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Characterization of MHC class I and β_2-microglobulin sequences in Atlantic cod reveals an unusually high number of expressed class I genes

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Abstract Degenerate polymerase chain reaction (PCR) primers based on conserved residues from alignments of species with already characterized major histocompatibility complex (MHC)-encoded sequences were used in the search for class I and β_2-microglobulin (b_2m) genes in Atlantic cod (*Gadus morhua* L.). After PCR amplification and subsequent sequencing a putative class I sequence was identified, from which a probe was designed and used to screen a spleen cDNA library from one single individual. The full-length clone obtained was sequenced and shown to be a classical *Mhc* class I-encoded sequence. It revealed the characteristic α1-, α2-, and α3-domains and transmembrane and cytoplasmic region, with several conserved amino acids. A PCR amplification from the α2-domain to the CY-region was performed on the same library, using a proofreading enzyme. At least 11 unique additional sequences were isolated. Moreover, sequencing of the additional cDNA clones resulted in a total of 17 different *Mhc* class I sequences in this individual. A Southern hybridization of DNA from four different individuals using an α3-specific probe confirmed this large number of genes. Interestingly, based on differences mainly in their transmembrane region, the sequences obtained could be divided into two distinct groups. Within the groups no support could be obtained for any further subdivision. Southern experiments using an α1-specific probe gave almost the same restriction fragment length polymorphism with a high number of hybridizing bands, suggesting a low divergence in this part of the gene. Sequencing of PCR clones obtained with a proofreading enzyme confirmed this at the nucleotide level. PCR amplification to isolate and characterize the b_2m gene resulted in a sequence which was used to screen a thymus cDNA library. Two different alleles were obtained and these showed the characteristic features of known teleostean β_2m sequences. A Southern hybridization with genomic DNA from four different individuals suggested the presence of one b_2m locus in Atlantic cod.

Key words *Mhc* class I · β_2-microglobulin · Atlantic cod

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Introduction

Atlantic cod is an important fish for the countries around the Atlantic ocean, the North sea, and the Baltic sea, and the fish industry is of importance to several of these countries (Kurlansky 1997). A further dimension has emerged with the suggestion by the International Council for Exploration of the Sea (ICES) that cod should be used as a model for predicting the effects of climatic change on the marine life of the North Atlantic (ICES 1991). However, cod stocks are threatened by excessive exploitation due to improved fishing techniques (Cook et al. 1997) as well as increased pollution of the sea. Pollution is known to affect the defence mechanisms against parasites in fish (Khan 1987a, b; Kiceniuk and Khan 1987). Cod fish farms, though being merely experimental today, might become a reality in the near future, and due to the high densities of fish in aquaculture, the risk of infectious diseases increases.

In aquaculture of other fish species, e.g., rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*), vaccination is a commonly used method for protection against pathogens. After immunization, an increase of the immunoglobulin (Ig) concentration has been noted (Wilson and Warr 1992) and an immuno-

logical memory has been shown in rainbow trout resulting in an enhanced secondary antibody response, but without affinity maturation, as seen in mammalian secondary responses (Arkoosh and Kaattari 1991). In general, fish have various levels of natural antibodies against commonly used haptens like TNP (trinitrophenyl) or DNP (dinitrophenyl) and mammalian blood cells (Ingram 1980; Vilain et al. 1984). Cod also has natural antibodies which react with a multitude of different antigens, i.e., cod immunoglobulins are "sticky" (Magnadóttir et al. 1999; Pilström and Petersson 1991). Upon immunization, no increase of the already relatively high Ig levels could be observed, but cod show increased protection against the pathogen used (Bjørgan-Schrøder et al. 1992). The immune defence of cod may thus take advantage of other mechanisms, perhaps cellular in nature. Therefore it is important to study and understand more of molecules such as the major histocompatibility complex (MHC)-encoded cell surface proteins and T-cell receptors (TcR) in this species.

The mammalian *Mhc* includes both *Mhc* class I and class II genes separated by a cluster of class III genes that encode, e.g., complement factors. The role of the class II gene products, composed of the α- and β-chain, is to present processed extracellular antigens. The classical class I α-chain (Ia), noncovalently associated with β_2-microglobulin (β_2m), presents processed, intracellular-derived proteins. The classical class I genes are highly polymorphic and are codominantly expressed on all nucleated cells. The nonclassical MHC class I, or class Ib, also associates with β_2m and is coded for in the mammalian *Mhc* region. They show, in addition to sequence dissimilarity to the classical class I, comparatively low or no polymorphism and a tissue-specific expression, suggesting a function different from that of the classical ones (Klein and O'hUigin 1994).

Indirect evidence, such as allograft rejection and mixed leukocyte reactivity, shows that fish possess *Mhc* similar to those in warm-blooded vertebrates (Kaastrup et al. 1988, 1989; Stet and Egberts 1991). In 1990, partial genomic sequences of both *Mhc* class I and class II were described in the common carp (*Cyprinus carpio*) (Hashimoto et al. 1990). Further characterization of the fish *Mhc* has revealed numerous sequences from several species (Dixon et al. 1995; Stet et al. 1996). Evolutionary relationships and differences in the pattern of expression suggest the existence of both classical and nonclassical *Mhc* class I loci in fish (Shum et al. 1999; van Erp et al. 1996).

The cod immune system deviates from the teleost norm with respect to its functionality. This led us to characterize the genes encoding MHC class I molecules in this species.

Materials and methods

Animals and collection of tissue

Atlantic cod (*Gadus morhua* L.) were caught by trawling and netting in the Gullmaren fjord on the Swedish west coast. Organs were immediately removed from anesthetized fish, frozen in liquid nitrogen, and stored at –70 °C for later use.

Genomic DNA (from blood samples) was prepared according to Sambrook and co-workers (1989).

Cod cDNA libraries

An amplified cod spleen cDNA library from one individual, constructed in λgt11 (Bengtén et al. 1991) was used as template in all polymerasechain reactions (PCRs) of and screening for *Mhc* class I.

In addition, a new cDNA library was made from a single individual. Total cod thymus RNA and poly(A)-selected mRNA were prepared using the QuickPrep Total RNA Extraction Kit (Pharmacia Biotech, Uppsala, Sweden) followed by the QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech, Uppsala, Sweden). Cod thymus cDNA was prepared from the poly(A)-selected mRNA using the cDNA Synthesis Kit (Pharmacia Biotech) and ligated into λgt10. A total of 3.5×10^6 PFU/ml was obtained and one-fifth of the library was amplified to a final of 2.6×10^{10} PFU/ml. This library was only used in PCR amplification of and screening for β_2m.

Polymerase chain reaction

A number of PCRs were performed and in Fig. 1 the primers used for each reaction are shown. The concentrations of primers, $MgCl_2$ alternatively $MgSO_4$, and nucleotides were optimized for each amplification. Two different thermostable enzymes were used [*Taq* (MBI Fermentas, Vilnius, Lithuania) and *Pfu* (Stratagene, Calif.) polymerase] in the buffer conditions recommended by the manufacturers. The reactions started with denaturation at 94 °C for 5 min, followed by 30 PCR cycles, and ended with 10 min of extension at 72 °C. Each cycle profile began with 94 °C for 1 min, followed by annealing for 1 min, and extension at 72 °C for 2 min + 3 s/cycle. The different annealing temperatures used are shown in Fig. 1.

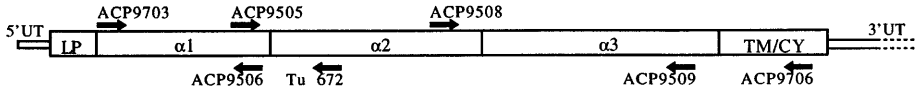
The PCR fragments were either gel-filtered on a Sephadex S-400 column (Pharmacia) or gel-purified on an ethidium bromide-supplemented 1% agarose gel and recovered on glass wool (Heery et al. 1990). After ligation into pMOS Blue T-vectors (Amersham, Buckinghamshire, UK) or suitably digested pUC19 (Pharmacia), the positive clones were picked and sequenced.

Screening of cDNA libraries

The PCR products from reactions 3 and 6 (see Fig. 1) were purified (Wizard PCR Preps, DNA Purification System, Promega, Wis.) and labeled with ^{32}P by the random primer method (Megaprime DNA labeling system, Amersham).

A total of 3×10^5 PFU of the spleen cDNA library were transferred to nitrocellulose filters and probed with an α-chain probe. The same amount of the thymus cDNA library was probed with a β_2m probe. Hybridization was done at 65 °C overnight in a buffer [modified from Church and Gilbert (1984)] containing 0.5 M NaH_2PO_4/Na_2HPO_4 (pH 7.2), 1 mM ethylenediaminetetraacetate, and 7% (w/v) sodium dodecyl sulphate (SDS). The filters were washed at high stringency in 0.1 × standard sodium citrate (SSC) and 0.1% SDS at 65 °C for 60 min. Positive clones were identified and after further screening, under the same conditions, single positives were isolated. After amplification in bacteria (*Escherichia coli* Y1089 or C600), clones were excised from the phage arms, ligated into pUC19 (Pharmacia), and sequenced.

no.	Primer	Primer sequence	Position, aa	°C	Enzyme	Comments
1	Tu 672 λgt11 Fw λgt11 Rev	GC DAT RAA RTC CTY KCC RTC RTA GGTGGCGACGACTCCTGGAGCCCG TTGACACCAGACCAACTGGTAATG	125-118	55	*Taq*	Amplification of LP, α1 and partly α2
2	ACP9505 λgt11 Fw λgt11 Rev	AG CGC TTT AAC CAG ACA GGA GG (see above) (see above)	83-91	55	*Taq*	Amplification of α2, α3, TM/CY and 3'UT
3	ACP9505 ACP9509	(see above) T CAG CTG AC CAC ACA CTC GTA CCT CC	(see above) 264-255	60	*Taq*	Designing probe, α2 and partly α3
4	ACP9508 ACP9706	AC YAM ACC RWG GWK TGT GTT G A GGA GCC GGA ATT CTG CCC CTC	159-166 338-332	50	*Pfu*	From α2 via α3 to TM/CY
5	ACP9703 ACP9506	C TCT CTG CAT TAC TTC TAC AC CC TCC TGT CTG GTT AAA GCG CT	3-10 91-83	50	*Pfu*	Amplification of α1



6	B2M-B λgt10 Fw λgt10 Rev	CTC BCC GTT CTT CAG CAG TTGAGCAAGTTCAGCCTGGTTAAG GGCTTATGAGTATTTCTTCCAGGG	44-39	52	*Taq*	Amplification of 5' part of β2-microglobulin
7	B2M-B λgt10 Fw	(see above) (see above)	(see above)	55	*Taq*	Designing probe, 5' part of trunc. clone

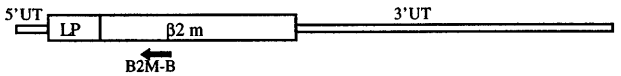


Fig. 1 The different PCR reactions used to obtain *Mhc* class I α and β_2m in Atlantic cod. Position refers to those in Fig. 2 and °C to the annealing temperature

Sequencing

All sequencing was done by the dideoxy nucleotide triphosphate chain termination method (Sanger et al. 1977).

Nucleotide sequences were analyzed with the computer package DNAStar (DNASTAR Inc., Wis.), whereas alignments and trees were constructed using CLUSTAL W Multiple Sequence Alignment Program ver. 1.7 (Thompson et al. 1994).

Southern blot

Products from PCR 4, 5, and 7 (see Fig. 1) were excised from an ethidium bromide-supplemented 1% agarose gel, recovered (see above), and labeled with ^{32}P using the random primer method (see above).

Genomic DNA from four individuals was digested with *Ssp* I, separated on a 0.8% agarose gel (7 μg DNA/lane) and transferred to a Hybond N^+ nylon filter (Amersham) using 0.4 M NaOH. The filter was hybridized with the different probes in a buffer modified from Church-Gilbert (see above) at 65 °C overnight. The filter was then washed at 65 °C in 0.1 × SSC and 0.1% SDS for 30 min, and exposed to X-ray film at –70 °C for 5 h.

Results

Polymerase chain reaction and screening of the cDNA library

A fragment of approximately 450 base pairs (bp) was obtained from a cod spleen cDNA library from one individual, using anchored PCR with a reverse degenerate primer in exon 3 (Tu 672) (Takeuchi et al. 1995) together with a λgt11 vector-specific primer (see Fig. 1, reaction no 1). Subsequent cloning and sequencing suggested it to be a partial *Mhc* class I cDNA, containing part of the α2-domain, α1-domain, leader peptide and 5' untranslated (UT). Based on this sequence, a specific forward primer could be designed (ACP9505) to amplify the 3' part of the gene (see Fig. 1, reaction no 2). From this sequence, consisting of the α2-domain, α3-domain, transmembrane (TM) region, cytoplasmic tail (CY) and 3'UT, a specific reverse primer was designed (ACP9509) and together with ACP9505 a fragment of 541 bp was obtained (reaction no. 3). This fragment was used as a probe for screening the cDNA library and several positive clones were obtained (n = 10), of which the longest one (no c28; accession number AJ132511) was sequenced. The cDNA sequence contained 1104 bp of coding region flanked by 21 bp of 5'UT region and 562 bp 3'UT region, truncated 5' of the poly(A) signal. The deduced amino acid sequence appears to be a complete *Mhc* class I cDNA. The coding region could be divided into the traditional domain classification (α1, α2, α3, TM, and CY). The sequence shares most of the conserved features of other vertebrate MHC class I sequences (Fig. 2). Several amino acid (aa) residues inferred to interact with antigen peptides [Y7, Y59, Y159, and Y171 for the N-termini of the peptides and R84, Y98, T142, K145, and W146 for the C-termini of the peptides (Saper et al. 1991)] are conserved, except that the tyrosine at position 84 is replaced by an arginine. The important cysteine residues at position C100 and C164 forming a disulphide bridge in the α2-domain and at position C200 and C259 in the Ig-domain (α3) are present as well as the well-conserved W216. Other conserved motifs for the Ig-domain, e.g., YxCxVxH (HLA aa pos. 257–263), are also conserved. Cod has, however, an L263 instead of histid-

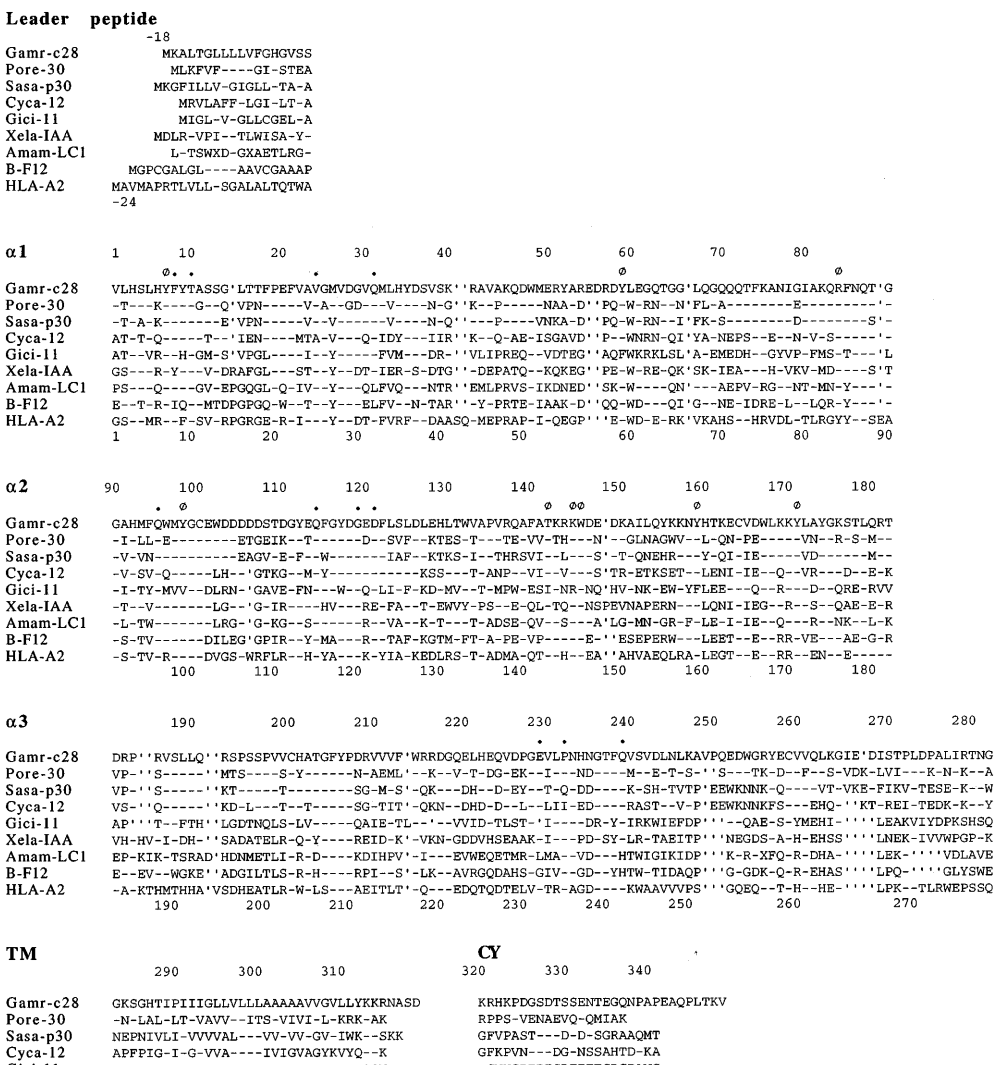


Fig. 2 Amino acid sequence of the Atlantic cod full-length cDNA clone (c28) aligned with *Mhc* class I sequences from other species. Pore-30 denotes *Poecilia reticulata*, guppy; Sasa-p30, *Salmo salar,* Atlantic salmon; Cyca-12, *Cyprinus carpio*, common carp; Gici-11, *Ginglymostoma cirratum*, nurse shark; Xela-IAA, *Xenopus laevis*, African claw frog; Amam-LC1, *Amieva amieva*, Amieva lizard; B-F12, *Gallus gallus*, chicken and HLA-A2 from human. *Hyphens* (–) indicate amino acid identity to the cod sequence and *apostrophes* (′) are introduced to maximize the sequence alignment. Important residues for binding peptide side-chains are indicated by (ø) and contact sites between the α-chain and β_2m are indicated by (•). *Numbering above* the alignment is based on the cod sequence and *numbering below* is based on HLA. The EMBL/GenBank accession numbers for each sequence are: guppy (Z54085), Atlantic salmon (L07606), carp (X91015), nurse shark (AF028557), frog (L20733), lizard (M81094), chicken (M31012), and human (K02883)

ine. Pairs of residues forming salt-bridges are conserved (H3/D28 and R41/E61 within α1, H92/D118 and K143/D147 within α2, and D217/R256 within α3) (Saper et al. 1991) and as is the potential N-linked glycosylation site at N86. The important contact sites for class I and β_2m are conserved in all three domains, as well as a salt-bridge between the two chains (αE229/βK6) (Saper et al. 1991). For the contact with the T-cell co-receptor (the CD8-binding site), it has been shown that the negatively charged loop consisting of residues 220–229 in HLA is a central site (Bjorkman and Parham 1990). The cod sequence has four amino acids with acidic side chains, and only one with a basic side chain within a stretch of 10 aa at position 217–226.

These observations, combined with the absence of any obvious defects in the nucleotide sequence, indicate that this cDNA clone is likely derived from an mRNA transcribed from a functional *Mhc* class I gene.

The deduced amino acid sequence of clone c28 was compared with MHC class I sequences from other species in a distance tree (Fig. 4A). Cod clusters with known teleostean classical MHC class I molecules, supported by a high bootstrap value, outside the other vertebrate groups. Within the teleostean group, cod clusters together with pufferfish and guppy.

Mhc class I loci in cod

Part of the α2- and α3-domain and part of the TM/CY region were PCR-amplified from the spleen cDNA library, using specific primers (ACP9508 and ACP9706), designed from the full-length clone (see Fig. 1, reaction no. 4). The PCR product obtained by the use of *Pfu* polymerase was cloned and sequenced. This analysis revealed 11 unique sequences out of 14 clones. To confirm the presence of multiple *Mhc* class I sequences in a

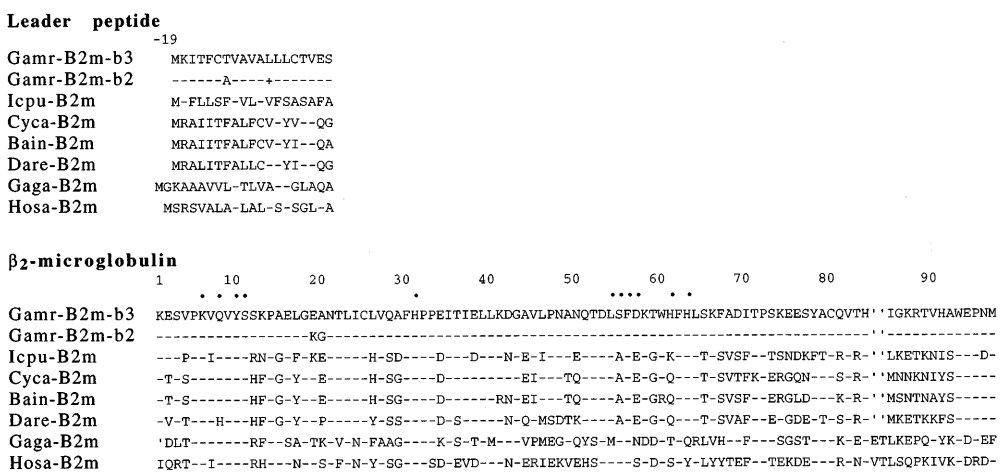


Fig. 3 The two Atlantic cod (*Gadus morhua*) β_2m amino acid sequences aligned with β_2m from other species: Icpu, channel catfish; Cyca, common carp; Bain, *Barbus intermedius,* large African barb; Dare, zebrafish; Gaga, chicken; Hosa, *Homo sapiens*, human. Hyphens (–) indicate identity with the cod sequence Gamr-B2m-b3 and *apostrophes* (′) are gaps introduced for maximizing the alignment. A synonymous nucleotide substitution within the two cod alleles in the leader peptide are indicated by a *plus* sign (+). Contact sites between the α-chain and β_2m are indicated by a *dot* (•). Numbering is based on the cod sequence. EMBL/GenBank accession numbers are for channel catfish: AF016041, carp: L05536, barb: Z54151, zebrafish: L05384, chicken: M84767, and human: M30683

single individual, genomic DNA from four individuals was digested with *Ssp* I and blotted. Hybridization with an α3-specific probe under high-stringency conditions revealed between 21 and 32 scorable fragments per individual (Fig. 5A), although more bands were observed with different intensity in the higher relative molecular mass region. Restriction fragment length polymorphisms (RFLP) were observed between the individuals analyzed.

The high number of hybridizing fragments in the Southern blot analysis prompted us to sequence the remaining nine clones from the screening of the cDNA library. Sequence analysis showed that these clones had all been truncated during the cDNA synthesis, mainly in the α2-domain, but revealed five additional unique sequences. Based on differences observed mainly in the TM region, the sequences could be divided into two groups (Fig. 6, group A and B). Four of these sequences were closely related to the full-length clone c28 (group A), whereas the other three showed similar differences from c28 and formed the other group (group B). Two of the cDNA clones were confirmed by the α2-TM/CY PCR (reaction no. 4) and the remaining nine PCR fragments all fitted into either group A or B (Fig. 6). Two of the PCR fragments (p37 and p55) showed high similarities to group B, mainly in the TM region, but in the α3-domain (aa pos. 206) they showed a characteristic residue found in group A sequences. cDNA clone c32 shows some deviations from the conservation of peptide binding residues shown by c28. These are Y98→D98, K145→R145, and W146→L146. A salt-bridge forming residue, D147, is also altered to E147, as all sequences in group B (Fig. 4). In addition, c20 shows R145 instead of K145, and c24, p37, p93, and p55 have changed the amino acid at position 171 (Fig. 6).

The relationship between the amino acid sequence (Gamr-c28 aa pos. 166–334) of both cDNA clones and PCR fragments is also shown in a neighbour-joining tree in Fig. 4B. The tree clearly shows two major clusters. Comparison of the 3'UT region of the cDNA clones also supported this pattern, (data not shown). The nucleotide sequence of clones c28, c39, and c41 (part of group A) are above 80% identical in this part, and the cDNA clones in group B (c32, c24, and c20) are also over 80% identical to each other, and 60–70% identical to group A. One of the 3'UT sequences (clone c33) was shown to contain a CA dinucleotide repeat and could not be assigned to either the A or B group sequences.

These data indicated the presence of a large number of class I sequences. However, this conclusion is mainly based on the number of hybridizing fragments in the Southern experiment, using an α3-specific probe. To further investigate the nature of this multitude of class I loci, a study of the characteristics of the α1-domain was performed using similar techniques, i.e., Southern hybridization and PCR analyses.

A probe was obtained by PCR (Fig. 1, reaction no. 5), which was used to probe the same filter, containing genomic DNA from four different individuals, as used in the previous hybridization experiment (Fig. 5A). The sequence of this probe was identical to the α1-encoding domain of clone c28. The results revealed a similar high number of fragments as seen in the α3 probing, albeit with a minor difference in the RFLP pattern (Fig. 5B). This observation suggested low divergence in this part of the class I sequences. To substantiate this, PCR fragments were cloned from the cDNA library (Fig. 1, reaction no 5) and sequenced. This analysis revealed the presence of at least nine

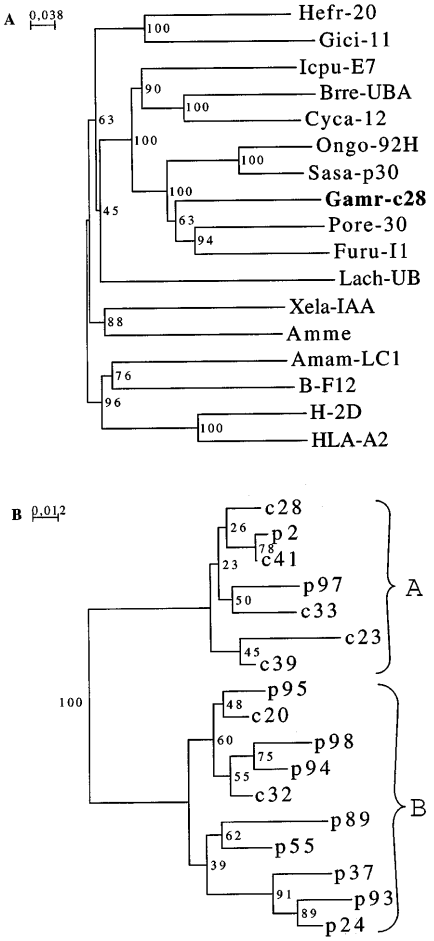


Fig. 4 **A** Neighbor-joining tree of MHC class I amino acid sequences in different species. Hefr-20 denotes *Heterodontus francisci*, horned shark; Gici-11, nurse shark; Icpu-E7, *Ictalurus punctatus*, channel catfish; Dare-UBA, *Danio rerio*, zebrafish; Cyca-12, common carp; Ongo-92H, *Oncorhynchus gorbuscha*, pink salmon; Sasa-p30, Atlantic salmon; Pore-30, guppy; Furu-I1, *Fugu rubrupes*, pufferfish; Lach-UB, coelacanth; Xela-IAA, frog; Amme, *Ambystoma mexicanum*, axolotl; Amam-LC1, Amieva lizard; B-F12, chicken; H-2D, *Mus musculus*, mouse and HLA-A2 from human. EMBL/GenBank accession numbers for those sequences not used in Fig. 2 are: horned shark (AF028559), channel catfish (AF053549), zebrafish (Z46777), pink salmon (D58386), pufferfish (AF001215), axolotl (U83137) and mouse (L36068). **B** Neighbor-joining tree based on amino acid sequences of the cDNA (c) and PCR (p) clones from one single Atlantic cod (see Fig. 6). Bootstrap values are shown in percentage from 1000 replications

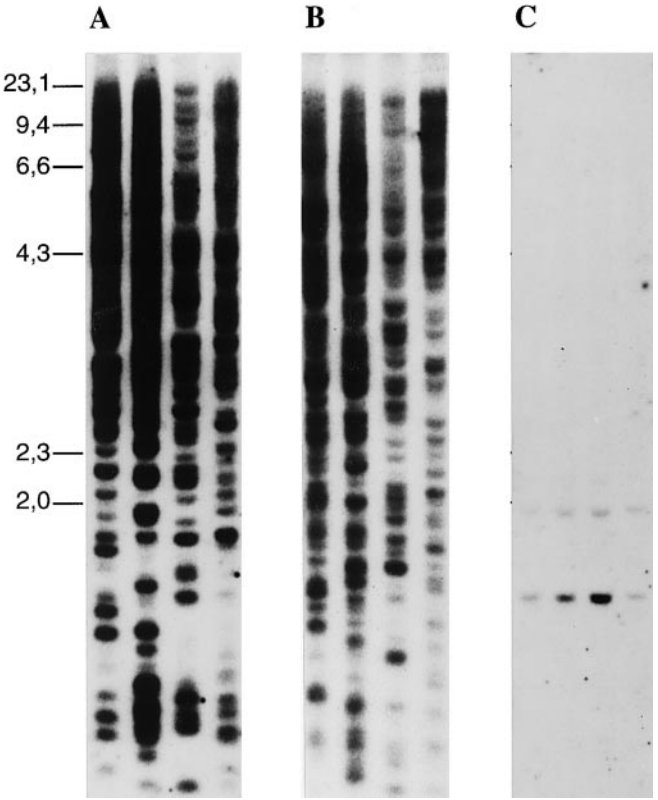


Fig. 5 A–C Southern blot of genomic DNA from four different Atlantic cod individuals. Erythrocyte DNA was digested with *Ssp* I, and the blot was hybridized with **A** an α3-specific, **B** an α1-specific, or **C** a β_2m-specific probe under high stringency conditions. The size of the DNA standard marker is indicated in kilobases (kb)

unique expressed sequences coding for the α1-domain in this individual. At the nucleotide level the unique sequences were between 80% to 99% identical (data not shown). However, the inferred amino acid sequences (Fig. 7) showed similarities between 57% and 99%, with the majority of the sequences around 70%.

β_2-microglobulin

A 200 bp fragment was obtained from a thymus cDNA library by anchored PCR with a degenerate reverse primer, B2M-B (Dixon et al. 1993) and λgt10 vector primer (Fig. 1, reaction no. 6). Cloning and subsequent sequence analysis indicated that this fragment was part of the coding sequence of cod *b_2m*. The fragment was used to screen the library, and of the 10 positive clones the longest five were sequenced. The obtained full-length cDNA clone (accession number AJ132752) contained 9 bp of 5'UT, 351 bp coding region, and 737 bp of 3'UT with a canonical polyA signal 418 bp downstream of the stop codon. The clone is not poly-adenylated, but a potential poly-adenylation site can be found 20 bp downstream of the poly(A) signal. The deduced amino acid sequence for the leader peptide shows that it is 19 aa long, the same length as in other fish β_2m sequences (Fig. 3). The mature protein, which is 97 aa long, showed that the important cysteines involved in the Ig-folding of the molecule are conserved at amino acid position C25 and C80, the YxCxVxH Ig-motif (aa pos. 78–84) and the contact residues with the α-chain are also conserved (Fig. 3).

In one of the five cod cDNA clones, K19 and G20 were exchanged with E19 and A20. The two types of sequences were found to be equally represented in the cDNA library, based on PCR analysis (Fig. 1, reaction no 6).

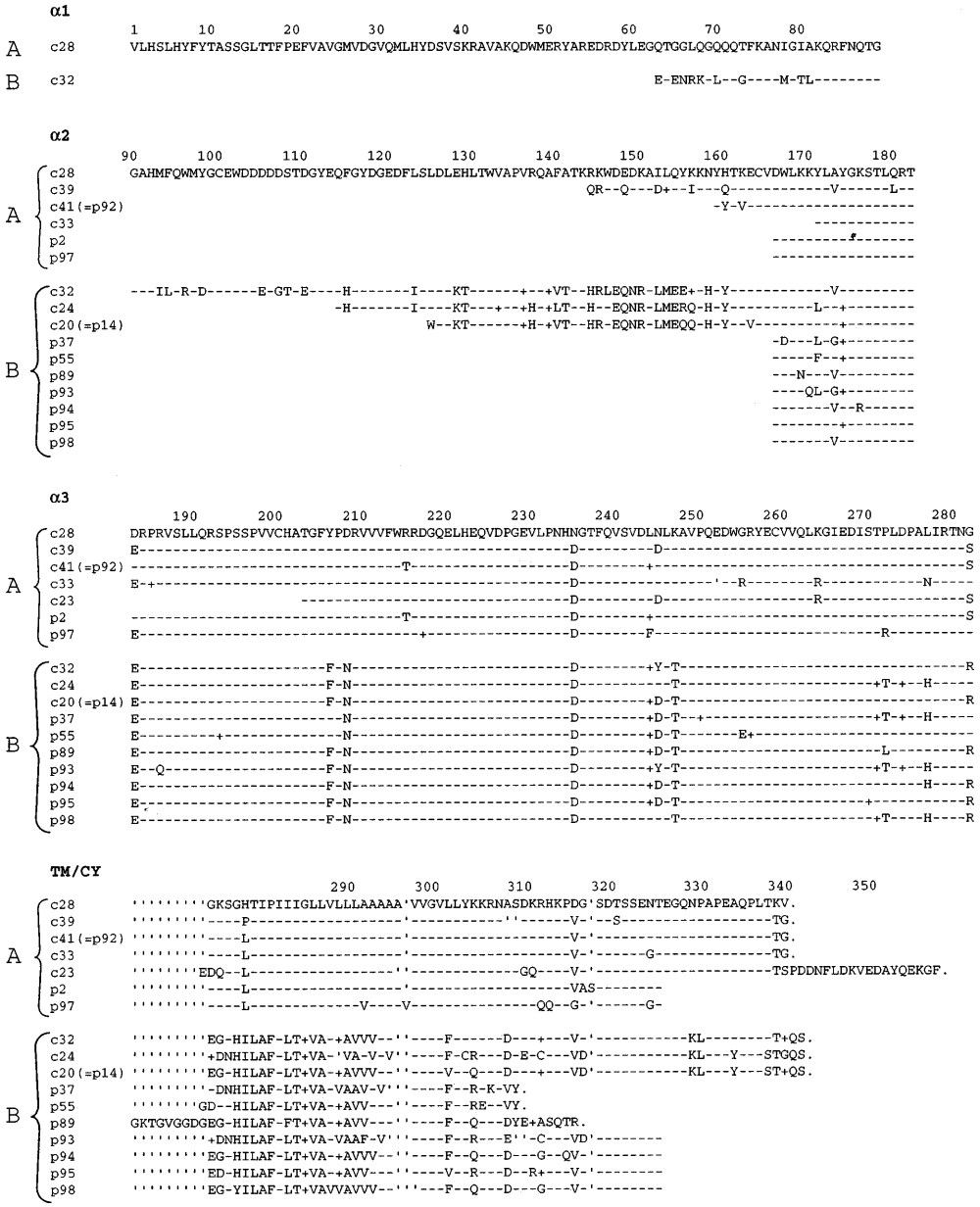


Fig. 6 Amino acid sequence of different cDNA clones (c) and PCR fragments (p) obtained from a single individual cod. *Hyphens* (–) depict identity to clone c28 and a *plus* sign (+) indicates synonymous nucleotide substitutions. Gaps introduced to maximize the alignment are indicated by an *apostrophe* (′). The clones have EMBL/GenBank accession numbers AJ132511-529

A distance tree of the β_2m amino acid sequence from Atlantic cod and from other species shows that cod β_2m is related to other fish *b*$_2$*m* genes (Fig. 8). The cod sequence falls into the teleost cluster and outside the other sequences used to construct the neighbour-joining tree, including the representative of the Chondrostei (Siberian sturgeon). Within the teleost cluster, cod, a species belonging to the Paracanthopterygii (Nelson 1994), falls outside both the Ostariophysi (common carp, zebrafish, large African barb, and channel catfish) and the Protacanthopterygii (rainbow trout).

One of the PCR fragments obtained consisted of a portion of the coding region (aa pos. 5–44) of cod *b*$_2$*m*, making it suitable for use as a probe. A Southern filter from four individual cod was probed under high-stringency conditions and showed two bands in all lanes and at the same position in each individual (Fig. 5C).

Discussion

Atlantic cod show no or low increase in antibody levels after immunization (Bjørgan-Schrøder et al. 1992; Espelid et al. 1991). This suggests that cod perhaps preferentially uses some other mechanisms for immune protection. Studying the genes of the T-cell receptor and the MHC might shed some light on this phenomenon. Here we report on the MHC class I genes and β_2m, and these findings may contribute to a better understanding of the immune responses in Atlantic cod.

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                  20        30        40        50        60        70        80
c28      ASSGLTTFPEFVAVGMVDGVQMLHYDSVSKRAVAKQDWMERYAREDRDYLEGQTGGLQGQQQTFKANIGIAK
pa1.9    --------------------------------------------------------------------------
pa1.27   G-----A-----V---------F-------------------------------RAL-T------------
pa1.12   ----------------------F-----------------QVTK------DRE--TA--H---------TL-
pa1.8    G---------------------F-----------------QVTK------DRE--TA--H---------TL-
pa1.13   G----S------S--------IDY---NTQ-V+L------LVTI--P----RN--KA--A------------
pa1.3    -----S---------------IDY---NTQ-++L-----KQAT-D-G----RN--IY--S---Y------L-
pa1.6    -----S---------------FYY---NTQ-++P------QVTSG------RE-RVS--S--A--VS-VNL-
pa1.2    G-----A-------------------------P--------T-DHPEV--RDN-N+--A---Y--GV--L-
pa1.4    ---+-EA-----------------F--F--KT-L------QLT-DHPS---RSNELSR-S--A--D--WTL-
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Fig. 7 Alignment of amino acid sequences of different α1 PCR clones, obtained from a single individual cod. *Hyphens* (–) indicate identity with the α1-domain sequence from the cod full-length clone c28. Synonymous nucleotide exchanges are indicated by a *plus* sign (+). The clones have EMBL/GenBank accession numbers AJ133441-450

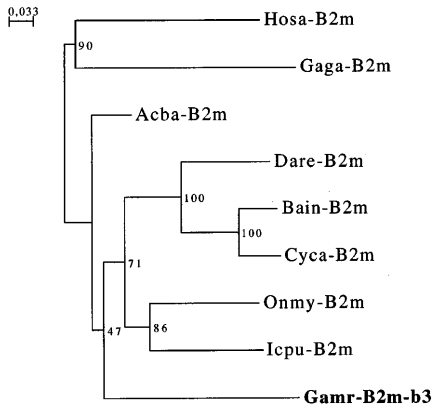

Fig. 8 Neighbor-joining tree of amino acid sequences from β_2m of different species. Designation for those sequences not used in Fig. 3: Onmy, *Oncorhynchus mykiss*, rainbow trout and Acba, *Acipenser baeri,* Siberian sturgeon. Bootstrap values are shown in percent from 1000 occasions. EMBL/GenBank accession number are for rainbow trout: L47354 and sturgeon: AJ132766

The full-length clone described in this study shows high similarities to *Mhc* class I genes already characterized in other species (Fig. 2). In contrast to the observed conservation of inferred important residues there are some striking substitutions, such as the N235 present in the full-length clone c28 where all other sequences have D235 (Figs. 2 and 6). There is also a consistent substitution of the W60 with L60 in all cod sequences obtained (Fig. 7). This W60 residue is even present in non-classical class I sequences (Stet et al. 1998). The implications of these substitutions are as yet unclear, as the importance of the conservation of these residues has not been identified (Kaufman et al. 1994).

In a phylogenetic tree, the cod full-length cDNA clone c28 clearly clusters with all of the other teleost class I sequences (Fig. 4A). Cod groups together with guppy and pufferfish, which is to be expected, since cod is considered a relatively recently diverged species. Cod belongs to the Paracanthopterygii and guppy and pufferfish to the Acanthopterygii, which are more related to each other than to the Ostariophysi (Nelson 1994). The common carp, zebrafish, and channel catfish belong to the latter superorder.

A high number of different cDNA sequences were obtained, which seems to point to the presence of many class I loci in cod. In other studies only a limited number of expressed class I sequences were found, e.g., in guppy, in a library comprising eight individuals, only three class I sequences were obtained (Sato et al. 1995). Screening of cichlid and pufferfish libraries produced a maximum of four different class I sequences (Sato et al. 1997; Timón et al. 1998). Thus, in combination with studies in carp (van Erp et al. 1996), Atlantic salmon (Grimholt et al. 1993), and nurse shark (Bartl et al. 1997), the norm seems to be that there are between one and four expressed classical class I sequences in the fish species studied. In contrast, Southern hybridizations, which have been performed as part of the studies, seem to point to a high number of classical class I loci present in the genome of some of these fish species (Sato et al. 1995, 1997; Timón et al. 1998). Estimates vary between 9 and 19 hybridizing fragments observed in Southern hybridizations, using either the full-length cDNA or an α3-specific probe. Similar observations have been obtained using a nonclassical carp class I probe (Stet et al. 1993). Although seemingly similar to the above-described hybridization patterns, the results for cod revealed a maximum of 32 legible hybridizing fragments. The difference between the present study and those in other fish species are the hybridization conditions. The high number of fragments reported in other fish experiments were, with a few exceptions, performed under low-stringency conditions. A prime example of the difference that can be observed between the outcome of high- and low-stringency conditions is given by studies in sharks (Bartl et al. 1997). The results presented for Atlantic cod were, however, obtained at high-stringency conditions, indicating the presence of multiple genomic sequences which seem to have a high degree of similarity. This is also supported by the uniformity of the intensity of the hybridizing bands (Fig. 5A). An over-estimation of the number of class I loci in cod may result if some of the loci are heterozygous. Another factor which could influence such estimates is the ploidy status of the fish under discussion. Atlantic cod with a DNA content of 1.8 pg per cell (L. Pilström, unpublished data), which is comparatively low, and with only

46 chromosomes (Nygren et al. 1974), is considered a diploid species.

A striking observation was the fact that Southern blot filters with DNA from four individual cod, now probed with an α1-specific sequence, revealed a similar pattern to that observed with the α3 probe (Fig. 5). This is in contrast to observations in carp, where an exon 3/4 probe produced at least five bands, whereas the exon 1/2 probe only produced one single hybridizing fragment (van Erp et al. 1996). This seems to suggest that in cod the divergence between the different α1-encoding sequences, belonging to different class I loci, is relatively low. To test this possibility, PCR analysis was performed of α1-encoding sequences in the cDNA library, which was prepared from a single individual cod. The sequences (n = 9) obtained were indeed very similar at the nucleotide level (80%–99%). This fact apparently resulted in the extensive cross-hybridization of the probe with the different α1 sequences present in the genome of the four individuals tested. Although the nucleotide sequences have not diverged to a large extent, the inferred amino acid sequences differed markedly (Fig. 7), with the lowest similarity at only 57% (pa1.4 compared to pa1.13). This set of sequences is probably not complete in this individual, because in the initial experiments 17 different α3-encoding cDNA sequences were obtained. The latter observation suggests the presence of at least 17 expressed class I genes, which is also underpinned by the estimates from the Southern hybridization experiments. Although some studies in other species suggest the presence of a large number of class I genes, this is not paralleled by a similar number of expressed genes (Sato et al. 1995, 1997; Timón et al. 1998).

The 17 sequences isolated could be divided into two groups, based mainly on differences in the transmembrane region. Invariably fish and axolotl classical class I sequences show little divergence in this region (Sammut et al. 1997; Takeuchi et al. 1995; Timón et al. 1998; van Erp et al. 1996). Moreover, in the shark (*Triakis scyllia*) two different classical class I loci have been identified, which also show little variability in the transmembrane region (Okamura et al. 1997). Thus, the observation of the differences in the cod cDNA sequences is unique in this sense, and resembles more the situation seen in human classical HLA-A and -B class I sequences (Parham et al. 1995). This suggests the presence of two paralogous class I loci in the Atlantic cod. Interestingly, these two loci are represented by multiple sequences as indicated in Fig. 6. Phylogenetic analysis of the sequences obtained supported, with a high bootstrap value, the division into group A and B type class I sequences (Fig. 4B). However, the low bootstrap values supporting a high number of nodes within the two main clusters prevented us from assigning loci designations. Thus, the only firm conclusion that can be drawn from this analysis is the presence of two groups, substantiating the fact that the class I genes in Atlantic cod have likely been derived from two paralogous loci. The class I sequences observed are most probably the result of duplications of these two loci. The phenomenon of expansion and contraction of class I genes is not an uncommon event in the evolution of the vertebrate MHC (Klein et al. 1993; Parham 1994). Although the sequences were found as cDNA, it is uncertain whether all are actually translated into functional class I heavy chains.

The high number of different class I genes expressed is a situation that resembles the expression pattern of nonclassical class I genes. The cod class I sequences described here, however, show all the structural features of a classical class I gene. Moreover, in the phylogenetic analysis the cod sequence clusters with the classical teleost class I sequences (Fig. 4A). Studies on the nature of classical and nonclassical class I sequences in the common carp have shown that these sequences form distinct clusters within a neighbor-joining tree (Stet et al. 1998). The newly characterized nonclassical sequence in rainbow trout shows a similar distinction from classical ones (Shum et al. 1999).

A functional MHC class I molecule on the cell surface needs to be noncovalently associated with β_2m (Rock et al. 1990). Thus, the isolation of the coding sequence for the cod β_2m would support the functionality of the MHC class I sequences described in this study. The PCR primer used to obtain the initial sequence has been shown to be highly successful in several fish species, such as Nile tilapia, common carp, and Siberian sturgeon (Dixon et al. 1993; Lundqvist et al. 1999). The mature protein in cod is 97 amino acids long, which is consistent with all of the other teleost β_2m sequences described. In contrast, the mammalian mature protein is 99 aa long (Güssow et al. 1987). All of the well-known conserved features, such as the presence of cysteines and contact residues with the class I heavy chain, are also present in the cod sequence. Although Criscitiello and co-workers (1998) described an N-linked glycosylation site at aa position 89–91 (NIS), this site is not present in any other teleost sequence, including the Atlantic cod. Similar to the topology of the phylogenetic tree based on the class I heavy chain sequences, the cod β_2m is found on a branch within the teleost cluster, but separate from those species which belong to other superorders.

The result from the Southern hybridization shows two hybridizing fragments which were found at the same position in the four individuals analyzed. This is in contrast to observations in zebrafish (Ono et al. 1993), and catfishes (Criscitiello et al. 1998), where only one single hybridizing fragment could be demonstrated. However, there are examples of studies in fish which have shown multiple hybridizing fragments using β_2m probes. In the case of the common carp two hybridizing bands were detected, which may reflect the tetraploid nature of this species (Dixon et al. 1993). The situation in rainbow trout is undoubtedly unique, as it contains multiple β_2m sequences, giving rise to extensive RFLP (Shum et al. 1996). The fragments observed in cod most

likely represent two different alleles at the same locus. There are two arguments to support this conclusion. First, two cDNA sequences were obtained, which showed minor variations in the coding region (aa pos. -13, -8, 19, and 20). Second, Atlantic cod has been demonstrated to be a diploid species (Nygren et al. 1974).

A noncovalent association of β_2m with the MHC class I α-chain has been demonstrated on the surface of cells from channel catfish (Antao et al. 1999). Atlantic cod seems to possess both the classical class I and the b_2m genes and therefore is likely to be able to express a functional class I molecule on the cell surface. However, the most striking observation is the unusually high number of expressed class I sequences. This has not been shown in any of the other teleost species studied. The classical nature of the class I sequences obtained makes it likely that they are constitutively expressed in all organs, including the thymus. Negative selection of T cells in the thymus, which has been demonstrated in mammals, is influenced by the number of class I sequences expressed (Parham 1994). If a similar mechanism of negative selection were to occur in the cod thymus as well, the abundance of expressed class I sequences might have a profound impact on the cod T cells. This could lead to a substantial deficit in the T-cell repertoire of the cod immune system. The likely penalty for expression of too many class I sequences must be compensated in cod. One possible compensatory mechanism may be the expression of natural antibodies which react with a multitude of different antigens, i.e., cod immunoglobulins are "sticky" (Magnadóttir et al. 1999; Pilström and Petersson 1991). Additionally, cod also has a high serum level of immunoglobulins ranging from 5 up to 44 mg/ml serum, depending on the size of the individuals (Magnadóttir et al. 1999), whereas other teleost species in general show a lower Ig concentration (Israelsson et al. 1991). The Ig level in cod does not increase as a response to immunization, and no secondary response with increased levels of specific antibodies has been demonstrated (Bjørgan-Schrøder et al. 1992; Espelid et al. 1991). The aberrant expression of the Ig molecules is neither due to problems at the gene level nor with the molecules themselves. Molecular studies have shown that both the heavy and the light chain locus are encoding *bona fide* immunoglobulins involved in humoral defence (Bengtén et al. 1991, 1994; Daggfeldt et al. 1993). This increased level of natural "sticky" antibodies may be the compensatory mechanism to ensure the survival of the species. In addition, it has been noted that Atlantic cod does have a good immune defence, since cod specifically vaccinated with killed pathogenic bacteria are more protected against the pathogen than unvaccinated cod (Bjørgan-Schrøder et al. 1992). Thus, there seem to be mechanisms in addition to the antibodies, which appear to be involved in the adequate immune protection of this interesting species.

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