Distinct antifreeze polypeptides (AFP) were isolated from the skin of the winter flounder, Pleuronectes americanus, by gel filtration and reverse phase high performance liquid chromatography. In parallel, several cdNA clones were isolated from a skin cdNA library using a liver AFP cdNA probe. Both protein and DNA sequence analyses indicate that flounder skin contains several distinct but homologous alanine-rich AFPs. Although the skin type AFPs contain 11 similar amino acid repeats found in the secretory liver type AFPs, the skin type AFPs are mature polypeptides lacking both the signal and prosequences, indicating that they may function intracellularly. The skin type AFP is significantly less active in thermal hysteretic activity than the liver type AFP. Genomic Southern analysis indicates that like the liver type AFP genes, there are multiple copies (30–40 copies) of skin type AFP. Although the liver type AFP genes are specifically expressed in the liver and to a lesser extent in intestine, the skin type AFP genes are expressed in all tissues examined including the liver and abundantly in exterior tissues, i.e. skin, scales, fin, and gills, suggesting an important protecting role in these exterior tissues.

Many species of Antarctic and polar fish secrete macromolecular antifreezes into their plasma in order to avoid freezing (DeVries, 1983; Davies and Hew, 1990). At present, four distinct types of antifreeze proteins have been characterized from a variety of fish: the antifreeze glycoproteins and three types of AFPs1 (Davies and Hew (1990) and Griffith and Ewart (1995) and references therein). The antifreeze glycoproteins, which are found in three families of Antarctic fish and polar cods, largely consist of a tripeptide repeat (Ala-Ala-Thr) with a disaccharide attached to the threonyl residue. Type I AFPs are alanine-rich, $\alpha$-helical polypeptides found in many righteye flounders and sculpins. Type II AFPs are enriched with halfcystine and are found in sea raven, smelt, and herring. Type III AFPs are globular proteins found in several Zoarcoid families including eelpout and wolfish. Although the different AFPs and antifreeze glycoproteins are structurally distinct, they share an unusual ability to inhibit ice crystal growth by binding to the ice surface and thus lowering the freezing temperature. At present, most if not all of the antifreeze glycoproteins and AFPs have been isolated from serum, and their DNA sequences have been deduced by cdNA cloning from the liver. Invariably, all of these proteins described to date are synthesized as larger precursor polypeptides containing the signal peptides, which is consistent with a secretory role.

The AFP from the winter flounder, Pleuronectes americanus, has been studied extensively in terms of its protein structure and function, gene organization, gene expression, and regulation. The genome of the winter flounder contains multiple copies of AFP genes, most of which are arranged as regular tandem repeats (Scott et al., 1985).

The flounder AFP mRNAs have recently been found in many tissues. In addition to liver, they can be detected at relatively high concentrations in skin, scales, fin, and gills. Furthermore, the liver and nonliver-derived AFP mRNAs respond differently to seasonal and hormonal treatment (Gong et al., 1992). Although the liver AFP mRNAs are tightly regulated with a 1000-fold difference between their summer and winter levels, the nonliver derived AFP mRNAs are more than 40-fold, with little induction for the nonliver AFP mRNAs (Gong et al., 1995). These experiments suggest that there may be distinct sets of AFP genes that respond differently to a wide variety of stimuli. This prompted us to re-examine the nature of the nonliver AFP mRNAs and their presumptive gene products.

**EXPERIMENTAL PROCEDURES**

Materials—Winter flounder (P. americanus) were collected from Conception Bay, Newfoundland, Canada. Several tissues were removed and frozen in liquid nitrogen and stored at $-70^\circ$C before use.

Isolation of Skin AFPs—Skin scrapings (46 g) were homogenized in 500 ml of 0.1 M NH$_4$HCO$_3$ using a Polytron homogenizer. After low speed centrifugation, the supernatant was lyophilized (1 g), redissolved, and chromatographed in a Sephadex G-75 column (2.5 x 80 cm) in 0.1 M NH$_4$HCO$_3$. Active fractions, measured with a nanoliter osmometer, were pooled and rechromatographed once in the same column. The repurified materials (approximately 50–100 mg) were further fractionated on a Bondclone 10 C$_18$ column (Phenomenex, Torrance, CA) using a 0.5% trifluoroacetic acid and acetonitrile gradient. Individual fractions were pooled and lyophilized.

Measurement of Antifreeze Activity—Antifreeze activity was measured as thermal hysteresis (the difference between the melting and freezing temperatures) essentially following the procedure of Chakrabarty et al. (1989). Proteins were dissolved in 0.1 M NH$_4$HCO$_3$ and their concentrations were determined by duplicate amino acid

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1 The abbreviations used are: AFP, antifreeze protein/polypeptide; HPLC, high performance liquid chromatography.
analyses. Activity measurements were performed on a series of dilutions with concentrations ranging from 0.1 to 7.0 mM for each protein using a Clifton Nanolitre Osmometer (Clifton Technical Physics, Hartford, NY). Prior to the series of measurements for each protein, measurements were made from three sample wells using buffer alone, and their average gave the background hysteresis. For each dilution, measurements were made from three wells, and the average was taken. The background hysteresis was then subtracted from this value in order to obtain the antifreeze activity.

Structural Analysis of Skin AFP—Purified skin AFPs from reverse phase HPLC were used for amino acid analysis, protein sequencing, and mass spectroscopy. Both amino acid analysis and protein sequencing were performed by the Biotechnology Service Centre, Hospital for Sick Children, Toronto, and mass spectrometry was done by the Carbohydrate Centre, University of Toronto, Toronto. For amino acid analysis, the samples were hydrolyzed in 6 N HCl at 110°C for 24 h and analyzed using the Waters Picotag System. Because of the blockage of the N- terminal methionine, skin AFPS were pretreated with cyanogen bromide (in 5% formic acid, 24 h, with 200-fold molar excess of CNBr) prior to protein sequencing in a Protein Mass Spectrometer.

Isolation of Skin AFP cDNA Clones—Total RNA was isolated using the skin tissues containing scales and a dorsal fin from a single individual fish collected in winter by the acid guanidium thiocyanate-phenol-chloroform extraction method as originally described by Chomczynski and Sacchi (1987) and modified for fish tissues by Gong et al. (1992). Poly(A)+ RNA was then selected by oligo(dT)–Sepharose as described by Sambrook et al. (1989). The cDNA library was constructed using the lambda Uni-ZAP XR vector system from Stratagene (La Jolla, CA). About 2.9 x 10^6 primary clones were obtained from 2 μg of poly(A)+ RNA. The skin AFP cDNA clones were screened by hybridization using a liver AFP cDNA clone, pkenc17, which encodes the most abundant serum AFP component A or HPLC-6 (Pickett et al., 1984). Because of the high representation of AFP clones in the skin cDNA library, some AFP clones were obtained by colony hybridization after bulk in vivo excision. For bulk in vivo excision, about 10^8 phages from amoebina skin cDNA library were used to infect plating bacteria together with the helper phage ExAssist according to the manufacturer’s manual (Stratagene). About 200 colonies were transferred to a new plate and hybridized with pkenc17 after transferring onto a nylon membrane, Colony/Plaque Screen (Du Pont NEN). Both strong and weak hybridized colonies were characterized for potential distinct AFP clones. Hybridization was performed as described previously by Gong et al. (1992) but washed at a less stringent condition (0.3 x NaCl, 60°C).

Double strand DNA sequencing was performed by dideoxynucleotide chain termination using the T7 DNA sequencing kit according to the manufacturer’s instructions (Pharmacia). Each clone contained about a 240–300-base pair cDNA insert, and complete sequences were obtained by sequencing from both ends using SK and T7 primers.

Primer Extension—A 22-mer oligonucleotide, 5'-GGCTGTGGCCGTCATGTGGATG-3', complementary to the region starting 7 bases upstream of the ATG codon in sAFP2 (clone S3) (see Fig. 5), was synthesized by the Biotechnology Service Centre, Toronto. The oligonucleotide was labeled using γ-32P-ATP by T4 DNA kinase. The primer extension experiment was carried out as described by Sambrook et al. (1989).

Genomic Southern Blot Hybridization—Genomic DNA was isolated from a testis collected from a single fish by the method of Blin and Stafford (1976). Briefly, about 0.4 g of tissue was digested with proteinase K in 50 ml Tris, pH 8, 100 mM EDTA, 0.5% SDS overnight at 50°C. The mixture was then extracted extensively with phenol/chloroform. The aqueous phase was dialyzed against TE (10 mM Tris, pH 8.1, 1 mM EDTA), and high molecular weight DNA was collected by the addition of NaCl to 0.1 M and 2 volumes of ethanol. Restriction-digested genomic DNA was separated by electrophoresis on a 0.7% agarose gel. The gel was soaked in 0.4 M NaOH, 0.6 M NaCl for 30 min, and blotted onto a Hybond membrane (Amersham Corp.) in the same solution. Hybridization was performed as described previously (Gong et al., 1992).

Northern Blot Hybridization—Total RNAs from selected tissues were extracted as stated above. Total RNAs were separated by formaldehyde agarose gel electrophoresis and Northern blot hybridization was performed as described previously (Gong et al., 1992). The relative levels of AFP mRNAs were estimated by densitometric scanning of autoradiograms using Scanjet 3P (Hewlett Packard) and analysis using a computer program (NIH Image 1.52).

RESULTS

Isolation of Skin Type AFP—The HPLC profile of AFPS isolated from skin scrapings is shown in Fig. 1. The HPLC profile of gel filtration chromatography-purified serum AFP is also included for comparison. The serum AFPS contain only two major components, HPLC-6 and HPLC-8 (Fig. 1A). Except for HPLC-5, -7, and -9, which represent post-translational modifications and minor serum AFPS, the other earlier elution peaks are not related to AFPS, as examined by amino acid analysis (Fournier et al., 1984). However, the HPLC profile of AFP isolated from the skin scrapings was obviously more heterogeneous and contained at least 5–6 components (Fig. 1B). The major skin AFPS are designated as sAFP1, sAFP2, sAFP3, HPLC-6*, and HPLC-8*, respectively. In addition, there were several peaks eluted early in the HPLC. These early elution peaks were minimal in antifreeze activity. Furthermore, amino acid compositions of these materials were not significantly enriched with alanine and were not further investigated. However, HPLC-6* and HPLC-8* had retention times identical to those of HPLC-6 and HPLC-8, respectively. Subsequent amino acid compositions and protein sequencing analyses confirmed that they were in fact HPLC-6 and HPLC-8, respectively, and indicated some contamination of the skin samples from the blood.

Amino acid analyses indicate that the skin AFPS are enriched with alanine, with 60.2% of alanine comparable with 62.2% to HPLC-6 and HPLC-8 (data not shown). In addition, the skin AFPS contain methionine and proline, two amino acid

FIG. 1. Reverse phase HPLC profiles of flounder AFPS. A, serum AFP, B, skin AFP. Sephadex G-75 purified antifreeze from sera (A) and skin scrapings (B) were fractionated on a Bondclone 10 C18 column (2.1 x 30 cm); the flow rate was 4.5 ml/min with a 20–40% acetonitrile, 0.1% trifluoroacetic acid gradient.

Fig. 1
Intracellular AFPs of P. americanus

residues absent in serum AFP, and are relatively low in leucine, aspartic acid, and glutamic acids compared with HPLC-6 or HPLC-8. One of the skin AFPs (sAFP1) contains histidine, which is absent in serum AFP. Unlike the serum AFP (HPLC-6 and HPLC-8), the skin AFP N termini were blocked. sAFP1, sAFP2, and sAFP3 were analyzed by atmospheric pressure ionization mass spectroscopy to determine the nature of the blocking group. Based on their cDNA sequences to be described in the later sections, these peptides had M, of 3376.56, 3480.61, and 3482.67, respectively. Atmospheric pressure ionization mass spectroscopy analyses indicated that larger M, ions of 3419.06, 3480.61, and 3525.41 were present in sAFP1, sAFP2, and sAFP3, accordingly. The difference (42 M,) was consistent with the addition of an acetyl group at the N terminus. sAFP2, in addition, contained a minor M, of 3366.87 and might indicate slight contamination by sAFP7 (M, of 3324.46). Their amino acid sequences were confirmed by protein sequencing after CNBr cleavage of the N-terminal methionine. Like the serum AFPs, skin AFPs also contain similar threonine 11 amino acid repeats. Interestingly, the skin AFPs resemble two previously sequenced serum AFPs, SS-3 and GS-5 from the shorthorn sculpin and grubby sculpin, respectively (Hew et al., 1985; Chakrabartty et al., 1988), particularly in their N-terminal regions (Fig. 2). Both SS-3 and GS-3 are virtually identical in their N-terminal structures more in line with the AFP isolated from the shorthorn and grubby sculpins (Sicheri and Yang, 1995).

In Fig. 3, activities of the skin AFPs plotted as a function of concentration form hyperbolic curves consistent with those typically observed for type I AFPs (Scott et al., 1987; Chakrabartty et al., 1989; Wen and Laursen, 1992a) and most other fish AFPs (Kao et al., 1986). The activity curve of the major serum AFP, HPLC-6, was in line with those previously obtained using a synthetic analogue of this protein (Chakrabartty et al., 1989) and a mixture of AFPs from flounder serum (Kao et al., 1986). In contrast, the AFPs isolated from skin appeared far less active. The skin AFPs displayed lower activities than the serum AFP in all but the lowest (0.1 mM) concentration measured, and activity curves appeared to be approaching saturation plateaus at lower concentrations than the serum protein (Fig. 3). The curves for two of the skin AFPs, sAFP2 and sAFP3, were virtually coincident with activities less than half of that observed for HPLC-6. A detailed structural and functional analysis of the skin type AFP will be reported elsewhere.2

Isolation of Skin AFP cDNA Clones—A winter flounder skin cDNA library was constructed using Stratagene's lambda Uni-ZAP XR vector system. When a small portion of the primary library was screened with a liver AFP cDNA, one winter flounder (Davies and Gauthier, 1992). Part of the F2 sequence is shown in Fig. 5. The sequence of 11–3, which is almost identical to that of F2, in the region shown in Fig. 5 had only 5 nucleotide changes and a 5-base deletion (Davies and Gauthier, 1992). Due to the presence of in frame stop codons in the 5' upstream region corresponding to the presequence in the liver AFP gene and the lack of a typical TATA box in the putative promoter region, these two genes have been previously assigned as pseudogenes (Davies and Gauthier, 1992). As shown in Fig. 4, sAFP2 is identical to the protein encoded by the "pseudogene" F2 and is different from

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the AFP encoded by 11–3 by one amino acid. One of the cDNA clones encoding sAFP2 (S3) has only two nucleotide substitutions compared with F2 (Fig. 5). The two major transcription start sites, as determined by primer extension in Fig. 6, are indicated by carets. The sequence before the transcription start sites are shown in lowercase letters. The position of an intron is indicated by an arrowhead. The putative TFIID binding site, aataa, the oligonucleotide for primer extension, and the polyadenylation site AATAAA are underlined.

In order to confirm the isolated clones contain full-length coding sequences and to map the transcription start site of the skin type AFP genes, primer extension was carried out as shown in Fig. 6. There are two major extension products using skin total RNA as templates. The extension is 17 or 18 base pairs from the 5' end of S3 cDNA clones. Only one extension product corresponding to the long extension product in the scales was detected using the liver RNA as templates. The two extension products map the transcription start site at bases 130 and 131 of the F2 gene (Fig. 5). A putative TFIID binding motif AATAAAT is found 25 nucleotides upstream of the first start site, further indicating that F2 and 11–3 are functional. These data also confirm that the skin AFP clones contain a full-length coding sequence, without the signal peptide and prosequences. The two extension products may indicate two transcriptional initiation sites for the skin type AFP genes. Alternatively, because the primers we used hybridize to all skin AFP genes, the two extension products could be the result of genetic polymorphism.

Multiple Copies of Skin Type AFP Genes in the Genome—Because 9 different skin AFP cDNA clones encoding eight distinct skin type AFPs were identified, it is likely that the skin type AFP genes belong to a multigene family similar to the liver type AFP genes. In order to confirm this, a genomic blot hybridization was performed. Genomic DNA was isolated from the testis collected from a single individual fish and cut with four different restriction enzymes, HindIII, EcoRI, SstI, and BamHI. Two identical blots were made and probed with the liver type (pkenc 17) and skin type (S3) AFP probes, respectively. As shown in Fig. 7, both probes hybridized to multiple bands. It is interesting to note that the skin probe recognizes most, if not all, liver AFP fragments, whereas the liver AFP probe only hybridized to a limited number of DNA fragments. Because most of the liver AFP genes are regular tandem repeats and most liver AFP fragments contain multiple copies of AFP genes (up to 40 copies), it is likely that even a weak hybridization from the skin probe produces a strong signal. The cross-hybridization was also observed in the Northern blot analyses (Fig. 8A). Another possibility is that some skin type AFP genes may be clustered together with the liver type AFP genes. The skin AFP-specific fragments can be recognized by the comparison of the two blots as shown in Fig. 7 and are indicated by dots. Most of the skin type AFP genes are also most likely linked as indicated by the hybridization pattern in BamHI-digested DNA, in which the skin type AFP genes are restricted to two high molecular weight fragments (over 23 kilobases). These data, together with the identification of at least nine distinct skin AFP cDNA sequences, unequivocally support the presence of a skin type AFP multigene family. The hybridization pattern of the liver probe is similar to the previously published genomic blot by Scott et al. (1985), which estimated there were about 40 copies of AFP genes in the winter flounder genome. This estimation probably did not include the skin type AFP genes. By comparison of the hybridization intensity of skin and liver AFP DNA fragments, the number of skin type AFP genes is almost the same as the number of liver type AFP genes. Therefore, it is likely that the skin type AFP genes constitute an additional 30–40 copies of AFP genes in
the genome. So far, we have isolated nine different skin AFP cDNA clones encoding eight distinct AFPs. At the protein level, at least three different antifreeze polypeptides have been identified by reverse phase HPLC.

Tissue Distribution of AFP Transcripts—The tissue distribution of AFP transcripts were previously examined using a liver cDNA probe in the winter flounder (Gong et al., 1992). Because the hybridization was performed at a relatively low stringency, the nonliver AFP transcripts were likely to be detected due to cross-hybridization. With the availability of the skin AFP probe, the tissue distribution of AFP mRNAs were re-examined using a high stringency wash condition (0.015 M NaCl, 72°C). Preliminary experiments in which the liver and skin cDNA clones were cross-hybridized indicated that this wash condition resulted in a minimal cross-hybridization between the skin and liver genes. As shown in Fig. 8, total RNAs were isolated from selected tissues collected from a single fish and probed with the skin (S3, A) and liver (pkenc, B) AFP probes respectively. 10 μg of total RNA was loaded on each lane except for the liver RNA in B, which was loaded with 1 μg. C, the blot was probed with a mixture of liver and skin AFP probes. The positions of liver type and skin type AFP mRNAs are indicated.

FIG. 6. Transcription start sites. Primer extension was performed using a skin AFP-specific oligonucleotide as described under “Experimental Procedures.” 5 μg of scale total RNA and 30 μg of liver total RNA from the winter flounder (WF) and 30 μg of total liver RNA from Atlantic salmon (AS) were used as templates as indicated at each lane. The size of primer extended products were determined by a set of sequence ladders as shown on the left (nucleotides). The two major extended products are indicated on the right.

FIG. 7. Genomic Southern blot analysis of AFP gene family. The genomic DNA was prepared from a single individual and cut by four different restriction enzymes: HindIII, EcoRI, SstI, and BamHI. Two identical blots were made and probed with the inserts of skin clone S3 and the liver clone pkenc17, respectively. The skin-specific fragments as indicated by caret are defined as those only present in the skin blot or weakly in the liver blot. Molecular weight marker lambda DNA/HindIII (Life Technologies Inc.) are shown on both the left and the right sides.

FIG. 8. Tissue distribution of AFP mRNAs by Northern blot analysis. Total RNAs were prepared from eight different tissues as indicated, separated by electrophoresis, blotted, and probed with the skin (S3, A) and liver (pkenc, B) AFP probes respectively. 10 μg of total RNA was loaded on each lane except for the liver RNA in B, which was loaded with 1 μg. C, the blot was probed with a mixture of liver and skin AFP probes. The positions of liver type and skin type AFP mRNAs are indicated.

The level of AFP mRNAs in the liver and skin tissues, a blot containing liver, scale, and stomach total RNAs was hybridized with a mixture of liver and skin AFP probes that were mixed and labeled in the same reaction to ensure the same specific activity for the two probes (Fig. 8C). The level of scale AFP mRNA is about 10–20% of the liver level, indicating that the skin and other exterior tissues such as gills are also major sites of AFP.
In the present study, we have isolated a family of skin type AFPs and their corresponding cDNA genes. The skin type AFP genes are ubiquitously expressed in all tissues examined, with high levels in the exterior tissues such as skin, scales, fin, and gills. Structurally, the skin type AFP lacks both the pre- and prosequences and might imply an intracellular role in freeze protection. These studies confirm and extend our previous suggestion that there are at least two different sets of AFP genes, i.e., liver type and skin type, in the winter flounder (Gong et al., 1992, 1995).

Function of Skin Type AFPs—In almost all AFP-producing fish species studied, AFPs are synthesized in the liver and secreted into the blood circulation. The AFPs thus function through lowering the plasma freezing temperature to prevent fish from freezing in ice-laden sea water. The presence of the skin type AFPs provides an additional twist to our understanding of the function of AFPs. Because the skin type AFP genes encode polypeptides without the signal peptide, they may function as intracellular proteins, although the possibility that these small polypeptides use an alternate pathway for secretion cannot be presently ruled out (Mignatti et al., 1992). However, analysis of the HPLC profile (Fig. 1A) of the plasma AFP did not reveal the presence of any skin type AFP in the circulation and thus would argue against any significant secretion of the skin type AFPs. The intracellular localization of AFPs may imply an unusual role in the protection of fish from freezing. A cytosolic AFP could play a significant antifreeze role by providing a barrier to ice crystal passage or growth through the skin. Valerio et al. (1992) found that isolated skin from winter flounder was a significant barrier to ice crystal growth, which would highlight a significant protective function of the skin type AFPs. The very high levels of skin type AFP mRNA in the exterior tissues (i.e., 20% of total skin mRNA) may reflect a need for high concentrations of AFP to achieve effective protection from freezing. In many interior tissues, the expression is relatively low but significant, about 1–10% of the skin level. These tissues may already be protected by circulating plasma AFPs that are produced in liver. Low levels of intracellular AFPs may also have other physiological roles in these tissues.

Activity of the Skin AFP—The activity of the fish skin AFPs falls within the range of fish AFP activities in general (Kao et al., 1986). However, they are not as active as the serum AFP from winter flounder (Fig. 3). The presence of AFPs with different activities within a species is not without precedent. Comparison of AFPs from the shorthorn sculpin revealed that the longer 45-residue AFP, SS-8, is highly active, with an activity close to that of winter flounder serum AFP, whereas the curve for the shorter 32-residue AFP, SS-3, from the same species is much lower (Kao et al., 1986) and consistent with those of the winter flounder skin AFPs, sAFP2 and sAFP3.

The skin AFPs lack several residues that, in the serum AFP, are known to contribute to activity (Wen and Laursen, 1992b). A large number of the skin type AFPs are potential ice crystal interaction sites because their helical repeats are generally Thr-Xaa10 instead of Thr-Xaa2-(Asn/Asp)-Xaa7. The presence of any skin type AFP in the circulation and thus the absence of any skin type AFP induces an additional potential ice-binding motif in the crystal structure of flounder serum HPLC-6 consisting of Thr/Asp or of Thr/Asn. The proposed structural basis for the presence of skin type AFPs in the circulation and thus the absence of any skin type AFP induces an unusual role in the protection of fish from freezing. A cytosolic AFP could play a significant antifreeze role by providing a barrier to ice crystal passage or growth through the skin. Valerio et al. (1992) found that isolated skin from winter flounder was a significant barrier to ice crystal growth, which would highlight a significant protective function of the skin type AFPs. The very high levels of skin type AFP mRNA in the exterior tissues (i.e., 20% of total skin mRNA) may reflect a need for high concentrations of AFP to achieve effective protection from freezing. In many interior tissues, the expression is relatively low but significant, about 1–10% of the skin level. These tissues may already be protected by circulating plasma AFPs that are produced in liver. Low levels of intracellular AFPs may also have other physiological roles in these tissues.

Evolution of Type I AFP Genes—It is clear that the liver and skin type AFP genes belong to multigene families. Most, if not all, of the liver genes appear to be tandemly repeated with regular spacing (Scott et al., 1985). The skin type AFP genes (30–40 copies) appear to be also linked. The ubiquitously expressed skin type AFP genes may represent a primitive AFP gene in this multi-gene family. Gene duplication of the primordial skin type AFP genes and subsequent divergence or selection may give rise to two distinct AFP genes, i.e., liver type and skin type. One skin type AFP gene may have gained a signal peptide sequence, prosequence, and liver regulatory elements to become a liver-specific gene encoding secreted AFPs. Selection during ocean cooling events may then have led to the amplification of genes encoding highly active AFP forms expressed in liver. The emergence of a liver-specific AFP gene and its subsequent amplification were likely relatively recent events and occurred after the divergence of the winter flounder and sculpins, which also produce type I alanine-rich AFPs. Although the type II fish AFPs found in three different taxonomic groups are homologous to the C-type lectins and appear to have evolved from the carbohydrate-recognition domains of these proteins (Davies et al., 1993; Ewart and Fletcher, 1993), the evolutionary origins of the other fish AFPs remain unclear. The type I AFPs do not appear to be homologous to known protein families. However, many proteins with the structural characteristics of the skin type AFPs are present in the lower vertebrates and the invertebrates. For example, the skin from many amphibians contains antimicrobial proteins that form small amphiphilic helices (Kreil, 1994). If similar proteins occur in fish skin, they may be progenitors of the type I AFPs or they may be among the proteins that we now identify as skin type AFPs. These possibilities and their implications for the roles and properties of the type I AFPs should be examined.

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Differential Expression of AFP Gene Family—There are about 70–80 copies of AFP genes including both the liver and skin type in the winter flounder genome. The liver type AFP genes appear to be expressed predominantly in liver and to a much lesser extent in intestine. Consistent with this, we have not isolated any liver type AFP cDNA clones from the skin cDNA library. In contrast, the skin AFP genes are ubiquitously expressed. Because there are 30–40 copies of skin AFP genes, whether or not some of these skin type genes are specifically expressed in certain tissues remains a question. It will be inter-
testing to characterize AFP cDNA clones from other tissues such as gills, heart, kidney, and spleen. The discovery of non-liver AFP genes in the winter flounder suggests that variants with different structures and tissue specificities may occur in the AFP gene families of other AFP-producing species, particularly those species producing different types of AFPs. For example, like the winter flounder, the ocean pout (*Macrozoarces americanus*), which synthesizes type III AFPs, has an AFP multigene family (Hew et al., 1988) and expresses AFP mRNAs predominantly in the liver but also in many nonliver tissues (Gong et al., 1992). It will be interesting to determine whether there are different sets of AFP genes with modified or different structures and functions in this species.

Our observation, to our knowledge, represents the first report that two distinct types of AFPs are present within the same species and has raised new questions regarding the evolution and gene regulation of these proteins. Our success in identifying the new skin type AFP will stimulate further research on the identification of similar AFPs in other fish species and the role of these AFPs in intracellular function.

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