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Wolffish Antifreeze Protein Genes Are Primarily Organized as Tandem Repeats That Each Contain Two Genes in Inverted Orientation

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The antifreeze protein genes of the wolffish (*Anarhichas lupus*) constitute a large multigene family of 80 to 85 copies, which can be classified into two sets. One-third of the genes were linked but irregularly spaced. The other two-thirds were organized as 8-kilobase-pair (kbp) tandem direct repeats that each contained two genes in inverted orientation; DNA sequence analysis suggests that both genes are functional. Except for a single region specific to each gene, the genes and their immediate flanking sequences were 99.2% identical. This degree of identity ended soon after a putative transcription termination sequence; as the 3′ ends of the genes were only 1.3 kbp apart, these sequences might confer mutual protection from interference by transcriptional runoff. A Southern blot of wolffish DNA restricted with enzymes that do not cut within the tandem repeats indicated that the repeats were clustered in groups of six or more. The organization of antifreeze protein genes in the wolffish was very similar to that in the unrelated winter flounder, which produces a completely different antifreeze. This similarity might reflect common dynamics by which their progenitors adapted to life in ice-laden sea water.

The colligative effect of solutes in the blood of marine teleosts is sufficient to prevent freezing down to −0.7°C, but seawater may freeze at temperatures as low as −1.9°C depending on its salinity. Fish can survive in seawater below the freezing point of their serum in a supercooled state but only in deep water, as contact with ice crystals results in rapid freezing and death (17). Some teleost species have adapted to life in shallow ice-laden seawater by producing macromolecular antifreezes. These proteins or glycoproteins are produced in the liver and exported into the circulation, where they act noncolligatively to depress the freezing point of blood by binding to ice crystals and inhibiting their growth (4). Concentrations as high as 20 mg/ml may be required to protect fish at the freezing point of seawater (−1.9°C).

To date, three very different types of antifreeze proteins (AFPs) have been described: the alanine-rich, alpha-helical AFP (type I) of sculpins and righteye flounders; the cystine-rich AFP (type II) of the sea raven (*Hemitripterus americanus*); and the type III AFP of the eel pouts, which is neither alanine nor cystine rich (10, 19). In addition, the glycoprotein antifreeze of the cods and nototheniids is structurally distinct from the three AFPs (5). The marked differences in the structures of these antifreezes indicate that they are of independent origin, and their limited distribution in phyletic lineages led Scott et al. to suggest that they originated comparatively recently (19). If so, the impetus to produce antifreeze proteins is most likely to have been the sea-level glaciations of the Cenozoic ice ages, as the world's oceans are thought to have been free of ice for the preceding 250 million years (9).

We report here on the organization of the AFP genes in the wolffish (*Anarhichas lupus*), an eel pout producing the type III AFP, and point out its remarkable similarity to the AFP gene organization in the unrelated winter flounder (*Pseudopleuronectes americanus*). Both fish have a set of linked but irregularly spaced genes and a larger tandemly repeated series. In the wolffish each tandem repeat contains two genes in inverted orientation. DNA sequence analysis suggests that these genes are fully functional and that they may use a specific DNA structure to prevent interfering with each other's transcription. We speculate that the similarity in organization between wolffish and winter flounder AFP gene families reflects their common solution to intense selective pressure to produce high levels of AFP.

MATERIALS AND METHODS

Wolffish tissue was collected at the Marine Sciences Research Laboratory (St. John's, Newfoundland, Canada). Genomic DNA was prepared by pulverizing testis tissue in liquid N_2 by the method of Blin and Stafford (2) with modifications as described previously (20). Restriction digests of genomic DNA (15 μg) were blotted onto nitrocellulose (21). Phage digests were blotted onto GeneScreen Plus (Du Pont) by a variation of the alkaline transfer method (15), in which fragments are transferred to the membrane by the fluid in the gel. A wolffish DNA library was prepared in Charon 35 as follows. Wolffish DNA (400 μg) was partially digested with restriction endonuclease *Sau*3A and centrifuged on a 10 to 40% sucrose gradient. Fractions containing DNA fragments 15 to 20 kbp long were pooled and precipitated in 2.5 volumes of ethanol. Annealed *Bam*HI arms of Charon 35 were prepared as described by Maniatis et al. (12) and ligated to the genomic DNA. The ligated DNA was packaged by using a lambda DNA in vitro packaging kit from Stratagene. A second wolffish library was constructed in Charon 30 by digesting genomic DNA to completion with *Bam*HI and treating it as above, except that fragments from 7 to 10 kbp in size were selected for insertion into Charon 30 arms. The libraries were plated on *Escherichia coli* K802 and screened directly (1) by using a ^{32}P-labeled clone of ocean

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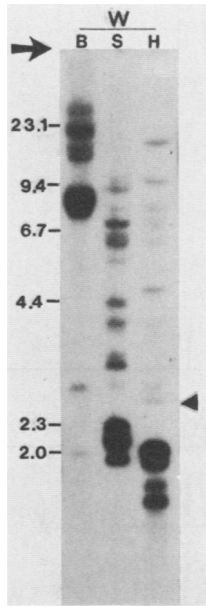

FIG. 1. Genomic Southern blot of wolffish DNA. Samples (15 μg) of genomic wolffish (W) DNA were digested to completion with *Bam*HI (B), *Sst*I (S), or *Hin*dIII (H), electrophoresed on a 0.7% agarose gel, and blotted onto nitrocellulose. The blot was probed with a nick-translated ocean pout cDNA clone. The arrow marks the origin, and the positions of fragments from a *Hin*dIII digest of lambda DNA are noted on the side. The arrowhead marks the 2.5-kbp *Hin*dIII fragment used to calibrate densitometry plots.

pout AFP cDNA (11) or a wolffish 0.4-kbp *Bam*HI-*Hin*dIII genomic DNA fragment (see below) as probes. Restriction fragments of genomic clones were subcloned into pUC19 by standard methods (12). Fragments to be sequenced were subcloned into M13 and sequenced by the dideoxy method (13).

RESULTS

Type III AFPs are encoded by a large multigene family in the wolffish. When a Southern blot of restricted genomic DNA from the wolffish was probed with a type III AFP cDNA clone, 10 to 20 different bands of hybridization were seen in each lane (Fig. 1). One or a few bands showed intense hybridization. The total amount of hybridization indicates that AFP genes are present in high copy number in the wolffish, and the distribution of the signal implies that the vast majority of the genes have similar flanking sequences, being contained within *Hin*dIII and *Sst*I fragments ranging from 1.8 to 2.3 kbp and *Bam*HI fragments in a narrow size range, ca. 8.0 kbp. This pattern is often indicative of tandem repeats. For ease of presentation, the genes contained in the intensely hybridizing *Bam*HI bands will be referred to as the major component of the AFP genes (see below), and the remaining genes as the minor component.

Cloning of the minor-component AFP genes of the wolffish. To investigate the genomic arrangement of wolffish AFP genes, the *Sau*3A Charon 35 library of wolffish genomic DNA was screened with a cloned type III AFP cDNA. Of 1.6×10^4 library plaques examined, 13 hybridized intensely, and 12 of these clones were analyzed in detail. They variously contained from one to five hybridizing regions. The restriction patterns for 8 of the 12 can be arranged to produce a continuous stretch of 60 kbp which contains nine AFP gene regions in total (Fig. 2). While there was no suggestion of a regularly repeated substructure, there was some evidence for a duplication in this region, as the restriction pattern between the *Hin*dIII site at 17.4 kbp and the *Sst*I site at 23.9 kbp was very nearly reproduced in the region from 30.3 to 37.0 kbp. This linked but irregular pattern is highly reminiscent of the genomic arrangement of the minor-component AFP genes in the winter flounder (3, 18) and in general the α- and β-globin genes of vertebrates (6).

We believe that each of the hybridizing regions contains a single gene for two reasons. First, AFP cDNA bound to the different hybridizing regions with the same intensity on Southern blots (data not shown), a fact which argues that none of these regions contains either multiple genes or isolated exons. Second, while the patterns of restriction sites flanking and within the hybridizing regions varied, they were either the same as or a simple derivative of the *Sst*I and *Hin*dIII sites flanking, and the *Sal*I site within the 5′ end of, the major-component AFP genes (Fig. 3), two of which have been sequenced (Fig. 4).

When bands on the genomic Southern blot (Fig. 1) were correlated with cloned fragments containing genes (Fig. 2), it was apparent that all of the cloned genes were from the

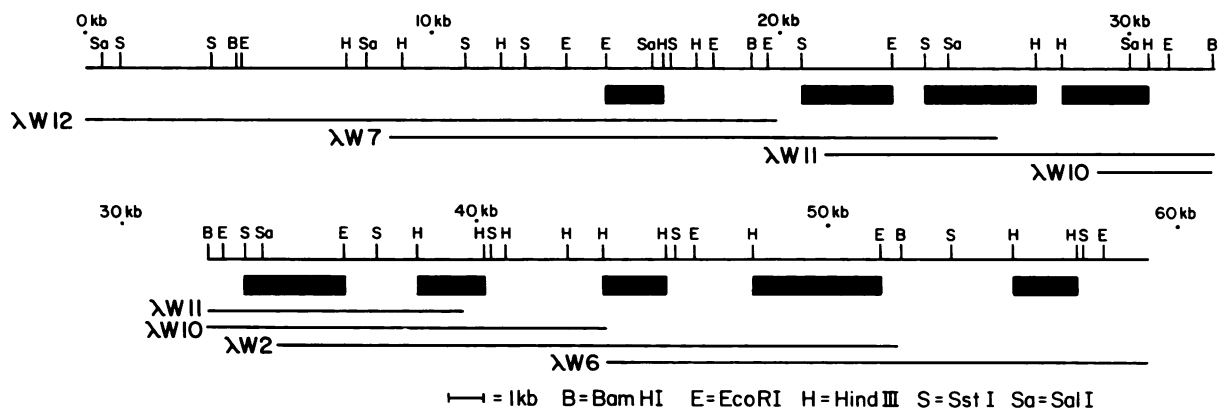

FIG. 2. Restriction map of ≈60-kbp gene sequence which contains nine AFP genes. Thick black bars beneath the map identify hybridizing fragments, and horizontal lines beneath the bars show the extent of representative clones from the region. The restriction pattern between 17.4 and 23.9 kbp (H-E-B-E-S-E-S) is very similar to that between 30.3 and 37.0 kbp (H-E-B-E-S-Sa-E-S) in sequence and spacing (see text for discussion).

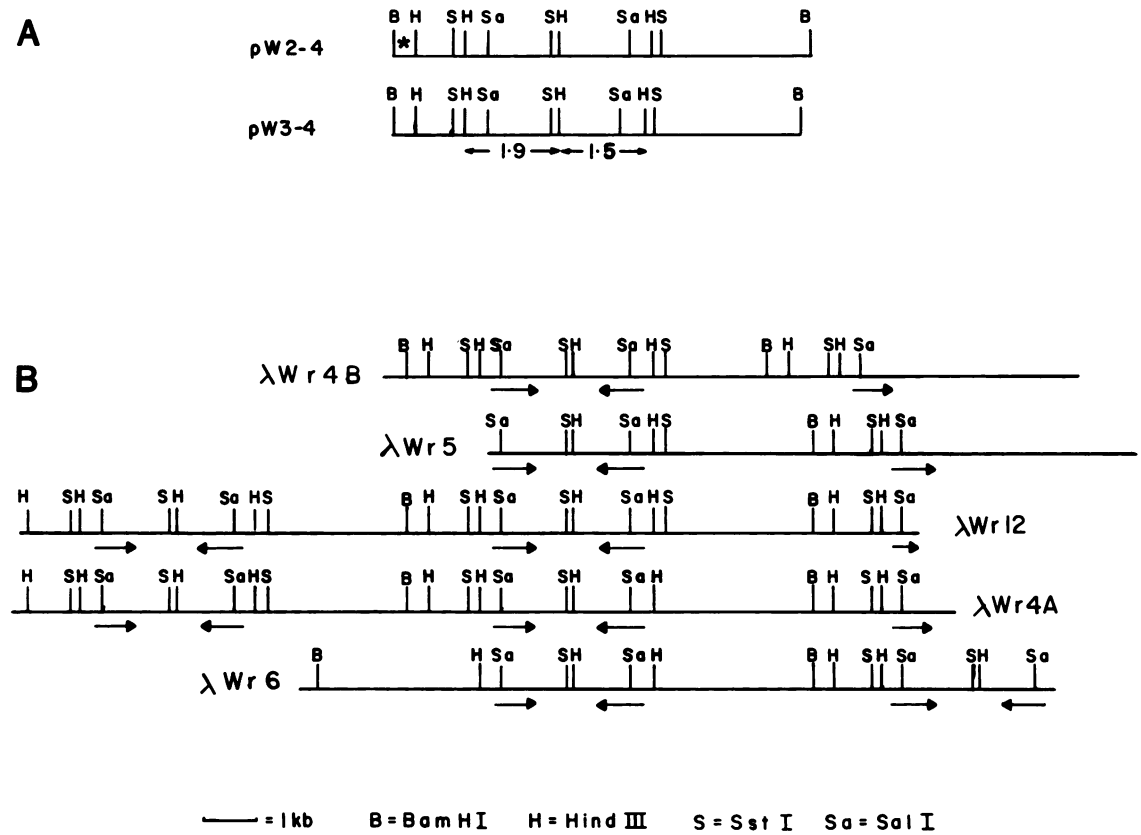

FIG. 3. (A) Restriction maps of subclones from the *Bam*HI Charon 30 library which contained AFP genes. The 0.4-kbp *Bam*HI-*Hin*dIII fragment of p2-4 marked by an asterisk did not contain sequences from the 60-kbp sequence illustrated in Fig. 2 and was used to probe for major-component genes. The 1.9- and 1.5-kbp *Hin*dIII fragments and the *Sst*I-*Sal*I fragment spanning the central *Hin*dIII site were further subcloned from pW3-4 for sequencing (see Fig. 4). (B) Restriction maps of genomic clones containing major-component genes from the partial *Sau*3A Charon 35 library. Arrows beneath the clones show the positions and directions of transcription of AFP genes. See text for discussion.

minor component. *Bam*HI sites were relatively rare in the clones, and three *Bam*HI fragments contained eight of the nine genes in the 60-kbp stretch; they fell at 13.5 kbp (three genes), 14.5 kbp (one gene), and 19.5 kbp (four genes), all well above the major-component band at 8 kbp. The ninth gene in the stretch lay within an *Sst*I fragment of 3.3 kbp which appeared to be present as a single copy. Thus, major-component genes were not represented in this initial library screen and appeared to be refractory to cloning.

Cloning the major-component genes. To improve the chances of cloning a major-component gene, a second library was constructed from a total *Bam*HI digest of wolffish genomic DNA by using fragments from 7 to 10 kbp in length as inserts. Charon 30 was chosen as the vector, as it can accommodate slightly smaller inserts than Charon 35. The library was probed with cloned AFP cDNA, and two plaques showed very strong signals. Both clones were found to contain two *Bam*HI fragments of approximately 8 kbp each, only one of which hybridized to AFP cDNA. The hybridizing *Bam*HI fragments were subcloned into pUC19 for restriction analysis (Fig. 3A). They had very similar internal restriction patterns, each containing two AFP genes located in *Sst*I and *Hin*dIII fragments which corresponded to intensely hybridizing bands in Fig. 1. They thus appeared to be iteration units of a repeated sequence which contained the major-component AFP genes.

Genomic organization of the major component. To isolate a hybridization probe that might specifically identify genomic clones containing major-component repeat units, we screened clone pW2-4 for sequences not present in the minor component. Charon 35 clones spanning the 60-kbp stretch in Fig. 2 were pooled, nick translated, and used to probe a blot of restriction digests of clone pW2-4. The *Bam*HI-*Hin*dIII 0.4-kbp fragment marked with an asterisk in Fig. 3A failed to hybridize to the minor-component sequences; it was subcloned and used to screen the initial Charon 35 library for major-component genes. Six plaques were found to hybridize strongly to both the *Bam*HI-*Hin*dIII 0.4-kbp probe and to AFP cDNA. Five of these clones were characterized further, and their restriction maps are shown in Fig. 3B. The clones contained direct tandem repeats of the basic pattern seen in the subclones, with minor length and restriction site polymorphisms. Two clones (λWr4B and λWr5) ended in sequences not present in the 8-kbp repeats, as judged by the absence of specific restriction sites. Whether these sequences define the ends of tandem repeat stretches or are aberrant spacer regions between repeats is not known. One clone, λWr6, contained a variant symmetric restriction pattern which appeared to be the result of a recombination event between the "left gene" of one repeat and the "right gene" of another to generate an inverted repeat containing two "right" genes. The combination of direct and inverted repeats in these clones might explain why major-component genes are underrepresented in library screens relative to their copy number in the genome (23, 25).

To investigate further the extent of the tandem repeat

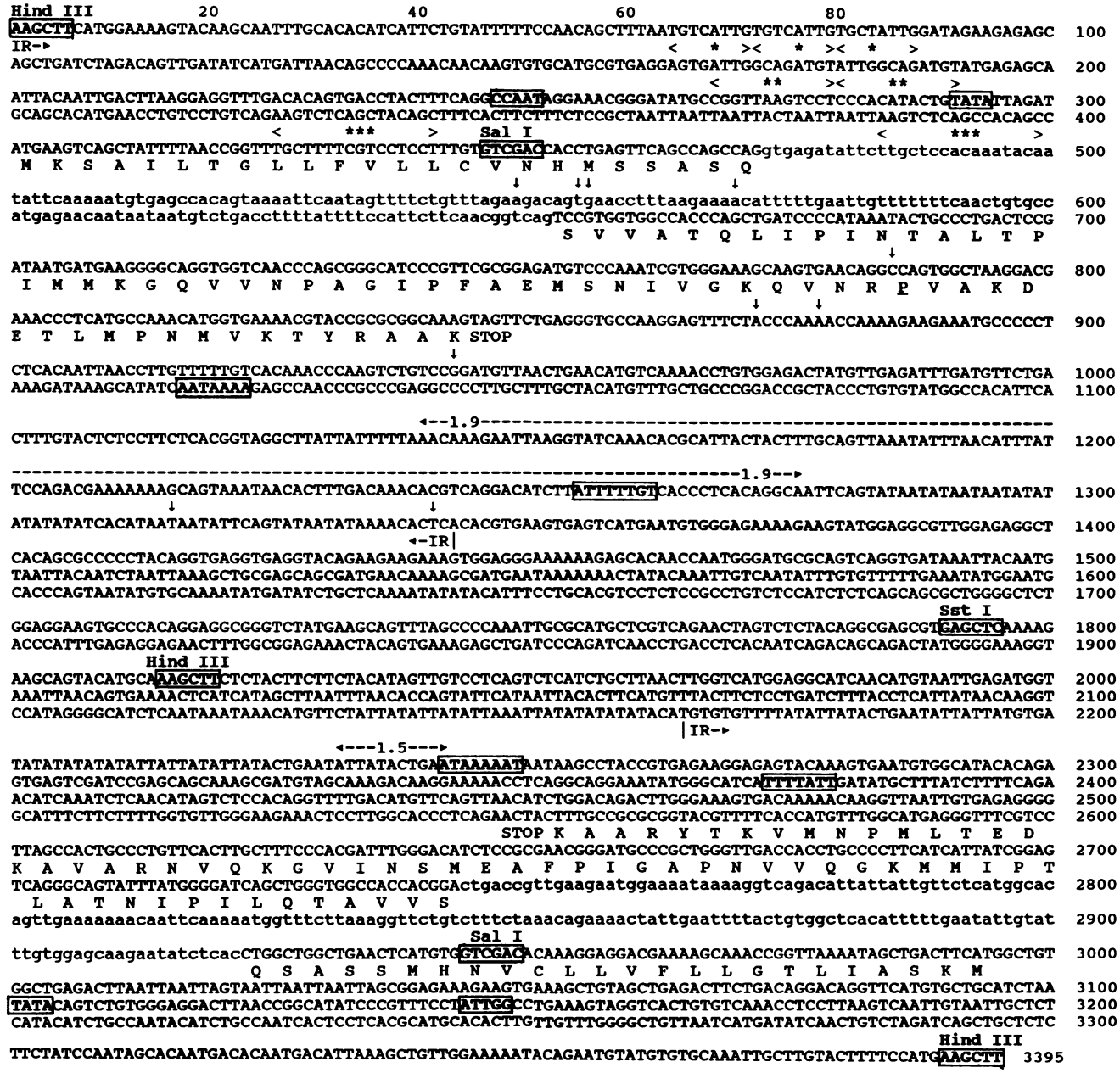

FIG. 4. Continuous sequence of the 1.9- and 1.5-kbp *Hin*dIII fragments noted in Fig. 3A. The sequence given is that of the coding strand for the 1.9-kbp gene (and hence the noncoding strand of the 1.5-kbp gene). Restriction sites are boxed and identified above the sequence. The inverted repeat (IR) sequences extend to (and presumably beyond) the outer *Hin*dIII sites; the internal limits at positions 1342 and 2164 are marked below the sequence by ←IR| and |IR→. The amino acid sequences of the genes are indicated below the DNA sequences; in both genes the intron is given in lowercase letters. CAAT and TATA boxes, the polyadenylation signal sequences (AATAAAA), and the putative termination sequences (ATTTTTNT, see text) are enclosed in boxes. Base pair differences between the 1.9- and 1.5-kbp gene IR sequences are noted by ↓ above the 1.9-kbp sequence. The single difference in the coding regions of the genes is at position 785, resulting in a proline (CCA) in the 1.9-kbp gene and an alanine (GCA) in the 1.5-kbp gene. The 137-bp sequence unique to the 1.9-kbp gene (1140 to 1276) and the 11 bp of seemingly unrelated sequence (2242 to 2232) in the 1.5-kbp gene are indicated by dashed lines above the sequence. The difference results in nonhomologous "termination" sequences in the two genes. Repeated oligonucleotide sequences in the 5′ noncoding region of the genes are noted beneath the 1.9-kbp sequence: <*>, three 8-bp tandem sequences, the first two of which are identical, while the last shares six of eight bases; <**>, 12-bp tandem repeat; <***>, sequences sharing 15 of 16 bases.

clusters, genomic DNA was digested with *Bam*HI and enzymes known not to cut within the repeat unit (Fig. 5A), blotted, and probed with type III AFP cDNA. The resulting autoradiograph is shown in Fig. 5B. After digestion with *Pst*I or *Eco*RI, the vast majority of AFP genes were found in fragments of 45 kbp or longer. *Kpn*I digestion produced hybridizing fragments of even greater size. These results suggest that the tandem repeats are clustered in groups of six or more.

AFP genes as pairs of inverted repeats. The disposition of restriction sites in the major-component subclones suggests that the two AFP genes are inverted relative to each other.

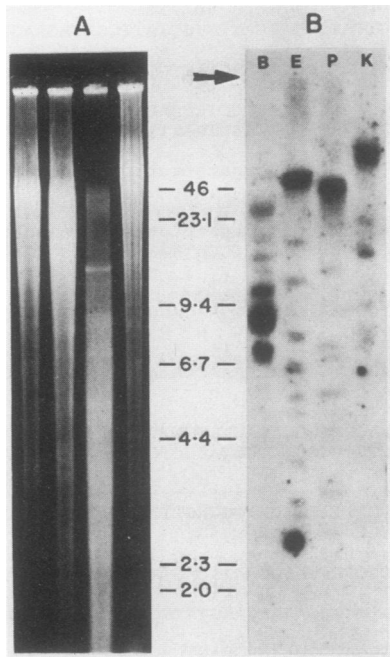

FIG. 5. Agarose gel (A) and Southern blot (B) analyses of wolffish genomic DNA digested with *Bam*HI as a reference and enzymes known not to cut within the repeat unit. B, *Bam*HI; E, *Eco*RI; P, *Pst*I; K, *Kpn*I.

To confirm this, the hybridizing 1.9-kbp and 1.5-kbp *Hin*dIII fragments and the *Sst*I-*Sal*I fragment that spans the central *Hin*dIII site were subcloned from pW3-4 and sequenced. As can be seen in Fig. 4, the two genes were in a tail-to-tail orientation, with 1.3 kbp separating their polyadenylation signal sites. With one major exception, their coding and flanking sequences showed 99.2% sequence identity over a stretch of 1.34 kbp from the outer *Hin*dIII sites inward. The anomaly was seen 3′ to the polyadenylation signals, where a 137-bp sequence in the 1.9-kbp gene was replaced in the 1.5-kbp gene by 11 bp of seemingly unrelated sequence. The wolffish genes were interrupted by a single intron so that the 5′ exon coded for the signal polypeptide and the first two amino acids of the mature AFP.

Estimating the number of wolffish AFP genes. The band marked with an arrowhead in the *Hin*dIII digest of Fig. 1 corresponds to the *Hin*dIII fragment from 28.0 to 30.5 kbp in Fig. 2; this 2.5-kbp fragment contained a single gene and was used to estimate the total number of wolffish AFP genes from a laser densitometry scan of the digests in Fig. 1. The *Hin*dIII digest gave an estimate of 82.7 genes, the *Bam*HI digest gave one of 83.3 genes (54 in the major component, 29 in the minor), and the *Sst*I digest gave one of 105.5 genes. As the estimate from the *Hin*dIII digest can be considered the most accurate because of the internal standard, it seems reasonable to conclude that there are 80 to 85 AFP gene copies in the wolffish genome, with approximately two-thirds of them residing in *Bam*HI fragments of 8 kbp.

DISCUSSION

The large number of wolffish and winter flounder AFP genes is surprising in view of the fact that most eucaryotic genes are present in a single copy. While we have no data on the minor-component genes, it appears likely that the genes of the major-component tandem repeats are functional, since the two which have been sequenced possess conventional CAAT and TATA boxes and splice junctions and code for type III AFP precursors (11). When the tandem repeat genes are considered as a pair, however, a further concern arises: because of the convergent nature of transcription and the short distance between the genes, it seems possible that run-on transcription from one gene might interfere with processing of the other. It is worth noting in this respect that 0.3 to 0.4 kbp downstream from the polyadenylation site, the genes contained the sequences ATTTTTGT (1.9-kbp gene) and ATTTTTAT (1.5-kbp gene), which match the consensus ATTTTTNT sequence, where N is any nucleotide, implicated in the termination of transcription in vaccinia viruses and other organisms (26).

The products of other amplified gene families are often required in massive amounts in a limited time span within the cell cycle (e.g., the histone genes) or ontogeny (e.g., the *Xenopus laevis* 5S RNA genes, *Drosophila melanogaster* chorion genes). By contrast, the time constraints for AFP production are not so severe, since AFPs are either expressed year-round or gradually accumulate in the serum over a period of months (10), and they are needed throughout development and adult life. They are, however, required in massive amounts (10 to 20 mg/ml of serum), and a direct correlation between AFP gene dosage and serum AFP levels has been observed (18). Thus, gene amplification rather than the enhancement of transcription from single-copy genes seems to have met the need for large amounts of AFP during the evolutionary history of the wolffish and winter flounder lineages.

It appears that different forms of amplification have contributed to the minor and major components of the wolffish AFP gene family. By the disposition of internal *Sal*I sites and flanking *Hin*dIII and *Sst*I sites, neighboring pairs of minor component genes in Fig. 2 appear to be variously found in head-to-head, head-to-tail, and tail-to-tail arrangements. The seemingly random orientation of nearest neighbors and the variable distance between genes suggests either extensive rearrangement of an initially simple pattern or a complex amplification event(s). A number of related models involving disproportionate DNA replication have been proposed to explain qualitatively similar results from other systems (reviewed in reference 16).

The major-component genes are found in direct tandem repeats, and two models have been proposed to explain the origin of such patterns. One model involves unequal crossing-over in meiosis; after an initial direct duplication of the amplification unit, mispairing of chromatids followed by recombination can generate a large array of tandem repeats, provided that certain criteria are met (24). In the second model (14), the tandem repeats are generated by a process analogous to that seen in the amplification of the 2μm circle in *Saccharomyces cerevisiae* (7). A circular DNA molecule containing a large inverted duplication is excised from the chromosome, and replication is initiated within the circle. Before replication is completed, a recombination event occurs between the replicated and unreplicated parts of the inverted duplication. The recombination event gives rise to a "double rolling circle," which then generates an expanded tandem array to be reincorporated into the chromosome. In this model, the inverted duplications are assumed to be large because of the requirement that recombination occur before the termination of replication in the excised circle. This feature accords well with results from mammalian tissue culture systems, in which amplicons of 1,000 kb are routinely seen (22), but makes the double-rolling-circle model an

unlikely candidate as an amplification method for the wolffish 8-kbp repeats. Nonetheless, in view of the inverted repeats within the tandem repeats, this model remains a formal possibility. The high degree of sequence identity between the two genes implies either that the amplification is a recent event or that a mechanism of sequence correction is operating.

We have previously commented on the remarkable diversity of macromolecular antifreezes in marine teleosts, which includes three different AFP types and one antifreeze glycoprotein (19). These structures are so diverse as to preclude their derivation from a common ancestral sequence. Given these separate origins, it is remarkable that the size and organization of the AFP genes coding for the type I AFP in winter flounder (20) and the type III AFP in the wolffish should be so similar. These fish belong to different orders and produce completely different AFPs, yet both have two-thirds of their AFP genes present in 8-kbp tandem direct repeats, with the remainder linked but irregularly spaced.

We have argued in the past, on the basis of the limited phylogenetic spread of AFP genes, that they arose in response to the Cenozoic glaciations (19), and we believe that the independent amplification of AFP genes in the Zoarcoidei (type III) and the Pleuronectidae (type I) is a further indication of this, reflecting a common reaction to the challenge posed by the sudden appearance of ice in seawater. Seawater cooled at the poles during the early Cenozoic to below the freezing point of teleost sera, and shallow-water and epipelagic fish must have died en masse with the appearance of sea ice. Subsequently, fish whose serum freezing points were below that of seawater colonized these waters, and the initial immigrants must have experienced a tremendous selective advantage from the lack of competition in the depleted faunal landscape (8). Evidently, amplification of genes coding for proteins with antifreeze activity allowed the progenitors of species in the Zoarcoidei and Pleuronectidae to rapidly achieve the requisite degree of freezing-point depression.

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