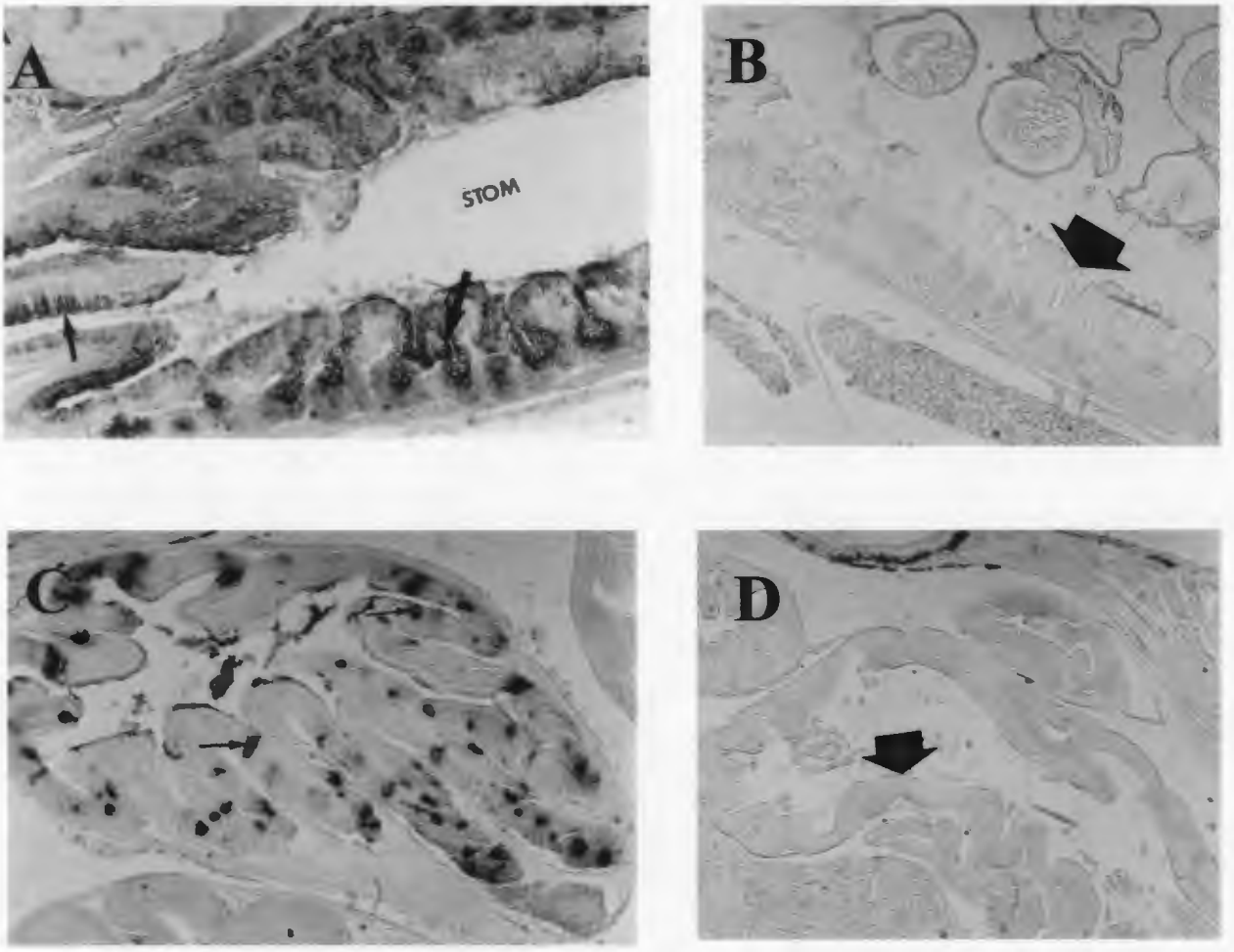


Figure 4.19 (A)Posterior esophagus and stomach of newly metamorphosed winter flounder juvenile following immuno-staining with antiserum for skin-type AFP . Note immunoreaction in mucosal layer of esophagus (medium arrow) and in association with mucous neck cells of stomach (large arrow). (X660). (B) Normal rabbit serum control. (C) Intestine of new juvenile showing distribution of immuno-reactive cells/goblet cells (arrows). (X660). (D) Normal rabbit serum control.

that the AFP is being secreted from the cells. As the gut develops further forming a distinct folded intestine and rectal region, immuno-reactivity also becomes extensive (Figure 4.18c). By the time the fish reach 25 days post-hatch a large portion of the cells lining the intestine and rectum have also become AFP immuno-positive showing evidence of the immuno-reactive product all along the luminal surface. At this stage elongate intestinal goblet cells also appear immuno-positive for AFP (Figure 4.18c).

At the early stages of metamorphosis (about 40 days post-hatch) the esophageal mucosal epithelial cells continue to show evidence of AFP immuno-staining. Immuno-reactivity also becomes obvious in the developing glandular and neck cell region of the stomach (Figure 4.19a). Further, at this stage immuno-reactivity in intestinal goblet cells becomes more prominent (Figure 4.19b). During late fall, juvenile fish exhibit a further proliferation of cells in the intestine which immuno-stain for AFP, the distribution of these cells and their co-localization with Alcian blue positive cells again strongly suggest that they are intestinal mucous cells (Figure 4.20ab). Within the stomach, glandular cells continue to immuno-stain along with occasional submucosal cells.

Immuno-histochemical results on adult stomachs in November showed a distribution of AFP positive cells very similar to that observed in the juveniles. The immuno-reaction was again associated with the neck cell region (Figure 4.21a). The intestine however offered a distinct divergence from that observed in the younger fish. Immuno-positive cells were not found to be associated with intestinal mucous cells at all at this stage. Cells appeared elongate with basal nuclei with staining distribution localized to the cytoplasm (Figure 4.21b). Intestinal mucous cells showed no comparable reaction

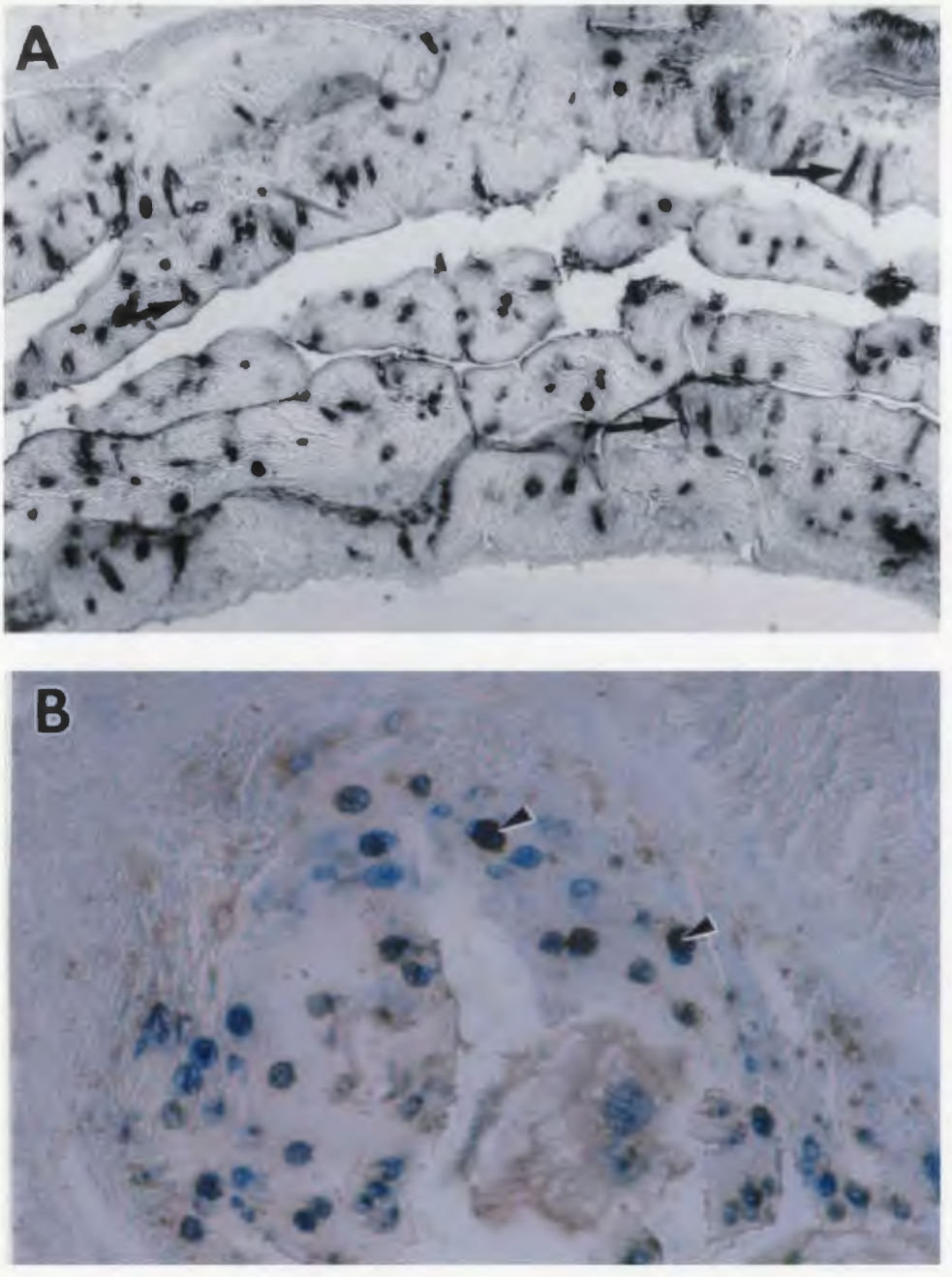


Figure 4.20 (A) Winter flounder juvenile intestine showing large distribution of AFP/mucous cells. (arrows) (X660). (B) Co-localization of AFP immuno-positive cells and cells staining with Alcian blue showing that the cells are one in the same (arrowheads). (X825)

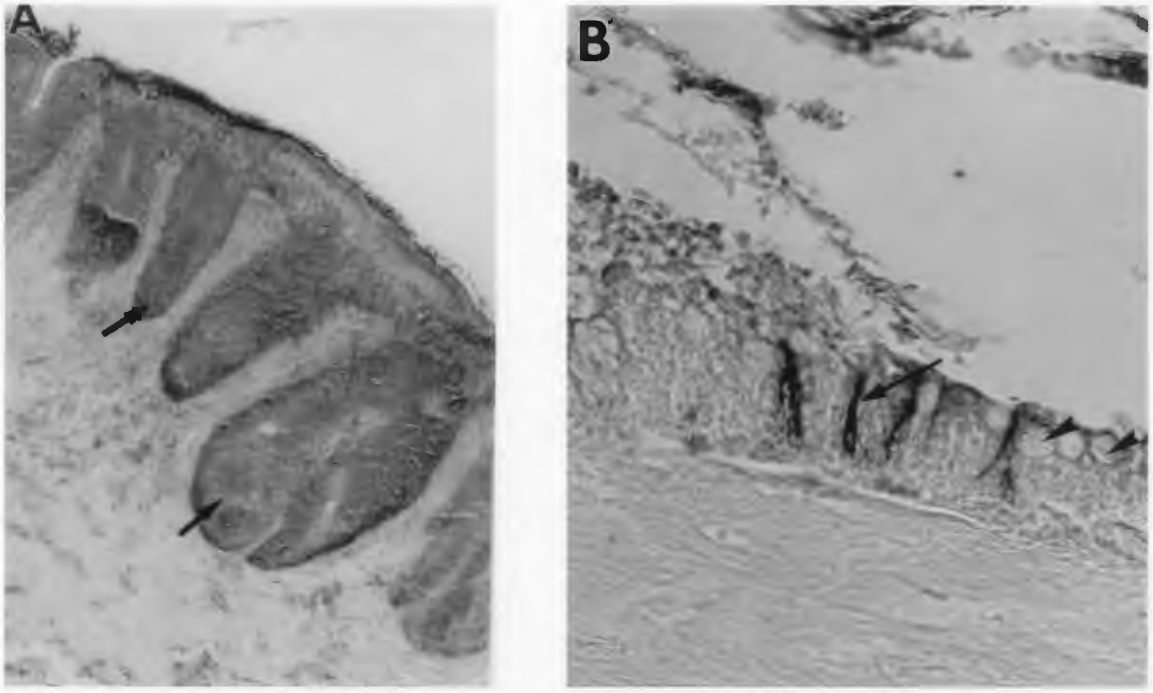


Figure 4.21 (A) AFP immuno-reaction on sections of adult winter flounder stomach. Note reaction in association with neck cell region (arrows). (X330). (B) Immuno-reaction on adult winter flounder intestine. Note distinction between immuno-positive cells (arrow) and mucous cells (arrowheads) at this stage. (X660).

(Figure 4.21b).

4.3.2.1 Controls

Whenever possible parallel sections or duplicate samples were run in control reactions. Four standard controls were used in this study 1. Elimination of the primary anti-sera 2. Substitution of primary anti-sera for normal rabbit serum (Figure 4.19B,D). 3. Substitution of an antiserum to an unrelated antifreeze protein (Type II) for the primary anti-sera. 4. Incubation of the anti-sera with a 100-fold excess of purified antifreeze protein. In all cases specific staining was eliminated.

4.4 DISCUSSION

Past work has indicated that three epithelial tissues could be important as potential sites where ice crystal nucleation could occur and possibly propagate ice into the fish i.e. the skin, the gills and the gut. Gong et al. (1992; 1995; 1996) found that significant levels of an epithelial specific AFP is expressed in all three of these tissues, suggesting that they are active in skin-type AFP production. Little is known however with regard to the role of AFP in the gut and it's development through ontogeny.

RT PCR analysis from the present study using gene specific primers for skin-AFP indicated that the clones isolated from stomach and intestine of adult flounder are skin-type but did exhibit some deviation from the original nucleotide sequence of the WFP9 skin AFP clone from which the primers were designed. Translation of the DNA sequence to the primary amino acid sequence showed that the substitutions likely did not affect the overall protein in structure or function (V. Ewart personal communication).

Valerio et al. (1992) surmised that the gut could be a region prone to epithelial ice

crystal propagation due to the single layer of cells partitioning this region from the vascular blood supply. Cells in this region are known to form very tight cellular junctions preventing movement of solutes and solutions into the extracellular and intercellular spaces (Murray et al. 1996). Considering this idea, in order for ice crystals to move into the intercellular spaces, the membranes and their respective junctions would have to be compromised. AFPs here would likely again act in protecting this region from the propagation of ice crystals across the epithelial membranes. O'Grady et al. (1982; 1983) observed thermal hysteresis activity and was able to isolate AFGP from the intestinal fluid of a variety of Antarctic fishes. Based on this data it was suggested that the AFGPs likely acted in the prevention of ice nucleation in the gut due to the ingestion of ice laden seawater.

The winter flounder digestive system undergoes significant change during the first 40 days prior to metamorphosis. The majority of this change is associated with gut development and maturity and is similar to that observed in other species (Boulhic and Gabaudan 1992; Bisbal and Bengtson 1995; Baglole et al. 1997; Calzada et al. 1998; Douglas et al. 1999; Gawlicka et al. 1995).

The digestive tract begins as a simple tube lined with a simple low columnar epithelium and continues this way for about four days. This compares to similar observations in the yellowtail flounder (Baglole et al. 1997). Structurally there appears to be little difference in the appearance of these cells along the tube, but as evidenced by AFP immuno-staining, an obvious functional difference exists at this stage in the foregut. At this point in larval gut development typical mucous secreting cells are not present.

These cells appear only after approximately 10 days post-hatch and then primarily in the esophagus, this indicates that the AFP producing cells actually arise before the occurrence of true mucous producers.

Mucous histochemical results from metamorphs and juveniles indicate a very similar pattern to that observed in the adult fish (Murray et al. 1994ab; 1996). In this case as well as in the case of the juveniles, mucous cells of the intestine and esophagus were observed to produce acid mucopolysaccharides whereas the stomach mucous cells contained only neutral mucin.

The AFP immuno-histochemical staining in the stomach is consistent between the adult and juvenile stages, however the stage specific cell type change in the intestinal mucosa is puzzling. Co-localization experiments have shown a distinct relationship between the mucous cell and AFP secretion in the juveniles, but the elongate AFP immuno-positive cells in the adult intestine show no obvious association with mucous secretion. These data appear to indicate an AFP related change in cellular function between juvenile and adult winter flounder.

Gong et al. (1996) showed that the epithelial AFPs did not have an associated secretion signal sequence, suggesting that the protein may not be secreted through known pathways and might function intracellularly. The association of AFPs with mucous secreting cells in both the stomach and the intestine of juveniles indicate an alternative mode of secretion allowing AFP "spiked" mucous to overlay the gut mucosa. This idea supports the ice-exclusion hypothesis (DeVries 1986), suggesting that a mucus layer containing AFP could be a very effective barrier to ice crystal invasion. DeVries (1986)

argued, that by inhibiting the growth of ice crystals, the antifreeze proteins could prevent them from entering the fish by blocking their propagation across epithelial membranes like skin, gills, and gut. Valerio et al. (1992) pointed out that in adult winter flounder, the gut could be particularly susceptible to ice crystal invasion due to its relatively thin epithelium. Passive consumption of seawater may be a direct source of exposure to ice. Histological data from this study and from Murray et al. (1994ab; 1996) certainly verifies the delicate structure of the epithelium and its close association with the vascular supply. so an additional barrier to ice propagation could be important. Data from this study indicates that in larvae and juveniles the co-secretion of mucous and AFP could have an important role in secreting AFP into the digestive tract. Further work is required to elucidate the importance of the intestinal cell type change from juvenile to adult and when that change actually occurs.

CHAPTER 5. ONTOGENY AND SEASONAL EXPRESSION PATTERNS OF ANTIFREEZE PROTEIN GENES IN WINTER FLOUNDER LIVER

5.1 INTRODUCTION

The liver is the primary source of serum antifreeze proteins (AFP) in the winter flounder (*Pleuronectes americanus*) (Fletcher et al. 1998; Ewart et al. 1999). In adult flounder these AFP (termed liver-type AFP, Gong et al. 1996) are synthesized and secreted into the blood during the winter when the fish can be exposed to freezing conditions. The timing of this annual cycle of liver type AFP gene expression is regulated by photoperiod acting through the central nervous system on the pituitary, and the active pituitary factor responsible for repression of the liver-type AFP genes during the summer has been identified as growth hormone (Fletcher et al. 1989). The current hypothesis is that during the winter when the flounder do not grow, serum growth hormone levels fall below the level required to suppress transcription, thus allowing AFP mRNA to be produced (Fletcher et al. 1989, Idler et al. 1989; Fletcher et al. 1998). The role of water temperature in regulating the annual cycle of AFP in plasma appears to be confined to modulating liver AFP mRNA and plasma AFP turnover rates (Fletcher 1981; Price et al. 1986; Vaisius et al. 1989).

A second family of AFP genes (skin-type AFP) has recently been discovered in winter flounder (Gong et al. 1996). In contrast to the liver-type AFP genes where expression is restricted to the liver, these skin-type AFP genes are expressed in most tissues including the liver. However by far the most abundant expression occurs in the external epithelial tissues such as gill and skin. These skin-type AFP differ from the liver-

type AFP in that they lack a secretion signal peptide suggesting that they are likely to remain and function intracellularly. In addition, the skin-type AFP genes differ from the liver-type in that they are expressed constitutively throughout the year (Gong et al. 1996).

Winter flounder spawn in the spring when water temperatures are low and the larvae develop and metamorphose by summer's end, enabling them to enter their first winter as newly developed juveniles. The purpose of the present study was to determine when during ontogeny expression of the liver-type and skin-type AFP genes is initiated in liver.

5.2 MATERIALS AND METHODS

5.2.1 Adult Maintenance

Winter flounder were captured by SCUBA (November, 1996; January, 1997; July, 1998) and transported to the Ocean Sciences Centre where they were held in tanks supplied with running seawater under ambient conditions of photo-period and temperature.

In early spring, animals were checked daily for signs of gonadal swelling and the presence of eggs or milt. When fish of appropriate condition were found they were immediately isolated from the main tank in preparation for spawning.

5.2.2 Spawning and Fertilization

5.2.2.1 Spawning

Eggs were collected by carefully drying the genital pore area and then applying gentle pressure, allowing the eggs to pour easily into a clean dry container. The container containing the eggs was then stored at 5^{0} C until fertilization. Sperm was collected by

placing a syringe near the genital pore and gently drawing up the milt. Following gamete collection all further procedures were carried out at 5 °C.

5.2.2.2 Fertilization

Five hundred to one thousand microlitres of eggs in ovarian fluid were placed in a dry sterile plastic Petrie dish and mixed gently with 100 ul of milt. The sperm were activated by adding a few drops of UV treated and filtered seawater (0.2 μm Millipore filter system). The eggs were then allowed to sit for 5 minutes to ensure fertilization and subsequently flooded with fresh filtered seawater and allowed to water harden for an additional 20 minutes.

Following water hardening the eggs were flushed once more to remove any surplus milt and allowed to develop. Water was changed every other day by decanting off old and adding new fresh filtered seawater. Following fertilization eggs were incubated at 5 ^{0}C and hatched within 14 days.

5.2.3 Hatching and Transfer to Rearing Containers

Following hatch, 1000 to 2000 larvae were transferred to 4 litre plastic buckets lined with black plastic bags to enhance contrast for prey visibility. Buckets were set in flowing seawater baths at ambient temperature/photoperiod. Temperatures ranged from 5 to 7 ^{0}C in May/June to as high as 14^{0}C in August. The buckets contained 3 litres of filtered (0.2 μm) and UV sterilized seawater plus 500 mls of mixed algae ie *Isochrysis* sp. and *Tetraselmis* sp. Water was part changed (50%) and replaced every 2 to 3 days with mixed algae. Animals were fed daily on a ration of 5000 rotifers (variety: *Sap)* per litre

and supplemented with a further 300 millilitres of algae until metamorphosis (about 60 days). The first metamorphs from early egg batches occurred from mid July and all fish had reached metamorphosis by the end of August. Fish reaching metamorphosis and thus settlement were transferred to an aquarium with flowing ambient seawater and aeration and weaned onto a ration of 100 000 *Artemia* per day. Subsequently, fish were further weaned onto a commercial dry diet (i.e.Biokyowa).

All animals were maintained and killed according to the guidelines set by the Canadian Council of Animal Care (Olfert et al. 1993).

5.2.4 Tissue Sampling

5.2.4.1 RNA Analysis

Larval and metamorphosed winter flounder were taken for total RNA extraction. These samples were flash frozen in liquid nitrogen by two methods. For larvae ranging in age from newly hatched to premetamorphosis, fish were anesthesized in MS-222 and transferred to a 1.5-ml eppendorf tube and subsequently exposed to brief centrifugation at 10,000 rpm for 30 to 90s to form a pellet. Water was carefully decanted without disturbing the pellet and the tube was immersed directly in liquid nitrogen. Metamorphs were frozen by first anesthesizing them and then immersing them directly into a bath of liquid nitrogen. All samples were stored at –70 °C until use.

Adult winter flounder were sampled in either December (winter) or July (summer). All fish were sacrificed by a blow to the head followed by cervical severance. Livers were removed and immediately flash frozen in liquid nitrogen. Tissues were stored at - 70 °C until analysis.

5.2.4.2 Histology, *In situ* hybridization and Immuno-histochemistry

Larval flounder were sampled every 10 days from hatch to metamorphosis. Following metamorphosis five to ten fish were sampled monthly up until the middle of the first winter. Specifically samples were taken on August 12/97, September 4/97, October 1/97, November 14/97, and February 13/98. Samples were also taken during the following summer (July 1998) and during December 1998. Similarly, 1-cm^2 blocks of liver were excised from adult winter flounder in fall (November 1996), winter (January 1997) and summer (July 1998).

Tissues were either fixed in 4 % paraformaldehyde in PBS for 30 minutes to 2 hours for *in situ* hybridization, 2.5% gluteraldehyde and 4.0% paraformaldehyde in 0.06 M sodium cacodylate, pH 7.2 for electron microscopy or 24-48 hours in Bouins fluid for histology, mucous histochemistry and immuno-histochemistry. All fixations were carried out at room temperature. Following fixation, tissue was processed for embedding in paraffin or epon resin. Staining for glycoprotein histochemistry followed the technique of Pearse (1985).

5.2.5 Total RNA Extraction and RT PCR

5.2.5.1Total RNA Extraction

Total RNA was extracted from whole scales using either the Qiagen®, Rneasy® Mini RNA prep kit following disruption and homogenisation using the QIAshredder ® and the included homogenisation buffer or alternatively with Trizol ® reagent, according to the manufacturer's suggested protocol. Following extraction, RNA was treated with Life Technologies amplification grade DNAse I according to the manufacturer's

recommendations, aliquoted and frozen at -70 ° C until use.

5.2.5.2 PCR Primers

5.2.5.2.1 Skin-type AFP

PCR primers (WFP9A and WFP9B) were designed to amplify a 250 base pair (bp) fragment analogous to an AFP cDNA clone (WFP9) originally isolated from a winter flounder skin cDNA library (Gong et al., 1996). The sequence of the primers corresponded to 5'-GTCGAACACTCAGAATCACTG-3' and 5'-AGCTTCTCAGGTTTATTCAGC-3' respectively.

5.2.5.2.2 Liver-type AFP

A second set of PCR primers (CT5preproA and CT5preproB) were designed to amplify a 150 bp fragment analogous to the "prepro-" sequence of the liver specific AFP cDNA clone CT5 (Gourlie et al., 1984). This region is identical to that of the liver AFP cDNA pKenc-17. The primers had the associated sequences, 5'-AAGTTCTCAAAATGGCTCTC-3' and 5'-TGGGGCGGCTGCGGCAGGGG-3', respectively.

5.2.5.3 RT PCR

One microgram of total RNA, primers, and the required components of the Life Technologies SuperScript ™ One-Step ™ RT-PCR System were mixed according to the manufacturer's recommendations. The mixture was incubated at 50 ° C for 30 minutes for the reverse transcription reaction and then carried through PCR on a Perkin-Elmer Model 480 thermal cycler. The amplification consisted of 35 cycles at 94 ° C, (30s), 60 ° C (30s), and 68 ° C (45s).

5.2.5.4 Sequencing of PCR Products

At least five consecutive RT PCR reactions were run and the reactions pooled, DNA ethanol precipitated and then resuspended in 50 ul of sterile water. This volume was run on a 2% agarose gel for at least 1 hour at 100 V and stained with ethidium bromide. The gel fragment was excised and the DNA purified using the QIAquick Gel Extraction Kit (Qiagen). Purified PCR products and appropriate primers were subsequently sent to the DNA Sequencing Facility/The Centre for Applied Genomics at the Hospital for Sick Children, Toronto Ontario. For comparison, obtained sequences were checked against known AFP sequences to verify the result.

5.2.6 *In situ* Hybridization

5.2.6.1 Ribo-probe Production

Riboprobes were generated using clones for a winter flounder skin-type antifreeze protein (WFP9) (Gong et al. 1996) and a liver specific antifreeze protein (pKenc-17) (Gourlie et al. 1984). Probes were made using *in vitro* transcription as described previously (Chapter 2 and 3). Whenever possible sense riboprobes were generated as controls for hybridization.

5.2.6.2 Section Pre-treatment

Seven-micron wax sections on Silane (Sigma) coated slides, were deparaffinized in two washes of xylene for 10 minutes each and subsequently hydrated through an ethanol series (2 minutes in each of 100 %, 95 %, 70 %, and 50 %). Further pre-treatment of tissue including protease digestion and blocking of non-specific binding due to electrostatic interactions between probe and tissue were as described previously

(Chapter 2 and 3). Subsequently, tissue was further rinsed in 2 x saline sodium citrate (2 x SSC) before being dehydrated in a graded series of ethanol and air dried for at least 10 minutes.

5.2.6.3 *In situ* Hybridization

Sixty to one hundred micro-litres of probe solution were added to each slide and cover slipped. Slides were placed in a moist chamber with 50 % formamide in 2 x SSC, covered and sealed with parafilm. Hybridization was allowed to occur for up to 24 hrs at 45 ^{0}C (See Chapter 3).

5.2.6.4 RNAase Treatment and Post-hybridization Stringency Washes

Cover-slips were removed by washing gently in 2 x SSC and the slides were subsequently treated in 50% formamide in the same salt solution for one hour at 45 ^{0} C. Following this wash slides were rinsed in 10 mM Tris, pH 8.0, 500 mM NaCl and then RNase treated as described previously (Chapter 3). Following RNAase treatment, slides were washed once more in the same buffer for 30 minutes at 37° C and stringency washed at 50 °C for 30 minutes in each of 2 x SSC, 0.5 x SSC and 0.1 x SSC.

5.2.6.5 Antibody Treatment and Immuno-histochemical Detection

For signal detection, slides were treated as described in Chapter 3. Briefly, sections were rinsed in 0.1M Tris, pH 7.5, 150 mM NaCl and then blocked with 1% BSA in the same buffer. Sections were subsequently treated with a 1/250 dilution of anti-digoxigenin antibody conjugated to alkaline phosphatase and incubated overnight at 4°C. Following antibody treatment, the slides were washed in buffer and the chromogenic reaction developed as outlined in Chapter 3. The reaction was stopped by washing slides

in 1 X FS and then dehydrating rapidly through ethanol series, clearing briefly in xylene and mounting in Cytoseal (Stephens Scientific) for observation and photo-microscopy.

5.2.7 Immuno-histochemistry

5.2.7.1 Tissue Preparation and Blocking of Non-specific Sites

Tissue sections were deparaffinized by washing twice in xylene for 10 minutes, rehydrated through ethanol series and washed three times 5 minutes in PBT. Non-specific sites were blocked by incubating tissue in 20% goat serum in buffer for 15 minutes at room temperature.

5.2.7.2 Antibody Treatment

Winter flounder skin AFP was secretory expressed in *E. coli* JM105 and purified to homogeneity by Lin et al. (1999). Polyclonal antisera to winter flounder skin AFP was produced in rabbits following conjugation to keyhole limpet hemocyanin (Lin et al. 1999). Polyclonal antisera to winter flounder liver-type AFP (HPLC-6) was also generated in rabbits following purification from fish serum and conjugation to keyhole limpet hemocyanin. Monoclonal antibodies for liver-type AFP were obtained from A/F Protein Canada, Inc. Antisera to sea raven Type II antifreeze protein was produced in rabbits without conjugation to keyhole limpet hemocyanin (Ng et al. 1986).

Polyclonal antisera to either skin-type AFP or liver-type AFP was diluted in PBT with 5% goat serum, dilutions ranged from 1/500 to 1/1000. Monoclonal antibodies to liver type AFP were diluted 1/100 in PBT. Solutions were applied to sections and incubated for 30 minutes at room temperature. Following antibody application, slides were washed four times in PBT for five minutes each.

The secondary antibody (goat-antirabbit (polyclonals only) or goat-antimouse (monoclonals only) conjugated to horseradish peroxidase (HRP) (Sigma)) was diluted in PBT with 5% goat serum, applied to sections and incubated in an identical manner to the above. Dilutions ranged from 1/100 to 1/500. Slides were again washed four times in buffer for five minutes each.

5.2.7.3 Reaction Development and Mounting

Following the final wash, sections were covered with 500 ul of Immuno-Pure Metal Enhanced DAB substrate (Pierce) and incubated at room temperature for 5 minutes or until the chromogenic reaction was of favourable intensity. Reaction was then stopped by washing in PBT, dehydrated in ethanol series, cleared in xylene and mounted in Cytoseal (Stephens Scientific) for observation and photography.

5.2.7.4 Controls

Standard immuno-histochemical controls were utilised in all experiments. Negative controls involved the replacement of the primary antibody with normal rabbit serum or antiserum for Type II AFP from sea raven. Similarly, tests for antibody specificity including competition studies and protein dot blotting protocols using purified AFP were described previously (Chapter 2).

5.3 RESULTS

5.3.1 Histological Ontogeny and Glycoprotein Histochemistry

During larval development (hatch to day 24), the liver appeared as a glandular extension from the anterior gut and consisted of cuboidal shaped cells (Figure 5.1a). Histochemically the cells stained lightly positive for periodic acid/Schiffs (PAS) reagent.

Very little change was observed during this stage with the exception of an increase in the overall size or volume of the organ (Figure 5.1b) Following metamorphosis PAS staining intensity increased dramatically especially in the fish sampled during fall and winter (Figure 5.1c).

Adult liver structure was very similar to that of older juveniles, appearing as a very compact organ with an organized cellular structure (Figure 5.1d). Little obvious change in cellular structure is apparent seasonally at the light microscope level. However, ultrastructurally winter hepatocytes contained large amounts of cytoplasmic glycogen stores and distinct lipid bodies that are not obvious in summer liver (Figure 5.2ab).

5.3.2 RT PCR

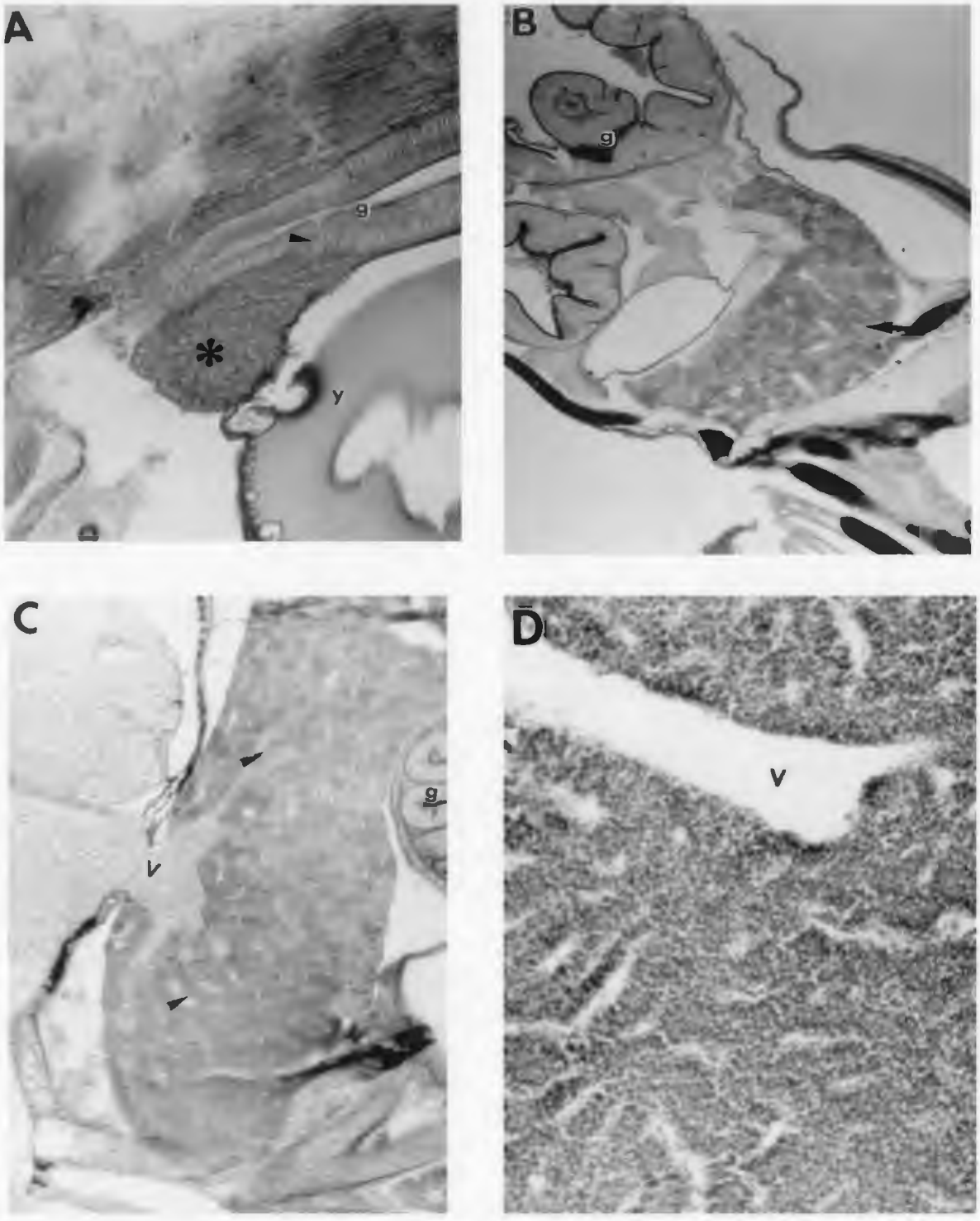
5.3.2.1 Testing Primers

Purified plasmid DNA containing the complete liver AFP clone pKenc-17 was reacted with primers CT5preproA and CT5preproB to test for specificity. Primers consistently showed good amplification of a 150-bp fragment that corresponded to the expected size of the prepro region of the liver AFP clone CT5 (Gourlie et al. 1984) (Figure 5.3). Skin primers WFP9A and WFP9B showed similar results as illustrated in Chapter 2.

5.3.2.2 RT PCR on Total RNA from Larvae, Juveniles and Adult Flounder

RT-PCR with liver AFP primers and total RNA isolated from winter flounder larvae did not produce a band. Juveniles in their first fall however produced a distinct 150-bp band corresponding well to the appropriate region of the original liver cDNA

Figure 5.1 (A) Relationship between the anterior gut and liver of a newly hatched winter flounder larva. Note the duct indicating the direct connection between the liver and the gut (g) lumen (arrowhead). X700. (B) Liver of a 24-day-old larva (arrow) showing the obvious increase in overall size of the organ. X350. (C) Liver of a metamorphosed juvenile winter flounder showing a cord like organization of the tissue (arrowheads) and large vascular supply (V). g, gut X350. (D) Section of liver from adult winter flounder in November showing overall cellular organization and vascular supply (V) X350. All tissue was stained with Periodic Acid/Schiff reagent. (asterix is liver) Y, yolk sac.


A
g
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g
v
D
v

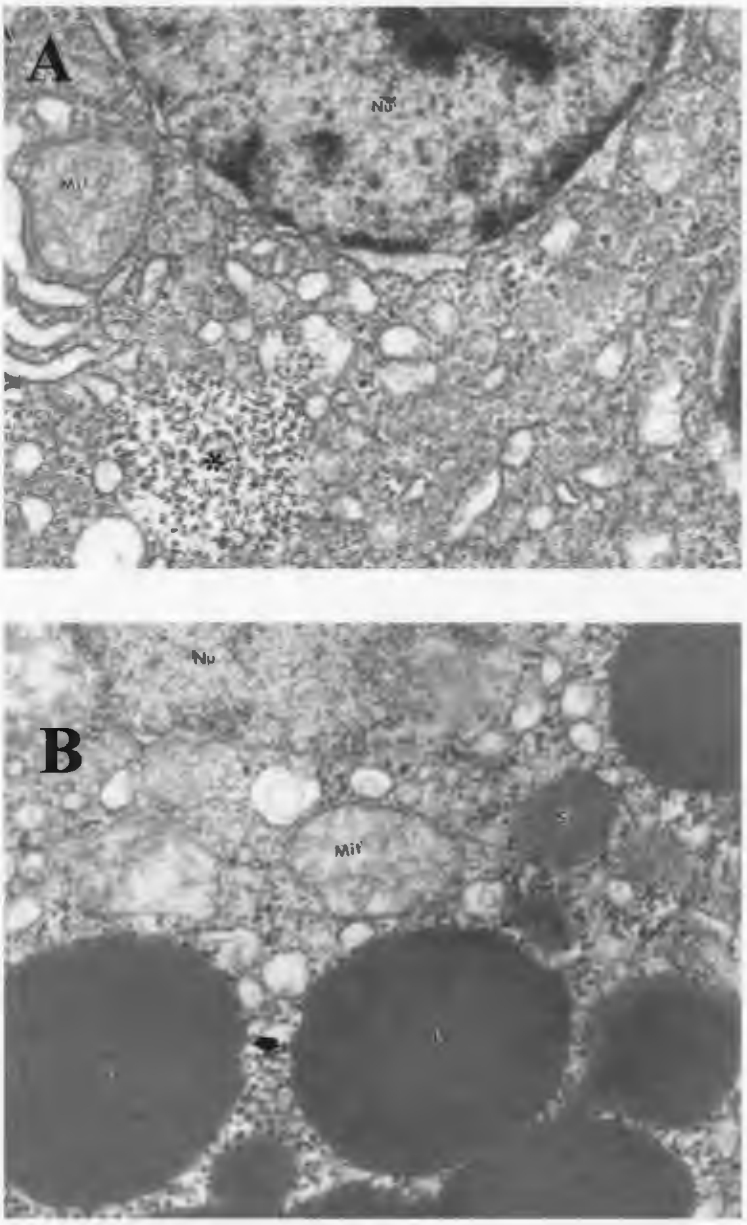


Figure 5.2 (A) Transmission electron micrograph of a typical hepatocyte from a winter flounder in summer season. Note extensive distribution of glycogen in the cytoplasm (asterix). (X42000). Mit, mitochondria. (B) Electron micrograph of a typical hepatocyte from winter flounder in winter. Note the presence of large electron dense cytoplasmic lipid droplets (L) and the decrease in observed cytoplasmic glycogen (arrowhead). Occasional membrane bound secretory vesicles are also present (S). (X42000).Nu, nucleus.

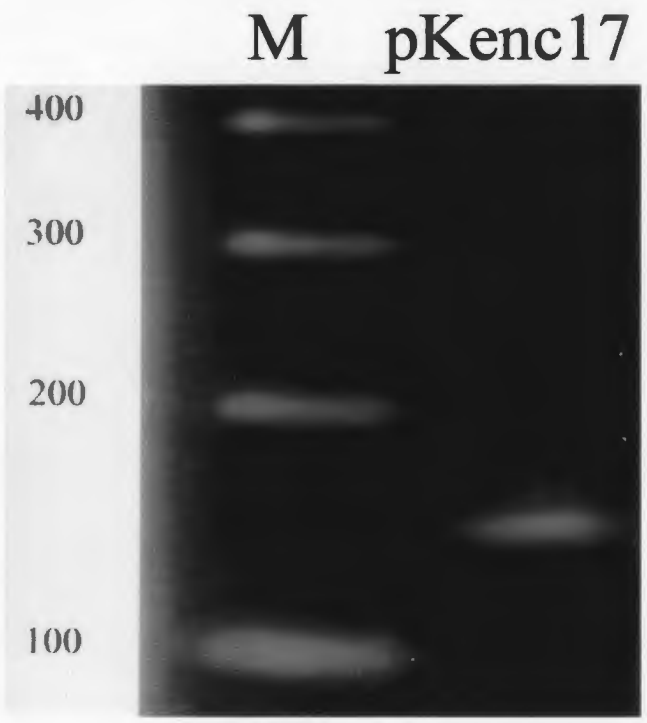


Figure 5.3 PCR verification of the specificity of primers for the "pre-pro" region of liver AFP clone CT5 with plasmid DNA for liver AFP clone pKenc-17. All samples were run on a 2% agarose gel at 100V for one hour and stained in ethidium bromide.

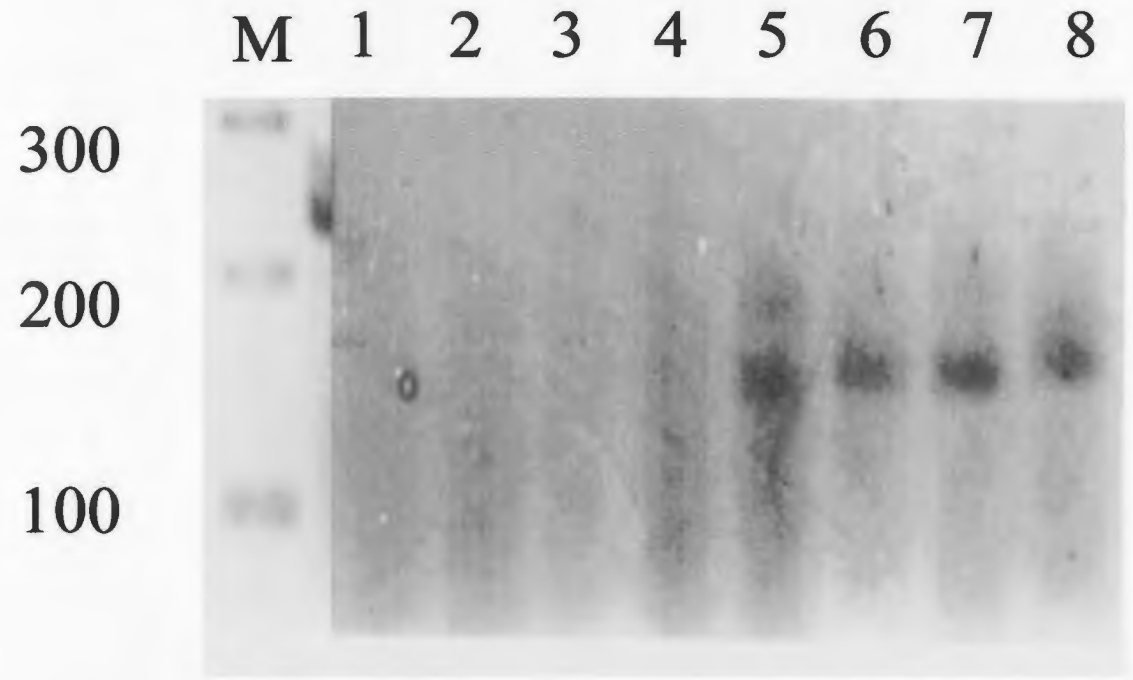


Figure 5.4 RT PCR results showing liver AFP expression over the postembryonic development of winter flounder. New hatch (lane 1), 4 days (lane 2), 10 days (lane 3), 20 days (lane 4), premetamorph (lane 5), metamorph (lane 6), first fall juvenile (lane 7) and first winter juvenile (lane 8). Samples were run on a 2% agarose gel at 100V and stained in ethidium bromide.

clone (CT5) (Figure 5.4). As shown in Chapter 2 primers to skin AFP clone WFP9 produced a distinct band throughout the post-embryonic stages.

RT-PCR results on total RNA isolated from adult winter flounder liver in January showed one distinct band for each relevant primer set. Once more, the band resulting from amplification with the skin primers was equivalent in size to that observed when the original cDNA was substituted for RNA in the PCR reaction (Figure 5.5a). No difference was observed following reactions using total liver RNA isolated in July (Figure 5.5a).

RT-PCR using the CT5prepro primer set and total RNA isolated in January consistently amplified a 150-bp band that was equivalent in size to that expected from the original clone (Figure 5.5b). Total RNA isolated in July did not consistently give a band for these primers (Figure 5.5b).

5.3.2.3 Sequence Comparison

All PCR products generated with the CT5prepro primers showed 100% identity to the original liver AFP clone CT5 (Figure 5.6a). The amino acid sequences translated from RT PCR products of fall juveniles and flounder adults in January showed 100% identity to the original clone (Figure 5.6b). Sequences generated from cDNAs amplified with skin AFP primers and total RNA from liver were identical to the original skin AFP clone WFP9. The only anomalies were a C to T substitution at nucleotide 107 and 120. A third substitution (G to A) was also observed at nucleotide 233 (Figure 5.7a). The amino acid sequences translated from RT PCR products were identical to the clone WFP9 with the exception of an Alanine to Valine substitution at amino acid 31(Figure 5.7b).

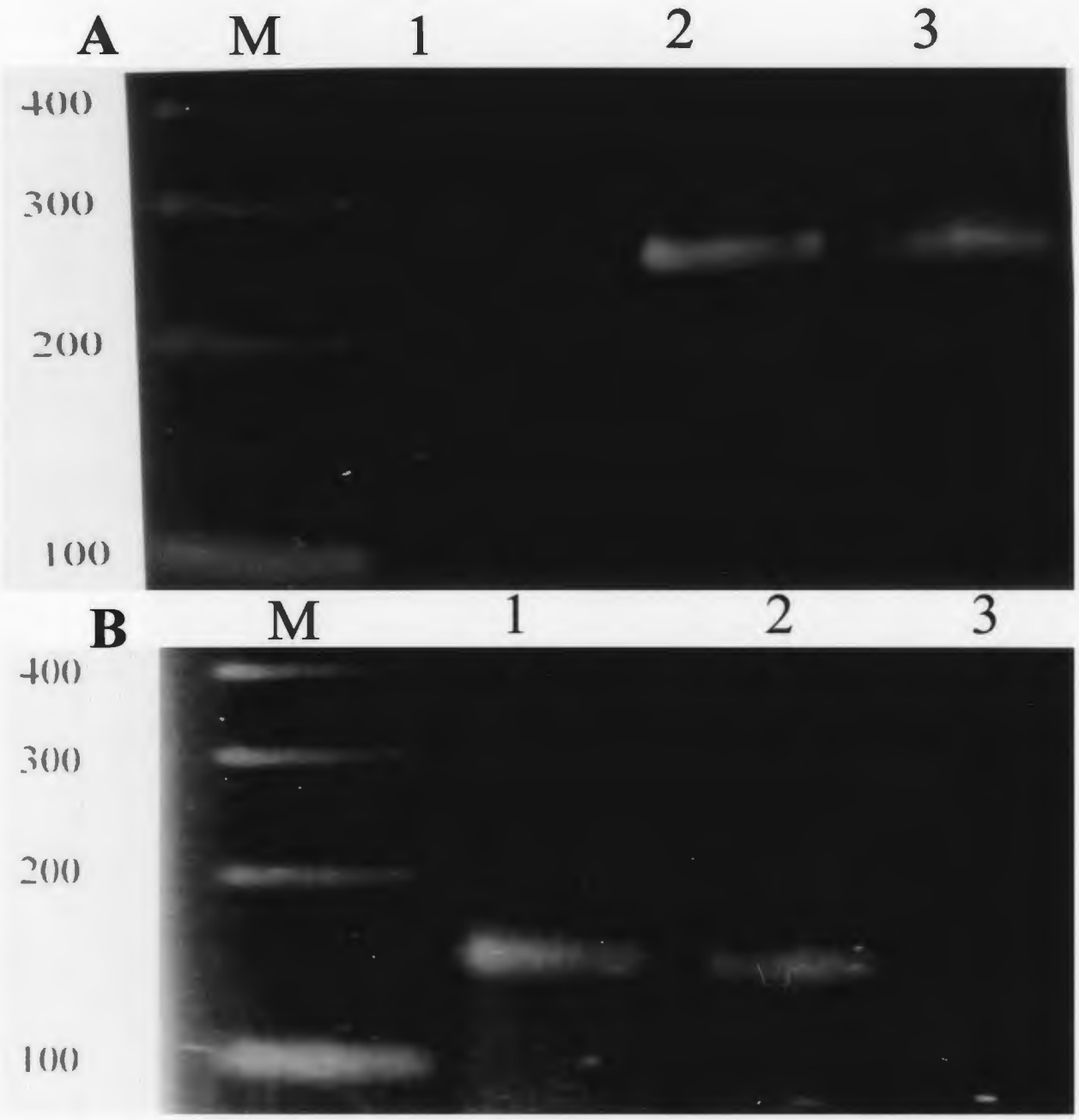


Figure 5.5 (A) RT-PCR results using primers for skin AFP clone WFP9, showing products amplified from total RNA isolated from winter flounder liver (winter), no reverse transcription (lane 1), summer (July) (lane 2), and winter (Janurary) (lane 3). (B) RT-PCR results using primers for the "pre-pro" region of liver AFP clone CT5, showing products amplified from total RNA isolated from winter flounder liver in winter (January) (lane 1), known pkenc-17 sequence (lane 2) and summer (July) (lane 3). Samples were run on a 2% agarose gel at 100V for one hour and stained in ethidium bromide.

(A)

Source

```
CT5PREPRO  1 A A G T T C T C A A A A T G G C T C T C T C A C T T T T C A C T G T
LiverAFP   1 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

CT5PREPRO 35 C G G A C A A T T G A T T T T C T T A T T T T G G A C A A T G A G A A
LiverAFP  35 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

CT5PREPRO 70 T C A C T G A A G C C A G C C C C G A C C C C G C A G C C A A A G C
LiverAFP  70 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

CT5PREPRO 104 C G C C C C A G C A G C A G C T G C C G C C C C T G C C G C A G C C
LiverAFP  104 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

CT5PREPRO 138 G C C C C
LiverAFP  138 - - - - -
```

(B)

Source

```
                1               10              20              30
CT5PREPRO  M A L S L F T V G Q L I F L F W T M R I T E A S P D P A A K A A P A A A A
LiverAFP   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
                40
CT5PREPRO  A P A A A A
LiverAFP   - - - - - -
```

Figure 5.6 (AB) Comparison of known liver AFP prepro-sequence from clone CT5 to that of PCR products amplified from total RNA isolated from adult winter flounder liver under different conditions and first winter juvenile flounder. Note the 100 % sequence identity for both nucleotides and amino acids.

(A)

Source

```
WFP9(skin) 1 GTCGAACACTCAGAATCACTGACATCAACATGGACG
Liver      1 ------------------------------------
WFP9(skin)37 CACCAGCCAAAGCCGCCGCAGCCACCGCCGCCGCC
Liver      37 -----------------------------------

WFP9(skin)72 GCCAAGGCCGCCGCAGAAGCCACCGCCGCCGCAGC
Liver      72 -----------------------------------

WFP9(skin)107 CGCCAAAGCAGCAGCCGACACCAAAGCCAAAGCAG
Liver      107 T--------------T-------------------

WFP9(skin)142 CCCGTTAAGGATCGTGGTCGTCTTGATGTGGGATC
Liver      142 -----------------------------------

WFP9(skin)177 ATGTGAACATCTGAGCAGCGAGATGTTACCAATCT
Liver      177 -----------------------------------

WFP9(skin)212 GCTGAATAAACCTGAGAAGCTGttt
Liver      212 ----------------------A----
```

(B)

Source

```
          1                  10                  20                  30
WFP9 MDAPAKAAAATAAAAKAAAEATAAAAAKAAADTKAKAAR
Liver ------------------------------V---------
```

Figure 5.7 (A) Nucleotide sequences of epithelial AFP cDNAs amplified from total RNA isolated from winter flounder liver using primers specific for the known sequence of skin AFP clone WFP9. Dashes represent regions of identity. (B) Translated protein sequence from the above cDNA showing regions of identity.

5.3.3 *In situ* Hybridization

5.3.3.1 Larval and Juvenile Winter Flounder

The mRNA for skin-type and liver type AFP was not detectable in the liver prior to November of year 0 fish (Figure 5.8ab). Following initial detection, AFP mRNA showed continued presence into February with a slight increase in intensity (Figure 5.8cd). At this stage the reaction product was evenly distributed among the hepatocytes.

5.3.2.2 Adult Winter Flounder

During fall and winter, mRNA in the liver of adult winter flounder was easily detectable using liver type and skin type AFP probes. Samples taken in November and January all indicated that expression products were present for both sets of genes (Figure 5.9ab). No hybridization product was observed in association with cells of the capsule or endothelial cells lining vascular structures. As the fish moved into summer there was a distinct decline in the intensity of hybridization product for both AFP mRNA probes, and by July the reaction product was absent all together (Figure 5.9cd).

5.3.3 Immuno-histochemistry

5.3.3.1 Larval and Juvenile Winter Flounder

As with the *in situ* hybridization data, immuno-detectable protein was not apparent in the hepatocytes until well beyond metamorphosis (November). AFP was detectable using antisera to winter flounder liver AFP and the monoclonal to liver AFP beginning in November of the flounder's first year (Figure 5.10a). Skin–type AFP in liver was never detectable using immuno-histochemistry (Figure 5.10b).

Figure 5.8 (A-B) Liver from juvenile winter flounder in November following *in situ* hybridization with riboprobe for liver-type AFP (A) or skin-type AFP (B). Note darkly stained regions throughout the liver (Liv) and the absence of staining in muscle (M). X350. (C-D) Liver from juvenile in February showing a similar staining pattern to the above. Liver AFP (C) and skin AFP (D). (E) Sense probe control tissue. (X700).

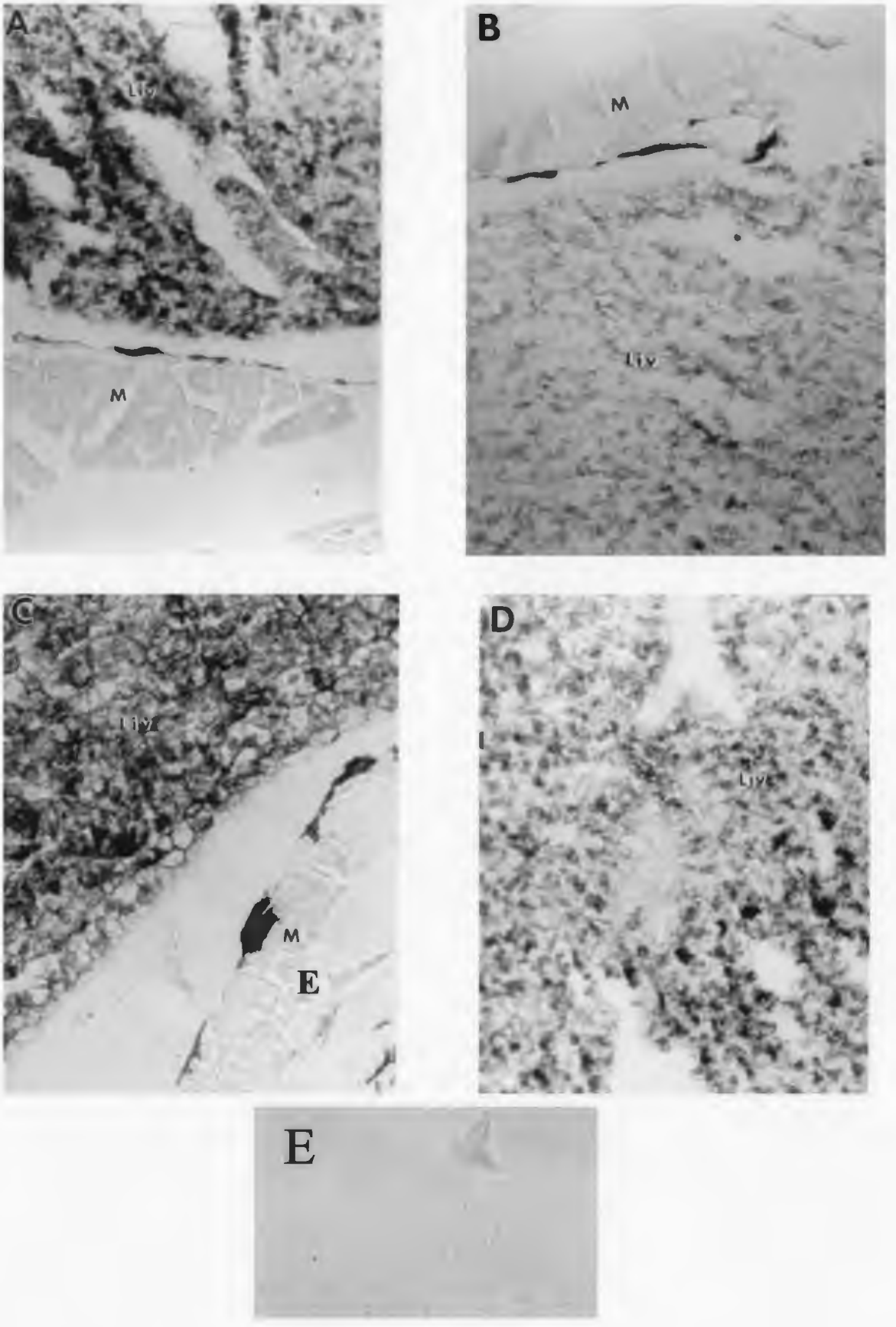
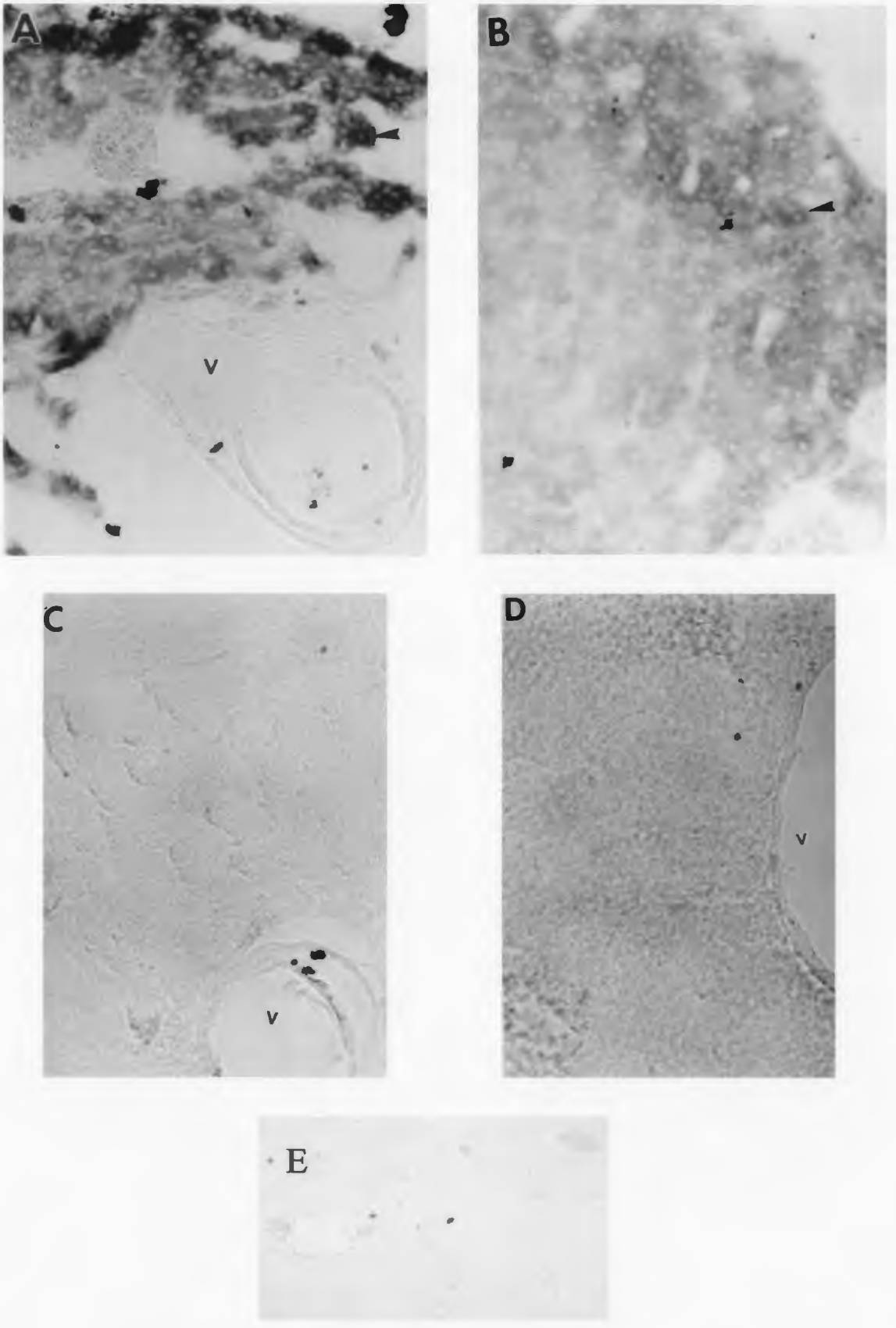

A
Liv
M
B
M
Liv
C
Liv
M
E
D
Liv
E

Figure 5.9 (A-B) Liver from adult winter flounder during November following *in situ* hybridization with riboprobe for liver AFP (A) or skin AFP (B). Note the cytoplasmic distribution of the stain outlining the nucleus and the absence of any reaction in the vascular tissue (V). X700. (C-D) Summer liver following *in situ* hybridization with the above riboprobes. Note complete absence of stain for both probes. (E) Sense probe control tissue. (X700).


A
V
B
C
V
D
V
E

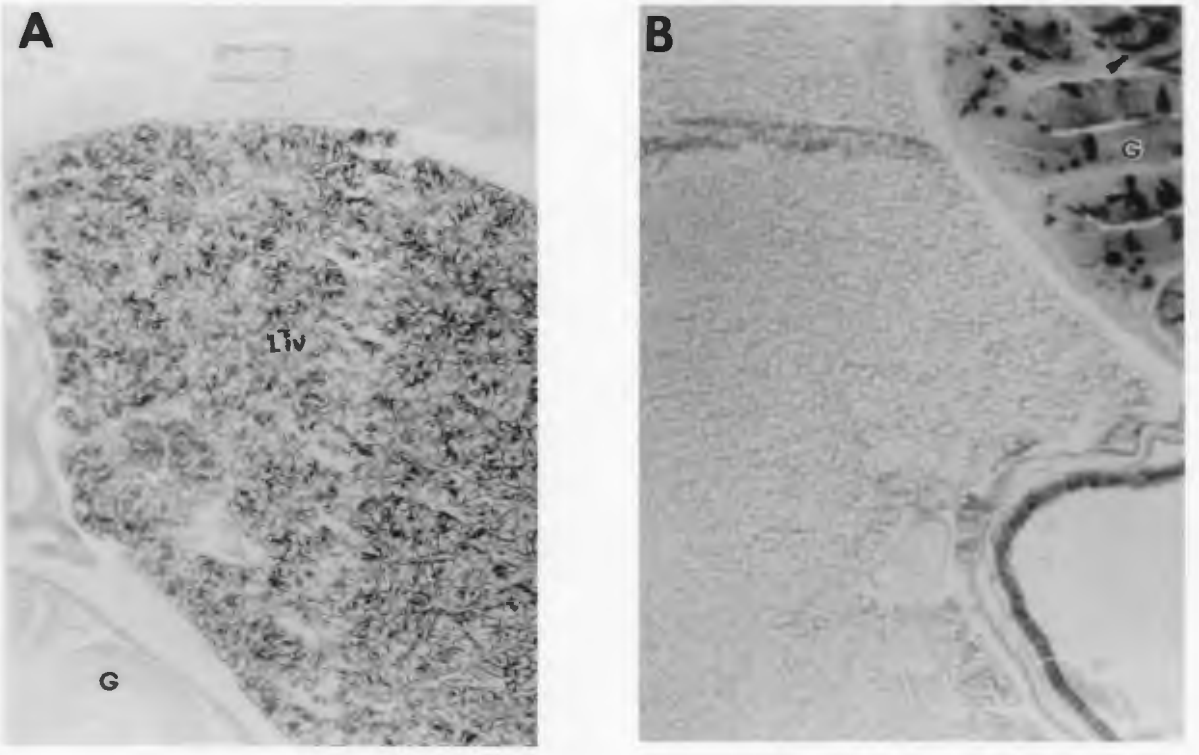


Figure 5.10 (A) Liver of a winter flounder juvenile in November following immuno-histochemistry with monoclonal antibody for liver type AFP. Note specificity of stain for liver tissue (Liv) and the lack of stain in surrounding tissues, i.e. Gut, G. (X350). (B) Liver of a winter flounder juvenile in December following immuno-histochemistry with antisera for skin type AFP. Note lack of significant stain in liver but specific reactions with cells in the gut (arrowheads). (X350).

5.3.3.2 Adult Winter Flounder

Liver-type AFP was detectable in adult liver in November and continuing through the winter until February (Figure 5.11ab) As with the juveniles, skin-type AFP was never detected in liver tissue using immuno-histochemistry (Figure 5.11c). Immuno-detectable liver-type AFP was only localized in hepatocytes. Tissue sections immuno-stained for liver-type AFP indicated that the AFP was localized to specific regions of the cytoplasm, possibly the endoplasmic reticulum or the Golgi apparatus (Figure 5.11ab). Antiserum to skin-type AFP did not show hepatocyte specific staining at any time of the year (Figure 5.11c).

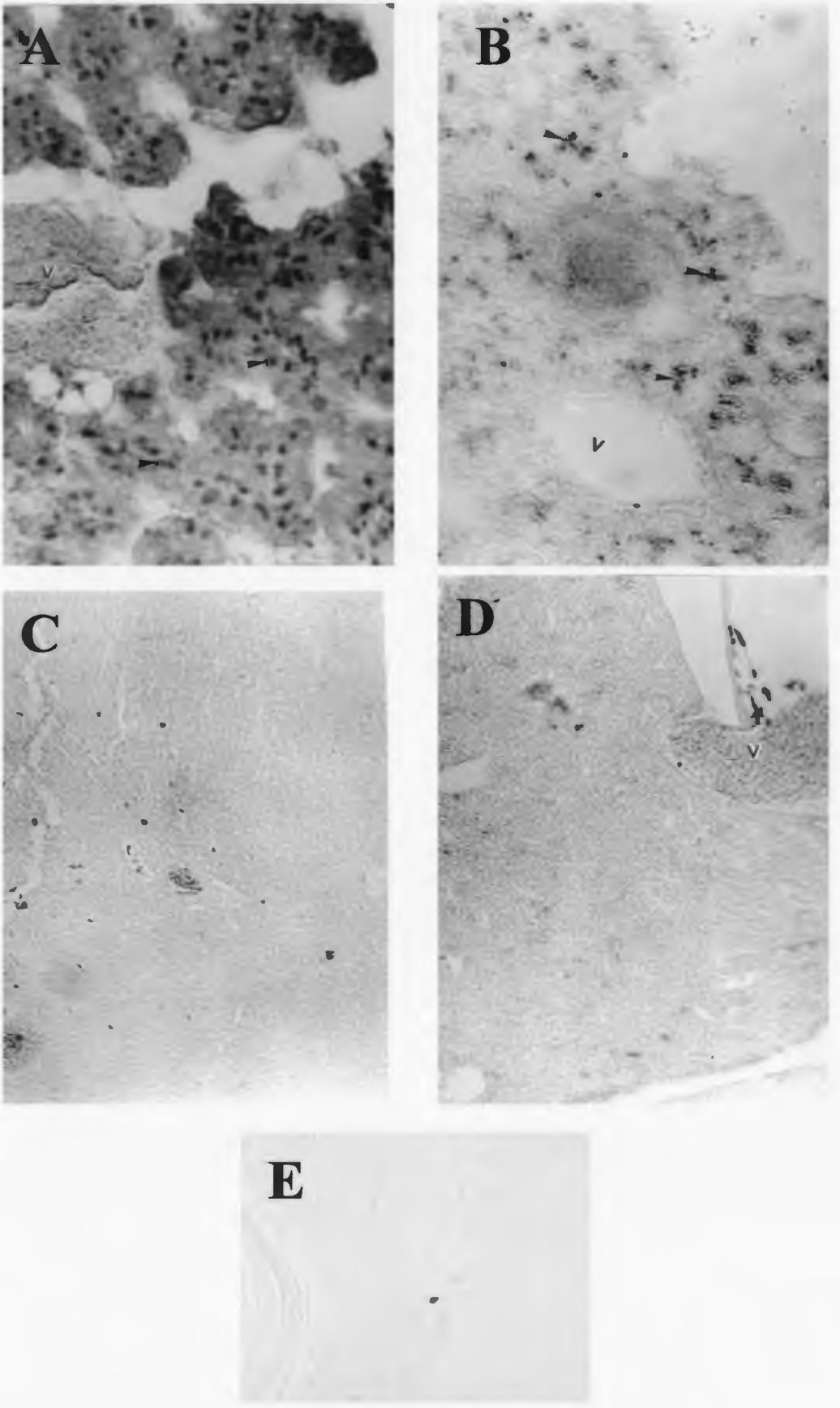
5.4 DISCUSSION

5.4.1 Liver-type AFP

The annual cycle of AFP production has been well established for liver type AFP in adult winter flounder inhabiting Newfoundland waters. Antifreeze proteins appear in the plasma in November when the water temperatures approximate 4-6 ^{0} C and disappear during May when the temperatures generally rise above 0 ^{0} C (Fletcher 1977). Liver AFP mRNA levels also show a similar annual cycle. However as would be expected, AFP mRNA can be detected in the liver during mid-October, in advance of antifreeze appearance in plasma, and it disappears from the liver by the end of April, almost two months earlier than the loss of antifreeze from the plasma (Fourney et al. 1984c). The early detection of liver AFP mRNA in new winter flounder metamorphs using RT PCR suggests preparation for the upcoming winter conditions.

In the present study on juvenile winter flounder that had never been exposed to

Figure 5.11 (A-B) Liver of adult winter flounder in November following immuno-histochemistry with polyclonal antisera for liver type AFP (A) or a monoclonal antibody for liver type AFP (B). Note that the stain product is localized to specific regions within the cell (arrowheads). X1400. (C) Liver of adult winter flounder in July following immuno-histochemistry with polyclonal antisera for liver type AFP. X350. (D) Liver of adult winter flounder in November following immuno-histochemistry with polyclonal antisera for skin type AFP. X350. Note lack of staining in association with hepatocytes for either C or D. (E) Normal rabbit serum control.


A
B
C
D
E

winter conditions, there was no evidence for AFP mRNA or antifreeze protein in the liver until the mid-November sample using *in situ* hybridization and immuno-histochemistry. This time of onset of liver type AFP production in these winter-naïve flounder is strikingly similar to that established for adults maintained under similar conditions of temperature and photoperiod. Therefore it is reasonable to suggest that the factors regulating the onset of AFP production in winter-naïve juveniles are the same as those established for adults. The inconsistency between when RT PCR detects first transcripts and when *in situ* hybridization detects first transcripts could be a question of technique sensitivity, non-specific amplification or even genomic contamination. Other interpretations could suggest an up regulation of transcription in late fall as a response to environmental cues. Further analysis at the molecular level is necessary to work out the details of this discrepancy.

The timing of liver-type AFP production in winter flounder can differ considerably between geographically distant populations. A study of the onset of AFP production in winter flounder from St. Margaret's Bay, Nova Scotia and Pasamaquoddy Bay, New Brunswick revealed that AFP did not appear in the plasma until one to two months later than they appeared in Newfoundland flounder. Moreover when the flounder were held under the same conditions of temperature and photoperiod they retained their differences in the time of onset of AFP production (Fletcher et al. 1985a; Fletcher unpublished data). These observations prompted the hypothesis that the timing of onset of antifreeze production is a genetically determined population characteristic which reflects the earliest date at which AFP would be essential to their survival (Fletcher et al. 1986).

However these studies were carried out on adult flounder that had been exposed to multiple winters. Therefore it is possible that the observed population differences in the time of onset of AFP production could have been the result of environmental selection for the most appropriate phenotype. However the results of the present study indicating that winter–naïve juveniles produced AFP at the same time of year as adults suggests that this is not the case, and that the timing of AFP production may indeed be genetically determined. This hypothesis can be investigated by examining the onset of AFP production in juveniles from the different habitats.

There is little or no difference in the primary structures of liver-type AFP found in the plasma of flounder inhabiting different geographical locations (Fourney et al. 1984a). However there is considerable evidence to suggest that winter flounder populations can differ with respect to antifreeze gene organization. Hayes et al. (1991), examined the AFP genes in winter flounder collected from nine distant locations and found that there were considerable differences in AFP gene copy number and arrangement between the populations. These authors suggested that the observed differences were the result of differences in environmental selection during the postglacial period.

5.4.2 Skin-Type AFP

In adult winter flounder, skin-type AFP is expressed in most tissues including the liver. However the most abundant expression, as judged by Northern blotting procedures, takes place in the external epithelia (Gong et al. 1996). In addition, current evidence suggests that the skin-type AFP genes are expressed year round in all of the epithelial tissues. Data indicating whether or not the expression of the skin-type AFP in the liver

changes seasonally is unclear.

In the present study it is evident, from the *in situ* hybridization results, that the liver did not begin to produce skin-type AFP mRNA until November when the flounder was a fully developed juvenile preparing to enter its first winter. This timing of skin-type gene expression is identical to that found for the skin of juvenile flounder where the skin AFP mRNA and protein appeared in November when the epidermis was thickening, and beginning to resemble that of adults (Chapter 3).

Since, in the adult, the skin-type AFPs are expressed year round, it is possible that the time of onset of skin-type AFP gene expression in skin and liver is developmentally controlled. That is, unlike the liver-type AFP, the skin-type AFP is not directly influenced by environmental factors such as temperature and photoperiod. Support for this suggestion comes from observations on the developing digestive tract. In this organ expression is initiated in epithelial cells by 10 days post hatch, and progresses throughout the summer to expression in the mucous cells lining the entire gut by November (Chapter 4).

The lack of evidence for the presence of skin-type AFP in the juvenile or adult flounder liver using immuno-histochemistry could indicate that the AFP mRNA does not get translated, or that cellular concentrations are below that sensitivity of the method. Gong et al. (1996), using Northern blotting procedures, estimated that the amount of skin-type AFP mRNA observed for liver was less than 10% the amount observed for skin. Therefore it seems likely that levels of skin-type AFP are too low to detect immunologically. Either way, no or very low levels of AFP call into question the

functional significance of the skin-type AFP in the liver.

In order to further understand the distribution of AFP expression in liver, especially during development, observations of the tissue structure and development of the liver in winter flounder from the present study were compared to that observed in other species (Kjorsvik et al. 1991; Segner et al. 1993). Govoni et al. (1986) observed that the liver and pancreas are already formed at hatch in most fish species. For the winter flounder from the present study, the liver and an associated duct were obvious at this stage, however the pancreas could not be identified. The increase in overall size of the liver during ontogeny has also been described previously for other species of marine fish. Both Segner et al. (1993) and Kjorsvik and Reiersen (1992) described enlargements of the liver during the development of the turbot and the Atlantic halibut, respectively. These changes were correlated with alterations of the hepatic energy stores of lipid and glycogen (Segner et al. 1993). The histochemical localization of an abundance of glycogen in the liver of juvenile winter flounder entering into the winter season certainly compares to these observations. Histology of the adult liver was also comparable to that previously described for the plaice (*Pleuronectes platessa*) (Timashova 1981). Large lipid droplets were evident in hepatocytes during the winter but were characteristically absent in summer and no distinct difference was observed between males or females. Interestingly, March and Reisman (1995) described the opposite seasonal pattern of lipid droplets in winter flounders from Long Island. Bodammer and Murchelano (1990) observed that flounder from Boston harbor in late winter showed extensive lipid deposits. These investigators suggested that the synthesis and deposition of lipids begins in later

winter and continues through the summer with the resumption of normal feeding habits. The differences seen in the livers from the present study could be related to the latitudinal differences between populations.

While according to the above data the liver is intact at hatch and likely functional by the end of the yolk-sac stage, it is clearly evident that AFP expression does not appear in this organ until much later. Further work is necessary to elucidate the factors important in the initial activation of low level expression of these genes at metamorphosis.

CHAPTER 6. LOCALIZATION AND ONTOGENY OF CELLS FROM THE WINTER FLOUNDER GILL EXPRESSING A SKIN TYPE ANTIFREEZE PROTEIN GENE

6.1 INTRODUCTION

Until recently, it was believed that the synthesis of antifreeze proteins was largely confined to the liver. It was from here that they were secreted into the blood to be distributed throughout the extracellular space. They were thought to protect marine teleosts from freezing in ice-laden seawater by reducing the freezing temperature of the extracellular fluids to safe levels. The view that antifreeze protein synthesis was confined to the liver changed when Gong et al. (1992) demonstrated the presence of AFP mRNA transcripts in a wide range of tissues in winter flounder and ocean pout (*Macrozoares americanus*), indicating that AFP synthesis was widespread throughout the fish. However, a detailed examination of a skin cDNA library revealed that the AFP mRNA differed considerably from any found in the liver in that they lacked pre- and pro-sequences. This evidence led to the discovery of two distinct AFP gene families in the winter flounder; a "liver-type" whose expression is highly liver specific, and a "skin-type" that is expressed in most tissues including the liver.

Although the skin-type AFPs are expressed throughout the body, by far the most abundant expression occurs in external epithelia (Gong et al. 1992; 1996). External epithelial tissues would come into intimate contact with ice crystals in an ice-laden environment. Therefore, the abundance of AFP mRNA found in skin and gills suggests that these tissues have a requirement for additional freeze protection over and above that

conferred by AFP produced and secreted by the liver into the circulatory system. In addition, since skin-type AFP lack a secretory signal peptide (pre-sequence) it would appear that they might remain and function intracellularly.

A primary function of the gills is to facilitate the rapid diffusion of respiratory gasses between the fish and its aqueous environment. Therefore the diffusion distance between water and blood is kept to a minimum by limiting the epithelium directly involved in gas exchange to a cell layer a few microns in thickness (Randall 1970). The consequence of this is that gill epithelia may be more susceptible to ice propagation and damage, than any other external tissue. This risk would be exacerbated by the fish actively pumping ice water across the gills in order to meet its oxygen requirements.

The present study identifies the cells responsible for AFP gene expression in winter flounder gills, and determines when during development from larvae to adult they are first expressed.

6.2 MATERIALS AND METHODS

6.2.1 Adult Maintenance

Winter flounder were captured by Scuba (November, 1996; January, 1997) and transported to the Ocean Sciences Centre where they were held in tanks maintained with running seawater under conditions of ambient photo-period and temperature.

In early spring, animals were checked daily for signs of gonadal swelling and the presence of eggs or milt. When fish of appropriate condition were found they were immediately isolated from the main tank in preparation for spawning.

6.2.2 Spawning and Fertilization

6.2.2.1 Spawning

Eggs were collected by first carefully drying the genital pore area and then applying gentle pressure, allowing the eggs to pour easily into a clean dry container. The container with eggs was then stored in a cool place until fertilization. Sperm was collected by placing a syringe near the genital pore and gently drawing up the milt. Following gamete collection all further procedures were carried out at 5 ^{0} C.

6.2.2.2 Fertilization

Five hundred to one thousand microlitres of eggs in ovarian fluid were placed in a dry sterile plastic Petrie dish and mixed gently with 100 µl of milt. The sperm were activated by adding a few drops of UV treated and filtered seawater (0.2 µm Millipore filter system). The eggs were then allowed to sit for 5 minutes to fertilize and were subsequently flooded with freshly filtered seawater and allowed to water harden for an additional 20 minutes.

Following water hardening the eggs were flushed once more to remove any surplus milt and allowed to develop normally. Water was changed every other day by decanting off old and adding new fresh filtered seawater. Following fertilization, eggs were incubated at 5 ^{0} C and hatched within 14 days.

6.2.3 Hatching and Transfer to Rearing Containers

Following hatch, 1000 to 2000 larvae were transferred to 4 litre plastic buckets lined with black plastic bags to enhance contrast for prey visibility. Buckets were set in

flowing seawater baths at ambient temperature/photoperiod. Temperature ranged from 5 to 7 ^{0} C in May/June too as high as 14 ^{0} C in August gradually dropping off to approximately 5 ^{0} C in November and then further decreasing to near 0 ^{0} C in December, January, and February. The buckets contained 3 l of filtered (0.2 μm) and UV sterilized seawater plus 500 mls of mixed algae i.e. *Isochrysis* sp. and *Tetraselmis* sp. Water was part changed (50%) and replaced every 2 to 3 days with mixed algae. Animals were fed daily on a ration of 5000 rotifers (variety: *Sap*) per litre and supplemented with a further 300 mls of algae until metamorphosis (about 60 days). Fish reaching metamorphosis and thus settlement were transferred to an aquarium with flowing ambient seawater and aeration and weaned onto a ration of 100 000 *Artemia* per day. Subsequently, fish were further weaned onto a dry diet (BioKyawa). All animals were maintained and killed according to the guidelines set by the Canadian Council of Animal Care (Olfert et al. 1993).

6.2.4 Tissue Sampling

6.2.4.1 RNA Analysis

Three adult winter flounder were sampled in either January (winter) or July (summer). All fish were sacrificed by a blow to the head followed by cervical severance. Gill arches with attached filaments were removed and immediately flash frozen in liquid nitrogen. Tissues were stored at –70 until analysis.

6.2.4.2 Immuno-histochemistry and *In situ* Hybridization

Five to ten fish were sampled just prior to metamorphosis (late larval stage) and

then monthly from metamorphosis to the middle of the first winter (January/February 1998). Samples were also taken during the following summer (July 1998). All tissues were fixed in either MEMFA (0.1M MOPS, 2mM EGTA, 1mM $MgSO^4$ and 3.7% formaldehyde) (wholemount *In situ* hybridization), 4% paraformaldehyde in PBS (section *in situ* hybridization (3.2.4.2)), or Bouins Fluid (immuno-histochemistry (3.2.4.2)).

Six adult flounder (mixed male and female) were sampled during winter (January) or summer seasons (July). Fish were removed from a holding tank and euthanized via cervical severance. Gill arches with attached filaments were removed and immediately immersed in MEMFA (0.1M MOPS, 2mM EGTA, 1mM $MgSO_4$ and 3.7% formaldehyde) for whole-mount *in situ* hybridization or 4% paraformaldehyde in PBS for section *in situ* hybridization. Fixed gill filaments were subsequently dissected away from the arch and placed in fresh fixative. Fixation was allowed to continue for 2 hours at room temperature. Following fixation, tissue destined for whole-mount *in situ* hybridization was frozen in 100 % methanol and the samples stored at –20 °C, whereas those destined for section *in situ* were processed for paraffin embedding. Tissue for immuno-histochemistry and general histology was fixed in Bouins fluid for 24 to 48 hours and processed for paraffin embedding. Briefly, tissue was dehydrated through ethanol series, cleared in two changes of xylene, infiltrated and embedded in wax. Sections 6-8 um were mounted on Silane (Sigma) coated glass slides, dried overnight at 40°C and stored at room temperature.

6.2.4.3 Mucous Histochemistry and Histology

The examination of gill ontogeny, histology and mucous histochemistry was

performed using Alcian blue (AB) pH 2.5/periodic acid-Schiffs reagent (PAS) (Bancroft and Cook 1984). Histochemical reactions showed blue for acid mucins, red for neutral mucins and reddish purple for a combination of neutral and acid mucins.

6.2.5 Total RNA Extraction and RT PCR

6.2.5.1Total RNA Extraction

Total RNA was extracted from gill filaments using either the Qiagen®, Rneasy® Mini RNA prep kit following disruption and homogenisation using the QIAshredder ® and the included homogenisation buffer or alternatively with Trizol ® reagent, according to the manufacturer's suggested protocol. Following extraction, RNA was treated with Life Technologies amplification grade DNAse I according to the manufacturer's recommendations, aliquoted and frozen at -70 °C until use.

6.2.5.2 PCR Primers

PCR primers (WFP9A and WFP9B) were designed to amplify a 250 base pair (bp) fragment analogous to an AFP cDNA clone (WFP9) originally isolated from a winter flounder skin cDNA library (Gong et al. 1996). The sequence of the primers corresponded to 5'-GTCGAACACTCAGAATCACTG-3' and 5'-AGCTTCTCAGGTTTATTCAGC-3', respectively.

6.2.5.3 RT PCR

One microgram of total RNA, primers, and the required components of the Life Technologies SuperScript ™ One-Step ™ RT-PCR System were mixed according to the manufacturer's recommendations. The mixture was incubated at 50 °C for 30 minutes for the reverse transcription reaction and then carried through PCR on a Perkin-Elmer Model

480 thermal cycler. The amplification consisted of 35 cycles at 94 °C, (30s), 60 °C (30s), and 68 ° C (45s).

6.2.5.4 Sequencing of PCR Products

At least five consecutive RT PCR reactions were run and the reactions pooled, DNA was ethanol precipitated and then resuspended in 50 ul of sterile water. This volume was run on a 2% agarose gel for at least 1 hour at 100 V and stained with ethidium bromide. The gel fragment was excised and the DNA purified using the QIAquick Gel Extraction Kit (Qiagen). Purified PCR products and appropriate primers were subsequently sent to the DNA Sequencing Facility/The Centre for Applied Genomics at the Hospital for Sick Children, Toronto Ontario. Obtained sequences were checked against known AFP sequences to verify the result.

6.2.6 *In situ* Hybridization

6.2.6.1 Ribo-probe Production

Flounder skin-type antifreeze protein cDNA WFP9 was ligated to an EcoRI/XhoI site in $_p$Bluscript SK (+/-) plasmid vector (Gong et al. 1996). In order to make antisense or sense RNA probes, plasmids containing WFP9 were linearized with either EcoRI or XhoI respectively. Probes were produced by *in vitro* transcription (Melton et al. 1984; Jowett and Lettice, 1994). The reaction mixture contained 5 ul linearized plasmid (1 ug/ul), 5 µl 10x buffer, 2 µl 250 mM 1, 4-Dithiothreitol (DTT) (Sigma), 0.5 µl RNAsin (Sigma), 10 µl 2.5 mM NTP mix containing digoxigenin tagged UTP (Boehringer Mannheim # 1209) and 1.5 µl of the relevant RNA polymerase, i.e. T7 or T3. The

mixture was incubated at 37 ^{0} C for 15 minutes and then precipitated with 5.0 µl of 0.2 M Ethylenediaminetetraacetic acid (EDTA) , pH 8.0, 6.25 µl 4 M lithium chloride (LiCl), and 200 µl cold ethanol overnight at -20 ^{0} C. Pellets were resuspended in 1 ml of hybridization buffer (50 % deionized formamide, 5 x sodium chloride, sodium citrate (SSC), 1 mg/ml Torula RNA, 100 ug/ml Heparin, 1 x Denharts, 0.1 % Tween-20, and 0.1 % 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and stored at -20 ^{0} C.

6.2.6.2 Whole-mount *In situ* Hybridization

6.2.6.2.1 Hybridization

The whole-mount *in situ* hybridization protocol was modified from Hemmati-Brivanlou et al. (1990) and Jowett and Lettice (1994) (See Chapter 2). Gill filaments were rehydrated in single 5 minute washes of 100 % methanol, 75/25 methanol/water and finally 25/75 methanol/FTW (1 x Fish Saline (FS) (Valerio et al., 1992) + 0.1 % Tween-20), followed by four 5 minute washes in 100 % FTW. Tissue was then incubated in proteinase K (20 ug/ml) for 15 minutes, washed in 0.1 M Triethanolamine (pH 7-8) for 2 times 5 minutes, followed by a further two washes in the same buffer with acetic anhydride. Following the last wash gill filaments were refixed in 4% paraformaldehyde in FTW and then washed 5 times for 5 minutes in fresh FTW.

Tissues were subsequently incubated in 1 ml hybridization buffer for 10 minutes at 63 ^{0} C and then prehybridized for a further 6 hours in fresh buffer. Following prehybridization, buffer was replaced with the probe solution and hybridization was

allowed to occur overnight at 63 ^{0} C.

Following hybridization tissue was treated for 10 minutes at 63 ^{0} C with a series of increasing stringency washes containing 100 % hybridization buffer, 50/50 hybridization buffer/2 x SSC in 0.3 % CHAPS, 25/75 hybridization buffer/2 x SSC in 0.3 % CHAPS. These washes were followed by two further washes (20 minutes) in 2 x SSC in 0.3 % CHAPS at 37 ^{0} C. Tissues were then subsequently treated with RNAase at 37 ^{0} C for 30 minutes and washed once in 2 x SSC with 0.3 % CHAPS at room temperature for 10 minutes and then twice more in 0.2 x SSC with 0.3 % CHAPS at 63 ^{0} C for 30 minutes.

6.2.6.2.2 Immuno-histochemical Detection of Hybridization Products

Following stringency washes the tissue was washed twice in FTW with 0.3 % CHAPS for 10 minutes at 63 ^{0} C, then once without CHAPS. The final volume of FTW was replaced by FBT (FS + 2 mg/ml BSA + 0.1 % Triton-X-100) and the tissue was washed at room temperature for 15 minutes.

Tissue was blocked with Fresh FBT + 20 % heat treated lamb serum for 1 hour at room temperature, after which the solution was replaced with a duplicate solution containing a 1/1000 dilution of the affinity purified sheep anti-digoxigenin coupled to alkaline phosphatase antibody (Boehringer Mannheim) and rocked overnight at 4 ^{0} C.

Excess antibody was removed through five 1 hour washes with FBT, followed by two washes with chromogenic buffer (100 mM Tris, pH 9.5, 50 mM magnesium chloride ($MgCl_2$), 100 mM sodium chloride (NaCl), 0.1 % Tween-20, 1mM Levamisol).

The last chromogenic wash was replaced with 2 mls of the same solution

containing 4.5 ul NBT/ml (Nitro blue tetrazolium in 70 % Dimethyl formamide) and 3.5 ul/ml BCIP (5-bromo-4-chloro-3-indolyl-phosphate). Reaction was stopped by washing tissue in 4% paraformaldehyde. Tissue was dehydrated through ethanol series, cleared in Benzoate/benzyl alcohol, mounted on welled glass slides for observation and photomicroscopy.

6.2.6.2.3 Controls

Sense RNA probes were used in identical hybridizations as anti-sense probes.

6.2.6.3 Tissue Section *In situ* Hybridization

The procedure was modified from Uehara et al., (1993) as described in paragraph 3.2.6.3.

6.2.6.3.1 Section Pre-treatment

Seven-micrometer wax sections were deparaffinized in two washes of xylene for 10 minutes each and subsequently rehydrated through an ethanol series (2 minutes in each of 100 %, 95 %, 70 %, and 50 %). Tissue was then digested in proteinase K (20 µg/ml) for 15 minutes at 37 ^{0}C, rinsed in 1 x fish saline, refixed in 4 % paraformaldehyde in 1 x fish saline for 2 minutes and washed in 1 x fish saline with 2 mg/ml glycine for 2 minutes.

Nonspecific binding sites caused by electrostatic interaction between the probe and basic protein groups were blocked by equilibrating specimens for 5 minutes in 0.1 M triethanolamine buffer pH 7.5 and then rinsing in the same buffer with 125 µl acetic anhydride for 10 minutes at room temperature. Tissue was further rinsed in 2 x saline sodium citrate (2 x SSC) before being dehydrated in a graded series of ethanol and air

dried for at least 10 minutes.

6.2.6.3.2 Hybridization

Sixty micro-litres of probe solution were added to each slide and cover slipped. Slides were placed in a moist chamber with 50 % formamide in 2 x SSC, covered and sealed with parafilm. Hybridization was allowed to occur for up to 24 hrs at 45 °C.

6.2.6.3.3 RNAase Treatment and Post-hybridization Stringency Washes

Cover-slips were removed by washing gently in 2 x SSC and slides were washed in 50% formamide in 2 x SSC for one hour at 45 °C and subsequently rinsed in 10 mM Tris, pH 8.0, 500 mM NaCl for two minutes. Tissues were then incubated with RNAase T_1 (10 units/ml) and of RNAase A (20 μg/ml) in the same buffer at 37 ° C for 30 minutes. Following RNAase treatment, slides were washed once more in the same buffer for 30 minutes at 37° C. Slides were next washed at 50 °C for 30 minutes in each of 2 x SSC, 0.5 x SSC and 0.1 x SSC.

6.2.6.3.4 Immuno-histochemical Detection of Hybridization Products

Slides were rinsed briefly in 0.1M Tris, pH 7.5, 150 mM NaCl and then incubated in the same buffer for 30 minutes with 1% BSA at room temperature to block nonspecific sites. Sections were then treated with a 1/250 dilution of anti-digoxigenin antibody conjugated to alkaline phosphatase in the above buffer with 1% BSA and incubated overnight at 4 ° C. Following antibody treatment slides were washed for 30 minutes in 0.1M Tris, pH 7.5, 150 mM NaCl with 1% BSA at room temperature to remove any unbound antibody. The chromogenic reaction was as outlined for the whole-mount *in*

situ hybridization protocol (paragraph 2.2.7.3). Approximately, 500 µl of BCIP + NBT in chromogenic buffer was added to each slide and the reaction allowed to proceed in the dark until the desired background was achieved (usually within 20 minutes). The reaction was stopped by washing slides in 1 X FS and then dehydrated rapidly through ethanol series, clearing briefly in xylene and mounting in Cytoseal (Stephens Scientific) for observation and photo-microscopy.

6.2.6.3.5 Controls

Control experiments for *in situ* hybridization included 1) hybridization with antisense riboprobes on gill filament tissue from the ocean pout, *Macrozoarces americanus*, a type III antifreeze protein producer and 2) hybridization with sense riboprobes on winter flounder gill tissue.

6.2.7 Immuno-histochemistry

6.2.7.1 Tissue Preparation and Blocking of Non-specific Sites

Tissue sections were deparaffinized by washing twice in xylene for 10 minutes, hydrated through ethanol series and washed three times 5 minutes in PBT (1x Phosphate Buffered Saline, pH 7.4, 0.1% Triton-X-100, 2mg/ml BSA). Non-specific sites were blocked by incubating tissue in 20% goat serum in buffer for 15 minutes at room temperature.

6.2.7.2 Antibody Treatment

Antiserum to skin-type AFP was diluted in PBT with 5 % goat serum and applied to sections for 30 minutes at room temperature, dilutions ranged from 1/500 to 1/1000.

Following antibody application, slides were washed four times in PBT for five minutes each.

The secondary antibody (goat-anti-rabbit conjugated to horseradish peroxidase (HRP) (Sigma)) was diluted in PBT with 5 % goat serum, applied to sections and incubated in an identical manner to the above. Dilutions ranged from 1/300 to 1/500. Slides were again washed four times in buffer for five minutes each.

6.2.7.3 Stain Development and Mounting

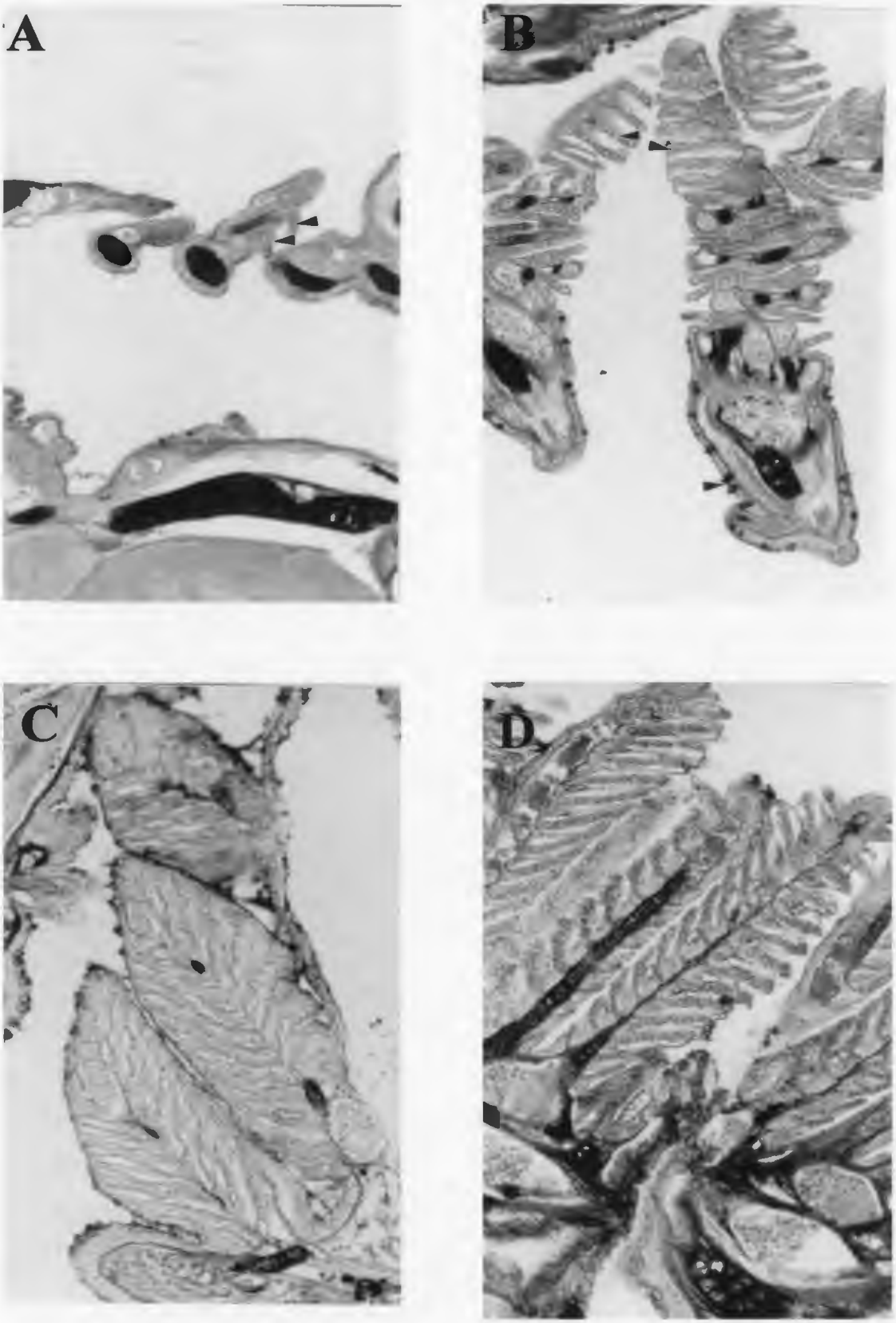
Following the final wash, sections were covered with 500 µl of Immuno-Pure Metal Enhanced DAB substrate (Pierce) and incubated at room temperature for 5 minutes or until the chromogenic reaction was of favourable intensity. Reaction was then stopped by washing in PBT, dehydrated in ethanol series, cleared in xylene and mounted in Cytoseal (Stephens Scientific) for observation and photography.

6.2.7.4 Controls

In parallel sets of experiments, negative controls involved the replacement of the primary antibody with normal rabbit serum or antisera for Type II AFP from sea raven. Further experiments showed that the cell specific staining could also be out competed with a 100-fold excess of purified AFP Type I. Immuno-blotting and Western blotting have previously shown antisera/protein specificity (C. Hew, personal communication).

As a tissue specificity control, winter flounder gill tissue was replaced by gill from fish known to not produce Type I AFP i.e. ocean pout or arctic cod. All previous controls also apply to this tissue

Figure 6.1 Development of the gill in winter flounder from late larval stage through the first winter (February). A) Section through the opercular region of a 30-day post hatch winter flounder larva showing the start of filament outgrowths from the early gill arch. Note also the occurrence of lamellar projections (arrowheads). (X175). B) Section through the opercular region of a newly metamorphosed juvenile winter flounder (August). Note the increase in abundance of lamellae and the appearance of goblet cells on the gill arch epithelia (arrowheads). (X175). C and D). Further illustration of the increase in lamellar complexity in gills from fish in December and February, respectively (X175).


A
B
C
D

6.3 RESULTS

6.3.1 Histology and Mucous Histochemistry

Prior to metamorphosis the gill structure consists of small outgrowths from the ventral pharyngeal cartilage (Figure 6.1a). Lamellar branching is minimal at this stage (Figure 6.1a). Following metamorphosis in August, the overall gill structure became considerably more elaborate showing extensive lamellar formation and the occurrence of mucous cells (Figure 6.1b). The complexity of lamellae increases as fish enter their first winter (December and February)(Figure 6.1cd). Mucous cells occurring on the filament and on the lamellae contain primarily acid or mixtures of acid and neutral mucins.

6.3.2 RT PCR

RT PCR results on total RNA isolated from winter flounder gill filaments consistently gave a band of DNA at about 250 bp (Figure 6.2). Occasionally the band did appear as a doublet or was slightly diffuse. No difference was observed between winter and summer. Generally, the band was consistent with that produced when purified WFP9 plasmid DNA was amplified with the same primers.

6.3.3 Sequence Comparison

All PCR products generated with the Skin AFP primers WFP9A and WFP9B and total RNA isolated from winter flounder gill tissue showed high similarity to the original skin clone WFP9 (Figure 6.3). The only sequence anomalies were a C to T substitution at positions 107 and 121. The amino acid sequences translated from these RT PCR products were identical to that of the clone WFP9 with the exception of an Alanine to

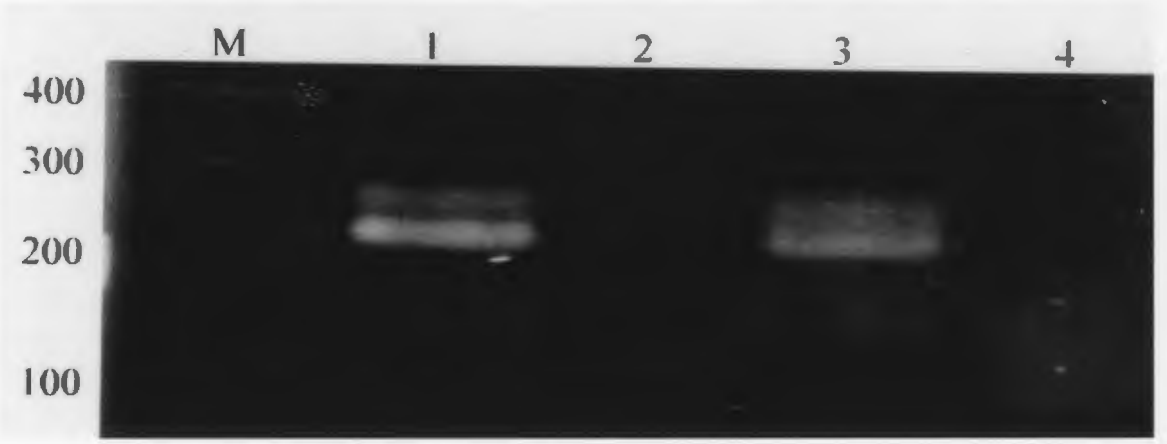


Figure 6.2 RT-PCR using primers for skin AFP clone WFP9, showing products amplified from total RNA isolated from winter flounder gill in winter (January) (lane 1) or summer (July) (lane 3). Lanes 2 and 4 used the same RNA but were not subjected to reverse transcription. Samples were run on a 2% agarose gel at 100V for one hour and stained in ethidium bromide.

(A)

Source

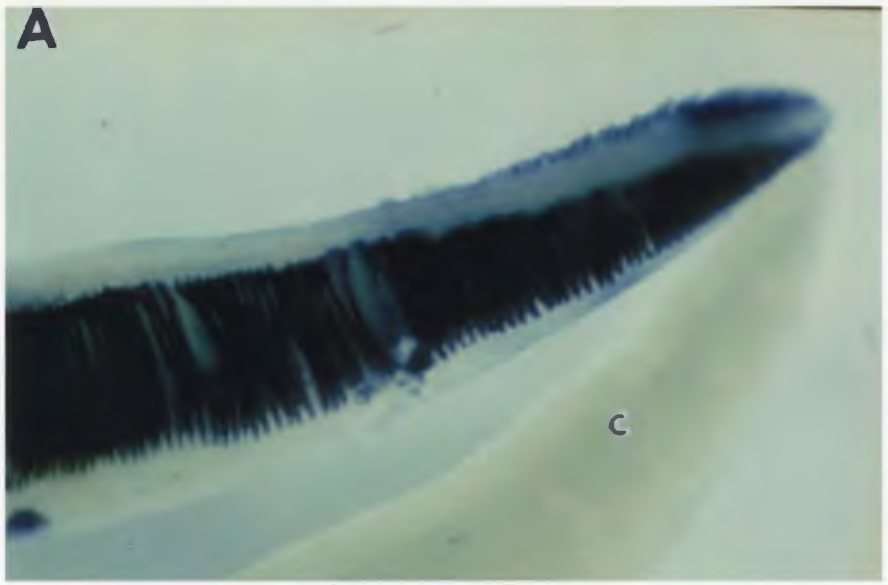
```
WFP9   1 GTCGAACACTCAGAATCACTGACATCAACATGGACGCA
GILL   1 ----------------------------------------

WFP9  39 CCAGCCAAAGCCGCCGCAGCCACCGCCGCCGCCGCCAA
GILL  39 ----------------------------------------

WFP9  77 GGCCGCCGCAGAAGCCACCGCCGCCGCAGCCGCCAAAG
GILL  77 ----------------------------T-------

WFP9 115 CAGCAGCCGACACCAAAGCCAAAGCAGCCCGTTAAGGA
GILL 115 ------T-----------------------------

WFP9 153 TCGTGGTCGTCTTGATGTGGGATCATGTGAACATCTGAG
GILL 153 ----------------------------------------

WFP9 192 CAGCGAGATGTTACCAATCTGCTGAATAAACCTGAGAA
GILL 192 ----------------------------------------

WFP9 229 GCTGttt
GILL 229 ---A---
```

(B)

Source

```
         1                  10                  20                  30
WFP9  MDAPAKAAAATAAAAKAAAEATAAAAAKAAADTKAKAAR
Gill  ----------------------------V---------
```

Figure 6.3 (A) Nucleotide sequences of skin-type AFP cDNAs amplified from total RNA isolated from winter flounder gill using primers specific for the known sequence of skin AFP clone WFP9. Dashes represent regions of identity. (B) Translated protein sequence from the above cDNA showing regions of identity.



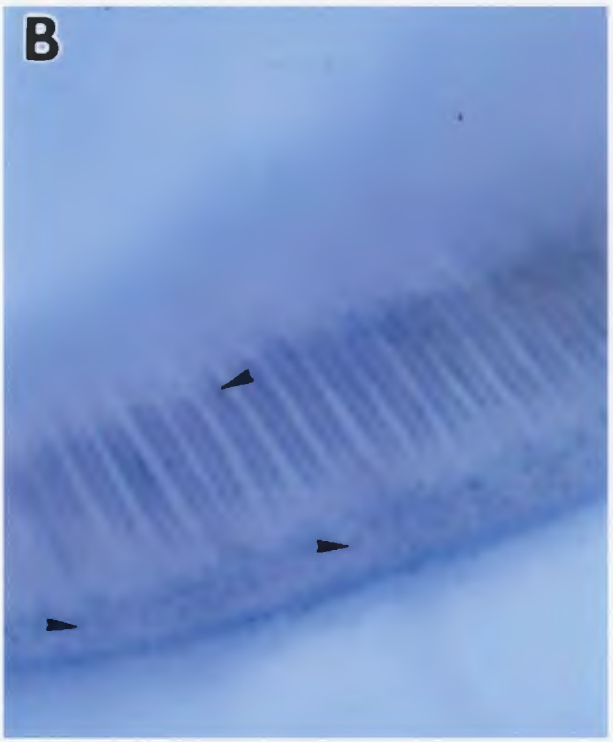


Figure 6.4 (A) Whole-mount *in situ* hybridization on adult winter flounder gill filament showing overall spatial distribution of skin type-AFP mRNA (November). The tissue was hybridizaed with an antisense RNA probe, synthesized *in vitro* using Digoxigenin tagged UTP and visualized via a anti-digoxigenin alkaline phosphatase antibody. Blue staining regions represent areas of skin type AFP mRNA positive cells. (X10). (B) High-resolution micrograph of a whole-mount gill preparation showing the distribution of skin type AFP mRNA positive cells (arrowheads) within the gill filament and lamellae of the winter flounder (November). (X140). C, sense probe control.

Valine substitution at amino acid 31. This change predicts a protein identical to that predicted from the cDNA isolated from RT PCR on liver total RNA.

6.3.4 *In situ* Hybridization

In situ hybridization on late larval and juvenile flounder gills did not at any time show evidence for the presence of AFP mRNA. Examination of whole-mounted adult flounder gill however, did reveal a strong AFP mRNA hybridization reaction in association with the gill lamellae (Figure 6.4a). The extent and intensity of hybridization reaction associated with the lamellae sharply contrasted with the low intensity of the stain associated with the non-lamellar areas of the filament. However, a more detailed examination of the filament using higher magnification revealed the presence of AFP mRNA positive cells on both the lamellae and filament itself (Figure 6.4ab). Identical results were observed for gills collected from flounder during the winter and summer. Parallel control experiments using a mRNA sense probe showed no evidence of hybridization (Figure 6.4a). Similarly, experiments using ocean pout gill filaments as controls were equally negative (Data not shown).

In situ hybridization on paraffin sections of gill revealed AFP mRNA hybridization products associated with numerous cells scattered over the entire external surface of the lamellae. This is illustrated in Figure 6.5 where the AFP mRNA positive cells are interspersed between mucous goblet cells.

6.3.5 Immuno-histochemistry

AFP immuno-positive cells identified using antisera to skin-type AFP, first became apparent in juvenile flounder gill tissue in October following metamorphosis

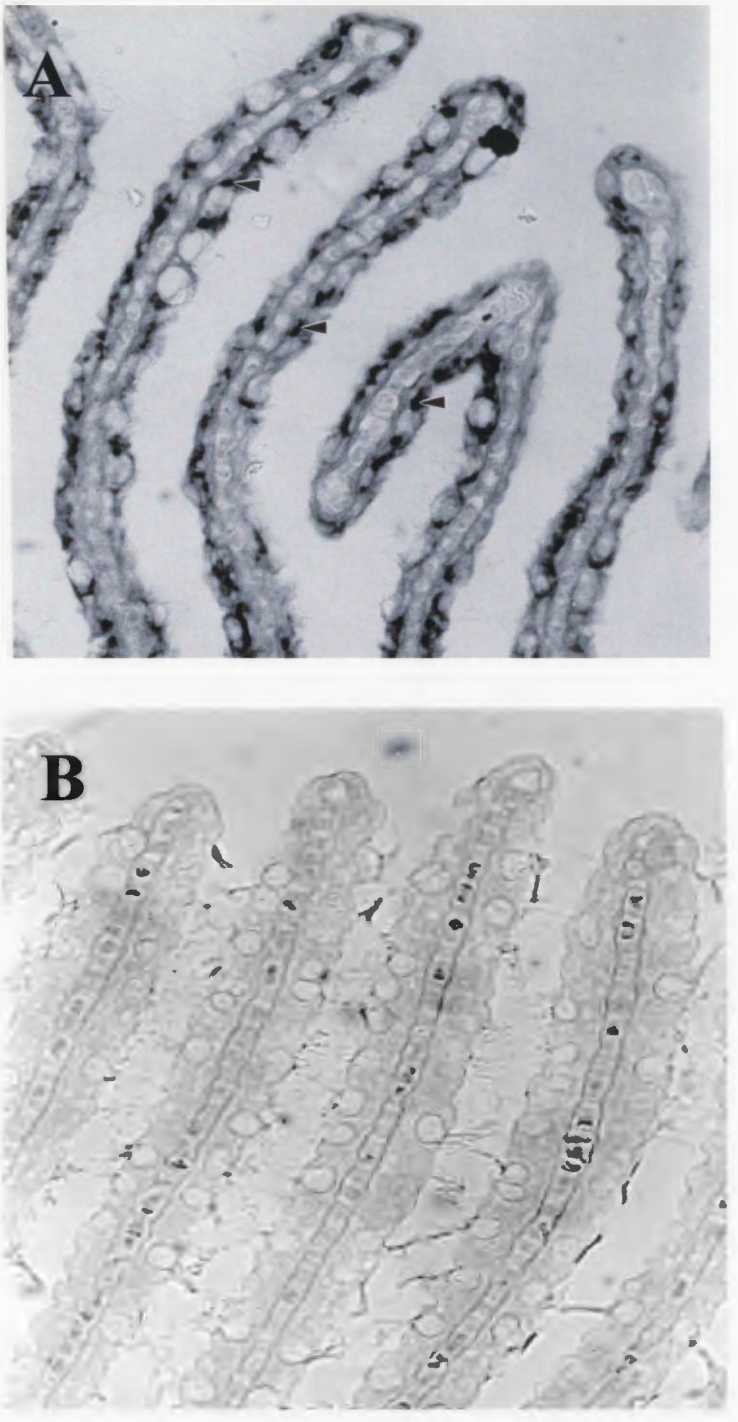
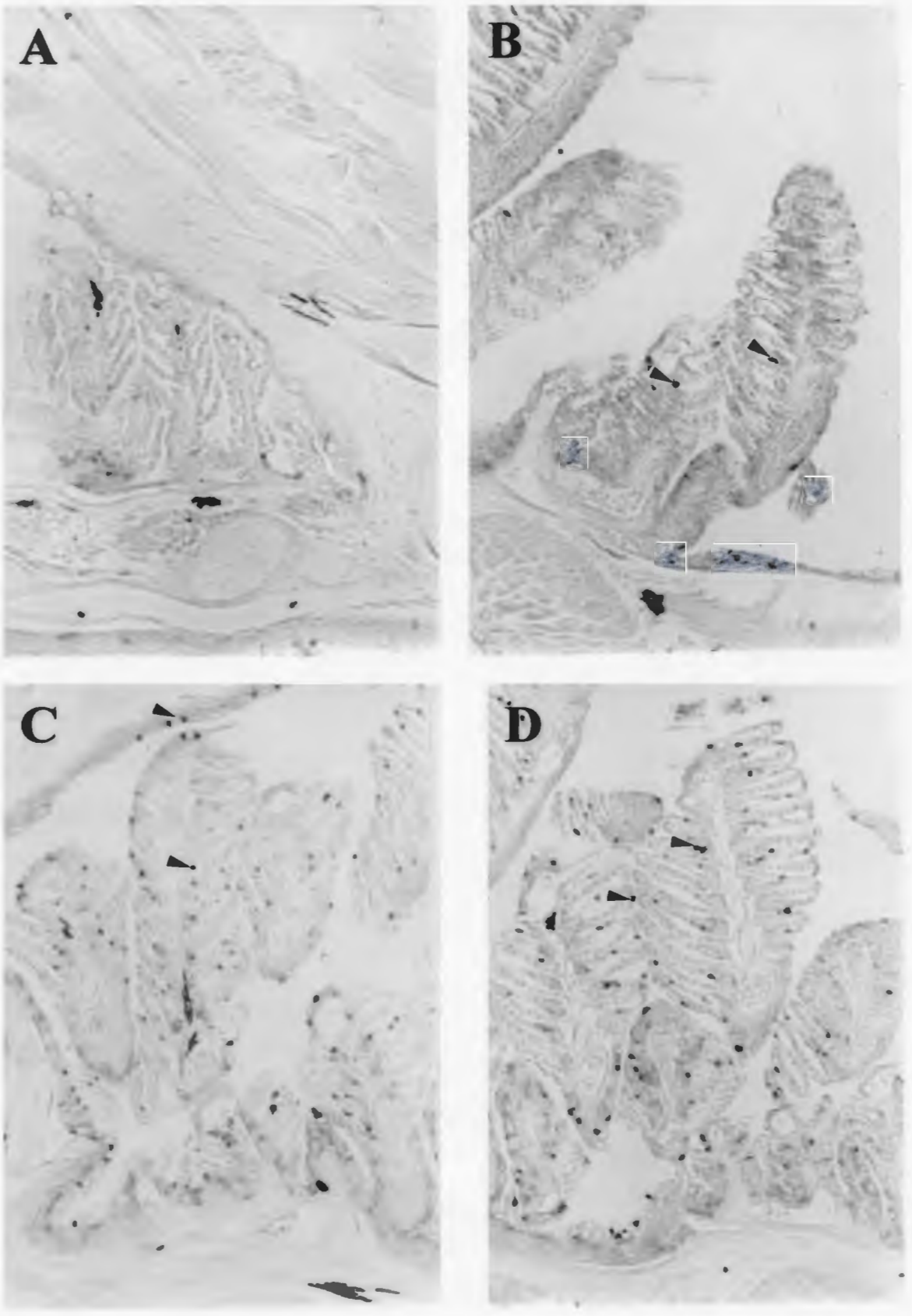


Figure 6.5 (A) *In situ* hybridization on paraffin sections of winter flounder gill (December) showing the cellular distribution of skin-type mRNA within the lamellae (arrowheads). (X430). (B) Sense control for *in situ* hybridization on paraffin sections of winter flounder gill. (X430).

Figure 6.6 A-D. The occurrence of AFP immuno-staining during the development of winter flounder gill from (A) early metamorphosis (August) to (D) the middle of the first winter (February). Note the gradual increase in the appearance of AFP immuno-positive cells during December (C) and February (arrowheads) (D). (B) shows the occurrence of the occasional AFP positive cell as early as October. (X175).


A
B
C
D

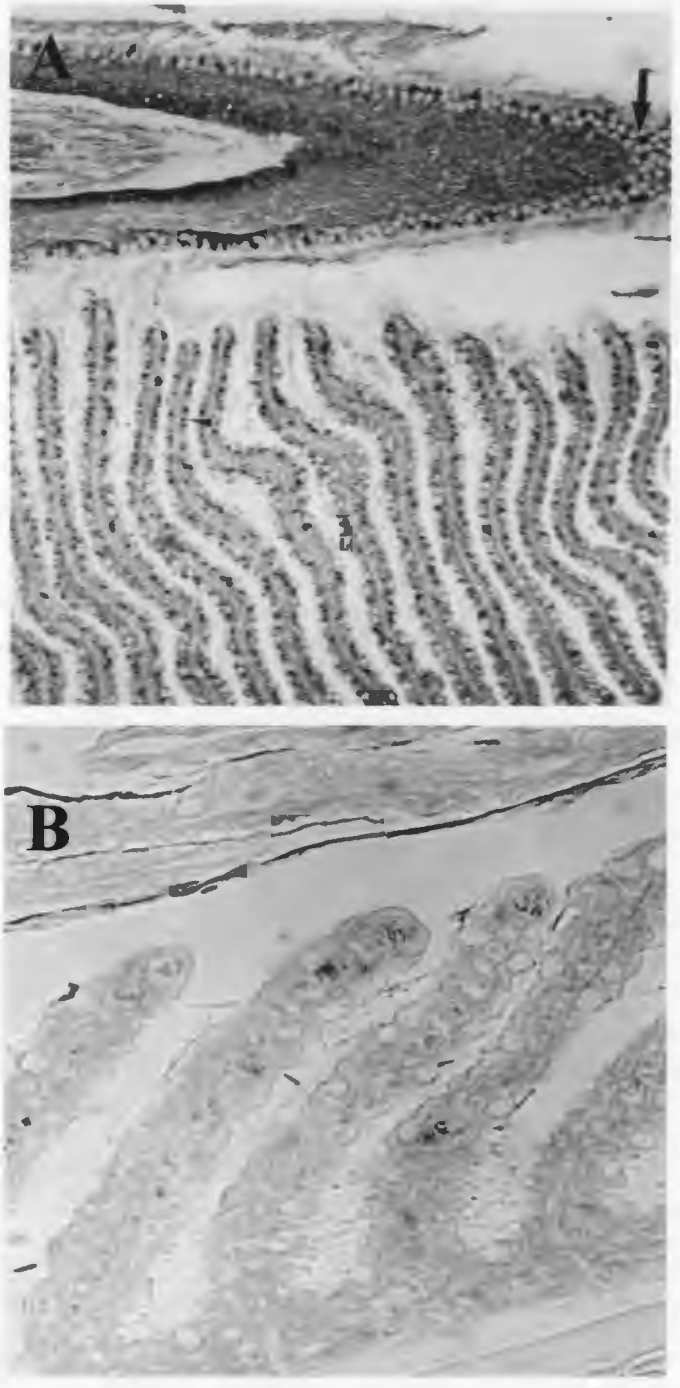


Figure 6.7 (A) Overall tissue distribution of skin-type AFP producing cells in the winter flounder gill as demonstrated using immuno-histochemistry. Rabbit anti-sera skin-type AFP was applied to paraffin sections of gill. Note the distribution of positive cells amongst goblet cells in the filament (arrow). Positive cells were visualized using a peroxidase conjugated secondary antibody and heavy metal enhanced DAB (arrowhead). F, filament; L, lamellae; (X100). (B) Normal rabbit serum control on winter flounder gill filament. (X430).

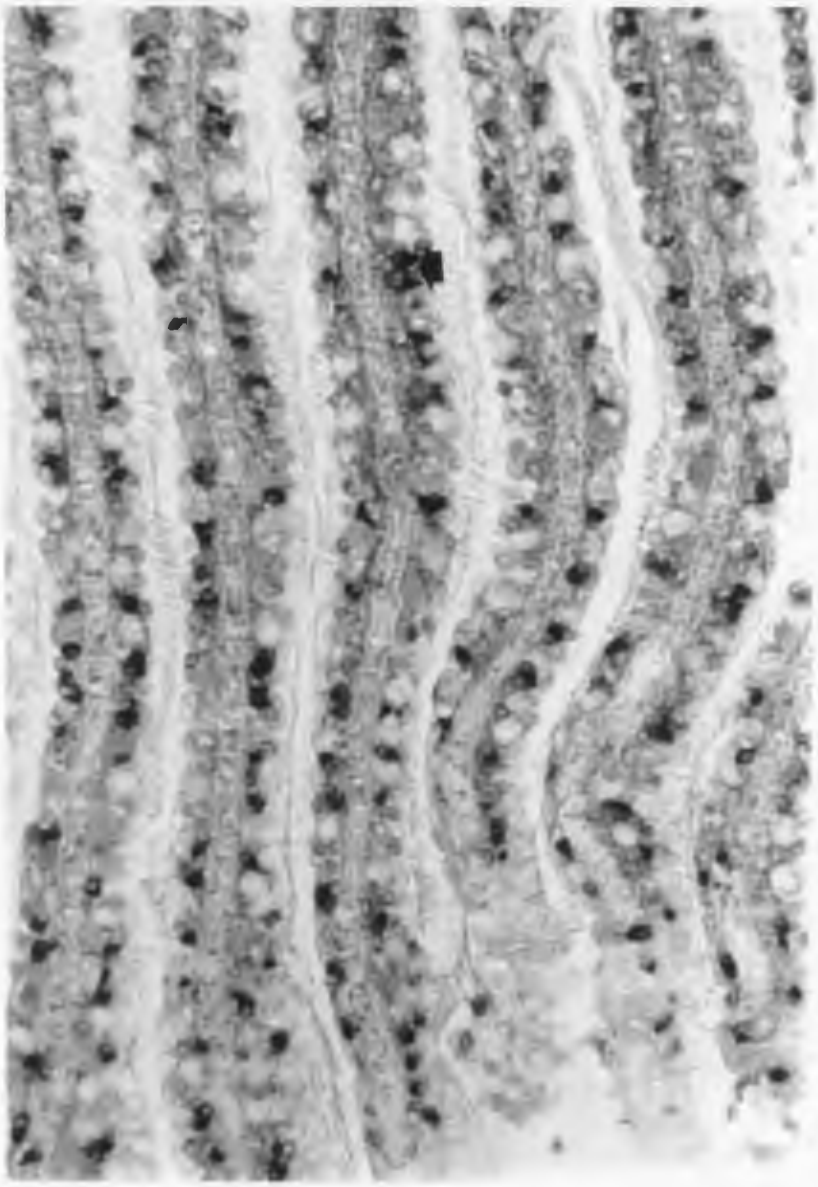


Figure 6.8 Higher magnification of gill lamellae showing cellular distribution of skin type AFP producing cells. Note concentration of stain deposit in the apical portion of cell (arrow). (X430).

Figure 6.9 High magnification of gill filament region showing skin-type AFP producing cells. Note the triangular shape of the cell and the cytoplasmic distribution of the stain (arrow). The nucleus remains stain free. (X430).

(Figure 6.6b). These cells gradually increased in number through February. The positive cells are located within the epithelia of the lamellae, filament and lining the opercular space (Figure 6.6bcd).

An examination of gill filaments from flounder adults using the same antibodies revealed the presence of AFP positive cells within the lamellae and the filament (Figure 6.7). The AFP positive cells appeared to have triangular or pyramidal shapes and were frequently observed to extend to the external surface (Figure 6.8).

The distribution of these AFP immuno-positive cells appears identical to that observed for the cells expressing AFP mRNA suggesting that both methods were identifying the same cell type. Mucous goblet cells appeared to be the only other abundant cell type on the epithelial surface. Close examination of the AFP positive cells indicated that the AFP was distributed throughout the cytoplasm, leaving the nucleus clear of reaction product (Figure 6.8). There was no evidence for the presence of AFP within the connective tissues or extracellular space.

Parallel experiments using a monoclonal antibody to avian sodium-potassium dependent ATPase revealed the presence of positive cells that appeared to be restricted to the basal region of the lamellae (Figure 6.10). The differences observed in distribution between the AFP positive cells and the ATPase positive cells suggest that they are not the same cell types.

6.4 DISCUSSION

The results of this study indicate the presence of skin-type AFP mRNA and skin-type AFP in the epithelial cells of the gill filaments and with the greatest concentration of

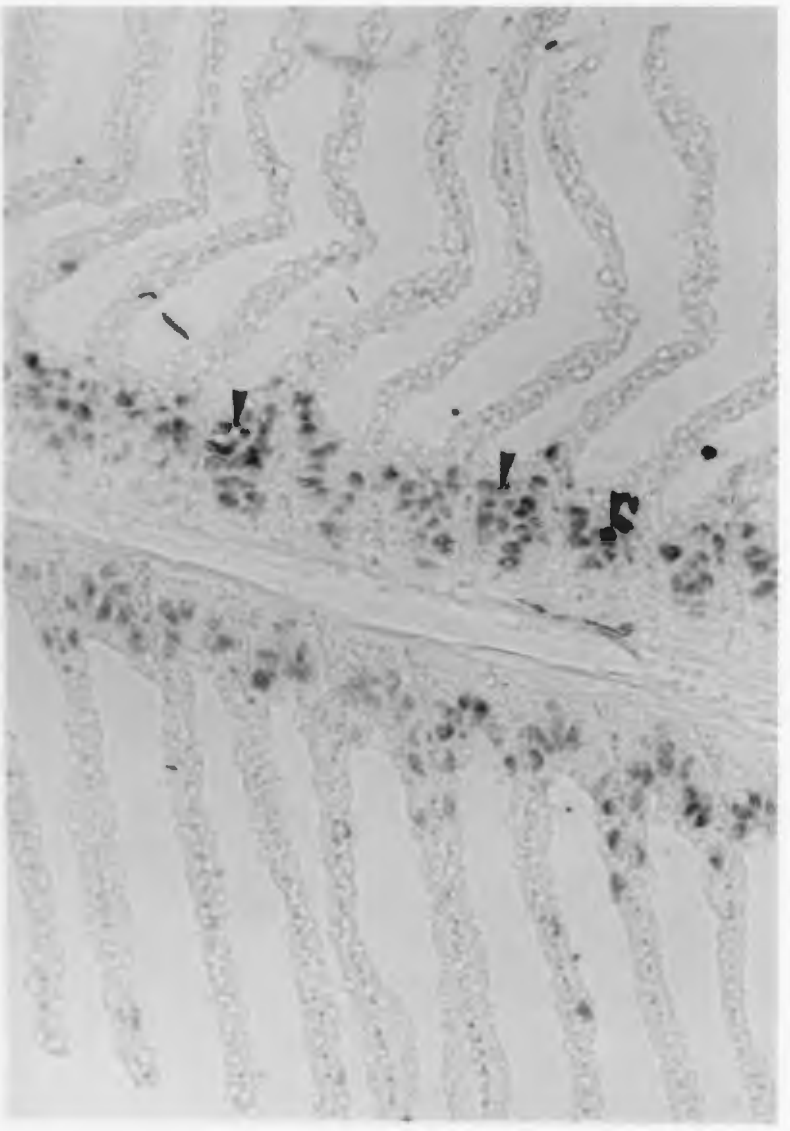


Figure 6.10 Immuno-histochemical localization of winter flounder gill chloride cells (arrowheads) using a monoclonal antibody for Na^+/K^+ ATPase. (X165).

these cells occurring within the epithelia of the lamellae where respiratory gas exchange takes place.

Of the four cell types known to comprise the gill epithelium, pavement cells, mucous cells, chloride cells and neuro-epithelial cells, the pavement cells appear to be the ones responsible for AFP synthesis. There was no evidence for the presence of AFP in the mucous cells. The chloride cells, as evidenced by the presence of Na^+/K^+ ATPase, differed in their distribution from the AFP positive cells and the neuro-epithelial cells are normally present in very low numbers and located in the epithelia of the filaments rather than the lamellae (Perry 1997, Bailly et al. 1992).

The discovery of the skin-type AFP gene family and their predominant expression in epithelial tissues raises important questions as to how AFPs function to protect marine teleost fish from freezing in an ice laden environment. What are the relative roles of these two AFP families? Is a central supply of AFP from the liver insufficient for complete freeze protection? Do the skin type AFPs function to block ice propagation into the body fluids of the fish, or is their primary function to protect the epithelial cells themselves?

Biological membranes have been shown to be very effective barriers to ice propagation. Valerio et al. (1992) found that the temperature at which ice could propagate across isolated winter flounder skin was as much as 1 ^{0}C lower then the freezing temperature of the blood. Since the temperature at which flounder freeze in the presence of ice approximates the freezing temperature of their blood, it is apparent that ice must propagate into the fish at a site other than the skin. The gills, and gill lamellae in particular, would appear to be the most likely route of ice propagation because the

epithelia involved in gas exchange consist largely of a thin, single layer of cells.

The lack of a secretory signal associated with the skin-type AFP genes suggests that the skin-type AFP remain intracellular (Gong et al. 1996). The results of the present study are consistent with this hypothesis, in that AFP could only be detected within the cytoplasm of the gill epithelial cells. There was no evidence for their presence within the extracellular space. However, these observations contrast to those found for the skin epidermis where the AFP appeared to be localized outside the epithelial cells in intimate contact with the plasma membrane. Therefore, despite the lack of a secretory signal sequence the AFP are exported from the skin epithelial cells. Alternative pathways for protein export that bypass the Golgi apparatus have been described by Mignatti et al. (1992), and Menon and Huges (1999).

As a general rule cells are well protected from intracellular freezing at high freezing temperatures due to the nature of the cell membrane and the spatial requirements for ice growth (see Valerio et al. 1992). Therefore, ice propagation into and through epithelial cells layers would, in general, be limited to the intercellular spaces. The localization of the skin-type AFP with in the extracellular space of the skin is consistent with the idea that the AFP would assist in blocking ice propagation into and through the skin. In this case localized concentrations of AFP could serve two functions; the first would be to prevent the damaging effects of ice within the epidermis, and the second being the deterrence of ice propagation through the epidermis and into the blood.

The localization of skin-type AFP within the gill epithelial cells rather than external to them suggests that the AFP in these cells function intracellularly. However,

the inability to detect AFP in the intercellular space between the epithelial cells may be related to the effective tight junctions that exist within this cell layer. The intercellular space between the skin epithelial cells is considerably greater than it is between the gill epithelial cells. Therefore, intercellular AFP may be detected more readily in the skin than it would be in the gill tissue.

The presence of AFP in the gill epithelial cells implies that there is a need to lower the freezing temperature of the cytoplasm in order to prevent it from freezing. This would suggest that there is a danger of ice being propagated into these cells when ice-laden seawater is pumped across the gill. Given the role that this epithelium plays in ion regulation and gas exchange, it seems unlikely that the cell membrane on the apical face of the epithelial cell wouldn't be any more permeable to ice propagation than any other cell. However the cell membrane between adjacent epithelial cells appears to be considerably more porous than the apical surface of the cell. Bartels and Potter (1993) have described communicating gap junctions between adjacent pavement cells of lamprey gills and Sandbacka et al (1998) presented evidence for the movement of Lucifer yellow across gap junctions between adjacent rainbow trout gill epithelial cells grown in culture. Such channels may render the cell membranes between adjacent pavement cells more permeable to ice propagation than the apical cell membranes. Therefore, should the apical surface of a pavement cell become damaged during winter, ice could propagate from this cell to the adjacent pavement cell, effectively causing a chain reaction that could result in considerable gill damage. Clearly the presence of AFP would lower the freezing temperature of the cytoplasm within the pavement cells and prevent such a

domino effect.

Studies carried out over the past decade suggest that antifreeze proteins can help to protect cold sensitive mammalian cell membranes from damage associated with hypothermic temperatures (see Fletcher et al. 1998 for review). Therefore, it is possible that the AFP found within gill epithelial cells could function to stabilise the cell membrane, and or the membranes of intracellular organelles. However, there is no data to indicate that AFPs do protect fish cell membranes (Fletcher et al. 1998). Consequently, it seems appropriate to favour a hypothesis that takes into consideration the well-documented evidence that AFP bind to and prevent the growth of ice.

The results of this study indicate that the onset of AFP gene expression in the gills of juvenile flounder entering their first winter occurred during October, a month earlier than was observed for the skin and the liver (Chapters 3 and 5, respectively). Since in the adult flounder the "skin" type AFP gene expression is developmentally, rather than environmentally controlled, the results obtained in the present study suggest tissue specific differences in the time of onset of "skin" type AFP gene expression. In any case it is clear that juvenile flounder entering their first winter begin to express "skin" type AFP genes well before there is any danger of exposure to subzero temperatures and the presence of ice. This implies the possibility of developmentally controlled preparation for winter conditions.

CHAPTER 7. GENERAL DISCUSSION

7.1 REVIEW OF PRIMARY OBJECTIVES

The discovery of skin-type or epithelial AFPs in the early to mid 1990's resulted in a need for a re-evaluation of how AFPs work to reduce cold associated injury in the fish that produce them (Gong et al. 1992; 1995). The work of Gong et al. (1996) suggested that skin-AFPs in winter flounder may act intracellularly. Further work by Chan et al. (1997), Miao et al. (1998), Lin et al. (1999) and Harding et al. (1999) examining the regulatory mechanisms of these AFP genes and the activity of the proteins, raised numerous questions regarding how these AFPs may work at the tissue and cellular levels. With the above in mind the present study was undertaken to gain an understanding of the cell types involved in "skin AFP" expression, their tissue distribution, relationship to liver AFP producing cells and when they arise during post-embryonic ontogeny.

Specifically, the objectives of this study were to (1) examine the spatial expression patterns of winter flounder AFPs during post-embryonic ontogeny using non-isotopic RNA probes and to further verify the presence of these transcripts using reverse transcription PCR. (2) use polyclonal and monoclonal antibodies and immuno-histochemical techniques to examine the tissue and cellular distribution of the associated proteins during the same developmental stages.

Through the course of this investigation these objectives were achieved, resulting in the generation of new data concerning the timing of Type I AFP expression in the life cycle of winter flounder, the types of cells involved and how they relate to each other

during the development of the animal.

7.2 EXPRESSION OF AFPS

Much of what is known regarding AFP expression in fish has centered on seasonal expression in winter flounder. This is likely due to the elaborate physiological mechanism behind the regulation of the gene in the liver (Pickett et al. 1983ab; Fletcher et al. 1984; Fletcher et al. 1989). Evidence of AFP expression in non-liver tissues like skin, amplify the importance of studying spatial expression patterns, as different tissues and cells have different physiological functions (Valerio et al. 1990; 1992; Gong et al. 1992; 1995; 1996). Much of the present thesis concentrated on comparing the temporal and spatial expression patterns of AFPs during larval development and in the adult fish.

The general conclusion that one should arrive at from examining the results in this thesis is that Type I AFPs are expressed with amazing tissue and cellular flexibility and in the case of epithelial types, are detectable at least as early as hatch for this species emphasizing their importance throughout the life cycle of the fish.

It has been recognized for some time that winter flounder AFPs are generated as products of a multigene family (Gourlie et al. 1984). Gong et al. (1992) reported that expression was also prevalent in a wide variety of tissues and that in flounder skin this expression was due to a family of very similar genes (Gong et al. 1996). The present study goes further to show that at least one of these "skin genes" is responsible for AFPs in all tissues examined, suggesting an overall physiological importance for this family. Considering the distribution of many similar genes across a variety of different tissues, one cannot ignore the importance of variation in cell type and function within these

tissues.

7.2.1 Larvae

Experiments using wholemount *in situ* hybridization with riboprobes for skin AFP clone WFP9 detected complementary transcripts in a band of paired spindle-shaped integumental cells running laterally along the larval fish prior to yolksac absorption (Chapter 2). Wholemount immuno-histochemistry using antisera for skin AFP showed immuno-signal in the same cells, thus providing good evidence that these cells are epithelial AFP producers. Experiments using RT PCR and primers specific to the original WFP9 clone resulted in an identical product on sequencing thus providing further support for the presence of skin-AFP in early larvae.

Comparing the spatial distribution of these AFP producing cells in larvae with the literature revealed that these cells were similar in position to superficial neuromast cells or closely associated cells, which in other species are important in early larval predator avoidance (Blaxter and Fuiman 1989). The loss of detectable expression in these cells at yolk-absorption suggests that AFPs are only important early on and/or they are of a maternal source. Determining the definitive role of AFPs in these cells is difficult, as little is known regarding the physiology of these proteins in epithelial cells. As discussed in Chapter 2, if these cells are indeed neuromast cells, then a possible scenario may involve a correlation between water temperatures, probability of ice interaction and successful detection of predators before exogenous feeding begins. Further work is necessary to more completely characterize these cells and the importance of their association with AFPs.

The detection of an AFP immuno-positive reaction in the randomly distributed integumental cells (Chapter 2) and gut epithelial cells (Chapter 4) of larval/juvenile flounder raised further questions since these cells did not appear to react with the riboprobe. Alcian blue staining indicated that these cells were glycoprotein (mucous) producing cells. Evidence of AFPs in these cells suggests the possibility of co-secretion with mucous, a situation previously observed for pleurocidin, a protein with antimicrobial properties that is known to be co-secreted with mucus in the skin of adult winter flounder (Cole et al. 1997). The present situation suggests that a layer of AFP spiked mucous could cover the luminal surface of the respective tissues thereby acting as a barrier to ice crystal penetration or damage. An interesting observation to make regarding these mucous/AFP-producing cells is that their respective embryological origins are totally different. Integument arises from the ectoderm whereas the gut and associated tissues develop from the endoderm (Slack 1991). The lack of AFP immuno-staining in other known mucous producing cells like those found in gill (Chapter 6) and interestingly in the gut of adult flounder (Chapter 4), raises many other questions regarding the physiological importance of this relationship in larvae and juveniles. It was not determined when the switch occurs in the adult fish or its importance, thus this area requires further investigation. The difficulty in obtaining an *in situ* hybridization reaction in these cells raises some questions as to the specificity and cross-reactivity potential of these antibodies. In both cases negative controls consistently gave no staining, indicating specificity for the protein. Further, preincubating the antisera with purified AFP also blocked the staining reaction, indicating that the reaction was likely due to an

AFP/antisera interaction. It is also important to note in this case that while tissue specific RT PCR is difficult in larval fish, where total RNA is extracted from whole animals, in adults AFP transcription products were detectable in both intestine and stomach using this technique but not with *in situ* hybridization.

Making the assumption based on these data, that these cells are AFP producers, then the lack of an *in situ* hybridization signal must be due to some other reason. Two potential explanations are possible. Firstly, considering the size of the AFP gene family in winter flounder, all of which are known to make similar AFPs, the possibility exists that a gene may be expressed that produces a cross-reacting protein but may have a transcript that is dissimilar enough to prevent hybridization with the riboprobe. Secondly, the tissue conditions for successful *in situ* hybridization are critical. Variations in fixation or cell morphology could present problems in getting probe to target (Speel et al. 1995; Hemmer et al. 1998). Considering that both types are glycoprotein or mucous secreting cells, large amounts of secretion product in the cytoplasm could create physical barriers to hybridization. Sequencing of RT PCR products from various adult tissues and early larval fish indicated that all clones were similar, so comparable probes should still work well given access to the target sequence. If this is the case then the second possibility is more likely. In order to show definitively that these larval cells are expressing AFP transcript, further experiments involving variations in tissue preparation technique may be required. Also, the production of monoclonal antibodies to epithelial AFPs may aid in more specific immuno-detection. In any case the discovery that expression occurs this early in the post-embryonic development of winter flounder raises

many further questions regarding the evolutionary origin and the original physiological function of AFPs.

7.2.2 Juveniles and Adults

7.2.2.1 Post-embryonic AFP Expression in Skin and Liver

One of the more significant observations in the present study involves the onset of AFP expression in the juvenile skin (Chapter 3). In this case a very interesting switch from those cells involved in AFP expression in the larval integument to expression in epidermal cells of juveniles and subsequently the adult is shown to occur (Chapter 2 and 3).

As discussed in the previous section and evidenced in Chapter 2 the larval winter flounder integument shows AFP expression in association with two basic cell types, the spindle shaped lateral cells and the more randomly distributed integumental cells. Both of these cell types appear restricted to the premetamorphic larva stages. At metamorphosis the epidermis shows few if any immuno-positive cells and are negative for *in situ* hybridization with AFP riboprobes. This pattern continues until the fish reaches the early autumn of its first year at which point epithelial AFP transcripts become detectable in association with the skin epidermis (Chapter 3). Similarly, immuno-signal also becomes detectable in association with this region. Histologically, an overall thickening of the epidermal layers and a diversification of cell types further define this period. A reorganization of AFP expressing cells at this stage would likely be correlated with the over all changes occurring due to metamorphosis.

Changes in patterns of gene expression or changes in cell type expressing a

specific gene at the time of metamorphosis are not unusual in organisms that undergo this process. Atkinson et al. (1996) describes a process in amphibians known as "cell-specific reprogramming of gene expression". This process occurs at the time of metamorphosis and may involve extensive cytological, biochemical and molecular remodeling. The liver of tadpoles has been shown to begin producing proteins that characterize the adult liver phenotype at the time of metamorphosis (Atkinson et al. 1996). The idea here is that specific metamorphosis inducing factors are also important in signaling the change from larval to adult gene expression. The switch from larval integumental AFP producing cells to adult epidermal AFP cells may also be a response to similar signals. Thyroid hormones have been shown to be important in the process of metamorphosis for many animals required to undergo this developmental change (Shi 1996). Thyroxine has been implicated in being involved in the metamorphic process in fish, including flounders (Miwa and Inui 1987; Miwa et al. 1988; Schreiber and Specker 1998; 1999). In the present investigation, RT PCR shows the first occurrence of liver source serum AFP transcripts at metamorphosis, however detection with *in situ* hybridization and immuno-histochemistry did not occur until well into the autumn (November) and coincided with the onset of expression in adult liver. This suggests that genetic reprogramming of hepatocytes due to metamorphosis could result in the initial start of transcription and then photoperiod response may act in the up-regulation of the gene later. Skin-type AFP transcripts are evident throughout post-embryonic development so it is difficult to determine when initial transcription begins in juvenile skin, however, detection with *in situ* hybridization and immuno-histochemistry appears to coincide with the start of AFP

expression in juvenile liver. This suggests that an up-regulation event could be occurring in this case as well, although the actually trigger may not be as clear as in the liver. Gong et al. (1995) showed that expression of skin AFPs do not appear to respond to hypophysectomy, however there is a distinct seasonality giving higher expression levels in winter versus summer. Further work is necessary to work out the specific regulatory mechanism for skin expressed AFPs.

The distribution of the actual protein in skin epidermis also presents an interesting problem. In this case the observed extracellular distribution is different from that in other tissues examined, as it is not a surface related phenomenon. Mucous cells in the gut and in the larval integument appear to secret AFP spiked mucous generally on to the luminal surface of the epithelia, whereas in skin the distribution appears more intimately associated with cells deeper in the epidermal layer. Considering the close proximity to the water and associated temperature effects this may make sense for the adult fish, but doesn't explain functionally why the larval integument appears to depend on a mucous secretion. Perhaps energetically co-secretion with mucous may be more "cost effective" for larval fish. It is also interesting to note that the outermost cell layer of the adult/juvenile skin does not appear to express AFPs but does show evidence of glycoprotein activity. In order to gain an overall understanding of how skin AFPs work to protect the fish, these questions and others generated by the present study will have to be addressed.

7.2.2.2 Post-embryonic AFP Expression in Gill

Data from Chapter 6 indicated that AFP expression occurs in association with

only one specific cell type in the gill. Based on the process of elimination using a monoclonal antibody for Na^+/K^+ ATPase it was shown that chloride cells are not involved and the overall numbers of other cells like neuro-epithelial cells are such that they do not observe the same numbers or distributions, so the gas exchange cell or pavement cell appears to be the best candidate. As with AFP expression in the juvenile skin and the liver, expression here also becomes detectable only following metamorphosis. This may be due to a number of factors, one of which being overall functional tissue maturity. It is well known that the gill does not become functional as a gas exchange organ until later in development (Laurent 1984). Prior to this period the majority of gas exchange likely occurs across the large surface area of the integument, so physiologically gill function is not important until later and potentially likewise the expression of AFPs in this organ. The onset of gill function at metamorphosis may also reflect the earlier detection of AFP immuno-positive cells. As observed in Chapter 6, occasional AFP cells become detectable as early as October of the animal's first year, an entire month before the observed occurrence in tissues like skin and liver. This may also reflect a particular sensitivity of this tissue to ice crystal exposure and perhaps indicate preparation for such exposure.

AFP distribution in this case is also very different from that observed in most other tissues. Here immuno-signal appears only in the cytoplasm of the cells involved, raising further questions as to the nature of this mode of action. Ideas raised in Chapter 6 regarding the importance of gap junctions in gill and possibly a "domino effect" resulting in the freezing of adjacent cells, require more investigation including immuno-

cytochemical studies at the ultrastructural level.

7.3 CONCLUSIONS

1. Expression of antifreeze proteins in winter flounder throughout its ontogeny shows enormous cellular flexibility in both form and function.
2. Expression of epithelial antifreeze proteins in winter flounder occurs at least as early as hatch during post-embryonic development. Sequence analysis of RT PCR products from different stages during development and in adults showed that very similar genes are responsible for expression of these proteins in a variety of tissues.
3. The onset of expression of liver secreted serum antifreeze proteins does not occur until metamorphosis.
4. The change in expressing cell types observed in skin at metamorphosis may be due to metamorphosis related signaling events similar to changes observed in other species also know to undergo metamorphosis (reprogramming of gene expression) i.e. Amphibians. As with liver AFP expression, data suggest that up regulation may also occur here following metamorphosis and in response to some environmental or metamorphic cue.
5. The AFP distribution in juvenile and adult skin suggests that the protein is released from the cell through some undetermined mechanism. Immuno-detection of the protein in the extracellular region indicates a function in slowing down or preventing ice crystal proliferation in this region. This is important in reducing the onset of osmotic intolerance and cellular dehydration due to extracellular freezing.
6. AFP expression in winter flounder gill appears to occur in association with pavement

cells. The intracellular localization of the AFP in these cells is consistent with the original described protein but not with observations of AFPs in other tissues i.e. juvenile/adult skin.

7.4 FUTURE DIRECTIONS

The above studies have generated numerous questions with regard to the ontogeny of antifreeze protein expression in winter flounder. Amongst others, the most intriguing questions relate to the variety of cells involved in expression and the apparent switches in cell type that were observed to occur at, and following metamorphosis as well as the apparent up regulation events in association with both skin AFP and liver source serum AFP.

One way to determine if photoperiod is important in the up regulation following metamorphosis is to maintain juveniles under long photoperiod and then quantitatively determine whether up regulation has occurred at the required time. A similar experiment with water temperature may be valuable. It would also be interesting to examine the effects of thyroxine on the onset of AFP expression in early and late metamorphs. These proposed experiments could be effective in elucidating how important the changes at metamorphosis are on AFP expression in juvenile and adult winter flounder. Further work is also necessary to elucidate the ultrastructural distribution of AFPs in gill pavement cells and why it appears different from that observed in other tissues.

CHAPTER 8. REFERENCES

Atkinson, B.G., Helbing, C., and Chen, Y. (1996). Reprogramming of genes expressed in amphibian liver during metamorphosis. In: In: Gilbert, L.I., Tata, J.R., Atkinson, B.G., eds. Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells. Cell Biology: A Series of Monographs. Academic Press Inc., pp 539-562.

Baardsnes, J., Kondejewski, L.H., Hodges, R.S., Chao, H., Kay, C., Davies, P.L. (1999). New ice –binding face for type I antifreeze protein. *FEBS Letters* 463: 87-91

Baglole, C.J., Murray, H. M., Goff, G.P. and Wright, G.M. (1997). Ontogeny of the digestive tract during larval development of yellowtail flounder: a light microscopic and mucous histochemical study. *J. Fish Biol.* 51: 120-134.

Bailly, Y., Dunel-Er, S., and Laurent, P. (1992). The neuroepithelial cells of the fish gill filament-indolamine-immunocytochemistry and innervation. *Anat. Rec.* 233: 143-161.

Bancroft, J.D. and Cook, H.C. (1984) Manual of Histological Techniques. New York. Longman Group Limited.

Bartels, H. and Potter, I.C. (1993). Intercellular junctions in the water-blood barrier of the gill lamella in the adult lamprey (*Geotria australis, Lampetra fluviatilis*). Cell Tissue Res. 274: 521-532.

Bereiter-Hahn, J., Richards, K.S., Elsner, L., and Voth, M. (1980). Composition and formation of flame-cell caps: a structure for the attachment of microorganisms to seahorse epidermis. *Proc. R. Soc. Edinb.* 79b: 105-111.

Bisbal, G.A. and Bengtson, D.A. (1995). Development of the digestive tract in larval summer flounder. *J. Fish Biol.* 47: 277-291.

Blaxter, J.H.S. and Fuiman, L.A. (1989). Function of the free neuromasts of marine teleost larvae. IN : The Mechanosensory Lateral Line: Neurobiology and Evolution (ed. by S. Coombs, P. Görner, and H. Münz) pp. 481-501, Springer-verlag, Berlin, Heidelberg.

Bodammer, J.E., and Murchelano, R.A. (1990). Cytological study of vacuolated cells and other aberrant hepatocytes in winter flounder from Boston Harbour. *Cancer Res.* 50: 8744-8756.

Boehringer Mannheim Canada (1994). The DIG System Technique and Product Guide.

Boulhic, M. and Gaboudan, J. (1992). Histological study of the organogenesis of the digestive system and swimbladder of the Dover sole, *Solea solea* (Linnaeus, 1758). *Aquaculture* 47: 305-319.

Brokken, L.J.S., Verbost, P.M., Atsma, W. and Wendelaar-Bonga, S.E. (1998). Isolation, partial characterization and localization of integumental peroxidase, a stress related enzyme in the skin of a teleostean fish (*Cyprinus carpio* L.) *Fish Phys. Biochem.* 18: 331-342.

Buckley, L.J. (1982). Effects of temperature on growth and biochemical composition of larval winter flounder, *Pseudopleuronectes americanus. Mar. Ecol. Prog. Ser.* 8: 181-186.

Burkhardt-Holm, P.L., Schmidt, H., Meier, W. (1998). Heatshock protein (hsp70) in brown trout epidermis after sudden temperature rise. *Comp. Biochem. Phys. Part*

A. **120**: 35-41.

Burton, D. and Burton, M.P.M. (1989a). Individual variability in the epidermis of prespawning inshore winter flounder, *Pseudopleuronectes americanus. J. Fish Biol.* **35:** 845-853.

Burton, D. and Burton, M.P.M. (1989b). Epidermal characteristics of reproductive subsets in an inshore population of winter flounder, *Pseudopleuronectes americanus. J. Zool. Lond.* **218**: 637-647.

Burton, D. and Everard, B.A. (1991a). The effect of androgen treatment on the epidermis of post-spawned winter flounder, *Pseudopleuronectes americanus* (Walbaum). *J. Fish Biol.* **38**:73-80.

Burton, D., and Everard, B.A. (1991b). A Comparison of the effect of captivity on the epidermis of prespawning and postspawned winter flounder, *Pseudopleuronectes americanus* (Walbaum). *J. Fish Biol.* **38**: 73-80.

Burton, D., and Fletcher, G.L. (1983). Seasonal changes in the epidermis of the winter flounder, Pseudopleuronectes americanus. *J. mar. biol. Ass. U.K.* 63:273-287.

Burton, D., Burton, M.P., and Idler, D.R. (1984). Epidermal condition in post-spawned winter flounder, *Pseudopleuronectes americanus* (Walbaum) maintained in the laboratory and after exposure to crude petroleum. *J. Fish Biol.* 25:593-606.

Burton, M.P. and Idler, D.R. (1984). The reproductive cycle in winter flounder, *Pseudopleuronectes americanus* (Walbaum). Can. J. Zool. 62: 2563-2567.

Calzada, A., Medina, A., and Gonzalez de Canales, M. L. (1998). Fine structure of the intestine development in cultured sea bream larvae. *J. Fish Biol.* 53: 340-365.

Chambers, R.C., and Leggett, W.C. (1987). Size and age at metamorphosis in marine fishes: an analysis of laboratory reared winter flounder (*Pseudopleuronectes americanus*) with a review of variation in other species. *Can. J. Fish. Aquatic Sci.* 44: 1936-1947.

Chan, S.L., Fletcher, G.L., and Hew, C.L. (1993). Control of antifreeze protein gene expression in winter flounder. In: Biochemical and Molecular Biology of Fishes. Ed. P.W. Hochaka and T.P. Mummsen, Vol. 2. Pp 293-305. Elsevier Science Publishers.

Chan, S-L, Miao, M., Fletcher, G.L. and Hew, C.L. (1997). The role of CCAAT/enhancer-binding protein α and a protein that binds to the activator-protein-1 site in the regulation of liver-specific expression of the winter flounder antifreeze protein gene. *Eur. J. Biochem.* 241: 44-51.

Cheng, C-H. C. (1998). Evolution of the diverse antifreeze proteins. *Current Opinion in Genetics and Development*. 8: 715-720.

Cole, A.M., Weis, P., and Diamond, G. (1997). Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder. *J. Biol. Chem.* **272** (18): 12008-12013.

Davies, P.L. (1992). Conservation of antifreeze protein-encoding genes in tandem repeats. *Gene*. 112: 163-170.

Davies, P.L. and Sykes, B.D. (1997). Antifreeze proteins. *Current Opin. Struct. Biol.* 7: 828-834.

Davies, P.L., and Gauthier, S.Y. (1992). Antifreeze protein pseudogenes. *Gene*. 112: 171-178.

Davies, P.L., Haugh, C., Scott, G.K., Ng, N., White, B.N., and Hew, C.L. (1984). Antifreeze protein genes of the winter flounder. *J. Biol. Chem.* 259 (14): 9241-9247.

Davies, P.L., Hew, C.L., and Fletcher, G.L. (1988). Fish antifreeze proteins: physiology and evolutionary biology. *Can. J. Zool.* 66: 2611-2617

Davies, P.L., Roach, A.H., Hew, C.L. (1982). DNA sequence coding for an antifreeze protein precursor from winter flounder. *Proc. Natl. Acad. Sci. USA*. 79: 335-339.

Deng, G., and Laursen, R.A. (1998). Isolation and characterization of an antifreeze protein from the Longhorn Sculpin, *Myoxocephalus octodecimspinosis. Biochimica et Biophysica Acta.* 1388:305-314.

Devries, A., Komatsu, S.K., Feeney, R.E. (1970). Chemical and physical properties of freezing point depressing glycoproteins from artic fishes. *J. Biol. Chem.* 245: 2901-2913.

DeVries, A.L. (1986). Antifreeze glycopeptides and peptides: Interactions with ice and water. In: Methods in Enzymology,(Packer L; ed.) vol. 127, pp. 293-303. New York: Academic Press.

DeVries, A.L., and Lin, Y. (1977). Structure of a peptide antifreeze and mechanism of adsorption to ice. *Biochem. Biophys. Acta*. 495: 388-392.

Douglas, S. E., Gawlicka, A., Mandla, D., and Gallant, J. W. (1999). Ontogeny of the stomach in winter flounder: characterization and expression of the pepsinogen and

proton pump genes and determination of pepsin activity. *J. Fish Biol.* 55, 897-915.

Duman, J.G., and DeVries, A.L. (1974). The effects of temperature and photoperiod on antifreeze production in cold water fishes. *J. Ex. Zool.* 190: 89-98.

Duman, J.G., and DeVries, A.L. (1976). Isolation, characterization, and physical properties of protein antifreezes from the winter flounder, Pseudopleuronectes americanus. *Comp. Biochem. Physiol.* 53B: 375-381.

Ewart, K.V., and Fletcher, G.L. (1990). Isolation and characterization of antifreeze proteins from smelt (*Osmerus mordax*) and Atlantic herring *(Clupea harengus harengus*). *Can. J. Zool.* 68: 1652-1658.

Ewart, K.V., and Fletcher, G.L. (1993). Herring antifreeze protein: Primary structure and evidence for a C-type lectin evolutionary origin. *Molecular Marine Biology and Biotechnology*. 2(1): 20-27.

Ewart, K.V., Lin, Q., Hew, C.L. (1999). Review: Structure, function and evolution of antifreeze proteins. *Cell. Mol. Life Sci.* 55: 271-283.

Ewart, K.V., Rubinsky, B, and Fletcher, G.L. (1992). Structural and functional similarity between fish antifreeze proteins and calcium-dependent lectins. *Biochem. and Biophys. Res. Com.* 185: 335-340.

Fletcher, G.L. (1976). Circannual cycles of blood plasma freezing point and Na and Cl concentrations in Newfoundland winter flounder (Pseudopleuronectes americanus): correlation with water temperature and photoperiod. *Can. J. Zool.* 55: 789-795.

Fletcher, G.L. (1977). Circannual cycles of blood plasma freezing point and Na+ and Cl- concentrations in NFLD. winter flounder (*Pseudopleuronectes americanus*) correlated with water temperature and photoperiod. *Can. J. Zool.* 55: 789-795.

Fletcher, G.L. (1979). The effects of hypophysectomy and pituitary repacement on the plasma freezing point depression, Cl^-, glucose and protein antifreeze in the winter flounder (*Pseudopleuronectes americanus*). *Comp. Biochem. Physiol.* 63A: 535-537.

Fletcher, G.L. (1981). Effects of temperature and photoperiod on the plasma freezing point depression, Cl^- concentration and protein "antifreeze" in winter flounder. *Can. J. Zool.* 59:193-201.

Fletcher, G.L., Cambell, C.L., and Hew, C.L. (1978). The effects of hypophysectomy on seasonal changes in plasma freezing point depression, protein "antifreeze" and Na^+ and Cl^- concentrations of winter flounder (*Pseudopleuronectes americanus*). *Can. J. Zool.* 56: 109-113.

Fletcher, G.L., Goddard, S.V., Davies, P.L., Gong, Z., Ewart, K.V., and Hew, C.L. (1998). New insights into AFP in Fish: physiological significance and molecular regulation. Society Experimental Biology, Seminar series. Cambridge University Press. pp 239-265.

Fletcher, G.L., Haya, K., King, M.J., and Reisman, H.M. (1985a). Annual antifreeze cycles in Newfoundland, New Brunswick and Long Island winter flounder, *Pseudopleuronectes americanus. Mar. Ecol. Prog. Ser.* 21: 205-212.

Fletcher, G.L., Hew, C.L., Li, X.L., Haya, K., and Kao, M.H. (1985b). Year round

presence of high levels of plasma anti-freeze peptides in a temperate fish, ocean pout (*Macrozoarces americanus*). *Can. J. Zool.* 63: 488-493.

Fletcher, G.L., Idler, D.R., Vaisus, A., and Hew, C.L. (1989). Hormonal regulation of antifreeze protein gene expression in winter flounder. *Fish Phys. and Biochem.* 7(1-4): 387-393.

Fletcher, G.L., King, M.J., and Hew, C.L. (1984). How does the brain control the pituitary's release of antifreeze synthesis inhibitor? *Can. J. Zool.* 62 (5): 839-844.

Fletcher, G.L., King, M.J., and Kao, M.H. (1987). Low temperature regulation of antifreeze glycopeptide levels in Atlantic cod (*Gadus morhua*). *Can. J. Zool.* **65 (2)**: 227-233.

Fletcher, G.L., Kao, M.H. and Fourney, R.M. (1986). Antifreeze peptides confer freezing resistance to fish. Can. J. Zool. 64: 1897-1901.

Fletcher, T.C., Jones, R., Reid, L. (1976). Identification of glycoproteins in goblet cells of epidermis and gill of plaice (*Pleuronectes platessa* L.), flounder (*Platichthys flesus* L.), and rainbow trout (*Salmo gairdneri* Richardson). *Histochem. J.* 8: 597-608.

Fourney, R.M. Joshi, S.B., Kao, M.H. and Hew, C.L. (1984a). Heterogeneity of antifreeze from the Newfoundland winter flounder (*Pseudopleuronectes americanus). Can. J. Zool.* 62: 28-33.

Fourney, R.M., Fletcher, G.L. and Hew, C.L. (1984b). The effects of long day length on liver antifreeze mRNA in the winter flounder (*Pseudopleuronectes americanus*). *Can. J. Zool.* 62: 1456-1460.

Fourney, R.M., Fletcher, G.L., Hew, C.L. (1984c). Accumulation of winter flounder antifreeze messenger RNA after hypophsectomy. *Gen. Comp. Endo.* 54: 392-401.

Gawlicka, A., The, S. J., Hung, S. S. O., Hinton, D. E., and de la Noue, J. (1995). Histological and histochemical changes in the digestive tract of white sturgeon larvae during ontogeny. Fish Physiology and Biochemistry **14,** 357-371.

Gong, Z., Ewart, K.V., Hu, Z.Z., Fletcher, G.L., and Hew, C.L. (1996). Skin antifreeze protein genes of the winter flounder, *Pleuronectes americanus*, encode distinct and active polypeptides without the secretory signal and prosequences. *J. Biol. Chem.* **271**: 4106-4112.

Gong, Z., Fletcher, G.L., and Hew, C.L. (1992). Tissue distribution of fish antifreeze protein mRNAs. *Can. J. Zool.* 70: 810-814.

Gong, Z., King, M.J., Fletcher, G.L. and Hew, C.L. (1995). The antifreeze protein genes of the winter flounder, *Pleuronectes americanus*, are differentially regulated in liver and nonliver tissues. *Biochem. and Biophys. Res. Comm.* 206: 387-392.

Gourlie, B., Lin, Y., Price, J., Devries, A.L., Powers, D., and Huang, R.C.C. (1984). Winter flounder antifreeze proteins: a multigene family. *J. Biol. Chem.* 259: 14960-14965.

Govoni, J.J., Boehlert, G.B., Watanabe, Y. (1986). The physiology of digestion in fish larvae. *Envir. Biol. Fishes.* 16:59-77.

Harding, M.M., Ward, L.G. and Haymet, A. D. J. (1999). Type I "antifreeze" proteins: Structure-activity studies and mechanisms of ice growth inhibition. *Eur. J. Biochem.* 264: 653-665.

Hawkes, J.W. (1974). The structure of fish skin. I. General Organization. *Cell Tiss. Res.* 149: 147-158.

Hayes, P.H., Davies, P.L., and Fletcher, G.L. (1991). Population differences in antifreeze gene copy number and arrangement in winter flounder. *Genome* 34: 174-177.

Hemmati-Brivanlou, A., Frank, D., Bolce, M.E., Brown, B.D., Sive, H.L. and Harland, R.M. (1990). Localization of specific mRNAs in *Xenopus* embryos by whole-mount *in situ* hybridization. *Development.* 110: 325-330.

Hemmer, M.J., Courtney, L.A., and Benson, W.H. (1998). Comparison of three histological fixatives on the immunoreactivity of mammalian P-Glycoprotein antibodies in the Sheephead Minnow, *Cyprinodon variegatus*. *The Journal of Experimental Zoology*. 281: 251-259.

Hew, C.L. and Fletcher, G.L. (1985). Biochemical adaptation to the freezing environment-structure, biosynthesis and regulation of fish antifreeze polypeptides. IN Circulation, Respiration and Metabolism (ed. by R. Gilles) pp. 553-563. Springer-verlag, Berlin, Heidelberg.

Hew, C.L. Wang, N.C., Yan, S., Cai, H.Y., Sclater, A. and Fletcher, G.L. (1986b). Biosynthesis of antifreeze polypeptides in winter flounder: characteristics and seasonal occurrence of precursor polypeptides. *Eur. J. Biochem.* 160: 267-272.

Hew, C.L., Chakrabartty, A., and Yang, D. (1987). Biochemical adaptation to the freezing environment: structure -function relationship of antifreeze polypeptides. IN: Integration and control of metabololic systems. ed. X. Con, O.I. Kon, M.C.M. Chung, P.L. Hwang, S.F. Leong, K.H. Loke, P. Thiyasararjah and P.T. Hwang.

Cambridge University Press. Cambridge pp. 299-309

Hew, C.L., Fletcher, G.L., and Ananthanarayanan, VS. (1980). Antifreeze protein from the shorthorn sculpin, *Myxocephalus scorpius*: isolation and characterization. *Can. J. Biochem.* 58: 377-383.

Hew, C.L., Scott, G.K. and Davies, P.L. (1986a). Molecular biology of antifreeze. In: Heller, H.C. et al. (eds) Living in the cold: physiological and biochemical adaptation. pp.117-123. Elsevier, New York.

Hochachka, P.W. (1986). Defence strategies against hypoxia and hypothermia. *Science* 231: 234-241.

Hochachka, P.W. (1988a). Channels and pumps - determinants of metabolic cold adaptation strategies. *Comp. Biochem. Physiol.* 90B (3): 515-519.

Hochachka, P.W. (1988b) Metabolic-channel-and pump-coupled functions: constraints and compromises of co-adaptation. *Can. J. Zool.* 66: 1015-1027.

Holmes, W.N., and Donaldson, E.M. (1969). The body compartments and distribution of electrolytes. In: Hoar, W.S., Randall, D.S. (Eds), Fish Physiology, Vol.1, Academic, New York, pp. 1-83.

Idler, D.R., Fletcher, G.L., Belkhode, S., King, M.J., and Hwang, S.J. (1989). Regulation of antifreeze protein production in winter flounder: a unique function for growth hormone. *General and Comparative Endocrinology.* 74(3): 327-334

Joweltt, J. and Lettice, L. (1994). Whole-mount *in situ* hybridization on Zebra fish embryos using a mixture of digoxigenin and fluorescein labeled probes. Trends in Genetics 10 (2): 73-74.

Kjorsvik, E. Meeren, T. Van Der, Kryvi, H., Arnfinnson, J., Kvenseth, P.G. (1991). Early development of the digestive tract of cod larvae, *Gadus morhua* L., during start feeding and starvation. *J. Fish Biol.* 38:1-15.

Kjorsvik, E., and Reiersen, A.L. (1992). Histomorphology of the early yolk-sac larvae of the Atlantic halibut (*Hippoglossus hippoglossus* L.)-an indication of the timing of functionality. *J. Fish Biol. 41*:1-19.

Kroese, A.B.A. and van Netten, S.M. (1988). Sensory transduction in lateral line hair cells. In: The Mechanosensory Lateral Line: Neurobiology and Evolution (ed. by S. Coombs, P. Görner, and H. Münz) pp. 147 - 161, Springer-verlag, Berlin, Heidelberg.

Laroche, W.A. (1981). Development of larval smooth flounder, *Liopsetta putnami* with a redescription of development of winter flounder, *Pseudopleuronectes americanus* (Family: Pleuronectidae). Fishery Bull. 78(4): 897-909.

Laurent, P. (1984). Gill internal morphology. In: Hoar, W.S. and Randall, D.J., eds. Fish Physiology, Volume X: metabolism. Part A: Anatomy, Gas transfer, and acid-base regulation. London: Academic press INC., Publishers, pp. 73-172.

Li, X.M., Trinh. K.Y., Hew, C.L., Buettner, B., Baenziger, J., and Davies, P.L. (1985). Structure of an antifreeze polypeptide and its precursor from the ocean pout, *Macrozoarces americanus*. *J. Biol. Chem.* 260: 12904-12909.

Lin, Q, Ewart, K.V., Yan, Q., Wong, W.K.R., Yang, D.S.C. and Hew, C. L. (1999). Secretory expression and site-directed mutagenesis studies of the winter flounder skin-type antifreeze polypeptides. *Eur. J. Biochem.* 264: 49-54.

Lin, Y. (1979). Environmental regulation of gene expression. *J. Biol. Chem.* 254: 1422-1426.

Lin, Y., and Long, D.J. (1980). Purification and characterization of winter flounder antifreeze peptide messenger ribonucleic acid. *Biochemistry*. 19: 1111-1116.

Loewen, M.C., Chao, H., Houston, M.E. Jr., Baardsnes, J., Hodges, R.S., Kay, C.M., Sykes, B.D., Sonnichesen, F.D. and Davies, P.L. (1999). Alternative roles for putative ice-binding residues in Type I antifreeze protein. *Biochemistry* 38: 4743-4749.

March, P.E. and Reisman, H.M. (1995). Seasonal changes in hepatocyte ultrastructure correlated with cyclic synthesis of secretory proteins in the winter flounder (*Pleuronectes americanus*). *Cell Tissue Res.* 281: 153-161.

Mazur, P. (1984). Freezing of living cells: mechanisms and implications. *Am. J. Physiol.* 247 (*Cell Physiol.* 16): C125-C142.

Melton, D.A., Kreig, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984). Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage Sp6 promoter. Nucleic Acid Research. 12 (18) : 7035-7056.

Menon, R.P. and Huges, R.C. (1999). Determinants in the N-terminal domains of galectin-3 for secretion by a novel pathway circumventing the endoplasmic reticulum-Golgi complex. *Eur J. Biochem.* 264:569-576.

Metcalfe, W.K.(1989). Organization and development of the Zebrafish posterior lateral line. In: The Mechanosensory Lateral Line: Neurobiology and Evolution (ed. by

S. Coombs, P. Görner, and H. Münz) pp. 147 - 161, Springer-verlag, Berlin, Heidelberg.

Miao, M., Chan, S.L., Hew, C.L., and Gong, Z. (1998). The skin-type antifreeze protein gene intron of the winter flounder is a ubiquitouis enhancer lacking a functional C/EBPα binding motif. *FEBS Lett.* 426: 121-125.

Mignatti, P., Morimoto, T., and Rifkin, D.B. (1992). Basic fibroblast growth factor, a protein devoid of secretory signal sequence, is released from cells via a pathway independent of the endoplasmic reticulum-Golgi complex. *Journal of Cellular Physiology* 151, 81-93.

Mittal, A.K. and Banerjee, T.K. (1980). Keratinization versus mucus secretion in fish epidermis. In: Spearman, R.I.C., Riley, P.A. (eds). The Skin of Vertebrates. Linn Soc. Symp. No. 9 Academic Press, London, New York, pp 1-12.

Miwa, S. and Inui, Y. (1987). Effects of various doses of thyroxine and triiodothyronine on the metamorphosis of flounder (*Paralichthys olivaceus*). *Gen. Comp. Endo.* 67: 356-363.

Miwa, S., Tagawa, M., Inui, Y., and Hirano, T. (1988). Thyroxine surge in metamorphosing flounder larvae. *Gen. Comp. End.* 70: 158-163.

Murray, C.K. and Fletcher, T.C. (1976). The immunohistochemical localization of lysozyme in plaice (*Pleuronectes platessa* L). tissues. *J. Fish Biol.* 9:329-334.

Murray, H.M., Wright, G.M. and Goff, G.P. (1994a). A study of the posterior esophagus in the winter flounder, *Pleuronectes americanus* and the yellowtail flounder,

Pleuronectes ferruginea: Morphological evidence for pregastric digestion? *Can. J. Zool.* 72: 1191-1198.

Murray, H.M., Wright, G.M. and Goff, G.P. (1994b). A comparative histological and histochemical study of the stomach from three species of pleuronectid, the Atlantic halibut, *Hippoglossus hippoglossus*, the yellowtail flounder, *Pleuronectes ferruginea*, and the winter flounder, *Pleuronectes americanus*. Can. J. Zool. 72: 1199-1210.

Murray, H.M., Wright, G.M. and Goff, G.P. (1996). A comparative histological and histochemical study of the post-gastric alimentary canal from three species of pleuronectid, the Atlantic halibut, *Hippoglossus hippoglossus*, the yellowtail flounder, *Pleuronectes ferruginea*, and the winter flounder, *Pleuronectes americanus*. *J. Fish Biol.* 48: 187-206.

Negulescu, P.A., Rubinsky, B., Fletcher, G.L., and Machen, T.E. (1992). Fish antifreeze proteins block Ca entry into rabbit parietal cells. *Am. J. Physiol.* 263 (cell physiol. 32): C1310-C1313.

Ng, N.F., Trinh, K.Y., and Hew, C.L. (1986). Structure of an antifreeze polypeptide precursor from the sea raven, *Hemitripterus americanus*. *J. Biol. Chem.* 261: 15690-15695.

Noakes, D.L. and Godin, J-G. J. (1988). Ontogeny of behavior and concurrent development changes in sensory systems in teleost fishes. IN: Fish Physiology (ed. by W.S. Hoar and D.J. Randall) pp.345-395, Academic Press, Inc. New York.

O'Grady, S.M., Ellory, J.C. and DeVries, A.L. (1982). Protein and glycoprotein

antifreezes in the intestinal fluid of polar fishes. *J. Exp. Biol.* 98: 429-438.

O'Grady, S.M., Ellory, J.C. and DeVries, A.L. (1983). The role of low molecular weight antifreeze glycopeptides in the bile and intestinal fluid of Antarctic fish. *J. Exp. Biol.* 104: 149-162.

Olfert, E.D., Cross, B.M., and McWilliam, A.A. (1993). Guide to the Care and Use of Experimental Animals. Vol. 1., 2nd edition. Canadian Council on Animal Care.

Ottesen, O. H., and Olafsen, J.A. (1997). Ontogenetic development and composition of the mucous cells and the occurrence of saccular cells in the epidermis of Atlantic halibut. *J. Fish Biol.* 50: 620-633.

Pearse, A.G.E. (1985). Histochemistry: Theoretical and Applied. Volume Two: Analytical Technology. Fourth Edition. Churchhill Livingston Edinburgh London Melbourne and New York.

Perry, S.F. (1997). The Chloride Cell: Structure and Function in the Gills of Freshwater Fishes. *Annu. Rev. Physiol.* 59: 325-347.

Petrie, B., Akenhead, S., Lazier, J., and Loder, J. (1988). The Cold Intermediate Layer on the Labrador and Northwest Newfoundland Shelves. *NAFO Sci. Counc. Stud.* 12:57-69.

Petzel, D., Reisman, H., and Devries, A.L. (1980). Seasonal variation of antifreeze peptide in the winter flounder, *Pseudopleuronectes americanus*. *J. Exp. Zool.* 211: 63-69.

Pickett, M.H., Hew, C.L., Davies, P.L. (1983a). Seasonal variation in the level of antifreeze protein mRNA from the winter flounder. *Biochimica et Biophysica Acta* 739: 97-104.

Pickett, M.H., Scott, G., Davies, P., Wang, N., Joshi, S., and Hew, C.L. (1984). Sequence of an antifreeze protein precursor. *Eur. J. Biochem.* 143: 35-38.

Pickett, M.H., White, B.N., and Davies, P.L. (1983b). Evidence that translational control mechanisms operate to optimize antifreeze protein production in the winter flounder. *J. Biol. Chem.* 258: 14762-14765.

Price, J.L., Gourlie, B.B., Lin, Y. and Huang, R.C.C. (1986). Induction of winter flounder antifreeze protein messenger RNA at 4 ^{0}C *in vivo* and *in vitro. Physiol. Zool.* 59: 679-695.

Price, J.L., Lyons, C.E. and Huang, R.C.C. (1990). Seasonal cycle and regulation by temperature of antifreeze protein mRNA in a Long Island population of winter flounder. *Fish Phys. and Biochem.* 8(3): 187-198.

Randall, D.J. (1970). Gas Exchange in Fish. In: Hoar, W.S., Randall, D.J. (eds). Fish Physiology, Volume 4. Academic Press. pp. 253-292.

Roberts, R.J., and Bullock, A.M. (1980). Histochemistry and kinetics of the epidermis of some British teleost fishes. In: Spearman, R.I.C., Riley, P.A. (eds). The Skin of Vertebrates. Linn. Soc. Symp. Ser. No. 9. Academic Press, London, New York, pp. 13-21.

Roberts, R.J., Bell, M. and Young, H. (1973). Studies on the skin of plaice (*Pleuronectes platessa* L.). II. The development of larval plaice skin. *J. Fish Biol.* 5: 103-108.

Roberts, R.J., Young, H. and Milne, J.A. (1972). Studies on the skin of plaice (*Pleuronectes platessa*). I. The structure and ultrastructure of normal plaice skin. *J. Fish Biol.* 4: 87-98.

Roper, D.S., (1981). Superficial neuromasts of the flatfish *Peltorhamphus novaezeelandiae* (Günther). J. Fish Biol. **18**. 753-758.

Rubinsky, B., Arav, A., and Fletcher, G.L., (1991). Hypothermic protection: a fundamental property of "antifreeze" proteins. *Biochem. Biophys. Res. Commun.* **18**, 566-571.

Rubinsky, B., Arav, A., Mattioli, M., and Devries, A.L. (1990). The effect of antifreeze glycopeptides on membrane potential changes at hypothermic temperatures. *Biochem. Biophys. Res. Commun.* 173: 1369-1374

Rubinsky, B., Mattioli, M., Arav, A., Barboni, B., and Fletcher, G.L. (1992). Inhibition of Ca2+ and K+ currents by "anti-freeze" proteins. *Am. J. Physiol.* 262 (regulatory integrative comp. physiology. 31: R542-R545.

Sandbacka, M., Lilius, H., Enkvist, M.O.K., and Isomaa, B. (1998). Rainbow trout gill epithelial cells in primary culture communicate through gap junctions as demonstrated by dye coupling. *Fish Physiol. Biochem.* 19: 287-292.

Schneppenheim, R. and Theede, H. (1982). Freezing-point depression peptides and glycoproteins from Artic-Boreal and Antarctic Fish. *Polar Biol.* 1: 115-123.

Scholander, P.F., VanDam, L., Kanwisher, J.W., Hammell, H.T., and Gordon, M.S. (1957). Supercooling and osmoregulation in Arctic fish. *Journal of Cellular and Comparative Physiology* 49:5-24.

Schreiber, A.M. and Specker, J.L. (1998). Metamorphosis in the summer flounder (*Paralichthys dentatus*): stage-specific developmental response to altered thyroid status. *Gen. Comp. Endo.* 111 (2): 156-166.

Schreiber, A.M. and Specker, J.L. (1999). Early larval development and metamorphosis in the summer flounder: changes in per cent whole-body water content and effects of altered thyroid status. *J. Fish. Biol.* 55: 148-157.

Scocco, P., Accili, D., Menghi, G. and ceccarelli, P. (1998). Unusual glycoconjugates in the oesophagus of a tilapine polyhybrid. *J. Fish Biol.* 53: 39-48.

Scott, G.K., Davies, P.L., Kao, M.H. and Fletcher, G.L. (1988). Differential amplification of antifreeze protein genes in the pleuronectinae. 27: 29-35.

Scott, G.K., Davies, P.L., Shears, M.A. and Fletcher, G.L. (1987). Structural variations in the alanine-rich antifreeze proteins of the pleuronectinae. *Eur. J. Biochem.* 168: 629-633.

Scott, G.K., Fletcher, G.L., and Davies, P.L. (1986). Fish Antifreeze Proteins: Recent Gene Evolution. *Can. J. Fish. Aquatic. Sci.* 43: 1028-1034.

Scott, G.K., Hew, C.L., and Davies, P.L. (1985). Antifreeze protein genes are tandemly linked and clustered in the genome of the winter flounder. *Proc. Natl. Acad. Sci. USA.* 82: 2613-2617.

Scott, W.B. and Scott, M.G. (1988). Atlantic Fishes of Canada. *Canadian Bulletin of Fisheries and Aquatic Sciences.* 219: pp 731.

Segner, H., Storch, V., Reinecke, M., Kloas, W., Hanke, W. (1993). The development of functional digestive and metabolic organs in turbot, *Scophthalmus maximus*.

Marine Biology 119: 471-486.

Shephard, K.L. (1994). Functions for Fish Mucous. *Rev. Fish Biol. Fish.* 4: 401-429.

Shi, Y-B. (1996). Thyroid hormone-regulated early and late genes during amphibian metamorphosis. In: Gilbert, L.I., Tata, J.R., Atkinson, B.G., eds. Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells. Cell Biology: A Series of Monographs. Academic Press Inc., pp 505-533.

Slack, J.M.W. (1991). From Egg to Embryo: Regional specification in early development. In: Developmental and Cell Biology Series 26. Eds. Barlow, P.W., Bray, D., Green, P.B., Slack, J.M.W.

Speel, E.J.M., Ramaekers, F.C.S., Hopman, A.H.N. (1995). Cytochemical detection systems for *in situ* hybridization, and the combination with immunocytochemistry. "Who is still afraid of Red, Green and Blue?" *Histochemical Journal* 27: 833-858.

Steponkus, P.L. (1984). Role of the plasma membrane in freezing injury and cold acclimation. *Ann. Rev. Plant Physiol.* 35: 543-584.

Timoshava, L.V. (1981). Seasonal changes in the structure of the liver of the plaice, *Pleuronectes platessa. J. Ichthyol.*, 21:145-151.

Uehara, F., Ohba, N., Nakashima, Y., Yanagita, T., Ozawa, M., Muramatsu, T. (1993). A fixative suitable for *in situ* hybridization histochemistry. *J. Hist. And Cytochem* 41(6): 947-953.

Vaisus, A., Fletcher, G.L. and Idler, D.R. (1988). Growth hormone represses transcription of an antifreeze gene in winter flounder. Proc. XVIth Int. Congr. Genetics. Aug.

20-27, 1988. Toronto. GENOME, 30: Suppl. 1:64.

Vaisus, A., Martin-Kearley, J., and Fletcher, G.L. (1989). Antifreeze protein gene transcription in winter flounder is not responsive to temperature. *Cellular and Molecular Biology* 35 (5): 547-554.

Valerio, P.F., Kao, M.H. and Fletcher, G.L. (1990). Thermal hysteresis activity in the skin of cunner, *Tautogolabrus adspersus*. *Can. J. Zool.* 68: 1065-1067.

Valerio, P.F., Kao, M.H., and Fletcher, G.L. (1992). Fish skin: an effective barrier to ice crystal propagation. *J. Exp. Biol.* 164: 135-151.

Webb, J.F. (1989). Developmental constraints and evolution of the lateral line system in teleost fishes. IN : The Mechanosensory Lateral Line: Neurobiology and Evolution (ed. by S. Coombs, P. Görner, and H. Münz) pp. 79 - 99, Springer-verlag, Berlin, Heidelberg.

Wen, D., and Laursen, R.A. (1992). Structure-function relationships in an antifreeze polypeptide- the role of neutral and polar amino acids. *J. Biol. Chem.* 267: 14102-14108.

Whitear, M. (1986). The skin of fishes including cyclostomes: epidermis. In: Biology of the Integument. 2. Vertebrates. pg 8-38. Bereiter-Hahn, J.. Matoltsy, A.G. and Richards, K.S. (eds). Berlin: Springer-Verlag.

Williams, P.J. and Brown, J.A. (1992). Developmental changes in the escape response of larval winter flounder *Pleuronectes americanus* from hatch through metamorphosis. Mar. Ecol. Prog. Ser. 88: 185-193.

Woon-Kai, L., Miao, M., Ewart, K. V., Yang, D. S. C., Fletcher. G. L., Hew, C.L. (1998).

Skin-type antifreeze protein from the Shorthorn Sculpin, *Myoxocephalus scorpius*. Expression and Characterization of A M_r 9,700 Recombinant Protein. *J. Biol. Chem.* 273: 23098-23103.

Yang, D.S.C., Sax, M., Chakrabartty, A., and Hew, C.L. (1988). Crystal structure of an antifreeze polypeptide and its mechanistic implications. *Nature Lond.* 333: 232-237.

Zhao, Z., Deng, G., Lui, Q., Laursen, R.A. (1998). Cloning and sequencing of cDNA encoding the LS-12 antifreeze protein in the longhorn sculpin, *Myxocephalus octodecim spinosis*. *Biochim. Biophys. Acta* 1382: 177-180.