THE IDENTIFICATION, CHARACTERIZATION, AND EXPRESSION ANALYSIS OF GENES RELEVANT TO IMMUNE RESPONSES IN ATLANTIC COD (Gadus morhua)

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The identification, characterization, and expression analysis of genes relevant to immune responses in Atlantic cod (*Gadus morhua*)

by

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ABSTRACT

The Atlantic cod (*Gadus morhua*) is an important species for global fisheries and aquaculture industries. A thorough knowledge of the genes and molecular pathways involved in Atlantic cod immune responses will likely lead to the development of new diagnostics, vaccines, and other methods of combating infectious diseases that threaten these industries. Using functional genomic approaches, this research investigated the innate immune response in immune tissues (head kidney and spleen) of Atlantic cod following treatment with bacterial antigens (i.e. formalin-killed, atypical *Aeromonas salmonicida*, referred to as ASAL) or a viral mimic (i.e. polyriboinosinic polyribocytidylic acid, referred to as pIC). This research led to the identification of 4154 expressed sequence tags (ESTs) that were generated from cDNA libraries enriched for transcripts dysregulated following stimulation with ASAL. From these transcripts, 10 genes with immune-relevant functional annotations were selected for quantitative reverse transcription – polymerase chain reaction (QPCR), and the full-length cDNA sequence of Atlantic cod IRF1 was obtained. This study, along with other concurrent studies, collectively identified the biological process of apoptotic regulation as one of the key mechanisms involved in Atlantic cod innate immune responses. Further studies led to the identification and characterization of anti-apoptotic Bcl-2 sub-family genes in Atlantic cod including orthologues of: NR-13, Mcl-1, and Bcl-X. For NR-13, Mcl-1, and Bcl-X1, the full-length cDNA, genomic sequence, and promoter regions were obtained and thoroughly analyzed. Although only partial cDNA and genomic sequences were obtained

for Bcl-X2, the presence of two Bcl-X-like genes in Atlantic cod was evident. In addition, the constitutive gene expression across tissues, and following treatments with ASAL and pIC, were also studied for these 4 genes.

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LIST OF FREQUENTLY USED ABBREVIATIONS

Abbreviations	Complete name
AA	amino acid
AMP	anti-microbial peptide
AP-1	activator protein 1
ARE	AU-rich element
ASAL	formalin-killed atypical *Aeromonas salmonicida*
Bcl-2	B cell lymphoma 2
Bcl-X	Bcl-2 related
BLAST	basic local alignment search tool
BH	Bcl-2 homology
bp	base pair
cDNA	complimentary DNA
CGP	Atlantic Cod Genomics and Broodstock Development Program
contig	contiguous sequence
CPE	cytoplasmic polyadenylation element
dsRNA	double stranded RNA
EST	expressed sequence tag
FTH	ferritin heavy subunit
GO	gene ontology
HK	hematopoietic kidney
HPI	hours post injection
IL	interleukin
i.p.	intraperitoneal
IRES	internal ribosome entry site
IRF	interferon regulatory factor
JAK	Janus kinase
LPS	lipopolysaccharide
Mcl-1/MCL1	myeloid leukemia sequence 1
NCBI	National Center for Biotechnology Information
NF-κB	nuclear factor kappa B
NR-13	neuroretina 13
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
pIC	polyriboinosinic polyribocytidylic acid
QPCR	quantitative reverse transcription - polymerase chain reaction
RACE	rapid amplification of cDNA ends
RQ	relative quantity
rRNA	ribosomal RNA
RTPCR	reverse transcription - polymerase chain reaction

S	spleen
SCYA	small inducible cytokine
SSH	suppression subtractive hybridization
STAT	signal transducers and activator of transcription
TM	transmembrane
UC	undisturbed control
UTR	untranslated region

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CO-AUTHORSHIP STATEMENT

The research described in this thesis was carried out by Charles Yu Feng, with guidance from Matthew L. Rise. Charles Yu Feng was responsible for data collection and analysis. Manuscripts resulting from this thesis were prepared by Charles Yu Feng, with editing assistance and intellectual input from co-authors as follows:

Authorship for publication arising from **Chapter 2** is **Charles Y. Feng, Stewart C. Johnson, Tiago S. Hori, Marlies Rise, Jennifer R. Hall, A. Kurt Gamperl, Sophie Hubert, Jennifer Kimball, Sharen Bowman, and Matthew L. Rise.** This manuscript has been published in *Physiological Genomics* 37: 149–163, 2009. PMID:19240301.

Additional notes regarding supplemental material

All Supplemental Figures supporting Feng et al. 2009 have been renumbered and incorporated into Chapter 2 of this thesis. Supplemental Tables supporting Feng et al. 2009 were not included in this thesis due to their large sizes. However, these Supplemental Tables are available at http://physiolgenomics.physiology.org/cgi/content/full/90373.2008/DC1.

Authorship for a publication arising from **Chapter 3** of this thesis is **Charles Y. Feng** and **Matthew L. Rise.** This manuscript has is in press in *Molecular Immunology* doi:10.1016/j.molimm.2009.10.011

CHAPTER 1

Introduction

*1.1 Bacterial and viral infections in Atlantic cod (*Gadus morhua*)*

The marine food fish industry is becoming increasingly dependent on aquaculture (22). Due to the decline of wild Atlantic cod populations, renewed interest in Atlantic cod aquaculture has been generated in countries like Canada, Norway, and the UK, where the cod fishery has traditionally been an important industry. Bacterial [e.g. (42)] and viral [e.g. (23)] outbreaks greatly threaten the health of cultured fish, causing heavy losses in the industry. In Atlantic cod, bacteria such as *Listonella* (*Vibrio*) *anguillarum* and *Aeromonas salmonicida*, are known causative agents for vibriosis and furunculosis, respectively (28, 29). In addition, a variety of viruses such as nodavirus, infectious pancreatic necrosis virus (IPNV), and viral haemorrhagic septicaemia virus (VHSV), also present great challenges to Atlantic cod aquaculture [reviewed in (29)]. For example, nodavirus infection has been identified in Atlantic cod populations along the coast of the UK and the east coast of North America (12, 34). Nodaviruses are the aetiological agents of viral nervous necrosis (VNN), which causes high mortalities in Atlantic cod, especially during larval and juvenile stages (12). To reduce the impact of infectious diseases on Atlantic cod aquaculture, the development of a comprehensive understanding of the Atlantic cod immune system is much needed. The study of genes and molecular pathways involved in the Atlantic cod immune responses will likely lead to the development of effective vaccines, molecular diagnostic tests, and targets for marker-assisted selection of disease-resistant breeding stocks.

1.2 Gene expression involved in innate immune responses of fish

Numerous studies have shown that the innate immune system is highly conserved throughout metazoan evolution [reviewed in (13) and (21)]. The innate immune system employs several families of pattern recognition receptors (PRR) that recognize and bind pathogen-associated molecular patterns (PAMPs) [e.g. (3)]. Lipopolysaccharides (LPS), peptidoglycan, flagellin, and unmethylated CpG motifs (bacterial DNA) are common PAMPs associated with Gram-negative bacteria, which can elicit innate immune responses through signalling pathways such as the Toll-like receptor (TLR) pathways [reviewed in (37)]. For example, the mammalian TLR4 is responsible for LPS recognition and downstream signalling (26), and its putative orthologue has been identified in zebrafish (19). When exposed to these PAMPs, fishes are capable of mounting innate immune responses that involve the altered expression of many genes encoding a variety of proteins such as: pro-inflammatory cytokines [e.g. interleukin-1b (IL1β)], complement components, chemotactic cytokines [e.g. interleukin-8 (IL8)], and anti-microbial peptides (e.g. hepcidin and cathelicidin), [e.g. (9, 10, 18, 30)]. Double stranded RNA (dsRNA) constitutes the genetic material of dsRNA viruses (e.g. members of Reoviridae and Birnaviridae families), a diverse group of viruses that infect a wide range of hosts from plants to mammals [reviewed in (16, 40)]. Single stranded RNA viruses [e.g. nodavirus (41)] are also known to produce dsRNA intermediates as part of their replication cycle. dsRNA or its mimics [e.g. polyriboinosinic polyribocytidylic acid (pIC)] are known to elicit a potent innate immune response in fishes [e.g. 20, 27)]. In addition, some transcripts that are responsive to viral mimic stimuli are also involved in

innate immune response to bacterial antigens [e.g. IL1β, and IL8, and IRF1 (8, 30)], which suggests that some common pathways/molecules are involved in innate immune responses to bacterial and viral pathogens. Prior to the launch of the Atlantic Cod Genomics and Broodstock Development Project (CGP) in 2006, only a small number of Atlantic cod immune-relevant genes had been identified and characterized [e.g. (35, 36, 39)], and no large scale functional genomic studies had been conducted in this species.

1.3 The roles of head kidney and spleen in fish

During an immune response, the change in gene expression varies among tissues depending on the specific roles they play. In fish, hematopoietic kidney (head kidney) serves as the major hematopoietic site. All types of mature leukocytes and their precursors (pluripotent hematopoietic stem cells) have been found in the head kidney, demonstrating its function as a primary lymphoid organ (24). In contrast to the mature B cells housed by posterior kidney, the head kidney of rainbow trout (*Oncorhynchus mykiss*) contains predominately B cell precursors (43). The spleen also houses mature B cells (43), and it is a secondary lymphoid organ that predominately functions as a filter for blood, removing aged erythrocytes and other apoptotic cells (32). In mammals, the splenic marginal zone serves as a site for the clearance of blood-borne pathogens as the blood passes through an open system of leukocytes (1, 14). Even though the splenic marginal zone is absent in teleosts (25), the periarterial macrophage sheaths (PAMS), also known as ellipsoids, are found in the red pulp of the spleen of teleosts and may trap blood-borne pathogens to allow phagocytosis by surrounding macrophages (33). The

study of gene expression involved in innate immune responses in fish spleen and head kidney can further reveal the different roles that these immune tissues play.

1.4 Using functional genomic tools to study Atlantic cod immune response

Suppression subtractive hybridization (SSH) cDNA (complimentary DNA, that is reverse transcribed from mRNA) libraries have been shown to be effective tools for the identification of genes that are differentially expressed during fish immune responses (27, 31, 38). One of the goals of the on-going Atlantic Cod Genomics and Broodstock Development Project (CGP) is to fully characterize the Atlantic cod immunome. CGP research has led to the identification of numerous Atlantic cod transcripts including many immune-relevant transcripts. By construction and sequencing of high complexity cDNA libraries (SSH and normalized cDNA libraries), the CGP has generated most of the Atlantic cod expressed sequence tags (ESTs, which represent portions of transcribed cDNA sequences) that are currently in public databases. Out of the 206,747 Atlantic cod ESTs that have been deposited into the NCBI EST database, 160,228 ESTs were generated by the CGP. In addition, an EST database has been created by the CGP to maintain access to these ESTs (available at www.codgene.ca). These ESTs generated by the CGP directly contributed to the development of a 20,000-gene (20K) oligonucleotide microarray that can be used as a platform for further functional genomic research in Atlantic cod.

The identification of ESTs provides information that can lead to the further characterization of immune-relevant genes. For example, full-length cDNA sequences

can be obtained using rapid amplification of cDNA ends (RACE), cloning, sequencing, sequence assembly, and annotation. Based on EST information, quantitative reverse transcription polymerase chain reaction (QPCR) assays can also be developed to study gene expression. Furthermore, based on the full-length cDNAs generated from RACE, using the genomic DNA as template, gene structure (i.e. introns and exons) can be determined by PCR-based approaches, and promoter regions can be obtained by genome walking PCRs followed by cloning, sequencing, and sequence analysis (e.g. to identify putative transcription factor binding sites). The identification and characterization of immune-relevant genes in Atlantic cod not only improve our understanding of cod immune responses, but also serve as an important step in identifying candidate biomarkers for marker-assisted selection for cod that are resistant to bacterial/viral infections. For example,,the presence of single nucleotide polymorphisms (SNPs) in immune-responsive genes (including introns, exons, and promoter regions) can lead to differences in their gene expression and/or activity of the encoding proteins, which may influence an individual's overall susceptibility to bacterial/viral infections. This research will also facilitate future investigations using a variety of techniques (e.g. microinjection-based targeted gene knockdown and gene overexpression) to reveal the specific functions of key immune-relevant cod genes and gene products.

1.5 Objectives of the research

The first objective of this research was to identify and analyze the genes involved in the innate immune responses of Atlantic cod following stimulation with a bacterial mimic

(i.e. formalin-killed atypical *Aeromonas salmonicida*, referred to as "ASAL" hereafter). In this research, construction of SSH libraries was employed to identify differentially expressed transcripts in the immune tissues (spleen and head kidney) of juvenile Atlantic cod, and the expression of selected transcripts was studied using QPCR. The annotation of these differentially expressed transcripts (i.e. ESTs) and biological process gene ontology analysis (i.e. functional categorization of cod putative gene products by the biological processes that are associated with orthologous proteins from other species) suggested that biological processes such as chemotaxis, regulation of apoptosis, antimicrobial peptide production, and iron homeostasis, are involved in the cod immune response to ASAL. Along with other concurrent research [e.g. (27)] within the CGP, this first research has led to the identification and analysis of ESTs that are involved in Atlantic cod immune responses.

The analysis of functional annotations associated with significant BLAST hits of previously generated Atlantic cod ESTs [mainly from (8, 27)] suggest that the apoptotic regulation may be involved in cod immune response to bacterial and viral stimuli. Therefore, the second objective of this M.Sc. research was to further characterize some of the key Atlantic cod genes that are involved in apoptotic regulation and to study their expression during immune response of Atlantic cod.

Members of the Bcl-2 family of genes and gene products are central regulators of apoptosis. They possess characteristic Bcl-2 homology (BH) domains, which account for their ability to dimerize and function as apoptotic regulators [reviewed in (4)]. The Bcl-2 family genes consists of three sub-families: the Bax-like pro-apoptotic sub-family, the

BH-3 only pro-apoptotic sub-family, and the Bcl-2 like anti-apoptotic sub-family [reviewed in (4)]. The pro-apoptotic Bcl-2 proteins [e.g. Bcl-2-associated X protein (Bax) and Bcl-2 antagonist/killer-1 (Bak)] are antagonized by their anti-apoptotic counterparts [i.e. the Bcl-2 like sub-family proteins, such as Bcl-2, Bcl-X_L, Mcl-1, and NR-13 (17)] which function by binding and sequestering the pro-apoptotic Bcl-2 proteins, thereby preventing mitochondrial membrane permeabilization (MMP) induced apoptosis [reviewed in (5)]. The NR-13 orthologue identified in zebrafish was shown to antagonize the pro-apoptotic Bax, and play a key role in development (2). Zebrafish orthologues of Mcl-1 and Bcl-X_L have also been identified (11). In addition to the anti-apoptotic functions of Mcl-1 and Bcl-X_L observed in zebrafish embryo (15), over-expression of either zebrafish Mcl-1 or Bcl-X_L protected beta-nodavirus-infected GL-av (a fish cell line) cells from necrotic cell death (6, 7).

Based on the immune-relevant ESTs that were generated from my first study and concurrent CGP studies [e.g. (27)], a variety of molecular biology techniques (e.g. RACE, genome walking) were employed to characterize the full-length cDNA, gene structure (e.g. intron-exon boundaries), and promoter regions (e.g. analysis of putative transcription factor binding sites) for Atlantic cod NR-13, Mcl-1, and Bcl-X putative orthologues. For these genes, the constitutive gene expression across six juvenile Atlantic cod tissues, and their expression following treatments with bacterial antigens (i.e. ASAL) or viral mimic (i.e. pIC) were studied using QPCR. This research presents the gene structure and promoter regions of teleost NR-13, Mcl-1, and Bcl-X for the first time.

In this research, the identification and analysis of immune-relevant genes in Atlantic cod improves our understanding of the genetic basis of Atlantic cod innate immune responses. A complete understanding of the molecular basis of cod innate immune responses will likely revolutionize disease diagnostics and prevention in both farmed and wild cod populations. In addition, this research will also be valuable to several research areas including comparative immunology and evolutionary biology.

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CHAPTER 2

Identification and analysis of differentially expressed genes in immune tissues of juvenile Atlantic cod (*Gadus morhua*) stimulated with formalin-killed, atypical *Aeromonas salmonicida*

2.1 Abstract

Physiological changes, elicited in animal immune tissues by exposure to pathogens, may be studied using functional genomics approaches. I created and characterized reciprocal suppression subtractive hybridization (SSH) cDNA libraries to identify differentially expressed genes in spleen and head kidney tissues of Atlantic cod challenged with intraperitoneal injections of formalin-killed atypical *Aeromonas salmonicida*. Out of a total of 4154 expressed sequence tags (ESTs) generated from 4 cDNA libraries, 10 genes with immune-relevant functional annotations were selected for quantitative reverse transcription – polymerase chain reaction (QPCR) studies using individual fish RNAs as templates to assess biological variability. Genes confirmed by QPCR as up-regulated by *A. salmonicida* included interleukin 1β (IL1β), interleukin 8 (IL8), a small inducible cytokine (SCYA), interferon regulatory factor 1 (IRF1), ferritin heavy subunit (FTH), cathelicidin, and hepcidin. This study is the first report on large-scale discovery of bacteria-responsive genes in cod, and the first to demonstrate up-regulation of IRF1 in fish immune tissues as a result of bacterial antigen stimulation. Given the importance of IRF1 in vertebrate immune responses to viral and bacterial pathogens, the full-length cDNA sequence of Atlantic cod IRF1 was obtained and compared with putative orthologous sequences from other organisms. Functional annotations of assembled SSH library ESTs showed that stimulation with the bacterial antigens caused changes in a wide range of biological processes including chemotaxis, regulation of apoptosis, antimicrobial peptide production, and iron homeostasis. In addition, differences in spleen and head kidney gene expression responses to the bacterial

antigens pointed to a potential role for the cod spleen in blood-borne pathogen clearance. Taken together, my data show that Atlantic cod immune tissue responses to bacterial antigens are similar to those seen in other fish species and higher vertebrates.

2.2 Introduction

Aeromonas salmonicida subsp. *salmonicida* is a Gram-negative bacterium that is the causative agent of typical furunculosis, a bacterial septicaemia of coldwater fish. In addition to this subspecies, a large number of other subspecies exist that produce atypical forms of this disease. These subspecies, which are referred to as atypical strains, infect a wide range of fish hosts in a wide variety of environments (87). Both typical and atypical *A. salmonicida* are known to infect and cause disease in aquaculture-reared Atlantic cod (*Gadus morhua*) (67).

Lipopolysaccharides (LPS), unmethylated CpG motifs, peptidoglycan, and flagellin are common pathogen-associated molecular patterns (PAMPs) associated with Gram-negative bacteria, which can be recognized by a variety of host pattern recognition receptors (PRRs). These PRRs include a number of Toll-like receptors as well as other cell-surface and cytosolic receptors that, upon stimulation, modulate immunity (58, 83). In higher vertebrates, these receptors, their signaling pathways, and the immunological pathways that they stimulate, are relatively well characterized when compared to our current understanding of lower vertebrates such as fish. To add to our knowledge of the immune system of fish we are undertaking research aimed at fully characterizing the Atlantic cod

immunome. This research is being conducted as part of the Genome Canada-funded Atlantic Cod Genomics and Broodstock Development Program (CGP, http://www.codgene.ca), and aims to develop a complete understanding of the genes and molecular pathways involved in Atlantic cod responses to pathogens, and thus to facilitate the development of management practices, markers and methods for selecting disease-resistant broodstock, and new vaccines and therapeutics to combat disease outbreaks in Atlantic cod aquaculture.

To this end, I constructed reciprocal suppression subtractive hybridization (SSH) cDNA libraries enriched for genes that were differentially expressed in the spleen and hematopoietic kidney (head kidney) of juvenile Atlantic cod following stimulation with formalin-killed, atypical *A. salmonicida*. Sequencing of the expressed sequence tags (ESTs), the development of an EST database, as well as the development of quantitative reverse transcription – polymerase chain reaction (QPCR) protocols, enabled us to partially characterize, functionally annotate, and study the expression of genes involved in primary immune responses (2 to 72 hours post-stimulation) to these bacterial antigens. The use of SSH libraries has been previously demonstrated to be an effective method for identifying Atlantic cod genes that are differentially expressed during immune responses to virus-like antigens (59, 71). I decided to study the head kidney and spleen due to their roles in fish as primary and secondary lymphoid organs, respectively (57). *Aeromonas salmonicida* was selected due both to its importance as a disease-causing organism, as well as the interest in developing vaccines against this pathogen for use in marine fishes (24, 43).

2.3 Materials and Methods

Bacterial antigen preparation

Single colonies of atypical *A. salmonicida*, originally isolated from a Norwegian Atlantic cod [Strain # aAs4099 (IMB # 05-2)] were grown in 100 ml of tryptic soy broth (Difco, Mississauga, ON) overnight at 17°C, with shaking (100 RPM). Cultures were centrifuged (10 min, 2000 x *g*, 4°C) and the resulting pellet was washed twice with 40 ml of cold, sterile phosphate-buffered saline (PBS). After washing, the bacteria were re-suspended in PBS to give an OD_{600} of 1.0, and inactivated by the addition of formalin (to a final concentration of 4% formalin w/v). Following inactivation, the bacteria were centrifuged to form a pellet (10 min, 2000 x *g* at 4°C), washed twice with 40 ml of PBS, and re-suspended in PBS to an OD_{600} of 1.0. Inactivation was confirmed by plating 0.5 ml of the suspension onto tryptic soy agar and incubating for 24 hours at room temperature. The inactivated cells were stored at -20°C until use.

Fish husbandry, bacterial antigen stimulation, and tissue sampling

One hundred and fifty Passive Integrated Transponder (PIT) tagged, juvenile, healthy-appearing Atlantic cod (~25 g) from a single family (Family 32, CGP 2006 year class) were divided equally into three 500 liter tanks, and maintained in flowing seawater (10°C, >90% O_2 saturation) under a 12 h light:12 h dark photoperiod. The fish were fed daily (at 1.5% body mass d^{-1}) with a commercial fish feed, and acclimated to the experimental system for 17 days before experimentation.

Immediately prior to stimulation, 8 individuals from each tank (0 h controls) were captured using a dip net and rapidly killed by a lethal dose (0.4 g/l) of tricaine methanesulphonate (TMS) (Syndel Laboratories, Vancouver, B.C., Canada). Brain, head kidney, and spleen tissues were placed individually in RNase-free 1.5 ml tubes, flash frozen in liquid nitrogen, and stored at -80°C until RNA extraction. Dissecting tools were cleaned with RNAseZap (Ambion, Austin, TX) for spleen and head kidney excision, or heat sterilized for brain excision, between individuals to prevent cross contamination between samples. After taking 0 h control samples, the remaining individuals received one of the following treatments: no handling (referred to as undisturbed control, or "UC"), an intraperitoneal (i.p.) injection of 100 µl of sterile PBS (referred to as "PBS"), or i.p. injection of 100 µl of formalin-inactivated *A. salmonicida* suspended in PBS (referred to as "ASAL"). Prior to injection, fish were captured using a dip net and lightly anaesthetized in an aqueous solution of 0.1 g/l TMS. At approximately 2 h, 6 h, 24 h, and 72 h post-injection (HPI), individuals (8 individuals per treatment per time point) from all three groups (UC, PBS, and ASAL) were captured, euthanized, and sampled as previously described for the 0 h control individuals (Figure 2-1A).

Nodavirus testing

To determine if individuals used in library construction were asymptomatic carriers of nodavirus, a reverse transcription-polymerase chain reaction (RTPCR) test for nodavirus (59) was conducted on individual brain RNA samples obtained from the UC and ASAL groups. This test was performed to avoid the use of nodavirus carriers as UCs (in SSH

library construction), and to determine if nodavirus carrier status of brain tissue influenced gene expression in immune tissues (in QPCR studies). The results of the nodavirus testing, and the nodavirus carrier status of individuals utilized in SSH library construction, are shown in Figure 2-1B.

Suppression subtractive hybridization (SSH) cDNA library construction and sequencing

Spleen and head kidney reciprocal SSH libraries were constructed to identify genes involved in the response to i.p. injection with bacterial antigens. For each tissue (spleen, head kidney) and condition (UC, ASAL), DNase I-treated, cleaned total RNA (10 µg per sample) from 5 individuals sampled at each time point (2, 6, 24, and 72 HPI) was used to generate the mRNA pools for the UC and ASAL groups (Figure 2-1B). Samples used to create the ASAL mRNA pool were taken from nodavirus carriers and non-carriers, whereas samples for the UC mRNA pool were taken only from non-carrier fish. This strategy was employed to maximize the difference between ASAL and UC pool transcriptomes, thereby maximizing the utility of resulting SSH libraries for immune gene discovery. The individuals utilized for building spleen and head kidney SSH libraries, with nodavirus carrier status information, are shown in Figure 2-1B. For each tissue, the ASAL mRNA pool was the tester in the forward subtraction, and the driver in the reverse subtraction; the UC mRNA pool was the driver in the forward subtraction and the tester in the reverse subtraction. Therefore, the forward SSH libraries were enriched for transcripts that were up-regulated due to injection stress, exposure to *A. salmonicida* antigens, and/or the presence of nodavirus (in brain) in an asymptomatic

carrier state. The reverse SSH libraries were enriched for transcripts that were down-regulated by these factors (Figure 2-1B).

Briefly, SSH library construction was performed using the PCR-Select cDNA Subtraction Kit (Clontech, Mountain View, CA) following the manufacturer's instructions as previously described (59). A brief description of the method and any differences from Ref. 59 follows. Total RNA was extracted from spleen and head kidney samples of individual fish (Figure 2-1) using TRIzol Reagent (Invitrogen, Burlington, ON, Canada). Total RNA was treated with DNase I (RNase-Free DNase Set, QIAGEN, Mississauga, ON, Canada) to remove residual genomic DNA and column purified (RNeasy MinElute Cleanup Kit; QIAGEN) as described (59). Poly(A)$^+$ RNA (mRNA) was isolated from UC and ASAL tissue total RNA pools (Figure 2-1B) using the MicroPoly (A) Purist Small Scale mRNA Purification Kit (Ambion, Austin, TX) following the manufacturer's protocol.

The SSH libraries were amplified using the Advantage 2 Polymerase Kit (Clontech) following the manufacturer's protocol, and purified using the MinElute PCR Purification Kit (QIAGEN). The resulting cDNA libraries were TA cloned into pGEM-T-Easy (Promega, Madison, WI), and the transformations were performed using MAX Efficiency DH5α Chemically Competent Cells (Invitrogen).

DNA sequencing, sequence assembly, and gene identification

The methods used for sequencing the SSH libraries, and for assembly and annotation of ESTs arising from these libraries, were previously described (59). Briefly, DNA extracted from individual bacterial clones was amplified using Templiphi^{TM} DNA polymerase and

sequenced using ET terminator chemistry (GE Healthcare, Piscataway, NJ) on MegaBACE capillary sequencers. The resultant ESTs were first trimmed with PHRED (20, 21), then screened and clustered using Paracel Transcript Assembler (PTA; Paracel Inc., Pasadena, CA). Both contigs (contiguous consensus sequences) and singletons (individual sequence reads) generated by the PTA clustering procedure were annotated using AutoFACT (36). In my AutoFACT annotation, BLASTn was used to identify ribosomal RNA sequences in LSU and SSU (large and small subunit, respectively) databases, while BLASTx was used for all other alignments. For both BLASTx and BLASTn, the BLAST hits with bit scores higher than 40 were considered significant. All EST sequences have been deposited in GenBank dbEST (See Table 2-1 for accession numbers and EST library statistics). In addition, these sequences and their AutoFACT annotations can be accessed through the CGP EST database (http://ri.imb.nrc.ca/codgene).

Quantitative reverse transcription – polymerase chain reaction (QPCR)

For 10 immune-relevant genes identified in the SSH libraries, transcript (mRNA) expression was studied in PBS and ASAL tissues (spleen and head kidney) from 5 time points (2, 6, 24, 72 HPI, and 0 h pre-injection control) using Power SYBR Green I dye chemistry and the 7300 Real Time PCR system (Applied Biosystems, Foster City, CA). With the exception of the 0 h control PBS group (n=5), 6 fish from each group, tissue, and time point were used in the QPCR study. QPCR primers, designed from EST sequences (Tables 2-2, 2-3, 2-4, 2-5 and 2-6) using the Primer 3 program (65) (available at http://frodo.wi.mit.edu), are listed in Table 2-6. Dissociation curves were run to ensure that

primer pairs amplified single products, and no-template controls were run to ensure that primer dimers were absent. The amplification efficiencies of primer pairs for SCYA, IRF1, and 18S ribosomal RNA were determined previously (59). The amplification efficiencies of the other primer sets were determined as described (59). Expression levels of the genes of interest were normalized to 18S ribosomal RNA, which was stably transcribed in all samples involved in the QPCR study.

For each sample, 1 µg of DNase I treated and column purified total RNA was reverse-transcribed in a final reaction volume of 20 µl as in Rise et al. 2008 (Ref. 59), and the resulting cDNA was diluted with nuclease-free H_2O to a final volume of 100 µl. PCR amplifications were performed using a 7300 Real Time PCR detection system (Applied Biosystems) using 25 µl reactions that contained 1 µl of diluted cDNA (10 ng input total RNA), 50 nM each of forward and reverse primer, and 1X Power SYBR Green PCR Master Mix (Applied Biosystems). The amplification program consisted of 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min (primer annealing and extension stage), with the fluorescent signal from SYBR green measured at the end of each 60°C step. For each sample, the target transcript (gene of interest) and the normalizer (18S rRNA) were each run in duplicate on the same plate. The fluorescence thresholds and baseline were determined automatically using the 7300 PCR Detection System SDS Software Relative Quantification Study Application (Version 1.2.3; Applied Biosystems). Thresholds were set manually if the software did not place them at the exponential phase of amplification with minimal variation between technical replicates. Cycle threshold (Ct) values were obtained and used for calculation of relative quantity (RQ) of each transcript with the $2^{-\Delta\Delta CT}$

quantification method and assuming 100% amplification efficiencies for gene of interest and normalizer primer pairs (41).

QPCR data analysis

All RQ data are presented as mean ± standard error (SE). RQ values were subjected to a two-way (main effects group and sampling time) analysis of variance (ANOVA). In addition, one-way ANOVA (for each group and sampling time) with Tukey post-tests were conducted to determine: 1) whether PBS control sample gene expression (RQ values) at 2, 6, 24, and 72 HPI differed significantly from gene expression in the 0 h control group from the PBS tank; 2) if gene expression of ASAL group at each time point differed significantly from levels of gene expression in the 0 h control group from the ASAL tank; and 3) if gene expression differed significantly between the PBS and ASAL group at each individual time point (2, 6, 24, 72 HPI, and 0 h). Differences in spleen and head kidney constitutive gene expression between asymptomatic carriers of nodavirus and non-carriers were examined by one-way ANOVA of RQ values obtained for all genes studied by QPCR at 0 h (prior to injection) as in Rise et al. 2008 (Ref. 59). All statistical tests were performed using Systat 12.0 (Systat Software Inc., San Jose, CA) with the p value set at ≤ 0.05.

Atlantic cod IRF1 characterization

The 5' and 3' ends of IRF1 cDNA were amplified using a commercial kit for RNA ligase-mediated-RACE, GeneRacer Kit (Invitrogen). IRF1-specific primers (Supplemental Table S2) were designed based upon IRF1 assembled EST sequences from the forward

spleen SSH library (contig sb_gmnlsfas.73.C1, containing ESTs with accession numbers EY975211 and EY975084). Briefly, 250 ng of the same mRNA from ASAL fish used in spleen SSH library construction (Figure 2-1B) was used as the RNA template. For 5'RACE, touch-down PCR was performed with GeneRacer 5' primer and IRF1_5'RACE1, followed by a nested PCR conducted with GeneRacer 5' nested primer and IRF1 _5'RACE2. For 3'RACE, only one round of touch-down PCR was carried out with GeneRacer 3'primer and IRF1_3'RACE. The cycling conditions of both touch-down PCR and nested PCR are as specified in the GeneRacer Kit manual with the extension time set to 3 minutes for all cycles. Nested primer pairs (IRF1_F1, IRF1_R1, IRF1_F2, and IRF1_R2; Supplemental Table S2) were designed in the 5' and 3' untranslated regions (UTRs) to amplify the open reading frame (ORF). The cycling conditions for both PCRs were 1 cycle of 2 min at 94°C, 25 cycles of (30 seconds at 94°C, 30 seconds at 70°C, 3 min at 72°C), and 1 cycle of 10 min at 68°C.

All PCR amplifications were performed using the Advantage 2 Polymerase kit (Clontech) and all PCR products were gel extracted using the QIAQuick Gel Extraction kit (QIAGEN), ethanol precipitated, washed and cloned into PCR®4-TOPO® (Invitrogen). The clones were transformed into One Shot® TOP10 competent cells, and plated on LB /carbenicillin (50 μg/ml). Individual colonies were grown overnight at 37°C in LB/carbenicillin (50 μg/ml), and plasmid DNA samples were isolated in the 96-well format using standard methods. The insert sizes of recombinant plasmids were determined by *Eco*RI (Invitrogen) digestion prior to sequencing. For each PCR product, 4 individual clones were sequenced in both directions using the ABI 3730 DNA Analyzer using standard techniques.

Atlantic cod IRF1 amino acid sequence analysis and phylogenetic tree construction

The amino acid (AA) sequence of Atlantic cod IRF1 was deduced based on the cDNA sequence using the SeqBuilder function of Lasergene 7.20 software package (DNASTAR, Madison, WI) and the polyadenylation signal was predicted using the RNA analyzer (4) (available at http://rnaanalyzer.bioapps.-biozentrum.uni-wuerzburg.de/). The IRF1 DNA binding domain model was predicted and visualized by Swiss-model and Swiss-PdbView software (2, 25, 35, 69) (available at http://swissmodel.expasy.org/). The deduced Atlantic cod IRF1 AA sequence was compared with the orthologous AA sequences from other vertebrates. The multiple alignments were performed using the CLUSTALX (version 2.09) program, and the unrooted phylogenetic tree for IRF1 was constructed by the neighbor-joining method and was bootstrapped with 10,000 replicates. The phylogenetic trees were plotted using MEGA4 (78).

2.4 Results

Screening of cod immune tissue transcripts responsive to stimulation with bacterial antigens

To identify genes important in the response of Atlantic cod to bacteria, juvenile fish were stimulated with formalin-killed, atypical *A. salmonicida*, and reciprocal SSH libraries from spleen and head kidney were constructed and sequenced. The single family of fish that was used was the same family utilized in a previous report (59). Although this family was selected due to their good growth and high survival in the laboratory, there were asymptomatic carriers of nodavirus within this family (Figure 2-1). Nodavirus screening by RTPCR on 32 individual fish brain samples from each of UC and ASAL group revealed that

20% and 32.5% of fish in the UC and ASAL groups, respectively, were carriers of nodavirus (Figure 2-1). For SSH library construction, I utilized *A. salmonicida*-stimulated spleen and head kidney samples from both nodavirus carriers and non-carriers. Undisturbed control (UC) tissues were obtained only from nodavirus negative fish. The resulting libraries, although biased toward genes involved in the response to the bacterial antigens, may also contain genes that are responsive to nodavirus carrier status and stress associated with the i.p. injection. With respect to immune-related genes, my selection of early time points (2, 6, 24, and 72 HPI) biased my results towards identification of genes involved in innate immunity.

I obtained a total of 4154 good quality (i.e. not rejected by PTA as described in Materials and Methods) ESTs including: 1048 from the forward spleen library (designated "sb_gmnlsfas" in Table 2-1, and the codgene website), 1087 from the reverse spleen library (sb_gmnlsras), 1033 from the forward head kidney library (sb_gmnlkfas), and 986 from the reverse head kidney library (sb_gmnlkras) (Table 2-1; www.codgene.ca). My ESTs are 3' biased and relatively short, averaging 300-500 bp in length (Table 2-1). The presence of short coding sequences is in part responsible for some of the higher E-values (up to 1e-4) reported in Tables 2-5. With the exception of the head kidney reverse library which showed 49.6% redundancy, the libraries were relatively complex (< 29% redundancy) (Table 2-1). Selected contiguous sequences (contigs) from the forward spleen, reverse spleen, forward head kidney and reverse head kidney libraries are shown in Tables 2-2, 2-3, 2-4, and 2-5, respectively. These data are limited to contigs and singletons with immune-related functional annotations. Complete lists of assembled sequences in these libraries, with contributing EST accession numbers and functional annotations, are found in on-line Supplemental Tables

S1A (forward spleen library), S1B (reverse spleen library), S1C (forward head kidney library), and S1D (reverse head kidney library).

The deepest contigs (i.e. having the highest numbers of contributing ESTs) in the forward spleen library were identified as a small inducible cytokine (SCYA, 12 contributing ESTs), and cathelicidin antimicrobial peptide (CAMP, 10 ESTs) (Table 2-2; Supplemental Table S1A). Other contigs present in this library were identified as ferritin heavy subunit (FTH, 5 ESTs), ferritin middle subunit (FTM, 4 ESTs), and myeloid cell leukemia 1 (MCL1, 3 ESTs). In the head kidney forward library, FTH (8 ESTs), FTM (6 ESTs), CAMP (3 ESTs), proteosome activator subunit 2 (3 ESTs) and goose-type lysozyme 1 (3 ESTs) were among the most common sequences, not including unclassified sequences (i.e. no significant BLAST hit) and transcripts found in both forward and reverse libraries (e.g. hemoglobin subunits) (Table 2-4; Supplemental Table S1C). Although the two forward libraries shared some transcripts in common, numerous genes were only identified in one of the libraries. For example, transcripts identified as SCYA, interferon regulatory factor 1 (IRF1), hepcidin antimicrobial peptide (HAMP), interleukin 8 (IL8), basic transcription factor 3 (BTF3), DNA-damage-inducible transcript 4, interleukin 1 beta (IL1β), and serum lectin isoforms 1 and 2, were unique to the forward spleen library (Table 2-2; Supplemental Table S1A). Transcripts identified as proteasome activator subunit 2, translationally-controlled tumor protein, CD84 molecule, lipopolysaccharide binding protein, interleukin 5 receptor alpha, and inhibitor of nuclear factor kappa B alpha, were unique to the forward head kidney library (Table 2-4; Supplemental Table S1C).

Within the spleen and head kidney reverse libraries multiple heat shock protein (HSP) transcripts were identified, including transcripts for HSP 90. Within the reverse libraries, several novel transcripts with gene names and functional annotations suggesting involvement with kinase or receptor activity were identified such as tyrosine kinase 2, mitogen-activated protein kinase 14a, scavenger receptor class B member 2, interleukin 1 receptor-like protein precursor (IL1R), lymphocyte antigen 75, complement receptor-like protein 1, a novel immune-type receptor 4, and Toll-like receptor 23 (Tables 3 and 5; Supplemental Tables S1B and S1D). In addition, transcripts with gene names and functional annotations suggesting involvement in apoptosis regulation (e.g. caspase 8, leukocyte elastase inhibitor, and cell division cycle and apoptosis regulator 1) were identified in the reverse spleen library (Table 2-3; Supplemental Table S1B).

Gene ontology (GO) annotation of non-redundant transcripts from SSH libraries

The non-redundant ESTs from the each of the SSH libraries were assigned biological process GO terms using AutoFACT and Goblet (26) as described (59). I was able to assign 57 and 49 GO terms for sequences from the forward and reverse spleen library, respectively (Figure 2-2A). For the forward and reverse head kidney libraries (Figure 2-2B), 59 and 28 GO terms were assigned, respectively. For the spleen libraries, the highest numbers of sequences were assigned to categories "immune response" (forward library) and "transport" (reverse library). For the forward head kidney library, the highest numbers of sequences were assigned to "protein biosynthesis", while for the reverse head kidney library, the highest numbers of sequences were assigned to "protein

biosynthesis" and "protein folding" (Figure 2-2B). In the spleen, "apoptosis"-annotated ESTs were more abundant in the forward library (Figure 2-2A), and in the head kidney, "regulation of apoptosis" and "apoptosis" were among the GO terms that were associated exclusively with ESTs in the forward library (Figure 2-2B). A comprehensive list of assembled ESTs from these libraries, and their GO annotations, can be found at http://ri.imb.nrc.ca/codgene/.

Gene expression patterns following injection of formalin-killed A. salmonicida *or PBS*

Nine genes (IRF1, CAMP, HAMP, SCYA, IL1β, IL8, FTH, MCL1, and BTF3) from the forward spleen and head kidney libraries and one gene (IL1R) from the spleen reverse library were subjected to QPCR to study the magnitude and timing of their expression following i.p. stimulation with formalin-killed, atypical *A. salmonicida* (Figure 2-3; Supplemental Table S4A-R). These genes were selected to investigate the influence of bacterial antigen stimulation on the expression of genes involved in the following biological processes: cytokine signaling (IL1β, IL1R, IL8, SCYA, and IRF1); apoptosis (BTF3, MCL1); iron homeostasis (FTH and HAMP); and antibacterial defense response (CAMP and HAMP). The CAMP QPCR was designed to study the overall expression of cathelicidin transcripts (i.e. all known paralogs) by utilizing primers in conserved regions (i.e. common to all cathelicidin-like ESTs represented in these SSH libraries).

The genes IRF1, CAMP, HAMP, and SCYA showed similar patterns of expression in the spleen and head kidney samples from the ASAL group, with highest levels of expression at 24 HPI, followed by a large reduction in expression by 72 HPI (Figure 2-3A-H). Of these,

at 72 HPI, only CAMP had significantly higher expression in tissues (both spleen and head kidney) of *A. salmonicida*-stimulated animals when compared to PBS controls (Figure 2-3C, D). The proinflammatory cytokines IL1β and IL8 had similar patterns of expression in the spleen and head kidney of stimulated animals. In both tissues, there were significantly higher levels of expression for both of these genes at 2 and 6 HPI in ASAL individuals when compared to the PBS controls (Figure 2-3I-L). Maximum expression of these genes relative to their appropriate 0 hour controls occurred at 6 HPI [IL1β (684.3 fold) and IL8 (33.8 fold) for spleen; IL1β (356.3 fold) and IL8 (70.6 fold) for head kidney] after which time levels of expression declined (Figure 2-3I-L). Expression of FTH was highest at 24 HPI in spleens from ASAL individuals (Figure 2-3M). Levels of FTH expression in head kidney were similar in magnitude between the PBS and ASAL groups and there was no trend in FTH expression over time in either group (Figure 2-3N). Levels of MCL1 expression were relatively low and similar in magnitude between spleen and head kidney samples (Figure 2-3O-P). In addition, there were no trends in expression over time in either tissue. Expression of MCL1 was significantly higher in spleens from ASAL individuals when compared to PBS controls at 6 and 24 HPI but significantly lower at 72 HPI (Figure 2-3O). Head kidneys from stimulated animals had significantly higher levels of expression when compared to the PBS controls at 6 hours, but significantly lower levels of expression before stimulation (0 h control) and at 72 HPI (Figure 2-3P). The two genes selected for QPCR studies from the spleen libraries (BTF3 and IL1R) were not significantly affected by *A. salmonicida*-stimulation (data given in Supplemental Table S4Q-R but not presented in Figure 2-3).

I also examined whether the presence of nodavirus, as detected by RTPCR in brain samples, had an effect on constitutive expression of these 10 genes in spleen and head kidney. For both immune tissues, there was no significant effect of nodavirus carrier status on the constitutive expression of these genes (data not shown).

Characterization and phylogenetic analysis of Atlantic cod IRF1

The putative translation of one EST from the spleen forward SSH library (EY975211) shared 40% identity to the IRF1 of snakehead (*Channa argus*) over 103 aligned AAs (GenBank accession number ABN42504). Additional sequence was obtained from this transcript by 5'RACE (807 bp) and 3'RACE (680 bp) using mRNA from the spleens of *A. salmonicida*-stimulated fish. These sequences were assembled to generate a 1465 bp cDNA sequence that encodes a 306-amino acid protein. The Atlantic cod IRF1 sequence characterized in this study was deposited in GenBank under accession number FJ346564. Structural modeling of Atlantic cod IRF1 shows that its DNA binding domain (DBD) consists of 3 α-helixes, 4 β-sheets, and 3 long loops (see Figure 2-4A for detailed information). Alignment of IRF1 amino acid sequences from Atlantic cod (deduced protein sequence) and several other vertebrate species shows that the sequences all contain a highly conserved (greater than 60% identity; Supplemental Table S5) DNA binding domain at the N-terminus with 6 conserved tryptophan residues (Figure 2-4B). Overall, the Atlantic cod IRF1 amino acid sequence is 42-55% identical to the IRF1 orthologs of other teleosts, and 34-37% identical to IRF1 orthologs from non-teleost vertebrates (Supplemental Table S5). A phylogenetic tree constructed based on the IRF1

multiple sequence alignment also shows that the Atlantic cod IRF1 is more closely related to other teleost IRF1 orthologs than to orthologs from other vertebrates (Figure 2-4C).

2.5 Discussion

Subspecies of the Gram-negative bacterium *Aeromonas salmonicida* are the causative agents of a serious disease in Atlantic cod (61, 67) and many other fishes (40, 54, 62). This disease is classified as either typical furunculosis, caused by *A. salmonicida* subsp. *salmonicida* or atypical furunculosis, caused by atypical variants of *A. salmonicida*. To date, a number of studies have examined the transcriptional response of salmonids challenged or vaccinated with the typical subspecies (*A. salmonicida* subsp. *salmonicida*) (19, 22, 47, 51). In addition, the immune response of zebrafish to challenge with the related species *Aeromonas hydrophila* has also recently been examined in detail (63). In this study I used an atypical variant of *Aeromonas salmonicida* that was obtained from an atypical furunculosis outbreak in Atlantic cod. To identify and characterize genes with transcriptional changes related to the early immune response (2-72 h) to bacterial antigens, Atlantic cod were injected with formalin-killed, atypical *A. salmonicida* (or control saline) and SSH libraries were constructed, sequenced and characterized for the spleen and head kidney. From these libraries 4154 ESTs were generated, enabling me to identify a large number of immune-related genes for which sequence information was previously not available for Atlantic cod.

Functional annotation and analysis of the ESTs generated from the SSH libraries revealed that the killed-*A. salmonicida* stimulation induced changes in the expression profiles of genes involved in a variety of physiological processes in spleen and head kidney. In addition to the physiological processes directly linked to the innate anti-bacterial immune response, such as antimicrobial peptide synthesis, chemotactic signaling, regulation of iron homeostasis, antigen processing and presentation, and complement pathway, other processes appeared to be dysregulated by the *A. salmonicida* stimulation including regulation of apoptosis, protein synthesis, proteolysis, DNA-dependent transcription, and stress response. Using QPCR with individual fish tissue templates (to assess biological variability), I investigated the expression of nine genes in the forward libraries with functional annotations representing a subset of these physiological processes.

One Atlantic cod contiguous sequence (contig) containing 2 ESTs from the forward spleen SSH library (enriched for genes up-regulated by bacterial antigens) had significant homology to the IRF1 of snakehead (*Channa argus*) and I obtained the full-length cDNA sequence using 5' and 3' RACE. The Atlantic cod IRF1 mRNA includes an ORF of 921 base-pairs that translates to 306 amino acid (AA) residues. This sequence has approximately 35% and 50% identity to the human (*Homo sapiens*) and rainbow trout (*Oncorhynchus mykiss*) IRF1s, respectively. Phylogenetic analysis placed Atlantic cod IRF1 sequence near to the branching point of the group containing IRF1 from teleosts. Structural modeling of Atlantic cod putative IRF1 DNA binding domain (DBD) suggests the presence of 3 α-helixes, 4 β-sheets, and 3 long loops which is consistent with the structure of human IRF1 (18). Therefore, based on its amino acid identity, the results of the phylogenetic analysis, and

structural modeling, I am confident that this sequence encodes the Atlantic cod IRF1 protein. Furthermore, the conserved DBD in Atlantic cod IRF1 suggests that it may have similar function and recognize similar DNA motifs (i.e. the interferon simulated response elements) as its human ortholog.

Most studies on fish have examined patterns of IRF1 expression following polyriboinosinic polyribocytidylic acid (pIC)-stimulation or virus challenge (13, 30, 55, 59, 77, 89). There are few reports of IRF1 expression in fish following stimulation with bacteria or bacterial antigens. Yabu *et al* (1998; Ref. 89) demonstrated induction of an interferon regulatory factor in the liver of Japanese flounders following intra-muscular injection with *Edwardsiella tarda*. Although these authors could not determine whether their sequence encoded IRF1 or IRF2 due to lack of representative sequences from fish, my more recent BLASTx analysis of their sequence shows homology to other IRF1 sequences (Figure 2-4). However, Collet and Secombes (2002; Ref. 13) reported that IRF1 expression was induced only by pIC and not by LPS in rainbow trout gonad cells. More recently, Ordas *et al* (2006; Ref. 55) described the IRF1 from turbot (*Scophthalmus maximus*) and seabream (*Sparus aurata*). In these species, IRF1 was reported to have low levels of constitutive expression in a variety of tissues, and its expression was induced by pIC stimulation and viral hemorrhagic septicaemia virus-challenge (VHSV). However, the increase in IRF1 expression in head kidney of turbot following *Vibrio pelagius* challenge was not statistically significant at the single time point they studied (8 h post-challenge). In this study, Atlantic cod IRF1 was constitutively expressed at low levels and expression was significantly elevated in spleen and head kidney at 24 h following *A. salmonicida* stimulation, with highest levels of induction

being observed in the spleen. In fish, the target genes of IRF1, as well as its importance in innate immune responses, are yet to be determined. However, in rainbow trout, the expression of IRF1 in macrophages can be induced by both IFN-γ and IL1β, with IFN-γ being a much more potent inducer of IRF1 than IL1β (48). As both IFN-γ and IL1β are known to be induced in fish following a challenge with bacterial antigens, it appears that similar pathways to those of higher vertebrates exist in fish (10, 12).

Chemotactic cytokines are directly involved in leukocyte trafficking and play an important role in the innate immune response. Interleukin 8, a CXC chemokine ligand (CXCL), was identified in the spleen forward SSH library and its sequence was homologous (96% AA identity) to the Atlantic cod IL8 recently described by Seppola *et al* (2008; Ref. 70). In my study, IL8 expression was up-regulated by formalin-killed *A. salmonicida* stimulation in both spleen and head kidney at 2 h, reaching its peak at 6 h, and returning to basal levels at 72 h. My results are in agreement with those of Seppola *et al* (2008; Ref. 70), who reported IL8 up-regulation in spleen and head kidney following i.p. injection with formalin-killed *Vibrio anguillarum* at the single time point they examined (24 h). Thus, it appears that expression of IL8 in Atlantic cod, like in other vertebrates (6, 10, 12), is induced rapidly as part of the inflammatory response to bacterial antigens.

Members of the CC chemokine subfamily are generally known for their activity targeting mononuclear cells rather than neutrophils (39). The largest contig in the spleen forward SSH library is homologous to a small inducible cytokine, SCYA104, from the African cichlid (*Paralabidochromis chilotes*). Analysis of Atlantic cod SCYA expression demonstrated that it was highly up-regulated in spleen, as well as up-regulated to a much

smaller magnitude in the head kidney, at 24 h following *A. salmonicida* stimulation. This gene has been previously reported to be highly up-regulated in the spleen of Atlantic cod at 6 and 24 h following stimulation with pIC (59). Using BLASTp analysis of Atlantic cod SCYA I determined that this gene is most closely related to the human monocyte chemotactic protein 2 (MCP2) (23/65 aligned AA for 35% identity). Human MCP2 is a known chemoattractant of monocytes, and its expression is induced in response to various immunogenic stimuli, such as IL1β, IFN-γ, and pIC (85, 86). However, due to relatively low levels of homology between the fish CC chemokines and putative orthologs in higher vertebrates, it is possible that they will have different functions. Further work is required to assign chemoattractant function to Atlantic cod SCYA.

Several putative apoptosis regulatory transcripts were identified in the forward SSH libraries, such as members of the caspase family and the Bcl-2 anti-apoptotic family. Of these, transcripts encoding myeloid cell leukemia sequence 1 (MCL1) were identified in both forward SSH libraries. My QPCR analysis indicated that Atlantic cod MCL1 has relatively low levels of constitutive and induced expression in both the spleen and head kidney. Furthermore, it presented no obvious trends over time following stimulation with bacterial antigens. In humans, 2 forms of MCL1 exist as a result of differential splicing, generating a longer anti-apoptotic form of MCL1 with all 3 exons and a shorter pro-apoptotic form of MCL1 containing exons 1 and 3 (8). In contrast to humans, both MCL1 paralogs identified in zebrafish have anti-apoptotic activity (37). The MCL1 identified in this study is a putative ortholog of the anti-apoptotic human MCL1 (87/223 aligned AAs for 39% identity) and it is more similar to zebrafish MCL1b (99/195 aligned AAs for 50% identity) than to MCL1a

(97/209 aligned AAs for 46% identity) described by Kratz *et al* (2006; Ref. 37). So far, there is no evidence that paralogs of MCL1 are present in the Atlantic cod genome, as all MCL1-representing sequences identified in the CGP database represent the same transcript. Even if another copy of MCL1 is present in cod, it is unlikely that the QPCR primers were amplifying both paralogs as the primers are specific to a region poorly conserved between the two zebrafish paralogs. In Atlantic salmon co-expression of MCL1 and IL1β in response to an ectoparasite infection has been documented (49).

Genes that encode for antimicrobial peptides (AMPs) and proteins that are involved in the regulation of iron homeostasis are commonly responsive in fish following stimulation with bacterial antigens or live bacterial challenge (11, 23, 45). Antimicrobial peptides, such as cathelicidins and hepcidins (HAMPs), are cationic peptides that lyse bacterial cells by disrupting the bilipid layer of their plasma membrane (3). In both the spleen and head kidney forward SSH libraries relatively high numbers of ESTs encoding cathelicidins were identified. Multiple alignment of these sequences indicates that they encode several putative forms of cathelicidins. Using data from these subtracted libraries, as well as other data for cathelicidins obtained from the CGP project website (www.codgene.ca), Maier *et al* (2008; Ref. 45) recently described 3 cathelicidins from Atlantic cod. These cathelicidins are very similar to each other with the majority of differences between them occurring within their antimicrobial peptide domain.

Due to the high similarity of the sequence encoding these cathelicidin anti-microbial peptides (CAMP), my QPCR assay was designed to amplify all forms of the cathelicidin transcripts. Using this assay, low levels of constitutive CAMP expression were evident in

both the spleen and head kidney. In both of these tissues expression levels peaked at 24 h post-stimulation with higher levels of expression seen in the spleen. Maier *et al* (2008; Ref. 44) studied the expression of one form of cathelicidin at 24 h following infection with an atypical strain of *Aeromonas* bacterium (*A. salmonicida ssp. achromogenes*) using RTPCR. These authors reported constitutive expression of CAMP in both the spleen and head kidney and no change in expression level in these tissues following infection. Interestingly, the cathelicidin that they studied was up-regulated in gill, liver, pyloric caeca and intestine but not in skin. Based on my results it appears that one or both of the other forms of cathelicidin are important in the spleen and head kidney response to bacterial stimulation. Maier *et al* (2008; Ref. 44) reported that DNase treatment of commercially purchased *Escherichia coli* LPS (with DNA contamination) or *A. salmonicida* lysate abolished their effects on CAMP expression in a Chinook salmon (*Oncorhynchus tshawytscha*) embryo cell line (CHSE-214), demonstrating that bacterial DNA, or perhaps both LPS and bacterial DNA, are required for the induction of fish CAMP expression. One of the defining characteristics of bacterial DNA is the presence an unmethylated CpG motif that can be recognized by the Toll like receptor 9 (TLR9) (66). Therefore, the up-regulation of CAMP by *A. salmonicida* observed in this study may be associated with the TLR9 signaling cascade.

Another AMP, hepcidin, was also identified in the spleen forward SSH library (Table 2-2). Some fish hepcidins have been shown to have antimicrobial activity (27, 28, 29), and the up-regulation of fish hepcidin expression has been observed in response to immunogens (e.g. Refs. 15, 29, 75), viral infection (e.g. Ref. 15) and bacterial infection (e.g. Ref. 28). Solstad *et al* (2008; Ref. 75) characterized an Atlantic cod hepcidin that was 100% identical

(over 98 aligned AA) to the sequence I obtained in this study. Up-regulation of this Atlantic cod hepcidin has been observed in peritoneum, blood, liver, and head kidney following stimulation with inactivated-*Listonella anguillarum* and pIC (75). In agreement with these findings, I report that the expression of HAMP was significantly up-regulated in both spleen and head kidney at 6 h with maximum levels of expression seen at 24 h post-stimulation. Besides their role as AMPs hepcidins are generally considered to be iron-regulatory hormones that modulate iron metabolism (53). In fish, several studies present evidence supporting this dual role for the hepcidins (27, 28, 29). The role that hepcidin plays in iron regulation in Atlantic cod remains to be determined.

The accumulation of free intracellular iron is toxic as it reacts with oxygen and creates H_2O_2 as a by-product (reviewed in Ref. 80). Peroxides can cause DNA damage and ultimately lead to cell death (e.g. as reviewed in Ref. 5). To maintain iron homeostasis, ferritin captures and stores free iron in a soluble nontoxic state thereby limiting cell damage. In this study, ferritin heavy subunit (H-ferritin) and ferritin middle subunit (M-ferritin) encoding transcripts were identified in both forward SSH libraries as deep contigs (4 or more contributing ESTs, see Table 2-2 and 2-4). H-ferritin is known to be a generic type of ferritin that is present in all animals, while M-ferritin has only been identified in fish and amphibians (1, 16). In contrast to H-ferritin, very little is known about the roles of M-ferritin in iron metabolism or the regulatory mechanisms of M-ferritin synthesis. It is known, however, to possess a conserved ferroxidase center as the one found in H-ferritin (1). In this study I found a significant increase in H-ferritin expression in the spleen at 24 h post-stimulation. In the head kidney, levels of constitutive expression were low and there was no significant

change in expression over time. In fish, previous studies have shown that the expression of H-ferritin in liver can be induced by *Edwardsiella ictaluri* infection (56), and Martin *et al* (2007; Ref. 48) further showed that IL1β caused up-regulation of both hepcidin and H-ferritin in trout macrophages.

Many of the dysregulated transcripts in this study were found in spleen but not head kidney SSH libraries. This could be a result of the degree of success of the subtractive hybridization, transcriptome complexity, or magnitude of transcription dysregulation. For all genes studied with QPCR with the exception of IL8, the magnitude of up-regulation was greater in spleen than in the head kidney. The difference in transcriptome shift between spleen and head kidney may reflect the distinct roles that these two immune tissues play in response to bacterial immunogen. Following the pathogen entry via blood stream, the resident leukocytes in spleen, predominately macrophages, trap and phagocytize the blood borne pathogens. The spleen also serves as a processing site for erythrocytes, as a result of which, the iron level within the spleen is relatively high (Reviewed in Ref. 74). Since the spleen is essential for both pathogen clearance and iron storage, it is critical that splenic macrophages withhold iron from pathogens (50). The more prominent HAMP up-regulation in spleen, coupled with the spleen-specific induction of ferritin by *A. salmonicida*, suggests that the fish spleen may play a key role in iron-withholding as an innate immune response to bacterial pathogens.

In this study, some of the cod used in my QPCR analysis were asymptomatic carriers of nodavirus as determined by RTPCR on brain tissues using nodavirus-specific primers. In asymptomatic carriers, nodavirus is carried in the brain and eyes, which are immune

privileged sites (31). Therefore, it is difficult to know what, if any, exposure the immune system has to this virus in the asymptomatic state. In this study, I found no significant differences in constitutive gene expression between nodavirus carriers and non-carriers in either spleen or head kidney. Using the same family of Atlantic cod that was used in the current study, Rise *et al* (2008; Ref. 59) reported that asymptomatic nodavirus carrier status of brain did not influence the constitutive expression of 13 immune-relevant genes in the spleen, including two genes of interest from the current report (IRF1 and SCYA). In addition, they reported that there was no apparent correlation between nodavirus carrier status and pIC response in the spleen for these genes. Unfortunately, I had insufficient samples to fully examine the correlation between nodavirus carrier status and responses to *A. salmonicida* in the spleen and head kidney.

The gene expression results may also be affected by the use of a single cod family. There is growing evidence for differences between families of fish (including Atlantic cod) in their susceptibility to disease (34, 88). In this study, I utilized a single family of Atlantic cod that were selected based on their survival and growth performance in culture. It is unknown whether this family contains individuals that are more or less susceptible to infection with atypical *A. salmonicida* than other families in the broodstock development program. Whether differences in disease resistance between families of cod will be related to differences in patterns of immune-related gene expression is unknown.

In summary, I have identified many genes in Atlantic cod that are known to be important in the innate immune response against bacteria, and have also characterized IRF1 in this species for the first time. The transcriptional innate immune response of Atlantic cod

to *A. salmonicida* observed in this study is similar to many previous studies in fish, and includes genes such as those involved in chemotactic signaling and AMPs.

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2.8 Tables

Table 2-1. Statistics for ESTs generated from the spleen (S) and head kidney (HK) SSH libraries.

Library Name	S_Forward	S_Reverse	HK_Forward	HK_Reverse
Tissue	spleen	spleen	head kidney	head kidney
Direction[1]	forward	reverse	forward	reverse
CGP identifier[2]	sb_gmnlsfas	sb_gmnlsras	sb_gmnlkfas	sb_gmnlkras
Accession Numbers	EY974820 - EY975867	EY975868 - EY976954	EY972492 - EY973450[3]	EY973692 - EY974677
Number of ESTs	1048	1087	1033	986
Average EST length[4]	375 bp	400 bp	333 bp	460 bp
Number of contigs[5]	136	157	172	229
Number of singletons	685	677	570	268
Number of non-redundant ESTs[6]	821	834	742	497
Percent redundancy[7]	21.7%	23.3%	28.2%	49.6%

[1]The forward SSH libraries were constructed to enrich genes up-regulated by the *A. salmonicida* injection, and the reverse SSH libraries were constructed to enrich genes down-regulated by the *A. salmonicida* injection.
[2]The identifiers of the SSH libraries in the CGP EST database: http://codgene.ca.
[3]For head kidney forward library, 74 ESTs were not submitted to GenBank as they were rejected during the PTA clustering process.
[4]The ESTs were trimmed with PHRED (20, 21) with the trim_alt and trim_cutoff fixed at 0.06, followed by the removal of known contaminant sequences and short sequences (<75bp), and the average EST length was calculated based on edited sequences.
[5]Sequences generated were then clustered using Paracel Transcript Assembler (PTA), with the cluster threshold set at 100 for relatively stringent clustering.
[6]The number of non-redundant ESTs is the sum of the number of contigs plus the number of singletons.
[7]The percent redundancy is the proportion of redundant ESTs in each library, calculated as [1 – (Number of non-redundant ESTs/total Number of ESTs)] multiplied by 100.

Table 2-2. Selected[1] transcripts identified in the forward spleen SSH library (designed to be enriched for genes up-regulated by bacterial antigens)

Accession Number[2]	BLASTx identification[3] of cod cDNAs			# of ESTs	Gene Ontology[4] or function of BLASTx hit[5]
	Name of BLASTx hit (species)	% ID (align)	E-value		
EY974899	Small inducible cytokine SCYA104 (African cichlid)	42% (27/63)	1e-10	12	CC chemokine activity
EY974843	Cathelicidin 1 (Atlantic cod)	97% (121/124)	9e-64	10	Defense response
EY975257	Ferritin heavy subunit (Atlantic salmon)	89% (157/176)	3e-89	5	Ferroxidase activity; iron ion homeostasis; oxidoreductase activity
EY975281	Ferritin middle subunit (Atlantic salmon)	75% (125/166)	2e-67	4	Ferroxidase activity; iron ion homeostasis; oxidoreductase activity
EY975262	Myeloid cell leukemia sequence 1b (zebrafish)	50% (61/121)	1e-23	3	Negative regulation of apoptosis (Lomo *et al* 1996; Ref. 42)
EY975464	Hepcidin (Atlantic cod)	100% (98/98)	9e-43	2	Iron homeostasis Antimicrobial activity (Shi and Camus 2006; Ref. 72)
EY975498	Interleukin 8 (Atlantic cod)	96% (96/99)	2e-43	2	CXC chemokine activity
EY975450	serum lectin isoform 4 (spotted halibut)	58% (31/53)	8e-12	2	Sugar binding; complement pathway activation (Nakao *et al* 2006; Ref. 52)
EY974936	Cathepsin L (barramundi)	41% (43/103)	4e-53	2	Cysteine-type endopeptidase activity; antigen processing (Zhang *et al* 2001; Ref. 90)
EY975712	DNA-damage-inducible transcript 4 (African clawed frog)	53% (50/94)	1e-17	2	DNA damage and/or p53 induced (Ellisen *et al* 2002; Ref. 17)
EY975030	Proliferating cell nuclear antigen (channel catfish)	96% (128/133)	1e-65	2	Regulation of transcription
EY975027	BH3 interacting domain death agonist protein (zebrafish)	33% (48/142)	8e-13	2	Positive regulation of apoptosis
EY975211	Interferon regulatory factor 1 (snakehead)	41% (43/103)	3e-13	2	Regulation of transcription
EY975863	Basic transcription factor 3 (African clawed frog)	87% (152/173)	4e-72	2	Regulation of apoptosis (Kusumawidjaja *et al* 2007; Ref. 38)
EY975549	Goose-type lysozyme 2 (Atlantic cod)	95% (89/93)	1e-44	1	Peptidoglycan catabolism
EY975542	Probable Bax	88%	9e-66	1	Negative regulation of apoptosis

	inhibitor 1 (Japanese flounder)	(136/153)			
EY975059	caspase 3B (Pufferfish)	72% (66/91)	2e-32	1	Apoptosis
EY975676	Interleukin 1 beta (Atlantic cod)	97% (41/42)	9e-16	1	Inflammatory response
EY975713	BCL2-like10 (zebrafish)	81% (53/65)	4e-22	1	Regulation of apoptosis (Kang *et al* 2007; Ref. 33)
EY975339	CC chemokine type 3 (Atlantic cod)	95% (21/22)	1e-04	1	Immune response; chemokine activity
EY975733	Toll-like receptor 8 (pufferfish)	84% (27/32)	1e-11	1	Transmembrane receptor activity
EY975550	CXC chemokine receptor type 3B (rainbow trout)	66% (110/166)	1e-45	1	CXC chemokine receptor activity
EY974897	CXC chemokine receptor (rainbow trout)	71% (66/92)	3e-15	1	CXC chemokine receptor activity
EY975110	serum lectin isoform 3 precursor (spotted halibut)	62% (43/69)	6e-19	1	Sugar binding
EY975124	Heme oxygenase 1 (European sea bass)	65% (31/47)	1e-08	1	Heme oxygenase activity
EY975780	Natural killer enhancing factor (Japanese flounder)	87% (131/150)	2e-75	1	Antioxidant activity

[1]Criteria for selection of contigs and singletons are discussed in the Results section.

[2]For each contig (i.e. cluster containing at least 2 ESTs), the accession number for a representative EST is given. All contigs and singletons from this SSH library were annotated using AutoFACT (36). The additional information (e.g. functional annotations, BLASTx statistics, and GenBank accession numbers for contributing ESTs) is listed in on-line Supplemental Table S1A .

[3]The top BLASTx hit with a gene name (e.g. not "hypothetical", "predicted", "unnamed" or "novel protein") is shown. The BLASTx statistics in this table were collected on October 8, 2008, and reflect the state of the GenBank non-redundant sequence databases on that date. The length of the BLASTx alignment (i.e. the number of amino acid residues translated from the cod cDNA that are aligned with the best BLASTx hit), percent identity (% ID) over the aligned region, and E-value are shown.

[4]Only the "molecular function" and "biological process" Gene Ontology terms are included in this table.

[5]Putative functions are assigned based on findings of previous studies.

Table 2-3. Selected[1] transcripts identified in reverse spleen SSH library (designed to be enriched for genes down-regulated by bacterial antigens).

Accession Number[2]	BLASTx identification[3] of cod cDNAs			# of ESTs	Gene Ontology[4] or function of BLASTx hit[5]
	Name of BLASTx hit (species)	% ID (align)	E-value		
EY976151	Acetylserotonin (zebrafish)	38% (81/210)	2e-21	4	LPS responsive in isolated macrophages (Goetz *et al* 2004; Ref. 23)
EY975983	Upstream transcription factor 1(USF1) (zebrafish)	71% (49/69)	2e-16	2	Regulation of transcription; immune response (Corre and Galibert 2005; Ref. 14)
EY976034	Interleukin-1 receptor-like protein precursor (Atlantic salmon)	65% (30/46)	5e-11	2	Interleukin-1 receptor activity
EY976089	Novel immune-type receptor 4 (rainbow trout)	50% (77/153)	6e-26	2	Receptor activity
EY976541	CD63 (rainbow trout)	65% (75/114)	3e-21	2	Protein binding; cell adhesion regulation
EY976556	Toll-like receptor 23 (pufferfish)	75% (104/138)	2e-58	1	Transmembrane receptor activity; protein binding
EY976136	Lymphocyte antigen 75 (cow)	39% (64/163)	2e-31	1	Receptor-based antigen processing for MHC class I presentation (Bonifaz *et al* 2002; Ref. 9)
EY976820	Cell division cycle and apoptosis regulator 1 (CCAR1) (zebrafish)	41% (71/170)	3e-22	1	Apoptosis (Rishi *et al* 2006; Ref. 60); apoptosis regulation (Majumdar *et al* 2007; Ref. 46)
EY976002	E3 ubiquitin-protein ligase Itchy (mouse)	81% (122/150)	1e-67	1	Regulation of p73 stability; down-regulated by DNA damage (Rossi *et al* 2005; Ref. 64)
EY976504	Leukocyte elastase inhibitor (rainbow trout)	70% (46/65)	5e-38	1	Involved in caspase-independent apoptosis (Torriglia *et al* 2000; Ref. 82)
EY976417	Caspase 8 (dog)	48% (35/72)	3e-08	1	Caspase activity; regulation of apoptosis
EY976348	Mitogen-activated protein kinase 14a (zebrafish)	73% (36/49)	4e-14	1	Kinase activity
EY976608	MIP1alpha (Japanese flounder)	43% (38/88)	4e-14	1	CC chemokine activity
EY976661	Heat shock protein 90 (pink stalk borer)	93% (28/30)	6e-08	1	Regulation of progression through cell cycle; protein folding

EY976096	Complement receptor-like protein 1 (rainbow trout)	40% (43/106)	1e-18	1	Receptor activity

[1]All contigs and singletons from this SSH library were annotated using AutoFACT (36). The additional information (e.g. functional annotations, BLASTx statistics, and GenBank accession numbers for contributing ESTs) is listed in on-line Supplemental Table S1B.
[1-5]See footnotes for Table 2-2.

Table 2-4. Selected[1] transcripts identified in forward head kidney SSH library (designed to be enriched for genes up-regulated by bacterial antigens).

Accession Number[2]	BLASTx identification[3] of cod cDNAs			# of ESTs	Gene Ontology[4] or function of BLASTx hit[5]
	Name of BLASTx hit (species)	% ID (align)	E-value		
EY972828	Ferritin middle subunit (Atlantic salmon)	81% (127/155)	2e-65	8	Ferroxidase activity; iron ion homeostasis; oxidoreductase activity
EY972657	Ferritin heavy subunit (Atlantic salmon)	90% (158/174)	3e-89	6	Ferroxidase activity; iron ion homeostasis; oxidoreductase activity
EY973285	Proteasome activator subunit 2 (common carp)	71% (113/158)	4e-45	3	Antigen cleavage and presentation (Sijts *et al* 2002; Ref. 73)
EY972595	Cathelicidin 1 (Atlantic cod)	98% (117/119)	3e-62	3	Defense response
EY972694	Cathepsin L (Japanese ricefish)	77% (98/126)	2e-58	3	Antigen processing (Zhang *et al* 2001; Ref. 90)
EY972725	Goose-type lysozyme 1 (Atlantic cod)	97% (143/146)	3e-73	3	Lysozyme activity
EY973198	Translationally-controlled tumor protein (common carp)	66% (104/156)	8e-55	2	B cell growth factor (Kang *et al* 2001; Ref. 32); Interleukin production (Bheekha-Escura *et al* 2000; Ref. 7)
EY972718	Probable Bax inhibitor 1 (Japanese flounder)	88% (105/118)	9e-54	2	Negative regulation of apoptosis
EY972894	cellular FLICE-like inhibitory protein (pig)	63% (26/41)	4e-07	2	Caspase activity; regulation of apoptosis
EY972979	Cyclin L1 (African clawed frog)	96% (24/25)	8e-07	1	Cell division
EY972692	Myeloid cell leukemia sequence 1b (zebrafish)	46% (49/106)	1e-16	1	Negative regulation of apoptosis (Lomo *et al* 1996; Ref. 42)
EY972562	CD84 (mouse)	26% (48/180)	1e-05	1	Lymphocytes proliferation macrophage activation (Tangye *et al* 2002; Ref. 79)
EY972791	Src family associated phosphoprotein 2 (Skap2) (*Astatotilapia burtoni*)	65% (78/120)	6e-25	1	Regulation of leukocytes adhesion (Togni *et al* 2005; Ref. 81)
EY973092	Lipopolysaccharide binding protein variant b (Atlantic cod)	98% (94/95)	6e-37	1	LPS binding (Stenvik *et al* 2004; Ref. 76)
EY973172	Interleukin 5 receptor alpha (rat)	25% (32/124)	2e-06	1	Interleukin-5 receptor activity
EY972529	Inhibitor of nuclear factor kappa B alpha (rainbow trout)	88% (23/26)	7e-05	1	LPS-inducible (Sangrador-Vegas *et al* 2005; Ref. 68)
EY972872	Complement receptor-like protein 1 precursor (rainbow trout)	44% (34/76)	8e-15	1	Receptor activity

[1]All contigs and singletons from this SSH library were annotated using AutoFACT (36). The additional information (e.g. functional annotations, BLASTx statistics, and GenBank accession numbers for contributing ESTs) is listed in on-line Supplemental Table S1C.

[1-5]See footnotes for Table 2-2.

Table 2-5. Selected[1] transcripts identified in reverse head kidney SSH library (designed to be enriched for genes down-regulated by bacterial antigens)

Accession Number[2]	BLASTx identification[3] of cod cDNAs			# of ESTs	Gene Ontology[4] or function of BLASTx hit[5]
	Name of BLASTx hit (species)	% ID (align)	E-value		
EY973973	Cyclin B2 (rainbow trout)	51% (82/158)	4e-30	5	Regulation of progression through cell cycle
EY974252	TRAF4 associated factor 1 (human)	30% (45/147)	6e-08	4	Signal transduction; down- regulated in nitric oxide-exposed human monocytic cells (Turpaev *et al* 2005; Ref. 84)
EY974460	Tyrosine kinase 2 (human)	67% (66/98)	1e-32	2	Signal transduction; Jak-STAT signaling pathway
EY974533	Heat shock 60kDa protein 1 (chicken)	57% (57/100)	4e-25	2	Response to stress
EY974525	Heat shock 90kDa protein 1 beta isoform b (rainbow trout)	98% (98/100)	8e-49	2	Response to stress
EY974305	Scavenger receptor class B member 2 (cow)	50% (45/90)	1e-23	1	Receptor activity
EY974340	HSP90 co-chaperone Cdc37 (green pufferfish)	74% (117/157)	3e-43	1	Regulation of cell cycle; protein folding
EY974279	Cathepsin B (Atlantic halibut)	46% (31/66)	8e-07	1	Regulation of catalytic activity; cysteine-type endopeptidase activity
EY974635	Cytochrome P450 (European sea bass)	60% (89/146)	2e-48	1	Monooxygenase activity; iron ion binding; oxidoreductase activity; heme binding

[1]All contigs and singletons from this SSH library were annotated using AutoFACT (36). The additional information (e.g. functional annotations, BLASTx statistics, and GenBank accession numbers for contributing ESTs) is listed in on-line Supplemental Table S1D.

[1-5]See footnotes for Table 2-2.

Table 2-6. Primers used for QPCR.

Primer name[1]	QPCR Oligonucleotide Sequences (5'-3')	Gene Name of the Top BLASTx hit	Amplicon size (bp)	Efficiency[2] (%)
CAMP-f	ATTGCAATTTCACCCTGAGC	Cathelicidin	118	94
CAMP-r	CCAGACCTGCTCCTTCTCAC			
IL8-f	CCAATCTGACGGCTCTCTGT	Interleukin 8	116	103
IL8-r	ATCGGCTCCCTACTGGTTCT			
FTH-f	TCGAGAAAGTGGGTCTCGAT	Ferritin heavy subunit	168	97
FTH-r	AGACGTCAGGAAGCCAGAAA			
IRF1-f	AGAAGGACGCCAGTCTGTTCAA	Interferon regulatory	100	86
IRF1-r	GCGGAAGTTGGCTTTCCATT	factor 1		
SCYA-f	CTCAAACCTCTGCATCGTCA	Small inducible	188	96
SCYA-r	CACGGAGAGGTAAGCAGCTC	cytokine SCYA 104		
IL1βR-f	ACATCATGCAGCGCTTCTC	Interleukin 1β receptor	101	86
IL1βR-r	TTTGCCCTCAAGGTCCTG	like precursor		
IL1β-f	ACAGGAAGTGCACCATGTCA	Interleukin 1β	107	95
IL1β-r	GTCGTGCACACAGAAAGCAG			
MCL-f	CGCAGACAGCACAACAAACT	Myeloid cell leukemia	102	101
MCL-r	GACACGCAGCCTTCTTTACC	sequence 1		
HAMP-f	CCACAGGCTCCTCTCAAGTC	Hepcidin	146	89
HAMP-r	CTGCAACTGCAATGCTGAAT			
BTF3-f	AGCTCGGCGTCAACAATATC	Basic transcription	159	89
BTF3-r	GCATCTCTGTCAGCTGCTTG	factor 3		
18S-f	ATGGCCGTTCTTAGTTGGTG	18S ribosomal RNA	180	109
18S-r	GGACATTTAAGGGCGTCTCA	(normalizer gene)		

[1]Primer direction is denoted by "f" or "r" following the gene name for forward or reverse, respectively.
[2]The calculation of amplification efficiency using a standard curve is described in Materials and Methods.

2.9 Figures

Figure 2-1

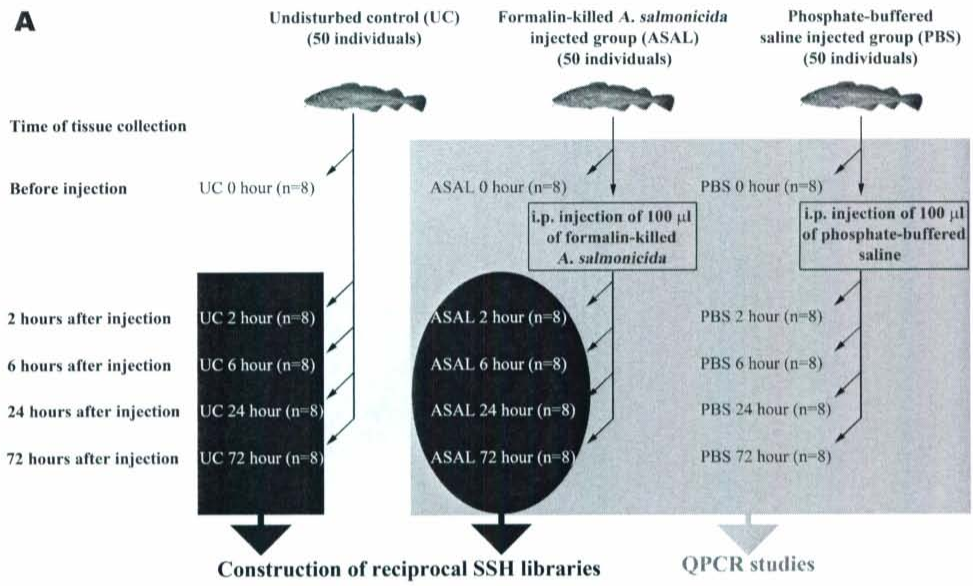


Figure 2-1. The experimental design, sampling strategy, and the construction of reciprocal SSH libraries. (A) The sampling strategy for the tissue collection from 3 experimental groups (UC, PBS, and ASAL). For UC and ASAL groups, individuals from the 2 h, 6 h, 24 h, and 72 h were sampled and selected for the construction of reciprocal SSH libraries (highlighted in black). For the PBS and ASAL groups, the individuals from all 5 time points were selected and used for QPCR studies (highlighted in grey, see Materials and Methods for additional information). (B) The selection of individuals for construction of reciprocal SSH libraries enriched for immune tissue transcripts that are responsive to bacterial antigen (formalin-killed, atypical *A. salmonicida*). The UC and ASAL individuals selected for the construction of the SSH libraries are represented by circles and squares, respectively. The presence of a product in the gel image indicates positive nodavirus carrier status (see Materials and Methods for information on accessment for nodavirus carrier status).

Figure 2-2

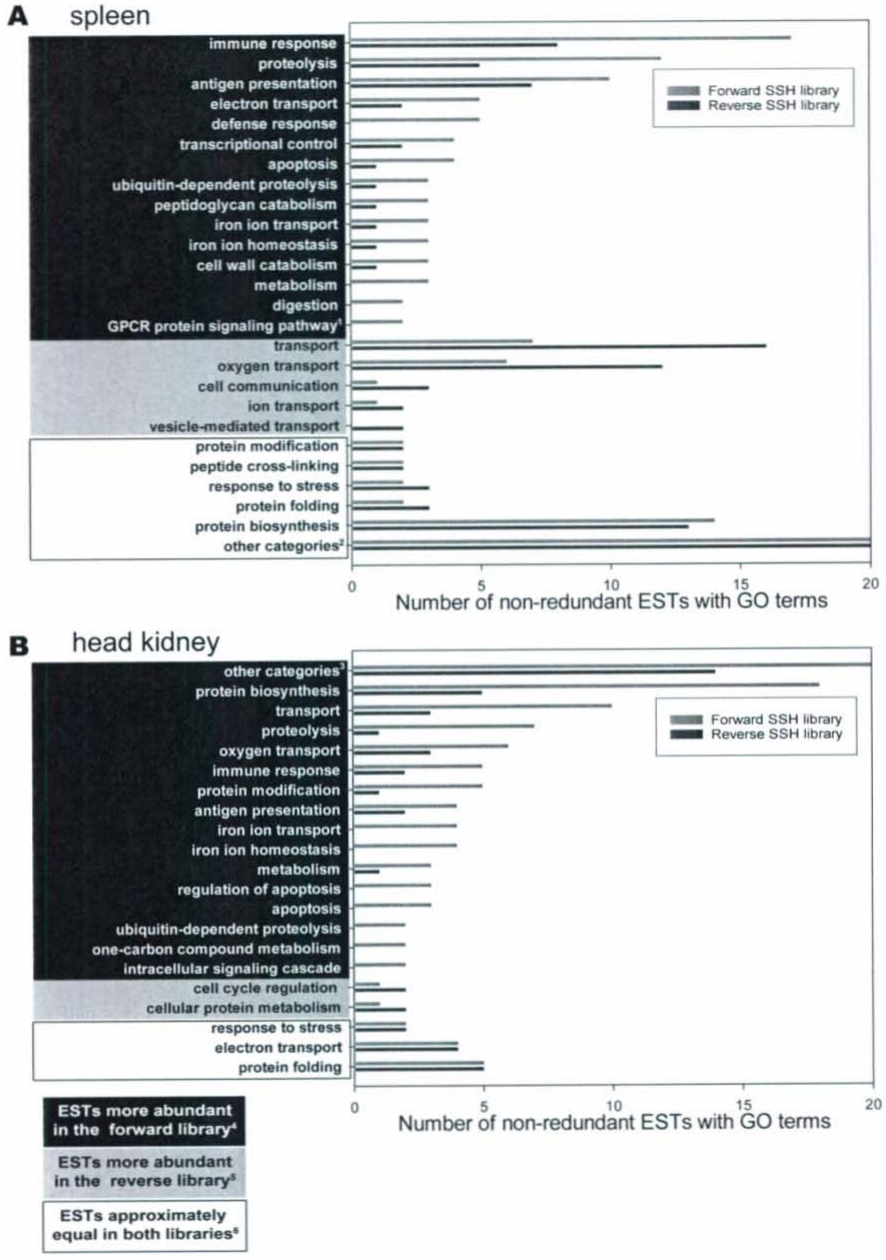

A spleen
immune response
proteolysis
antigen presentation
electron transport
defense response
transcriptional control
apoptosis
ubiquitin-dependent proteolysis
peptidoglycan catabolism
iron ion transport
iron ion homeostasis
cell wall catabolism
metabolism
digestion
GPCR protein signaling pathway[1]
transport
oxygen transport
cell communication
ion transport
vesicle-mediated transport
protein modification
peptide cross-linking
response to stress
protein folding
protein biosynthesis
other categories[2]
Forward SSH library
Reverse SSH library
0
5
10
15
20
Number of non-redundant ESTs with GO terms
B head kidney
other categories[3]
protein biosynthesis
transport
proteolysis
oxygen transport
immune response
protein modification
antigen presentation
iron ion transport
iron ion homeostasis
metabolism
regulation of apoptosis
apoptosis
ubiquitin-dependent proteolysis
one-carbon compound metabolism
intracellular signaling cascade
cell cycle regulation
cellular protein metabolism
response to stress
electron transport
protein folding
Forward SSH library
Reverse SSH library
0
5
10
15
20
Number of non-redundant ESTs with GO terms
ESTs more abundant in the forward library[4]
ESTs more abundant in the reverse library[5]
ESTs approximately equal in both libraries[6]

Figure 2-2. Gene classification of spleen (A) and head kidney (B) reciprocal SSH libraries of Atlantic cod based on Gene Ontology (biological process terms). GO annotations were obtained using AutoFACT and Goblet analysis of clusters. For the complete lists of GO annotations by biological process, see Supplemental Table S3A-D. Superscripts: [1]G protein coupled receptor (GPCR) protein signaling pathway. [2]For the head kidney SSH libraries, "other categories" include 38 and 14 assembled ESTs with GO biological process terms in the forward and reverse libraries, respectively. [3]For the spleen SSH libraries, "other categories" include 33 and 28 assembled ESTs with GO biological process terms in the forward and reverse libraries, respectively. [4]For a given GO biological process term, if the number of ESTs present in the forward SSH library was 2 and/or 50% more than in the reverse SSH library. [5]For a given GO biological process term, if the number of ESTs present in the reverse SSH library was 2 and/or 50% more than in the forward SSH library. [6]For a given GO biological process term, if the difference between the numbers of ESTs present in the SSH libraries was less than 2 and/or 50% of the smaller number.

Figure 2-3 page 1

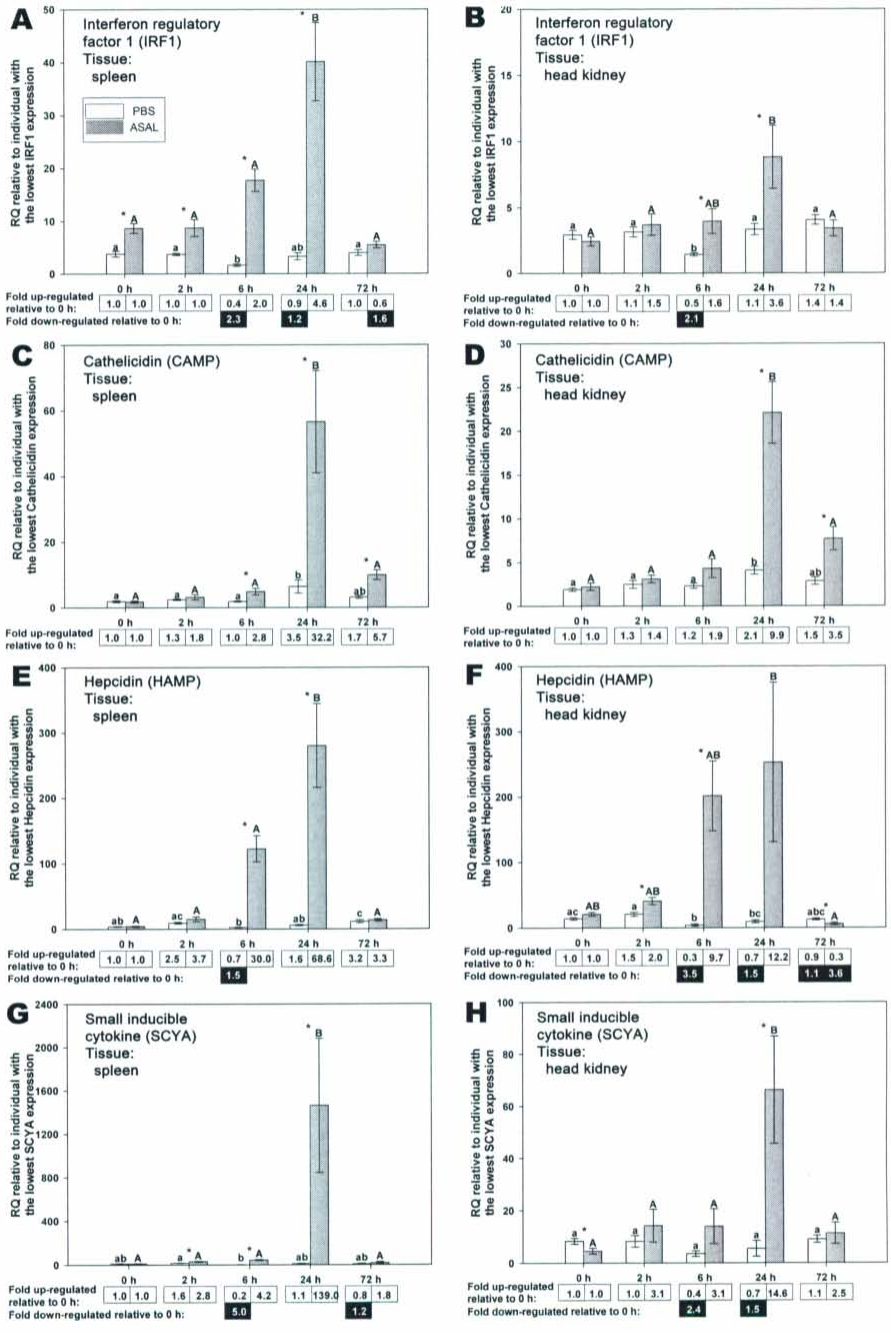


Figure 2-3 page 2

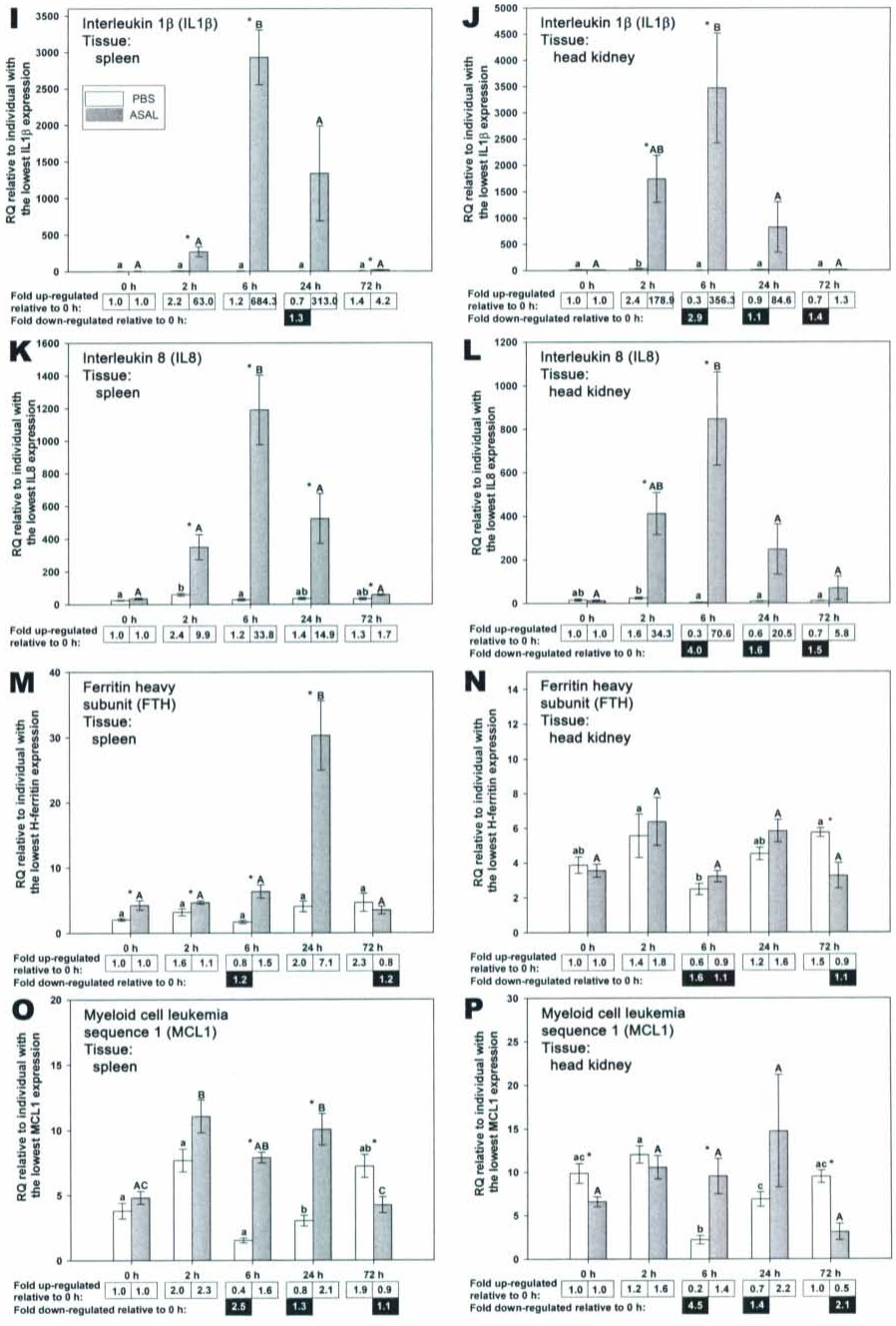
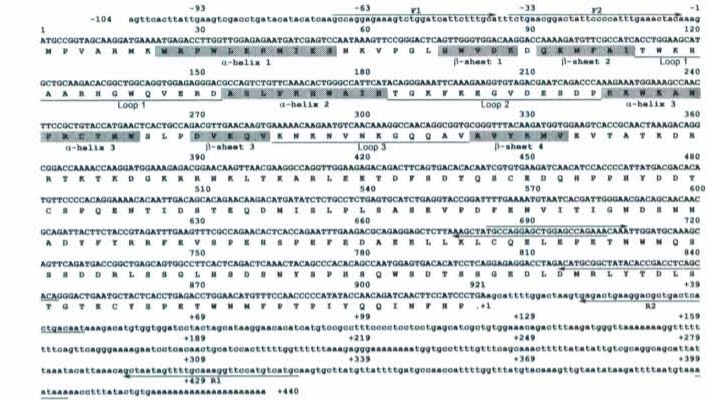


Figure 2-3. QPCR analyses of selected genes identified in the SSH libraries. Gene expression data are presented as means (± standard error). RQ (relative quantity) values were normalized to 18S ribosomal RNA and calibrated to the individual with the lowest gene of interest expression. Within each gene of interest study, identical letters (upper case for *A. salmonicida*-treated (ASAL) gene expression data, lower case for saline-injected (PBS) control data) indicate no significant difference ($p > 0.05$) between the groups at the different time points post-injection. Asterisks identify significant ($p \leq 0.05$) differences between *A. salmonicida*-treated and saline- injected control groups at a particular time point. For each condition and time point (e.g. ASAL, 24 h), fold up-regulation was calculated as (average RQ)/(average RQ for the appropriate 0 h control group), and fold down-regulation where appropriate was calculated as the inverse of fold up-regulation.

Figure 2-4 page 1

A



B

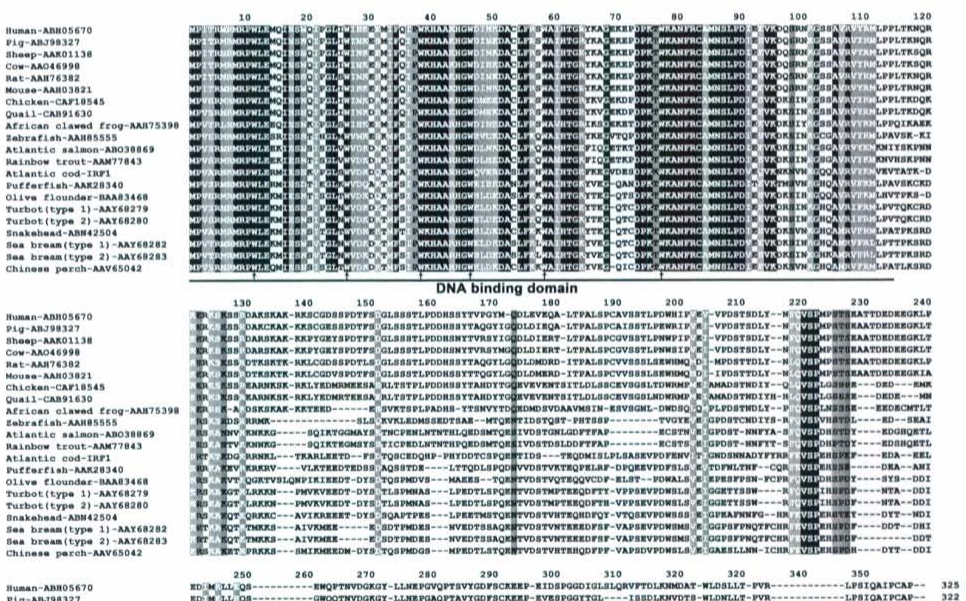


Figure 2-4 page 2

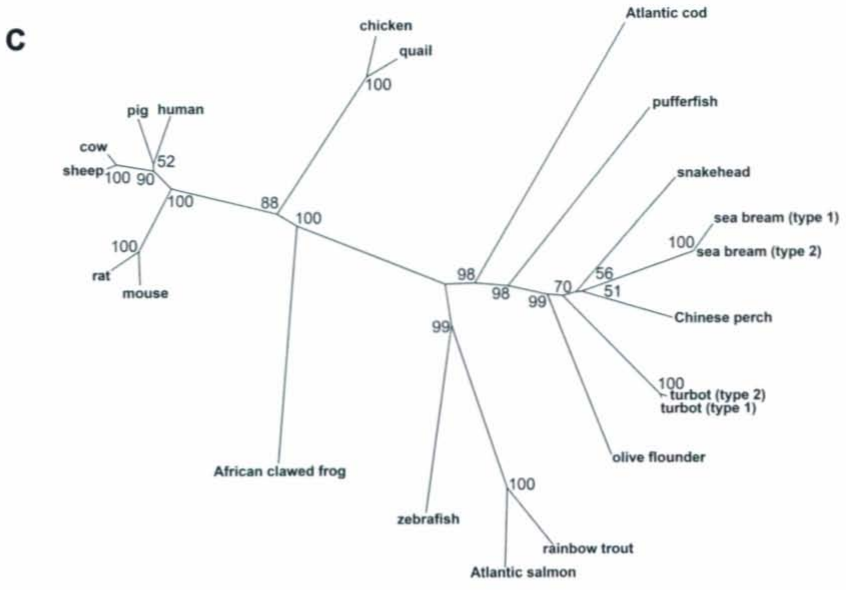


Figure 2-4. Characterization and molecular phylogenetic analysis of Atlantic cod (*Gadus morhua*) interferon regulatory factor 1 (IRF1). (A) Nucleotide and deduced amino acid sequences of Atlantic cod IRF1. The 5' and 3' untranslated regions (UTR) are shown in lower case letters, and the coding region is shown in upper case letters. Above the nucleotide sequence, the UTR 5' of the start codon is numbered 3' to 5', and distinguished by a negative (-) sign. The open reading frame and the 3' UTR are numbered 5' to 3' separately, with the numbering for the 3' UTR distinguished by a positive (+) sign. The polyadenylation signal is underlined and labelled. Forward priming sites are marked by 5'→3' arrows above the nucleotide sequence, and reverse priming sites are marked by 3'→5' arrows below the nucleotide sequence. The predicted structural features of the DNA binding domain are marked as follows: the AA residues comprising the 3 a-helixes and 4 b-sheets are highlighted in grey; the AA residues belonging to the 3 long loops are underlined. (B) Multiple alignment of amino acid sequences of Atlantic cod IRF1 with homologous sequenes from other species retrieved from the NCBI protein database (see Supplemental Table S4). The conserved tryptophan residues are marked by arrows below the amino acid alignment. Identical residues and conservative substitutions (printed in white letters) are highlighted in black and grey, respectively. Semi-conservative substitutions (printed in black letters) are highlighted in grey. Individual sequences are designated by the abbreviation of species names followed by GenBank accession numbers. The scientific names are as follows: Human, *Homo sapiens*; Pig, *Sus scrofa*; Sheep, *Ovis aries*; Cow, *Bos taurus*; Rat, *Rattus norvegicus*; Mouse, *Mus musculus*; Chicken, *Gallus gallus*; Quail, *Coturnix japonica*; African clawed

frog, *Xenopus tropicalis*; Zebrafish, *Danio rerio*; Atlantic salmon, *Salmo salar*; Rainbow trout, *Oncorhynchus mykiss*; Atlantic cod, *Gadus morhua*; Pufferfish, *Takifugu rubripes*; Olive flounder, *Paralichthys olivaceus*; Turbot, *Sparus aurata*; Snakehead, *Channa argus*; Sea bream, *Scophthalmus maximus*; Chinese perch, *Siniperca chuatsi*. (C) The relationship between Atlantic cod IRF1 and putative orthologs from other vertebrates. Based on the multiple alignment of the amino acid sequences, the unrooted phylogenetic trees were constructed by the neighbour-joining method using the CLUSTALX, and were bootstrapped 10,000 times. The bootstrapped phylogenetic trees were plotted using MEGA4 (76), and the bootstrap values (percentages) are marked at the branch point.

CHAPTER 3

Characterization and expression analyses of anti-apoptotic Bcl-2-like genes NR-13, Mcl-1, Bcl-X1, and Bcl-X2 in Atlantic cod (*Gadus morhua*)

3.1 Abstract

NR-13, Mcl-1, and BCL-X_L, are conserved anti-apoptotic proteins that belong to the anti-apoptotic Bcl-2 sub-family, which inhibits cell death by preventing mitochondrial membrane permeabilization (MMP). Given the anti-apoptotic functions of these proteins in vertebrates (e.g. human, mouse, and zebrafish), and the involvement of apoptotic regulation in immune responses, I determined the sequences of these genes and studied their expression in Atlantic cod (*Gadus morhua*) in response to viral and bacterial stimuli. Based on previously generated Atlantic cod expressed sequence tags (ESTs), I identified partial cDNA sequences of putative orthologues of Atlantic cod NR-13, Mcl-1, and Bcl-X, and obtained the full-length cDNA, genomic, and promoter region sequences for these genes. The analyses of Atlantic cod cDNA sequences, and comparisons of the cod deduced amino acid sequences to putative orthologues in other species, revealed the presence of highly conserved Bcl-2 homology (BH) and transmembrane (TM) domains in the Atlantic cod sequences. Analysis of gene structure revealed conserved intron/exon boundaries within the coding regions of human and Atlantic cod putative orthologues. I found that an intron/exon boundary immediately following the codon for the 8^{th} residue (tryptophan) of the BH2 domain exists in all anti-apoptotic Bcl-2 sub-family genes regardless of vast evolutionary distance. I also identified a non-coding exon in the Atlantic cod NR-13-like gene, which appears to be absent in its putative mammalian orthologues. Quantitative reverse transcription – polymerase chain reaction (QPCR) involving six tissues (blood, brain, gill, head kidney, pyloric caecum, and spleen) was used to study the constitutive transcript expression in non-stressed juvenile cod;

NR-13 and Bcl-X2 were most highly expressed in gill, whereas Mcl-1 and Bcl-X1 were most highly expressed in blood. In cod challenged with intraperitoneal (IP) injections of the viral mimic polyriboinosinic polyribocytidylic acid (pIC), 1) NR-13 mRNA expression was significantly up-regulated (compared to both 0 hour pre-injection and time matched saline injected controls) in spleen at 6 hours post-injection and in head kidney at both 6 and 24 hours post-injection (HPI), and 2) both Mcl-1 and Bcl-X2 were significantly up-regulated (compared to both 0 hour pre-injection and timed saline injected controls) in spleen at 6 HPI. QPCR was used to show that, in cod challenged with IP injections of formalin-killed, atypical *Aeromonas salmonicida* (ASAL), only NR-13 appeared to be responsive (significantly up-regulated in spleen at 6 HPI compared to 0 hour pre-injection controls). Interestingly, QPCR showed that saline injection had a mild (less than 3-fold) but significant inductive effect (compared to 0 hour pre-injection controls) on both NR-13 and Mcl-1 transcript expression in spleen at 2 HPI. Although I only obtained partial cDNA and genomic sequences for Bcl-X2, sufficient evidence was accumulated to show that two Bcl-X paralogues exist in Atlantic cod, possibly due to the teleost-specific genome duplication event. Promoter regions for NR-13, Mcl-1, and Bcl-X1 were obtained and analyzed for the first time in fish, and potential regulatory sites (e.g. putative NF-κB binding sites) that were found in the promoter regions of NR-13 and Mcl-1 may account for their transcriptional activation by pIC.

3.2 Introduction

Apoptosis is an evolutionarily conserved mechanism of programmed cell death, which is critically important for many biological processes such as development and homeostasis. A variety of pathogens have evolved abilities to either promote or inhibit apoptosis as part of their pathogenic mechanisms [reviewed in (31, 70)], and not surprisingly, vertebrate hosts have evolved mechanisms to manage apoptosis as part of their responses to pathogens [reviewed in (41)] and symbionts [e.g. (56)].

Members of the Bcl-2 family of genes and gene products are central regulators of apoptosis. They possess characteristic Bcl-2 homology (BH) domains, which account for their ability to dimerize and function as apoptotic regulators [reviewed in (9)]. The Bcl-2 family of genes consists of three sub-families: the Bax-like pro-apoptotic sub-family, the BH-3 only pro-apoptotic sub-family, and the Bcl-2 like anti-apoptotic sub-family [reviewed in (9)]. Most of the anti-apoptotic family members possess three or four BH domains, and most of them also possess a C-terminal transmembrane (TM) domain that is responsible for their localization to the cytoplasmic sides of intracellular membranes. The pro-apoptotic Bcl-2 members facilitate or directly trigger the permeabilization of the mitochondrial membrane, which leads to the release of caspase activators from the mitochondria thereby causing apoptosis. The pro-apoptotic Bcl-2 proteins [e.g. Bcl-2-associated X protein (Bax) and Bcl-2 antagonist/killer-1 (Bak)] are antagonized by their anti-apoptotic counterparts [i.e. the Bcl-2 like sub-family proteins, such as Bcl-2, Bcl-X_L, Mcl-1, and NR-13 (45)] which function by binding and sequestering the pro-apoptotic Bcl-2

proteins, thereby preventing mitochondrial membrane permeabilization (MMP) induced apoptosis [reviewed in (10)].

In human and mouse, there are 6 known members of the Bcl-2-like anti-apoptotic sub-family [Bcl-2, Bcl-X_L, Bcl-2-A1, Bcl-w, Mcl-1, and NRH (aliases for NRH: Bcl-2L10/Bcl-B/Diva/Boo)], which are crucial to the development and survival of lymphoid and myeloid cells [reviewed in (48)]. To date, orthologues of Bcl-2, Bcl-X_L, Mcl-1, and NRH have been identified in fish (39), and duplications of some members [e.g. Mcl-1 in zebrafish (39)] have also occurred possibly as a result of teleost-specific genome duplication (68). NR-13 was identified in quail neuroretina cells as a gene that was induced by Rous sarcoma virus (RSV) infection (27). NR-13 gene activation by $P60^{v\text{-}src}$ (a viral tyrosine kinase) or v-*rel* (a proto-oncogene) has been documented and accounts for the apoptotic inhibition involved in viral pathogenesis or oncogenesis, respectively (45, 47). Subsequent studies of NR-13 orthologues in mammals confirmed their roles as antagonists of apoptosis (3, 34, 37). Recently, the zebrafish NR-13 orthologue was identified, which was shown to antagonize the pro-apoptotic Bax, and play a key role in development (5). Zebrafish orthologues of Mcl-1 and Bcl-X_L have also been identified (35). In addition to the anti-apoptotic functions of Mcl-1 and Bcl-X_L observed in zebrafish embryo (39), over-expression of either zebrafish Mcl-1 or Bcl-X_L protected beta-nodavirus-infected GL-av (a fish cell line) cells from necrotic cell death (15, 16).

Many pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) and double stranded RNA (dsRNA), are known inducers of apoptosis in certain types of fish cells. For example, in goldfish (*Carassius auratus*) lymphocytes, LPS-induced apoptosis was found to be associated with

down-regulation of anti-apoptotic Bcl-2 expression and up-regulation in pro-apoptotic Bax expression (73). The apoptotic effect of polyriboinosinic polyribocytidylic acid (pIC, a synthetic dsRNA viral mimic) has also been demonstrated using rainbow trout (*Oncorhynchus mykiss*) macrophage RTS11 cells (23). In many *in vivo* studies, similar PAMPs have been used to elicit host immune responses. previous functional genomic studies [e.g. immune-relevant gene discovery and expression analyses using suppression subtractive hybridization (SSH) cDNA libraries and quantitative reverse transcription - polymerase chain reaction (QPCR), respectively] involving Atlantic cod stimulated with immunogens [e.g. formalin-killed, atypical *Aeromonas salminocida* (ASAL) or pIC] led to the identification of numerous expressed sequence tags (ESTs) that are involved in the Atlantic cod (*Gadus morhua*) innate immune response (24, 58). While the analysis of functional annotations associated with significant BLAST hits of these previously generated Atlantic cod ESTs suggested the involvement of apoptotic regulation in the cod immune response to bacterial and viral stimuli, further studies are needed to fully understand the role of apoptotic regulation in cod innate immunity. In this study, I report the identification and characterization of Atlantic cod putative orthologues of NR-13, Mcl-1, and Bcl-X1, and the identification and partial characterization of Atlantic cod Bcl-X2. For these four genes, I present constitutive gene expression in six juvenile Atlantic cod tissues, and their expression following intraperitoneal injection with ASAL, pIC, or control saline. In addition, I present the gene structure and promoter regions of teleost NR-13, Mcl-1, and Bcl-X1 for the first time. Throughout this paper, the word "orthologue" is used to describe the most similar known amino acid sequences

between species. While these sequences should be termed "putative orthologues", I often omit the word "putative" to improve the readability of the text.

3.3 Materials and Methods

Challenges with bacterial/viral mimics and tissue collection

Juvenile Atlantic cod (~ 25 g) from a single family [Family 32, CGP (Atlantic Cod Genomics and Broodstock Development Project, http://codgene.ca) 2006 year class] were divided into 3 tanks for 3 experimental groups, which were injected with phosphate-buffered saline (PBS, to control for the treatment), formalin-killed atypical *Aeromonas salmonicida* (ASAL) and polyriboinosinic polyribocytidylic acid (pIC). The antigen preparation, stimulation procedure, and tissue sampling were described previously (24, 58). Briefly, for each of the 3 experimental groups (referred to as PBS, ASAL, and pIC hereafter), the spleen and hematopoietic kidney (head kidney) tissues from 8 individuals were collected immediately prior to the injection (0 h time point), and at 2 h, 6 h, and 24 h post-injection (HPI). To study constitutive gene expression across tissues, the blood, brain, gill, head kidney, pyloric caecum, and spleen were collected for 6 non-injected (i.e. 0 h time point) fish from the same family. All sampled tissues were flash frozen in liquid nitrogen, and stored at -80°C until used for RNA extraction. RNA samples were DNase-I treated and column-purified as previously described (24, 58).

Identification of transcripts encoding anti-apoptotic Bcl-2 sub-family proteins

Mining of the CGP EST database (see Web Site References) revealed several transcripts representing anti-apoptotic Bcl-2 sub-family members from cDNA

libraries that were enriched for transcripts responsive to pIC and ASAL stimulations (Table 3-1). These transcripts were later identified as NR-13 and Mcl-1 by BLASTx (see the results section for details). In the search for additional anti-apoptotic Bcl-2 sub-family members, zebrafish Bcl-X_L (GenBank accession no. AAK81706) and Bcl-2 (GenBank accession no. AAI33849) amino acid sequences were used to query the CGP EST database using tBLASTn. This approach resulted in identification of clones representing two Bcl-2-related (Bcl-X) transcripts (referred to as Bcl-X1 and Bcl-X2 as discussed in the results section, also see Table 3-1). However, I was unable to find ESTs representing Bcl-2 in the CGP EST database. For each of the transcripts, contributing ESTs were assembled to produce a contiguous sequence (contig) using the SeqMan function of Lasergene 7.20 software package (DNASTAR), and the putative coding region within a given contig was determined based on BLASTx alignments.

Isolation of cDNA sequences for cod NR-13, Mcl-1, Bcl-X1, and Bcl-X2

The RNA ligase-mediated (RLM) rapid amplification of cDNA ends (RACE) approach was employed to obtain the full-length cDNA for target genes. Using a commercial RLM-RACE kit (Invitrogen), full-length RACE-ready cDNA was synthesized using 5 µg of the total RNA extracted from the spleen of an individual juvenile cod that was stimulated with ASAL 24 h before tissue collection (24). For all 4 genes involved in this study, gene-specific primers (GSPs) were designed based on appropriate contigs (Table 3-2), which were used for 5'-RACE, 3'-RACE, and open reading frame (ORF) PCRs (see Fig. 3-1 for schematics). All RACE PCRs were conducted using the same protocol, in which a touch-down PCR followed by a nested

PCR were conducted as specified in the GeneRacer Kit manual with the extension time set to 3 minutes for all cycles. Using the same full-length cDNA generated for RACE PCRs as template, nested PCRs were also conducted to obtain a 749 bp fragment of Bcl-X1 cDNA using the following cycling protocol: 1 cycle of 2 min at 94°C, 25 cycles of (30 seconds at 94°C, 30 seconds at 70°C, 3 min at 72°C), and 1 cycle of 10 min at 68°C (see Fig. 3-1C for details). To obtain the full-length cDNA for target transcripts, the overlapping RACE products and cDNA fragment were assembled using the SeqMan function of Lasergene 7.20 software package (Fig. 3-1).

Determining if the human-equivalent splice variant of the Mcl-1 transcript is present in spleen of bacterial antigen (ASAL)-stimulated Atlantic cod

The mRNA used was from the "ASAL-stimulated" pool used for SSH library construction as previously described (24). Briefly, pooled spleen RNA from a total of 20 ASAL-stimulated cod (with each fish contributing 10 µg of total RNA to the pool) was used for mRNA isolation using the MicroPoly (A) Purist Small Scale mRNA Purification Kit (Ambion, Austin, TX). Using 1 µg of the mRNA generated from that previous study as template, full-length cDNA was generated using the SMARTer RACE cDNA amplification kit following the manufacturer's instruction (Clontech), and the full-length cDNA (10 µl) was diluted to a final volume of 260 µl. Based on the gene organization of cod Mcl-1 (discussed in the subsequent section), primer pairs (Mcl-1_F1, Mcl-1_F2, Mcl-1_R6, and Mcl-1_R5; Table 3-2) were designed in the first and the third (last) exon for cDNA PCRs to determine if skipping of the second exon occurs in transcription of the cod Mcl-1 gene as previously observed in human (known as Mcl-1_S) (8). Using 2.5 µl of the full-length cDNA (~ 4 ng of input mRNA)

as template, the nested PCRs were conducted using the Advantage 2 Polymerase kit (Clontech) follow the manufacturer's instructions, and the same cycling protocol was followed as for the Bcl-X1 ORF PCR (described in the previous paragraph). The PCR product was visualized on a 1% agarose gel stained with ethidium bromide, and the 100 bp DNA ladder was used as the size marker (Invitrogen).

Isolation of promoter and genomic sequences

Genomic DNA was extracted from the fresh liver of a juvenile Atlantic cod (Family 32, CGP 2006 year class) using a genomic DNA isolation kit following the manufacturer's instructions (Promega, Madison, MI). Following a DNA integrity check by 0.6% agarose gel electrophoresis, 0.1 μg of the genomic DNA was used for genome-walking library construction using the GenomeWalker kit following the manufacturer's instructions (Clontech). Briefly, four aliquots of genomic DNA were restriction digested to completion by each of *Eco*RV, *Dra*I, *Pvu*II, and *Stu*I, followed by ligation with GenomeWalker adaptors (provided with the kit), creating 4 GenomeWalker libraries. In order to obtain the genomic and promoter region sequences for target genes, a combination of genome walking PCR and genomic approaches was utilized based on the sequence information generated using bi-directional RACE. (See Table 3-2 for the list of primers used, and Fig. 3-2 for schematics showing cloning strategies and primer locations.). All genome walking PCRs were performed following the GenomeWalker kit instructions. Briefly, the first round of PCR was performed using the adaptor primer 1 (AP1, Table 3-2) and a GSP, followed by a nested PCR performed using the adaptor primer 2 (AP2, Table 3-2) and a second GSP, and the extension time was set to 4 minutes for all cycles. The cycling

condition for all genomic PCRs were: 1 cycle of 2 min at 94°C, 5 cycles of (30 seconds at 94°C, 30 seconds at 72°C, 4 min at 72°C), 5 cycles of (30 seconds at 94°C, 30 seconds at 70°C, 4 min at 72°C), 25 cycles of (30 seconds at 94°C, 30 seconds at 68°C, 4 min at 72°C), and 1 cycle of 10 min at 68°C.

Cloning, sequencing, and sequence assembly

All PCR amplifications were performed using the Advantage 2 Polymerase kit (Clontech), and all PCR products were visualized on 1% agarose gels stained with ethidium bromide, and gel-extracted using the QIAQuick Gel Extraction kit (QIAGEN) following manufacturer's instructions. The extracted PCR product DNA was then ethanol precipitated, washed, air-dried, and resuspended in 7 µl of nuclease-free water using standard molecular biology techniques. To increase the cloning efficiency, large inserts (~ 1.2 kb or longer) were ligated into the pGEM-T-Easy vector (Promega, Madison, WI) at 4°C overnight. Smaller inserts (less than 1.2 kb) were cloned into PCR®4-TOPO® (Invitrogen) following the manufacturer's instructions. The recombinant plasmids were transformed into chemically competent One Shot® TOP10 competent cells (Invitrogen), and plated onto Luria broth (LB)/agar with 50 µg/ml carbenicillin. Individual colonies were grown overnight at 37°C in LB with 50 µg/ml carbenicillin, and plasmid DNA samples were isolated in the 96-well format using standard methods. The insert sizes of recombinant plasmids were determined by *Eco*RI (Invitrogen) digestion prior to sequencing. For each PCR product, 3 individual clones were sequenced as many times as needed to yield at least 6 fold coverage for every base pair by the ABI 3730 DNA Analyzer using the BigDye Terminator (Applied Biosystems) chemistry. For

each gene, all sequence fragments were assembled using the SeqMan function of the Lasergene 7.20 software package to generate the genomic assembly. Using the MegAlign function of the same package, the cDNA sequence obtained from bi-directional RACE was mapped to the appropriate genomic assembly to identify intron(s) and the upstream promoter region.

QPCR expression studies

The constitutive and treatment-induced expressions of Atlantic cod NR-13, Mcl-1, Bcl-X1, and Bcl-X2 were studied using quantitative reverse transcription – polymerase chain reaction (QPCR). The constitutive expression of each of the transcripts was assessed across 6 tissues (blood, brain, gill, head kidney, pyloric caecum, and spleen) collected from 6 non-stressed individuals (0 h controls). With PBS as a treatment control, mRNA expression of NR-13, Mcl-1, Bcl-X1, and Bcl-X2 in response to ASAL and pIC stimulations was studied in cod immune tissues (spleen and head kidney) at 4 time points (2, 6, and 24 HPI, and 0 h pre-injection control). All QPCRs were performed using Power SYBR Green I dye chemistry and the 7500 Real Time PCR system (Applied Biosystems). For all experimental groups, tissues, and time points, 6 individuals (i.e. those with consistent normalizer expression) from each group, tissue, and time point were used in the QPCR study. QPCR primers (Table 3-2) were designed using the Primer 3 program (60) (see Web Site References) based on the cDNA sequences generated with bi-directional RACE. Dissociation curves were run to ensure that primer pairs amplified single products, and no-template controls were also run to ensure that primer dimers were absent. The amplification efficiencies of primer pairs for Mcl-1 and 18S rRNA were determined previously (24,

58). The amplification efficiencies of the other primer sets were determined as previously described in (24, 58). Expression levels of the genes of interest were normalized to 18S ribosomal RNA, which was stably transcribed in all samples involved in the QPCR study.

For each sample, 1 μg of DNase I-treated and column-purified total RNA was reverse-transcribed using random primers (250 ng) and Moloney murine leukemia virus (M-MLV) Reverse Transcriptase (200 U, Invitrogen) at 37°C for 50 min in a final reaction volume of 20 μl [as described in Rise et al. 2008 (58)], and the resulting cDNA was diluted with nuclease-free H_2O to a final volume of 200 μl. PCR amplifications were performed using a 7500 Real Time PCR detection system (Applied Biosystems) using 13 μl reactions that contained 2 μl of diluted cDNA (10 ng input total RNA), 50 nM each of forward and reverse primer, and 1X Power SYBR Green PCR Master Mix (Applied Biosystems). The amplification program consisted of 1 cycle of 95°C for 10 min and 40 cycles of (95°C for 15 s and 60°C for 1 min), with the fluorescent signal measured at the end of each 60°C step. For each sample, the target transcript (gene of interest) and the normalizer (18S rRNA) were each run in duplicate (53) on the same plate. A small number of reactions (less than 1%, see the Results section for details) failed and were therefore removed from data analysis. The fluorescence thresholds and baseline were determined automatically using the 7500 Software Relative Quantification Study Application (Version 2.0; Applied Biosystems). In addition to the Ct (threshold cycle) values for each transcript, amplification efficiencies (Table 3-2) for each gene of interest and normalizer primer pairs were also incorporated into the calculation for relative quantity (RQ) using the 7500 software as described above, and the underlying

algorithm for the $2^{-\Delta\Delta CT}$ quantification method was explained in Livak and Schmittgen, (2001; Ref, 46).

QPCR data analysis

All RQ data are presented as mean ± standard error (SE). To compare gene expression across tissues (blood, brain, gill, head kidney, pyloric caecum, and spleen), the RQ values for each target gene were subjected to a one-way ANOVA with Tukey post-tests. To determine the effect of ASAL or pIC on gene expression, the RQ values were subjected to a two-way (main effects group and sampling time) analysis of variance (ANOVA). In addition, one-way ANOVA (for each group and sampling time point) with Tukey post-tests were conducted to determine: 1) whether PBS control gene expression (RQ values) at 2, 6, and 24 HPI differed significantly from that for the 0 h pre-injection control group from the PBS tank; 2) if gene expression of the ASAL group at each time point differed significantly from levels of gene expression for the 0 h pre-injection control group from the ASAL tank; 3) if gene expression of the pIC group at each time point differed significantly from levels of gene expression in the 0 h pre-injection control group from the pIC tank; and 4) if gene expression differed significantly among the PBS, ASAL, and pIC groups at each time point (2, 6, 24 HPI, and 0 h). All statistical tests were performed using Systat 12.0 (Systat Software Inc.) with the p value threshold set at ≤ 0.01.

Sequence analysis

The amino acid (AA) sequences of Atlantic cod NR-13, Mcl-1, Bcl-X1, and Bcl-X2, were deduced based on the cDNA sequence using the BLASTx and

SeqBuilder function of Lasergene 7.20 software package (DNASTAR). Using the pattern search function in SeqBuilder, the untranslated regions (UTRs) were scanned for RNA instability motifs [patterns: AUUUA and UUAUUUA(U/A)(U/A)], and polyadenylation signals (AAUAA). In addition, using the RegRNA (55) (also see Web Site References), the UTRs were also scanned for other regulatory features such as the cytoplasmic polyadenylation element (CPE) and IRES (internal ribosome entry site). The genomic sequence upstream of the transcription start was scanned for eukaryotic promoter elements using MatInspector Professional 8.0 [see both (11) and Web Site References], and only promoter elements with implications in immune response and regulation of apoptosis were identified. Each deduced amino acid sequence (Atlantic cod NR-13, Mcl-1, and Bcl-X1) was aligned with its putative orthologous amino acid sequences from other species (GenBank accession numbers listed in Table 3-3) using the CLUSTALX (version 2.09) program. The BH (Bcl-2 homology) domains were assigned based on amino acid sequence alignments, and BH domain signature sequences (PROSITE accession no. PS01080, PS01258, PS01259, and PS01260, see Web Site References), and the literature (1, 3, 14, 42). The putative transmembrane domains (TM) and nuclear localization signals (NLSs) were identified using PSORTII [(51), also see Web Site References]. For Mcl-1 amino acid sequences, PEST regions [amino acid sequences that are rich in proline (P), glutamic acid (E), serine (S), and threonine (T) residues] were predicted using PESTfind (see Web Site References). To determine the phylogenetic relationships among the anti-apoptotic Bcl-2 sub-family members, a phylogenetic tree was constructed using the MEGA4 (67). Briefly, an unrooted phylogenetic tree was constructed based on the multiple alignment of amino acid sequences by the neighbour-joining method,

bootstrapped 10,000 times, and the consensus tree was plotted using MEGA4. The intron/exon boundaries within the ORFs of Atlantic cod NR-13, Mcl-1, Bcl-X1, and Bcl-X2 were compared to their putative orthologues in human. To obtain the intron/exon boundaries for human putative orthologues, the cDNAs (see Table 3-3 for accession numbers) coding for human NRH, Mcl-1, and Bcl-X_L were BLASTn aligned against the human genome using the NCBI database (BLAST Assembled Genomes).

3.4 Results

Identification of Atlantic cod NR-13, Mcl-1, and Bcl-X transcripts

Twelve ESTs representing 11 clones from a total of 7 cDNA libraries enriched for immune-related transcripts (Table 3-1) were assembled into a contiguous sequence (contig) that was found (using BLASTx) to share significant similarity with chicken (*Gallus gallus*) NR-13 (GenBank accession no. AAK54806; 50% identical over 64 aligned amino acids, E-value = 3e-08). Forty-eight ESTs representing 47 clones from a total of 19 cDNA libraries (Table 3-1) were assembled into a contig with a translation that was significantly similar to Atlantic salmon (*Salmo salar*) Mcl-1 (GenBank accession no. CAJ90909, 46% identical over 252 aligned amino acids, E-value = 1e-53). In my search for additional anti-apoptotic Bcl-2 sub-family genes (see Materials and Methods for details), I assembled: 1) a contig based on 5 ESTs representing 4 cDNA clones from a total of 3 cDNA libraries (Table 3-1) with significant homology at the amino acid level to an Atlantic salmon Bcl-X (GenBank accession no. ACN11007; 61% identity over 201 aligned amino acids, E-value = 3e-53); and 2) a contig based on 3 ESTs representing 2 cDNA clones from a total of 2

cDNA libraries (Table 3-1) with significant homology at the amino acid level to a different Atlantic salmon Bcl-X (GenBank accession no. ACI68003, 84% identity over 64 aligned amino acids, E-value = 5e-25). To distinguish between these Bcl-X-like transcripts, the former was referred to as the Atlantic cod Bcl-X1 (Table 3-1) and the latter was referred to as the Atlantic cod Bcl-X2 (Table 3-1).

Cloning and sequence analysis of Atlantic cod NR-13, Mcl-1, and Bcl-X genes

I acquired and analyzed cDNA and genomic sequences (discussed in detail in the paragraphs below) to determine the genome organizations for NR-13, Mcl-1, Bcl-X1, and Bcl-X2 (note: Bcl-X2 was only partially resolved), which are schematically represented in Fig. 3-3. All introns identified in this study possess a classical "GT-AG" intron splicing motif.

Based on the NR-13 contig, primers were designed (Table 3-2) for 5' and 3' RACE. The overlapping sequences from RACE products (Fig. 3-1A) allowed the assembly of a full-length NR-13 cDNA that is 1428 bp long [excluding the poly (A) tail]. The transcript contains an ORF of 588 bp (195 AA), a 53 bp 5'-UTR, and a 787 bp 3'-UTR. The 3'-UTR of NR-13 contains 3 AUUUA pentamers (referred to as "AU pentamers" hereafter) that are embedded in two AU-rich (greater than 80%) regions (Fig. 3-4), which embody putative class I AU-rich elements (AREs) [reviewed in (7)]. In addition, near the poly (A) tail, a cytoplasmic polyadenylation element (CPE) is present which contains the canonical nuclear polyadenylation element (Fig. 3-4).

Following the isolation of full-length NR-13 cDNA, primers were designed to isolate the genomic region containing the Atlantic cod NR-13 gene (Table 3-2), from which a 4909 bp genomic sequence was compiled using overlapping genomic

sequences obtained from genome walking and genomic PCRs (detailed in Fig. 3-2A). Mapping of the 1428 bp [excluding the poly(A) tail] NR-13 full-length cDNA to the assembled genomic sequence revealed 3 exons and 2 introns that compose the NR-13 gene (Fig. 3-3, 3-4). The first exon is 49 bp in length, and encodes the entire 5'-UTR of the NR-13 mRNA. As this is the first report of the presence of a non-coding exon in a vertebrate NR-13 gene, the first intron (1257 bp in length) was verified by genomic PCR and sequencing (Fig. 3-2A).

To obtain the full-length Mcl-1 cDNA, primers (Table 3-2) were designed based on the Mcl-1 contig; a single 791 bp PCR product was obtained from the 5'-RACE, while two PCR products [881 bp and 464 bp excluding the poly (A) tail] were isolated from the 3'-RACE (Fig. 3-1B). The compilation of RACE PCR products resulted in two full-length Mcl-1 cDNA variants that were 1521 bp and 1104 bp in length. Although the Mcl-1 cDNA variants showed 100% identity over the 1104 bp aligned at the 5'-end, the longer variant possessed an extra sequence of 417 bp at the 3'-end and therefore had a longer 3'-UTR. Furthermore, for both cDNA variants, a polyadenylation element was located near the poly (A) tail (Fig. 3-5). Scanning of the Mcl-1 5'-UTR revealed an internal ribosomal entry site (IRES), while multiple RNA instability features were present in the 3'-UTR including: a total of 4 AU pentamers, an AU-rich region containing 2 of the AU pentamers, and two UUAUUUA(U/A)(U/A) nonamers (referred to as AU nonamers hereafter) [reviewed in (7)] (Fig. 3-5). To determine the genomic organization of Atlantic cod Mcl-1, a 2622 bp genomic DNA sequence containing the Mcl-1 gene was obtained (detailed in Fig. 3-2B), which allowed the mapping of Mcl-1 cDNA obtained from RACE. The

Atlantic cod Mcl-1 gene consists of 3 exons and 2 introns, and the alternative splicing of the third exon results in two cDNA variants of Mcl-1 (Fig. 3-3, 3-5).

Based upon the Bcl-X1 contig, primers (Table 3-2) were designed to amplify the putative ORF, followed by amplification of its 5'- and 3'- flanking sequences by bi-directional RACE PCRs (detailed in Fig. 3-1C) and a 1430 bp [excluding the poly (A) tail] full-length cDNA was assembled based on PCR products. The analysis of the Bcl-X1 cDNA reveals a 237 bp 5'-UTR, a 684 bp ORF (227 AA), and a 509 bp 3'-UTR [excluding the poly (A) tail]. The Bcl-X1 5'-UTR contains a 147 bp upstream ORF (uORF) and an IRES, while the 3'-UTR contains a canonical nuclear polyadenylation element 18 bp upstream of the poly (A) tail (Fig. 3-6). With primers designed based on the Bcl-X2 contig (Table 3-2), RACE PCRs were carried out, and 444 bp and 730 bp PCR products were obtained from 5'-RACE and 3'-RACE, respectively. The overlapping RACE PCR products allowed the assembly of a cDNA sequence of 1115 bp containing an in-frame ATG codon near the 5'-end. However, the sequence preceding the putative start codon appeared to be too short (16 bp) for a 5'-UTR (see Fig. 3-7 for details). In addition, the BLASTx analysis of this cDNA sequence indicated that the Atlantic cod Bcl-X2 putative ORF was missing 60-80 residues at the N-terminus based on a BLASTx alignment with Atlantic salmon Bcl-X (GenBank accession no. ACI68003, 83% identity over 156 aligned amino acids, E-value = 1e-71) and a variety of other vertebrate Bcl-X orthologues. Subsequent attempts to extend the 5'-RACE sequence with alternative primer sets (data not shown) were not successful. Based on a CLUSTALW (Megalign function of Lasergene 7.20) alignment of available sequence, Atlantic cod Bcl-X1 and Bcl-X2 are

56.7% and 68.3% identical to one another at the nucleotide and predicted amino acid level, respectively.

To determine the Bcl-X1 genomic organization, primers designed based on the Bcl-X1 cDNA (Table 3-2) were used for genome walking and genomic PCRs, and a 2684-bp genomic sequence including Bcl-X1 exons, introns, and 5'-flanking sequence was compiled (see Fig. 3-2C for details). Further analysis of the Bcl-X1 gene revealed a total of 3 exons, with the first exon encoding 90 bp of the Bcl-X1 5'-UTR. The putative Bcl-X1 ORF is encoded by the second and third exons, which are 687 bp and 653 bp in length, respectively (Fig. 3-6). Although similar approaches were utilized to obtain the Bcl-X2 genomic sequence (see Fig. 3-2D for details), the resulting 2580 bp Bcl-X2 genomic sequence included only part of the Bcl-X2 gene, consisting of an exonic sequence of 619 bp, preceded by an intronic sequence of 1961 bp (Fig. 3-7). Analysis of human and zebrafish Bcl-X genes {BLASTn of human Bcl-X_L cDNA (GenBank accession no. U72398) and zebrafish Bcl-X_L orthologue [alias zBIp1 in (39)] cDNA (GenBank accession no. AF317837) against the zebrafish and human genomes, respectively} indicated the presence of a large intron 2 (>55 kb and >25 kb for human and zebrafish Bcl-X genes, respectively). While the intron 2 for cod Bcl-X1 is relatively small (117 bp), considering the possible presence of a large intron in cod Bcl-X2, subsequent genome walking PCRs were not attempted for this gene.

NR-13, Mcl-1, Bcl-X1, and Bcl-X2 possess conserved BH and transmembrane domains

Multiple alignment of the deduced translations of Atlantic cod anti-apoptotic Bcl-2 sub-family transcripts with putative orthologous sequences from other species revealed many regions of homology (Fig. 3-8, 3-9, 3-10), especially within the BH domains that are characteristic of the Bcl-2 family. In addition, the PSORTII analysis (see Web Site References) revealed a conserved TM domain at the carboxyl-terminus for all putative proteins analyzed. Based on the overall amino acid identity (shown in Table 3-4), the putative Atlantic cod NR-13 protein shares the highest homology with other fish orthologues (above 60% identity, Table 3-4) followed by avian orthologues (~ 40% identity, Table 3-4), and was most diverged from its mammalian orthologues (~ 20% identity, Table 3-4). A conserved aspartate (D_{15}) residue within the BH4 region and a conserved arginine (R_{39}) residue within the BH3 region exist in all NR-13 putative orthologues, whereas a glutamic acid (E_{46}) residue is conserved only in the BH3 region of non-mammalian orthologues (Fig. 3-8). Furthermore, the BH and TM domains exhibited differences in degree of similarity across species (Table 3-4), with the BH2 domain being the most conserved of all domains across the NR-13 orthologues analyzed. The putative Atlantic cod Mcl-1 protein shares similarity with Mcl-1 orthologues from other species over the BH1-3 domains and a TM domain (Table 3-5 and Fig. 3-9). Although the N-terminus of the Mcl-1 protein displayed little amino acid identity across taxa, PEST sequences at the N-terminus were identified in all Mcl-1 orthologues (Fig. 3-9). With the exception of zebrafish Mcl-1a (13), a single PEST region (residue 74 to 111 in cod Mcl-1) was identified in putative fish Mcl-1 proteins, while the mammalian Mcl-1 proteins contain at least two PEST regions (grey highlighting in Fig. 3-9). Based upon multiple alignment analysis of predicted Bcl-X proteins (Fig. 3-10), highly conserved BH1-4 domains (above 60%

identity, Table 3-6), and a conserved TM domain (above 45% identity, Table 3-6) were evident across vertebrates. The putative Atlantic cod Bcl-X2 protein was not included in the multiple alignment due to possible truncation of the cDNA at the 5'-end (as discussed previously). The partial cod Bcl-X2 putative protein contains BH1-3 domains that were identified based on PROSITE BH domain sequences (PROSITE accession no.: PS01080, PS01258, and PS01259) (Fig. 3-7). Phylogenetic analysis of anti-apoptotic Bcl-2 sub-family proteins supported the contention that these Atlantic cod sequences were orthologues of NR-13, Mcl-1, and Bcl-X from other vertebrates (Fig. 3-11).

Conserved intron/exon boundaries exist within the coding regions of anti-apoptotic Bcl-2 sub-family genes

Schematic comparisons of domain structure and intron/exon boundaries in the coding regions of Atlantic cod anti-apoptotic Bcl-2 sub-family putative proteins and their human orthologues are shown in Fig. 3-12. In comparison with their human orthologues, the Atlantic cod genes not only encode conserved BH and TM domains, but also possess intron/exon boundaries at conserved locations within the coding regions (Fig. 3-12A). Furthermore, all 4 Atlantic cod anti-apoptotic Bcl-2 sub-family genes studied possessed an intron at the same location within their corresponding BH2 domain-encoding regions. To further investigate this phenomenon, the BH2 domains of representative members (NR-13, Mcl-1, Bcl-X, Bcl-2, Bcl-w, Bcl-2-A1, and CED-9) from the anti-apoptotic Bcl-2 sub-family were located and aligned (Fig. 3-12B), and both tBLASTn of protein sequences (accession numbers listed in Table 3-3) and BLASTn of their encoding cDNA sequences (see Table 3-3 for accession

numbers) against corresponding genome databases revealed a conserved intron/exon boundary immediately following the tryptophan (the 8th residue of the BH2 domain) codon (TGG).

Human-equivalent splice variant of the Mcl-1 transcript was not identified in spleen of bacterial antigen (ASAL)-stimulated Atlantic cod

It has been demonstrated in human that the skipping of the second exon in Mcl-1 transcription results in a cDNA variant containing only exons 1 and 3, referred to as the Mcl-1_S, which translates to a BH3-only protein with pro-apoptotic activity (6). Given the conserved genomic organization of Mcl-1 between cod and human, using cDNAs arising from the spleens of 20 fish that were stimulated with ASAL (see section *2.4* in Materials and Methods for details) as template, RT-PCR was performed with primer pairs located in exon 1 and 3 to search for an alternative product that does not contain exon 2. Agarose gel electrophoresis of the PCR product showed a single band at a size expected for the exon 2-containing product (Fig. 3-13), and a shorter product was not found.

Constitutive transcript expression analysis

Constitutive expression of anti-apoptotic Bcl-2 sub-family transcripts was evaluated by QPCR using tissues isolated from 6 individuals (Fig. 3-14A-D). The QPCR analysis showed that the 18S rRNA was transcribed at a similar level (all Ct values were within in 1 cycle, data not shown) in the following 6 tissues: blood, brain, gill, head kidney, pyloric caecum, and spleen. This finding supports the selection of 18S rRNA as the normalizer gene for quantifying the relative expression of target

genes in this study. The QPCR study (Fig. 3-14A, B, C, D) showed that all four genes were constitutively expressed at detectable levels in all six tissues examined. Constitutive NR-13 expression was highest in the gill, followed by blood and spleen; constitutive expression of NR-13 was significantly ($p < 0.01$) higher in the gill and blood than in the head kidney, brain, or pyloric caecum. Constitutive Mcl-1 expression was highest in the blood, followed by gill and spleen; constitutive expression of Mcl-1 was significantly higher in the blood and gill than in the head kidney, brain, or pyloric caecum. Constitutive Bcl-X1 expression was highest in the blood, followed by brain and gill; constitutive expression of Bcl-X1 was significantly higher in the blood, brain, and gill than in the head kidney or pyloric caecum. Constitutive Bcl-X2 expression was highest in the gill, although there were no significant differences in constitutive Bcl-X2 expression between the tissues that were studied. The Bcl-X1 and Bcl-X2 paralogues exhibited distinct constitutive expression profiles, with Bcl-X1 constitutive expression ranging widely across tissues (with significant differences between tissues as previously noted) and Bcl-X2 constitutive expression exhibiting a much more narrow range (with no significant differences between tissues) (Fig. 3-14).

Transcript expression analysis following in vivo exposures to viral and bacterial antigens

Quantitative reverse transcription – polymerase chain reaction (QPCR) was used to examine the expression of NR-13, Mcl-1, Bcl-X1, and Bcl-X2 in spleen and head kidney of juvenile Atlantic cod before treatment (0 h control) and at 3 time points (2, 6, and 24 h) following IP stimulation with a viral mimic (pIC), a bacterial

antigen (ASAL), or a sham injection control (PBS) (Fig. 3-15). In cod challenged with pIC, NR-13 mRNA expression was significantly up-regulated (compared to 0 h pre-injection controls) in spleen at 6 hours post-injection (HPI) (11.2 fold); 6 h pIC treated spleen NR-13 expression was also significantly higher than NR-13 expression in the 6 h PBS control or ASAL groups (Fig. 3-15A). In head kidney, the NR-13 expression was significantly up-regulated by pIC at both 6 HPI (3.6 fold) and 24 HPI (2.1 fold) in comparison to the 0 h control, and NR-13 expression at these time points was significantly higher than in the timed PBS or ASAL groups (Fig. 3-15B). In cod challenged with ASAL, NR-13 expression was significantly up-regulated as compared to 0 h (4.9 fold) in the spleen at 6 HPI. However, the NR-13 expression in the ASAL 6 HPI group was not significantly different (p = 0.056) from the time-matched PBS group (Fig. 3-15A). In spleen, Mcl-1 expression was significantly higher in the pIC group at 6 HPI in comparison to 0 h (3.1 fold) and timed PBS and ASAL groups (Fig. 3-15C). Mcl-1, Bcl-X1, and Bcl-X2 expression at 2, 6, and 24 HPI compared with 0 h was not significantly affected by either pIC or ASAL in head kidney (Fig. 3-15D, F, H), and Bcl-X1 was not significantly affected by either treatment in spleen (Fig. 3-15E). However, a significant increase in the expression of Bcl-X2 in spleen was observed in the pIC group at 6 HPI compared with both 0 h (2.6 fold) and time-matched PBS controls. Interestingly, QPCR showed that saline injection had a mild (less than 3-fold) but significant inductive effect (compared to 0 h) on both NR-13 and Mcl-1 transcript expression in spleen at 2 HPI.

Analysis of promoter regions of NR-13, Mcl-1, and Bcl-X1

The mapping of full-length cDNA sequences to corresponding genomic sequences identified transcription start sites for NR-13, Mcl-1, and Bcl-X1 (Fig. 3-4, 3-5, 3-6). For each gene, genomic sequence (797 bp for NR-13, 741 bp for Mcl-1, and 372 bp for Bcl-X1) 5' of the transcription start site was scanned for eukaryotic promoter elements based on MatInspector weight matrices (see Web Site References) and consensus sequences from previous studies. Analysis of the promoter regions showed that Atlantic cod NR-13, Mcl-1, and Bcl-X1 possess TATA-less promoters, as no consensus TATA box was found near the transcription start sites for any of these genes. In consideration of the putative anti-apoptotic roles of these genes, and the results of my constitutive and immune-relevant gene expression studies, I focused primarily on showing promoter elements with potential involvement in apoptotic regulation and immune responses (Fig. 3-4, 3-5, 3-6). The putative binding sites for GATA family transcription factors, cAMP-response element binding proteins [e.g. activating transcription factors (ATF, i.e. ATF-2 and ATF-6), and Tax/CREB], and CCAAT/enhancer binding protein beta (C/EBP-β) were identified in the promoter regions of all three genes analyzed. The putative binding sites for Rel/NF-κB (nuclear factor kappa B) transcription factors (e.g. c-Rel) and Ets transcription factors (e.g. PU.1 and Spi-B) were identified in the promoter regions of NR-13 and Mcl-1. Within the NR-13 5'-flanking region, other putative transcription factor binding sites commonly involved in immune responses and apoptosis included: 2 IRF (interferon regulatory factor)-7 sites, 2 STAT-5 sites, 2 STAT-6 sites, 2 p53 sites, and 1 AP-1 site (Fig. 3-4). In the Mcl-1 5'-flanking region, a putative IRFF (IRF-related factors) site was identified, and a total of 6 GM-CSF (granulocyte-macrophage colony-stimulating factor) binding motifs (a repeated sequence CAATW required for

promoter activity) (52) were also identified (Fig. 3-5). In the Bcl-X1 5'-flanking region, putative binding sites for RBP-J κ (recombination signal binding protein J κ) and Sp-2 were identified (Fig. 3-6).

3.5 Discussion

In this study, 4 anti-apoptotic Bcl-2 sub-family genes, NR-13, Mcl-1, Bcl-X1, and Bcl-X2, were identified in Atlantic cod by mining the CGP EST database. For cod NR-13, Mcl-1, and Bcl-X1, I sequenced the full-length cDNA, resolved the gene structure, and obtained and analyzed upstream promoter element-containing sequences. In addition, I obtained the partial cDNA sequence and partially resolved the gene structure of Atlantic cod Bcl-X2 (see "Results" and Fig. 3-3). To study the expression of Atlantic cod anti-apoptotic Bcl-2 sub-family genes, I examined constitutive gene expression in six tissues and studied the gene expression in immune tissues following the stimulations with bacterial antigens (formalin-killed, atypical *Aeromonas salmonicida*) or a viral mimic (pIC, a synthetic double-stranded RNA). Lastly, I screened upstream regions of NR-13, Mcl-1, and Bcl-X1 for potential regulatory motifs. The anti-apoptotic functions of orthologues of these cod genes, gene organisations, expression patterns, along with the presence of potential regulatory motifs were discussed separately for each gene, and then integrated to examine the potential roles of these genes in cod innate immune responses.

My analysis of ESTs generated from CGP cDNA libraries led to the identification of four Atlantic cod transcripts representing members of the anti-apoptotic Bcl-2 sub-family (Table 3-1). This allowed us to obtain the full-length cDNA sequences for NR-13, Mcl-1, and Bcl-X1, and a partial cDNA sequence for

Bcl-X2, using bi-directional RACE. Analysis of these cDNA sequences revealed high similarity between their predicted protein sequences and putative orthologous sequences from other vertebrates, especially within the Bcl-2 homology (BH) domains that are critical for their anti-apoptotic functions [reviewed in (2)]. In addition, all 4 Atlantic cod anti-apoptotic Bcl-2 sub-family cDNAs analyzed encode conserved transmembrane (TM) domains at their carboxyl termini (see Fig. 3-7, 3-8, 3-9, 3-10), which are required for localization to intracellular membranes such as the mitochondria outer membrane, the smooth endoplasmic reticulum, and the nuclear envelope [reviewed in (2)]. The cod Mcl-1 cDNA also encodes for a characteristic PEST region that is also found in other Mcl-1 orthologues (Fig. 3-9). The PEST regions are rich in proline, glutamic acid, serine and threonine amino acid residues, and contribute to the fast turnover rate of Mcl-1 protein in humans [reviewed in (20, 49)].

My phylogenetic analysis shows the relationships between the Atlantic cod anti-apoptotic Bcl-2 subfamily cDNA translations and related vertebrate proteins (Fig. 3-12). All NR-13 orthologues contain a conserved aspartate (D_{15}) residue within the BH4 region and a conserved arginine (R_{39}) residue within the BH3 region (Fig. 3-8). Using the approach of mutagenesis, Lalle et al (2002; Ref. 42) showed that these two oppositely charged residues are required for the ionic interaction between the BH4 and BH3 domains, and thus are essential for the anti-apoptotic activity of chicken NR-13 (42). In the same study, a glutamic acid (E_{46}) residue within the BH3 region was also shown to be an essential feature for the anti-apoptotic activity of chicken NR-13 (42) (the numbering for all residues refer to the cod NR-13 sequence). Interestingly, I noticed that the E_{46} is only conserved in bird and fish sequences (Fig.

3-8); the functional significance of this feature in fish NR-13 orthologues is yet to be determined. Zebrafish NR-13 orthologue [termed "Nrz" in (5)] functions as an apoptotic inhibitor that is localized to the mitochondria, and it plays a critical role in zebrafish development during somitogenesis and gastrulation (5). Given the high degree of similarity shared between the Atlantic cod and zebrafish NR-13 (61.5% overall identity, Fig. 3-8), Atlantic cod NR-13 may also function as an apoptotic inhibitor and play important roles in embryonic development. Functional studies for the Atlantic cod Bcl-2-like genes I describe here have not yet been conducted.

In addition to the typical features (BH1-3, PEST, and TM domains) possessed by Mcl-1 proteins (Fig. 3-9), a noteworthy feature of the predicted Atlantic cod Mcl-1 protein is that it contains a putative monopartite nuclear localization signal (NLS) with a single stretch of basic amino acids [RKPR, as reviewed in (43)], which was not identified by PSORTII in any other Mcl-1 orthologues with the exception of zebrafish Mcl-1a. Prior to this study, zebrafish Mcl-1a was the only Bcl-2 family protein identified with a NLS (Chen et al., 2000; Ref. 13). The NLS is crucial for the nuclear localization of zebrafish Mcl-1a protein (13).

My multiple sequence alignment analysis showed that the predicted Atlantic cod Bcl-X1 protein does not contain an aspartate residue (D_{61} in human Bcl-X_L) that is conserved in mammals, and the cod sequence contains only one of two threonine residues that are conserved in mammals (residue T_{115} but not T_{47} in human Bcl-X_L) (Fig. 3-10). This finding is consistent with previous observations made based on the zebrafish Bcl-X_L sequence (14). Given the critical involvement of the conserved threonine residues (T_{47} and T_{115}) and the aspartate residue (D_{61}) in phosphorylation (38) and caspase cleavage (17) of human Bcl-X_L, my results collectively suggest that

fish Bcl-X proteins may be subjected to different post-translational modification-based mechanisms from those in mammals.

The Atlantic cod NR-13, Mcl-1, and Bcl-X1 were each found to be encoded by 3 exons interrupted by two introns (Fig. 3-3), and have intron/exon boundaries within the ORF at conserved locations between human and cod putative orthologues (Fig. 3-12). Thus, these intron/exon boundaries are also likely to be conserved in other vertebrate orthologues. Furthermore, I identified a conserved intron/exon boundary immediately following the 2nd tryptophan codon (UGG) (the 8th residue) in the BH2 domain in all vertebrate Bcl-2-like genes, and it is in the same position of the *C. elegans* Bcl-2–like gene CED-9 (Fig. 3-12B). This conserved intron/exon boundary was also found in some pro-apoptotic members including Bak and Bax in humans (32). The conserved intron/exon boundaries within the ORF of these anti-apoptotic Bcl-2 sub-family genes bear functional significance as alternative splicing of these genes leads to functionally diverse proteins in apoptotic regulation (8, 36). Overall, the conserved intron/exon boundary within the BH2 domain and the conserved domains observed in these anti-apoptotic Bcl-2 sub-family proteins collectively suggest that these genes may have arisen from a common ancestral gene.

A non-coding exon for the first 90 bp of the 5'-UTR was identified in the Atlantic cod Bcl-X1 gene (Fig. 3-3), and a non-coding exon (encoding the first 102 bp of the 5'-UTR) was also identified in the zebrafish Bcl-X gene by BLASTn aligning the cDNA sequence (GenBank accession no. AF317837) against the zebrafish genome (data not shown). The presence of a non-coding exon appears to be a conserved feature of the vertebrate Bcl-X orthologues, as it was also identified in the mouse Bcl-X gene (29). In contrast, the non-coding exon may not be a shared

feature among the vertebrate NR-13 orthologues. My analysis of the cod NR-13 gene revealed a non-coding exon encoding the first 49 bp of the 5'-UTR (Fig. 3-3, 3-4). In contrast, a non-coding exon is not present in its human (aliases NRH/Bcl-B/Bcl-2L10/Boo/Diva, see footnotes of Table 3-3 for detailed explanation) (77) or mouse orthologues (GenBank accession no. NP_038507). Prior to this study, possibly due to the lack of full-length cDNA sequences (e.g. for chicken and zebrafish) or genomic sequence (e.g. for Atlantic salmon), the presence of a non-coding exon in non-mammalian NR-13 orthologues was not documented.

The conserved gene structure observed in Mcl-1 between human and Atlantic cod (Fig. 3-12A) raised the question whether the alternative use of exon 2 of the Mcl-1 gene also occurs in Atlantic cod as previously observed in human (8). The skipping of exon 2 ultimately leads to a pro-apoptotic BH3-only protein product, known as Mcl-1_S ("s" stands for "short") (8). Although my results indicated that the equivalent of the human Mcl-1_S splice variant was not identified in spleen of bacterial antigen (ASAL)-stimulated Atlantic cod (Fig. 3-13), I am not able to exclude the possible presence of this transcript in other tissues. This study revealed two cod Mcl-1 transcripts (both of which were polyadenylated) with variable 3'-UTRs resulting from alternative splicing of exon 3 (Fig. 3-3). With mounting evidence demonstrating the significance of the 3'-UTR in translational regulation of the human Mcl-1 by microRNA (50) and RNA binding protein (66), it is possible that the difference in the 3'-UTR of cod Mcl-1 variants dispose them to distinct translational control mechanisms.

Many key genes involved in the regulation of apoptosis possess IRES (internal ribosomal entry site), as this cap-independent translational mechanism is

capable of coping with cellular stress, where the cap-binding complex, the eIF4F (composed of eukaryotic translation initiation factors 4A, 4E, and 4G) is compromised [reviewed in (28)]. My analysis of Atlantic cod sequences revealed putative IRES in both Mcl-1 and Bcl-X1 (Fig. 3-5, 3-6). The IRESs of the human putative orthologues of these genes have been previously identified and studied (33, 75). In contrast, I found no IRES in the Atlantic cod NR-13 mRNA (Fig. 3-4), or in its mouse orthologue (GenBank accession no. NM_013479) using RegRNA.

AREs (AU-rich elements) are involved in targeting mRNA for rapid degradation, most of which contain ATTTA motifs with the exception of the Class III AREs [reviewed in (7)]. Analysis of the 3'-UTR of the cod NR-13 cDNA revealed 3 ATTTA motifs within AT-rich regions, which are characteristic of class I AREs [reviewed in (7)]. In contrast, no ATTTA motifs were identified in the 3'-UTR of the human NR-13 orthologue, and only one ATTTA motif was identified in the cDNA of the mouse orthologue (77). This observation suggests that the cod NR-13 mRNA may be less stable than its mammalian orthologues and, if so, that more dynamic transcription may be required to maintain the expression of the cod transcript. The functional significance of putative Class I AREs identified in the cod NR-13 cDNA needs to be further investigated. In addition, a putative cytoplasmic poly-adenylation element (CPE) was identified in both Atlantic cod NR-13 mRNA and its mouse orthologue (GenBank accession no. AF067660). The CPE is a critical feature required for translational activation of transcripts during oocyte maturation [reviewed in (57)]. It is likely that the presence of the CPE is a conserved feature of vertebrate NR-13 orthologues, which could be associated with the high expressions of NR-13 orthologues in ovaries of mouse and zebrafish (34, 39, 63).

Unfortunately, the gene structure for cod Bcl-X2 was not fully resolved in this study due to technical difficulties. Nonetheless, I accumulated sufficient evidence (Fig. 3-6, 3-7, 3-12, 3-16) to show that two Bcl-X genes exist in Atlantic cod. I have also identified 3 distinct Atlantic salmon Bcl-X transcripts using the Atlantic salmon full-length cDNA database (see Web Site References) (Fig. 3-12), providing further evidence of Bcl-X gene duplication in fish. Furthermore, my multiple sequence alignment and phylogenetic analysis based on partial predicted protein sequences clearly demonstrates that the Atlantic cod Bcl-X2 belongs within the branch containing Bcl-X orthologues (Fig. 3-12 and Fig. 3-16).

The constitutive gene expression of NR-13, Mcl-1, Bcl-X1, and Bcl-X2 was examined using QPCR in the following 6 tissues: blood, brain, gill, head kidney, pyloric caecum, and spleen (Fig. 3-14). Although highly variable, all transcripts displayed detectable constitutive expression in all tissues examined. The highest levels of NR-13 and Mcl-1 expression were detected in gill, blood, and spleen, suggesting that NR-13 and Mcl-1 may play important roles in maintaining the apoptotic balance in these tissues. In mammalian and avian systems, expression of NR-13 and Mcl-1 has been associated with the viability of cells of haemopoietic lineage (45, 47, 54, 65). Therefore, the high expression of these transcripts in Atlantic cod blood and spleen is not surprising. However, this study is the first to document high constitutive expression of NR-13 and Mcl-1 in fish gill tissue. This observation may be potentially linked to the prevalence of mitochondria-rich cells in gill (71), given that NR-13 and Mcl-1 both target mitochondria. In agreement with this study, Kratz et al. (2006; Ref. 39) performed semi-quantitative PCR with various zebrafish tissues (not including gill, blood, or spleen), and showed that NR-13 and Mcl-1 were

expressed at relatively low levels in brain, gut, and kidney. Although Atlantic cod Bcl-X1 and Bcl-X2 are very similar to one another at the predicted protein level (68.3% identity over 151 aligned amino acids, E-value = 6e-63), the constitutive expression data for these two paralogues are very distinct. Atlantic cod Bcl-X1 displayed highly variable expression (i.e. highest in blood followed by brain and gill, with relatively low levels of expression in spleen, head kidney, and pyloric caecum). In contrast, Bcl-X2 showed no significant differences in constitutive expression between any of the tissues that were studied. Differences in constitutive expression between these cod Bcl-X paralogues suggests that they utilize distinct transcriptional regulatory mechanisms.

QPCR was also used to study NR-13, Mcl-1, Bcl-X1, and Bcl-X2 gene expression in Atlantic cod immune tissues (spleen and head kidney) following intraperitoneal (IP) stimulation with bacterial antigens (ASAL: formalin-killed, atypical *Aeromonas salmonicida*), a viral mimic (pIC: a synthetic double-stranded RNA), or phosphate buffered saline (sham-injected control). The apoptotic effect of pIC on mammalian cells has been previously demonstrated, and a dsRNA-dependent protein kinase (PKR) has been identified as the key mediator of this effect (22). Multiple PKR-encoding genes have recently been identified in fish (59), and the apoptotic effect of pIC has also been demonstrated using the rainbow trout RTS11 macrophage cell line as the experimental model (23). Prior to this study, the activation of NR-13 expression was predominantly known as an apoptosis evasion mechanism involved in viral pathogenesis and oncogenesis (26, 45, 47). In this study, following IP injection of pIC as in Rise et al. (2008; Ref. 58), cod NR-13 mRNA expression was significantly elevated in both spleen and head kidney with the highest

level of induction occurring at 6 HPI for both tissues (Fig. 3-15A, B). While the magnitude of the induction was higher in spleen than in head kidney at 6 HPI, the induction of NR-13 expression persisted at 24 HPI in head kidney but not in spleen (Fig. 3-15A, B). The pIC-stimulated induction of NR-13 expression in cod immune tissues may offer transient protection for the immune cells (e.g. lymphocytes and neutrophils) from pIC-induced apoptosis. The rapid induction of NR-13 transcription has also been observed following treatment with PMA [phorbol myristate acetate, a carcinogen with mitogenic properties as reviewed in (69)] in cultured Japanese flounder kidney cells, and in a DT40 cell line derived from chicken bursal lymphoma (40, 45). Therefore, it is possible that the transcriptional activation of NR-13 is governed by a common pathway involved in both PMA and pIC treatments. In comparison to NR-13, less prominent induction of Mcl-1 and Bcl-X2 (but not Bcl-X1) expression following pIC stimulation was also observed, and significant induction was restricted to spleen for both genes (Fig. 3-15C, D, E, F, G, H). Collectively, my observations suggest that the induction of Atlantic cod NR-13, Mcl-1, and Bcl-X2 may be involved in maintaining apoptotic homeostasis and immune function in cod spleen following pIC stimulation.

Our previous studies have shown that both treatments (ASAL and pIC) successfully elicited potent innate immune responses in immune tissues of Atlantic cod, which were reflected by transcriptomic changes (24, 58). Prior to this study, there were no studies on the expression of Bcl-2-like genes in fish during innate immune responses to bacterial or viral immunogenic stimuli. In this study, I found that while NR-13, Mcl-1, and Bcl-X2 transcripts were all significantly up-regulated in pIC-treated spleen, the only significant change caused by ASAL (relative to 0 h

controls) was a moderate up-regulation of NR-13 in spleen at 6 HPI. In addition, the NR-13 expression level at this time point was significantly lower in the ASAL group than in the pIC group in both spleen and head kidney (Fig. 3-15A). Therefore, it is possible that the involvement of different pathways (e.g. TLR and PKR pathways) in response to pIC and ASAL accounts for the differences in the expression of these Bcl-2-like genes.

In order to further study the link between pIC stimulation and transcriptional activation of Atlantic cod NR-13 and Mcl-1, their promoter regions were scanned for potential regulatory motifs. I identified putative κB elements in the promoter regions of both cod NR-13 and Mcl-1 using MatInspector. Previous studies have shown that the NF-κB signalling pathway is highly conserved in vertebrates, and fish orthologues of the NF-κB family (e.g. c-Rel, RelA/p65, and NF-κB2/p100) recognize cognate κB elements (consensus sequence 5'-GGGRNWTTCC-'3) from mammals (12, 18, 61). In this study, I noticed that the κB element [identified as c-Rel (a NF-κB family member) by MatInspector] identified in the cod NR-13 promoter region perfectly matched the published consensus sequence (sense strand 5'-GGGAGATTCC-3' from -90 to -80, Fig. 3-4). On the other hand, both putative κB elements identified in Mcl-1 deviated (underlined bases) slightly from the κB element consensus sequence (sense strand '5-TGGTACTTCC-3' from -230 to -220, anti-sense strand 5'-GGTACTTCCC-3' from -229 to -219, Fig. 3-5). Such discrepancies could potentially lead to differences in binding affinity or preference by different NF-κB family members (12). The activation of the NF-κB pathway by bacterial (e.g. ASAL used in this study) LPS has been well documented in humans [reviewed in (76)].

Therefore, it is possible that the activation of NF-κB pathway plays a role in the up-regulation of NR-13 mRNA that I observed in spleen following the stimulation with ASAL (Fig. 3-15A). The activation of the NF-κB pathway by pIC has also been previously demonstrated using cultured HeLa cells, in which PKR-mediated activation and nuclear translocation of NF-κB was observed following pIC treatment (25). Furthermore, the involvement of NF-κB in transcriptional regulation of chicken NR-13 and human Mcl-1 has been demonstrated (30, 45). Based on these previous findings and my sequence analysis results, it appears that κB elements in the promoter regions of Atlantic cod NR-13 and Mcl-1 may be involved in the transcriptional activation of these genes in response to pIC in immune tissues (Fig. 3-15A, B, C).

Putative E4BP4/NFIL3 (nuclear factor, IL-3 regulated) binding sites were identified in both cod NR-13 and Mcl-1 promoters (Fig. 3-4, 3-5). This transcription factor is highly conserved throughout metazoan evolution, and it is responsible for IL-3-mediated anti-apoptotic effects in mammalian B-lymphocytes (19). Within the cod NR-13 promoter region, putative binding elements for STAT-5 and STAT-6 were also identified, both of which are known to be pro-survival transcription factors that are involved in transcription activation of Bcl-X_L in humans (21, 72). The induction of NR-13 expression by pIC could also be associated with transcriptional regulation by STAT-5 and/or STAT-6, which are involved in the JAK (Janus kinase)/STAT (signal transducer and activator of transcription) and MAPK (mitogen-activated protein kinase) signalling pathways. As putative IRF-7 binding sites were identified in the cod NR-13 promoter region, there may be a relationship between the induction of NR-13 expression and the previously reported up-regulation of IRF-7 transcript in

Atlantic cod spleen following pIC stimulation (58). Other regulatory motifs identified in the cod NR-13 promoter region that may be involved in immune responses include putative binding sites for AP-1, Ets1 (E26-transformation specific) transcription factors and CREBPs (cyclic AMP responsive element binding proteins). The transcription regulatory mechanisms of mammalian Mcl-1 have been extensively investigated, and suggest the involvement of MAPK, PI3K (phosphatidylinositol-3) and JAK/STAT signalling pathways in transcriptional regulation of Mcl-1 [reviewed in (20)]. Putative binding sites for the Ets1 transcription factors and CREBPs were identified in the cod Mcl-1 promoter region, suggesting that similar pathways may be involved in the transcriptional regulation of cod Mcl-1 as have been observed in humans (reviewed in (20)).

In this study, I identified two Atlantic cod Bcl-X genes with distinct constitutive and immune-responsive expression profiles, a finding which suggests that these cod Bcl-X paralogues may utilize different transcriptional regulatory mechanisms. Unfortunately, I was only able to obtain promoter region sequence for Bcl-X1. My analysis of the Bcl-X1 5'-flanking region revealed the presence of putative binding sites for Ets and AP-1 transcription factors (Fig. 3-6). In mammals, transcription factors belonging to the Ets, Rel/NF-kB, STAT and AP-1 families are known to be involved in the transcriptional control of the Bcl-X gene [Reviewed in (62)]. Consistent with the previously stated idea (based on sequence analyses) that the NF-κB pathway may be involved in the observed pIC-caused up-regulation of cod NR-13 and Mcl-1 transcripts, I did not identify any putative κB elements in the promoter region of cod Bcl-X1 and the transcription of cod Bcl-X1 was not affected by treatment with pIC (Fig. 3-15E, F).

Collectively, I obtained and analyzed the promoter regions of Atlantic cod NR-13, Mcl-1, and Bcl-X1 for the first time in fish. The sequence analyses suggest that there may be some similarities in the mechanisms of transcriptional regulation between cod anti-apoptotic Bcl-2 sub-family genes and their corresponding avian and mammalian orthologues. As the first analysis of the Atlantic cod NR-13 promoter region, this study revealed regulatory motifs that may be involved in the transcriptional regulation of this gene and may help to explain its significant up-regulation in pIC-treated spleen and head kidney. However, further functional characterisations of the promoters of Atlantic cod NR-13 and other Bcl-2 family genes will be required to verify their roles.

In this study, my expression analyses of cod NR-13, Mcl-1, Bcl-X1, and Bcl-X2 were conducted at the mRNA level. It is likely that mechanisms of translational regulation (e.g. IRES and microRNA) and post-translational modification (e.g. phosphorylation, caspase cleavage, and ubiquitination) also govern expression at the protein level for Mcl-1 [reviewed in (49, 74)] and possibly other Bcl-2-like genes. In support of this, I have identified putative IRESs in Atlantic cod Mcl-1 and Bcl-X1 mRNA sequence (as previously discussed). Therefore, investigations at the protein level will be needed to further study the involvement of Atlantic cod Bcl-2-like genes and gene products in innate immune responses.

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3.8 Web Site References

http://codgene.ca; the Atlantic cod genomics and broodstock development project (CGP)

http://frodo.wi.mit.edu; Web interface for Primer3

http://regrna.mbc.nctu.edu.tw/; Web interface for RegRNA

http://ca.expasy.org/prosite/; PROSITE database

http://psort.ims.u-tokyo.ac.jp/form2.html; Web interface for PSORTII

http://emboss.bioinformatics.nl/cgi-bin/emboss/pestfind; Web interface for PESTfind

http://www.genomatix.de/; Genomatix homepage (MatInspector 8.0 Professional)

http://web.uvic.ca/grasp/; consortium for Genomics Research on All Salmonids Project (cGRASP)

http://www.genomatix.de/online_help/help_matinspector/matinspector_help.html; Additional information for MatInspector 8.0 Professional

3.9 Tables

Table 3-1. Identification of anti-apoptotic Bcl-2 sub-family transcripts in the CGP EST database.

Gene	Library type[1]	CGP library identifier[2]	Tissue	Treatment[3]	No. of clones[4]
NR-13	SSH forward	gmnlsfic	spleen	pIC	2
	SSH forward	gmnlsfas	spleen	ASAL	1
	SSH reverse	gmnlsras	spleen	ASAL	1
	Normalized	gmnbspic	spleen	pIC	3
	Normalized	gmnlskic	spleen & head kidney	pIC	1
	Normalized	gmnbhkic	head kidney	pIC	1
	Normalized	gmnbhkas	head kidney	ASAL	2
Mcl-1	SSH forward	gmnlsfic	spleen	pIC	7
	SSH forward	gmnlsfas	spleen	ASAL	4
	SSH forward	gmnlkfas	head kidney	ASAL	1
	SSH forward	gmnlkfta	head kidney	thermal stress	2
	SSH forward	gmnlmfta	skeletal muscle	thermal stress	1
	SSH forward	gmnlpfic	peripheral blood	pIC	3
	SSH reverse	gmnlpric	peripheral blood	pIC	1
	Normalized	gmapht	heart	none	3
	Normalized	gmapov	ovary	none	1
	Normalized	gmnbgi	gill	none	1
	Normalized	gmnblits	liver	none	1
	Normalized	gmnbmd	pyloric caecum	none	1
	Normalized	gmnlem	embryo	none	5
	Normalized	gmnlla	larvae	none	5
	Normalized	gmnbbrts	brain	thermal stress	3
	Normalized	gmnbhkas	head kidney	ASAL	4
	Normalized	gmnbpcic	pyloric caecum	pIC	1
	Normalized	gmnbspic	spleen	pIC	1
	Normalized	gmnlskic	spleen & head kidney	pIC	2
BCL-X1	Normalized	gmnbbr	brain	none	2
	Normalized	gmnbhkic	head kidney	pIC	1
	Normalized	gmapov	ovary	none	1
BCL-X2	SSH forward	gmnllfta	liver	thermal stress	1
	Normalized	gmnblits	liver	thermal stress	1

[1]The forward SSH libraries were constructed to be enriched for transcripts that were up-regulated by an immune stimulus (ASAL: intraperitoneal injection of formalin-killed, atypical *Aeromonas salmonicida*; pIC: intraperitoneal injection of polyriboinosinic polyribocytidylic acid, a synthetic double-stranded RNA viral mimic) or thermal stress. Reverse SSH libraries were constructed to be enriched for transcripts that were down-regulated by a given treatment (ASAL, pIC, or thermal stress). Normalized cDNA libraries were constructed to normalize the abundance of transcripts in order to maximize gene discovery within the Atlantic cod genomics and broodstock development project (CGP).
[2]The identifiers of the SSH libraries in the CGP EST database (http://codgene.ca).
[3]The ASAL, pIC, and the heat stress stimulation procedures were described previously (24, 58).
[4]The number of clones does not necessarily equate to the number of ESTs, as some clones were sequenced more than once.

Table 3-2. Primers used for gene cloning and expression.

Primer name[1]	Oligonucleotide Sequences (5'-3')	Appplication[2]
NR-13_R1	CTCCATGTCCTGGGCCAGTCCCCTCAT	5'-genome walking and genomic PCR
NR-13_R2	AGAGCCTGGAAGCGAGCGTAGTGCTGC	5'-genome walking and genomic PCR
NR-13_R3	CTCTCGGGCGATCCTGGAGAACTTA	5'-RACE and genomic PCR
NR-13_R4	TAGCGCACCAAAAGGAACGTCAGG	5'-RACE and genomic PCR
NR-13_R5	CACCAACGTCTTGTTTGTCCACACTGGAG	genomic PCR
NR-13_R6	ACATGGTTTCAATTGGAGATTGGTCCCTGGT	genomic PCR
NR-13_F1	CTTCCGCCCTTGTACGTGAAGAGATCAGTCAG	genomic PCR
NR-13_F2	TGGCGGAGACGATAGCGGACTACCTA	3'-RACE and genomic PCR
NR-13_F3	AGAAGAGGGACTGGCTGCTGGAGAAC	3'-RACE and genomic PCR
NR-13_F4	TTTGGCTGTGCAGCTACTGGGCTTTACA	3'-genome walking
NR-13_F5	CAAATCTGGGGTTTTGTTGGTTGCCACA	3'-genome walking
NR-13_QPCR_F	CGGACTACCTAGGGGAGGAG	QPCR (97%, 171 bp)
NR-13_QPCR_R	GCACCAAAAGGAACGTCAG	QPCR (97%, 171 bp)
Mcl-1_F1	CTTCAAACTACGGAACCAGCTTAGT	cDNA PCR and genomic PCR
Mcl-1_F2	CCAGACCTGCTGGTTTAACAGCCC	cDNA PCR and genomic PCR
Mcl-1_F3	GAAGACATGAGTTTTGTCACGTCTGTGG	3'-RACE
Mcl-1_F4	TCTTCGCAGACAGCACAACAAACTG	3'-RACE
Mcl-1_F5	TGTTGTTTGGAATCTGGCCAGGCCCTAGCTCT	genomic PCR
Mcl-1_R1	GAGGGCTGTTAAACCAGCAG	5'-genome walking
Mcl-1_R2	TGTCGTGGTGTGTGAACCTT	5'-genome walking
Mcl-1_R3	CGTTGGTCTGAAAGGAGGTATGAGGAAA	5'-RACE
Mcl-1_R4	CCATTCCCGTTGGTCTGAAAGGAG	5'-RACE
Mcl-1_R5	CATGAGTGTATTTCTCACTGTCGTC	cDNA PCR and genomic PCR
Mcl-1_R6	AATACCAGCAAATCCAGCAAAG	cDNA PCR and genomic PCR
Mcl-1_R7	ATTTGGTGTGCGGGCCCTCAGCGTTTAC	genomic PCR
Mcl-1_QPCR_F	CGCAGACAGCACAACAAACT	QPCR (101%, 102 bp)
Mcl-1_QPCR_R	GACACGCAGCCTTCTTTACC	QPCR (101%, 102 bp)
Bcl-X1_F1	GTTGCCAACGACAGAAAGCCAATCAA	cDNA PCR and genomic PCR
Bcl-X1_F2	GAGCAGAAGCACCCAACCATGAAGTC	cDNA PCR and genomic PCR
Bcl-X1_F3	CGTACCCGGGACAGTCACAGGAGAT	3'-RACE and 3'-genome walking
Bcl-X1_F4	GGGCTCTGCTCGCCAAGAAACAT	3'-RACE and 3'-genome walking
Bcl-X1_R1	GCTGACAACTCGGTTCGGGTTATTCGTG	5'-genome walking
Bcl-X1_R2	GGCCCTGCGCTATACGATGCAACCAAA	5'-genome walking
Bcl-X1_R3	CTGATCGACATGCTTGTGTCAATGCT	5'-RACE and 5'-genome walking
Bcl-X1_R4	GGGCTGGAAGGGAATAGGCCTGTAAT	5'-RACE and 5'-genome walking

Bcl-X1_R5	AAACACGTGCTGACTCATCCGTCCTC	cDNA PCR and genomic PCR
Bcl-X1_R6	GTGCACAAGCAAACTGGGCCTTTGTA	cDNA PCR and genomic PCR
Bcl-X1_QPCR_F	AGGTGTTCAGGGACAGCATC	QPCR (98%, 157 bp)
Bcl-X1_QPCR_R	CAGTGGTCAATGTGGTCGTC	QPCR (98%, 157 bp)
Bcl-X2_F1	ATGGGAGCGTTTCTCCGAGGTGTTT	3'-RACE and genomic PCR
Bcl-X2_F2	TCCCAGGAGAGCTTCAGGAAGTGGTT	3'-RACE and genomic PCR
Bcl-X2_R1	ACGACCCCCGTGAACAAGGTCATC	5'-RACE and 5'-genome walking
Bcl-X2_R2	TCAGAGGCGTTTCTGGGCGAAGAGT	5'-RACE and 5'-genome walking
Bcl-X2_R3	GACATCACTTCCTGCCTCCTCGCCCTGA	genomic PCR
Bcl-X2-QPCR_F	AGCGTTTCTCCGAGGTGTT	QPCR (93%, 135 bp)
Bcl-X2-QPCR_R	GTTTCTGGGCGAAGAGTGAC	QPCR (93%, 135 bp)
GeneRacer 5'	CGACTGGAGCACGAGGACACTGA	5'-RACE
GeneRacer 5'-nested	GGACACTGACATGGACTGAAGGAGTA	5'-RACE
GeneRacer 3'	GCTGTCAACGATACGCTACGTAACG	3'-RACE
GeneRacer 3'-nested	CGCTACGTAACGGCATGACAGTG	3'-RACE
Adaptor Primer 1	GTAATACGACTCACTATAGGGC	genome walking
Adaptor Primer 2	ACTATAGGGCACGCGTGGT	genome walking
18S-QPCR_F	ATGGCCGTTCTTAGTTGGTG	QPCR (109%, 180 bp)
18S-QPCR_R	GGACATTTAAGGGCGTCTCA	QPCR (109%, 180 bp)

[1]Primer orientation is denoted by "F" or "R" following the gene name for forward or reverse, respectively. With the exception of QPCR primers, all gene-specific primers are numbered based on its target location relative to other primers of the same orientation within the gene.

[2] For QPCR primers pairs, the amplification efficiency (see Materials and Methods for more details) of the primer pair and the size of amplicon are in parentheses.

Table 3-3. Anti-apoptotic Bcl-2 sub-family sequences used for intron/exon boundary identification, multiple sequence alignment, and phylogenetic analysis.

Gene	Common name of the species[1]	Name of orthologue[2]	Amino acid accession no.	Nucleotide accession no.[3]
NR-13	human	NRH	CAD30221	AJ458330
orthologues	rat	Diva/Boo	AAK31792	AY029163
	mouse	Diva/Boo	AAC83150	AF067660
	chicken	NR-13	AAK54806	AF375661
	quail	NR-13	Q90343	
	turkey herpesvirus	vNR-13_L	AAG30102	
	zebrafish	NR-13	AAL32471	AF441285
	Atlantic salmon	NR-13 type 1	ACI32987	
	Atlantic salmon	NR-13 type 2	contig35013	
	Atlantic cod	NR-13		GQ380491
Mcl-1	human	Mcl-1_L	NP_068779	AF198614
orthologues	dog	Mcl-1	BAC21258	
	cat	Mcl-1	BAC77771	
	rat	Mcl-1	AAD13295	
	mouse	Mcl-1	AAC31790	U35623
	zebrafish	Mcl-1a	NP_571674	NM_131599
	zebrafish	Mcl-1b	NP_919375	NM_194394
	Atlantic salmon	Mcl-1 type 1	CAJ90909	
	Atlantic salmon	Mcl-1 type 2	Contig37696	
	green pufferfish	Mcl-1	CAF95150	
	Atlantic cod	Mcl-1		GQ387050
Bcl-X	human	Bcl-X_L	CAA80661	Z23115
orthologues	mouse	Bcl-X_L	AAC53459	U51278
	chicken	Bcl-X_L	AAB07677	U26645
	zebrafish	Bcl-X_L	AAK81706	AF317837
	African clawed frog	Bcl-X_L	BAB62748	
	Atlantic salmon	Bcl-X1	ACN11007	
	Atlantic salmon	Bcl-X2	ACI68003	
	Atlantic salmon	Bcl-X3	Contig33956	
	smelt	Bcl-X	ACO09883	
	pejerrey	Bcl-X	ACP19736	
	green pufferfish	Bcl-X	CAF96873	
	Atlantic cod	Bcl-X1		GQ387051
	Atlantic cod	Bcl-X2		GQ387052
				GQ387053
Bcl-2	human	Bcl-2	AAH27258	BC027258
orthologues	mouse	Bcl-2	AAH95964	BC095964
	chicken	Bcl-2	CAA78018	Z11961
	African clawed frog	Bcl-2	BAH28834	
	zebrafish	Bcl-2	AAI33849	BC133848
Bcl-2-A1	human	Bcl-2-A1	AAP36152	BT007484
orthologues	mouse	Bcl-2-A1	AAH28762	BC028762
	chicken	Bcl-2-A1	NP_990197	NM_204866
Bcl-w	human	Bcl-w	NP_004041	NM_004050
orthologues	mouse	Bcl-w	AAB09056	U59746
CED-9	*Caenorhabditis elegans*	CED-9	NP_499284	NM_066883

[1]In order to save space, the common names are provided for all species with the exception of *C. elegans* (nematode), only the scientific name of which is provided given its recognition as a model organism for studying apoptosis. The scientific names for the rest of the species are as follows: African clawed frog, *Xenopus tropicalis*; Atlantic cod, *Gadus morhua*; Atlantic salmon, *Salmo salar;* cat, *Felis*

catus; chicken, *Gallus gallus*; dog, *Canis lupus familiaris*; green pufferfish, *Tetraodon nigroviridis*; human, *Homo sapiens*; mouse, *Mus musculus*; pejerrey, *Odontesthes bonariensis*; quail, *Coturnix japonica*; rat, *Rattus norvegicus*; smelt, *Osmerus mordax*; turkey herpesvirus, *Meleagrid herpesvirus 1*; zebrafish, *Danio rerio*;

[2]The NRH nucleotide sequence (accession no. AJ458330) contains two possible ATG initiator codons that are 27 nucleotides apart. Despite the fact that the upstream start codon is less likely to be used than the downstream start codon (3), the putative translation of this protein has been previously reported as Bcl-B (37), Bcl-2L10 (77), and human Boo/Diva (44). Therefore, NRH, Bcl-B, Bcl-2L10, and human Boo/Diva are all derived from the same gene, which is referred to as human NRH hereafter. Other mammalian and viral NR-13 orthologues are named following previous reports (3, 4, 34, 64), the avian and fish orthologues are named as NR-13 based on homology. For human Mcl-1, the name "Mcl-1_L" is used to distinguish it from a shorter cDNA variant as a result of skipping the second exon in transcription (8). The naming of zebrafish Mcl-1 orthologues followed Kratz *et al* 2006 (39). The name "Bcl-X_L" is used to distinguish Bcl-X_L from other splice variants of the Bcl-X gene. However, in species where evidence for alternative splicing of the Bcl-X gene is yet to be found, the nomenclature "Bcl-X" is used. The "CED-9" is an acronym for "*C. elegans* death gene 9".

[3]The GenBank accession numbers are shown only shown for the nucleotide sequences that were used in this study. For Atlantic cod Bcl-X2, the GenBank accession numbers for partial genomic and mRNA sequences are GQ387052 and GQ387053, respectively.

Table 3-4. The amino acid sequence identity between putative Atlantic cod (*Gadus morhua*) NR-13 protein and its orthologues.

Common name	Scientific name	Name of orthologue[1]	Accession no.	Amino acid identity[2]					
				BH4	BH3	BH1	BH2	TM[3]	Overall
human	*Homo sapiens*	NRH	CAD30221	40.0	22.2	43.5	66.7	NA	23.5
rat	*Rattus norvegicus*	Diva/Boo	AAK31792	40.0	11.1	47.8	66.7	NA	23.5
mouse	*Mus musculus*	Diva/Boo	AAC83150	33.3	11.1	30.4	58.3	NA	21.1
chicken	*Gallus gallus*	NR-13	AAK54806	33.3	33.3	52.2	75.0	76.5	41.8
quail	*Coturnix japonica*	NR-13	Q90343	33.3	33.3	52.2	66.7	76.5	40.8
turkey herpesvirus	*Meleagrid herpesvirus 1*	$vNR\text{-}13_L$	AAG30102	40.0	33.3	34.8	75.0	41.2	33.8
zebrafish	*Danio rerio*	NR-13	AAL32471	46.7	66.7	82.6	91.7	94.1	61.5
Atlantic salmon	*Salmo salar*	NR-13 type 1	ACI32987	53.3	66.7	95.7	83.3	100.0	71.8
Atlantic salmon	*Salmo salar*	NR-13 type 2	contig35013	53.3	66.7	95.7	83.3	100.0	70.9

[1]The mammalian and viral orthologues are named following previous reports (3, 4, 34, 64), the avian and fish orthologues are named as NR-13 based on homology.

[2]The percentage of identity is calculated as the number (5) of identical amino acid residues divided by the total number of aligned amino acid residues. The BH4, BH3, and BH1 domains are assigned based on multiple sequence alignment (Figure 3-8) and a structural study of chicken NR-13 (42). The BH2 domain is assigned based on the PROSITE BH2 pattern sequence (PROSITE accession no. PS01258).

[3]Due to low levels of similarity between mammalian and other NR-13 orthologues, the mammalian TM domains failed to fully align with their non-mammalian counter parts. Therefore, for cod NR-13, the percent identity over the TM domains was only calculated in comparison to other non-mammalian orthologues.

Table 3-5. The amino acid sequence identity between putative Atlantic cod (*Gadus morhua*) Mcl-1 and its orthologues.

Common name	Scientific name	Name of orthologue[1]	Accession No.	Amino acid identity[2]				
				BH3	BH1	BH2	TM	Overall (%)
human	*Homo sapiens*	Mcl-1_L	NP_068779	46.7	60.0	66.7	64.7	22.5
dog	*Canis lupus familiaris*	Mcl-1	BAC21258	40.0	60.0	66.7	64.7	22.0
cat	*Felis catus*	Mcl-1	BAC77771	46.7	60.0	66.7	64.7	22.3
rat	*Rattus norvegicus*	Mcl-1	AAD13295	46.7	60.0	66.7	64.7	27.3
mouse	*Mus musculus*	Mcl-1	AAC31790	46.7	60.0	66.7	64.7	27.3
zebrafish	*Danio rerio*	Mcl-1a	NP_571674	53.3	80.0	66.7	58.8	49.9
zebrafish	*Danio rerio*	Mcl-1b	NP_919375	60.0	80.0	50.0	29.4	51.0
Atlantic salmon	*Salmo salar*	Mcl-1 type 1	CAJ90909	46.7	80.0	75.0	70.6	53.0
Atlantic salmon	*Salmo salar*	Mcl-1 type 2	Contig37696	40.0	90.0	83.3	76.5	53.5
green pufferfish	*Tetraodon nigroviridis*	Mcl-1	CAF95150	40.0	65.0	75.0	76.5	53.0

[1]For human, the name Mcl-1_L is used to distinguish it from a shorter cDNA variant as a result of skipping the second exon in transcription. The naming of zebrafish orthologues followed Kratz *et al* 2006 (39).

[2]The percentage of overall identity was calculated as the number of identical amino acid residues divided by the total number of aligned amino acid residues. The BH3, BH2, and BH1 domains are assigned based on multiple sequence alignment (Figure 3-9) and PROSITE signature sequences (PROSITE accession no. PS01080, PS01258, and PS01259).

Table 3-6. The amino acid sequence identity between putative Atlantic cod (*Gadus morhua*) Bcl-X1 and its orthologues.

Common name	Scientific name	Name of orthologue[1]	Accession No.	Amino acid identity[2]				
				BH4	BH3	BH1	BH2	Overall
human	*Homo sapiens*	Bcl-X_L	CAA80661	66.7	60.0	70.0	66.7	47.9
mouse	*Mus musculus*	Bcl-X_L	AAC53459	66.7	60.0	70.0	66.7	47.9
chicken	*Gallus gallus*	Bcl-X_L	AAB07677	61.9	60.0	65.0	66.7	45.9
African clawed frog	*Xenopus tropicalis*	Bcl-X_L	BAB62748	47.6	73.3	65.0	58.3	45.2
zebrafish	*Danio rerio*	Bcl-X_L	AAK81706	66.7	60.0	90.0	75.0	54.1
Atlantic salmon	*Salmo salar*	Bcl-X1	ACN11007	71.4	73.3	85.0	75.0	64.9
Atlantic salmon	*Salmo salar*	Bcl-X2	ACI68003	52.4	60.0	80.0	75.0	51.4
Atlantic salmon	*Salmo salar*	Bcl-X3	Contig33956	76.2	73.3	85.0	75.0	66.4
smelt	*Osmerus mordax*	Bcl-X	ACO09883	76.2	66.7	85.0	66.7	67.2
pejerrey	*Odontesthes bonariensis*	Bcl-X	ACP19736	61.9	53.3	90.0	75.0	52.9
green pufferfish	*Tetraodon nigroviridis*	Bcl-X	CAF96873	61.9	60.0	85.0	75.0	61.4

[1]The name "Bcl-X_L" is used to distinguish Bcl-X_L from other splice variants of the Bcl-X gene. However, in species where evidence for alternative splicing of the Bcl-X gene is not yet found, the nomenclature Bcl-X is used.

[2]The percentage of overall identity was calculated as the number of identical amino acid residues divided by the total number of aligned amino acid residues. The BH3, BH2, BH1, and BH4 domains are assigned based on multiple sequence alignment (Figure 3-10) and PROSITE signature sequences (PROSITE accession no. PS01080, PS01258, PS01259 and PS01260).

3.10 Figures

Figure 3-1

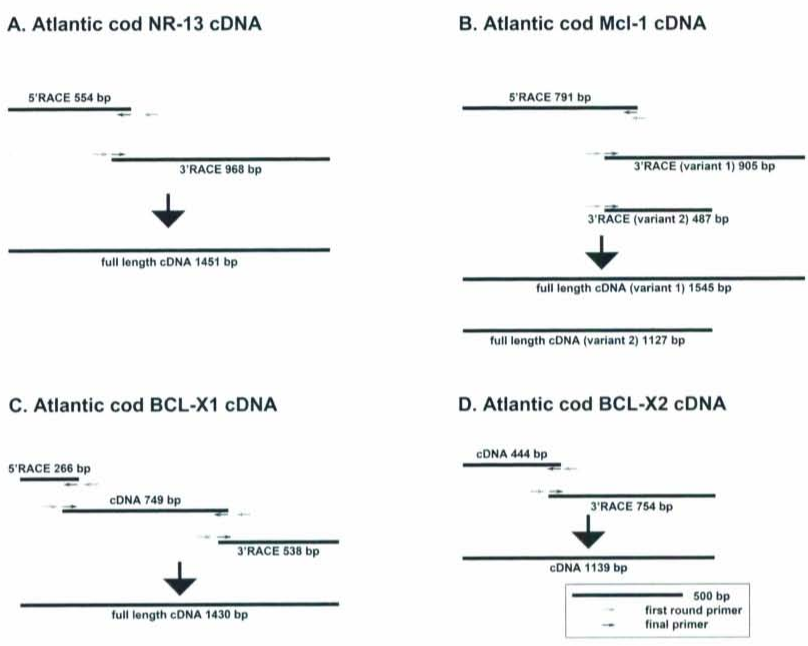

Figure 3-1. The cDNA cloning strategies for Atlantic cod NR-13, Mcl-1, Bcl-X1, and Bcl-X2 using RACE and cDNA PCRs. For all panels, the grey arrows indicate gene specific primers (GSPs) used for the first round of PCR, while the black arrows indicate the GSPs used to generate to final PCR product used for sequencing. The sequences of all primers can be found in Table 3-2. For each gene, the length of the final compiled sequence is shown on the bottom of each panel. (A) Cloning and compilation of the Atlantic cod NR-13 cDNA. Nested forward primers NR-13_F2 and NR-13_F3 were used for 3'-RACE PCRs with GeneRacer 3' primer and GeneRacer 3' nested primer, respectively. NR-13_R4 and NR-13_R3 were used for 5'-RACE PCRs in combination with GeneRacer 5' primer and GeneRacer 5' nested primer, respectively. The 71 bp overlap between the PCR products generated from the 5'-RACE and 3'-RACE allowed the compilation of the NR-13 cDNA. (B) Cloning and compilation of the Atlantic cod Mcl-1 cDNA. Nested forward primers Mcl-1_F3 and Mcl-1_F4 were used for 3'-RACE PCRs in combination with GeneRacer 3' primer and GeneRacer 3' nested primer, respectively. Nested reverse primers Mcl-1_R4 and Mcl-1_R3 were used in combination with GeneRacer 5' primer and GeneRacer 5' nested primer, respectively. The 151 bp overlap between the PCR products generated from the 5'-RACE and 3'-RACE allowed the compilation of the two forms of Mcl-1 cDNA. (C) Cloning and compilation of the Atlantic cod Bcl-X1 cDNA. Nested primer pairs Bcl-X1_F1, Bcl-X1_F2, Bcl-X1_R6, and Bcl-X1_R5 were used to amplify the sequence containing the Bcl-X1 ORF. Nested reverse primers Bcl-X1_R4 and Bcl-X1_R3 were used in combination with GeneRacer 5' primer and GeneRacer 5' nested primer, respectively. Nested forward primers Bcl-X1_F3 and Bcl-X1_F4 were used in combination with GeneRacer 3' primer and

GeneRacer 3' nested primer, respectively. The cDNA PCR product that overlaps with the PCR product from 5'-RACE by 69 bp, and the PCR product from 3'-RACE by 54 bp allowed the compilation of the Bcl-X1 cDNA. (D) Cloning and compilation of the Atlantic cod Bcl-X2 cDNA. Nested forward primers Bcl-X2_F1 and Bcl-X2_F2 were used for 3'-RACE PCRs in combination with GeneRacer 3' primer and GeneRacer 3' nested primer, respectively. Nested reverse primers Bcl-X2_R1 and Bcl-X2_R2 were used in combination with GeneRacer 5' primer and GeneRacer 5' nested primer, respectively. The 59 bp overlap between the PCR products generated from the 5'-RACE and 3'-RACE allowed the compilation of the Bcl-X2 cDNA.

Figure 3-2

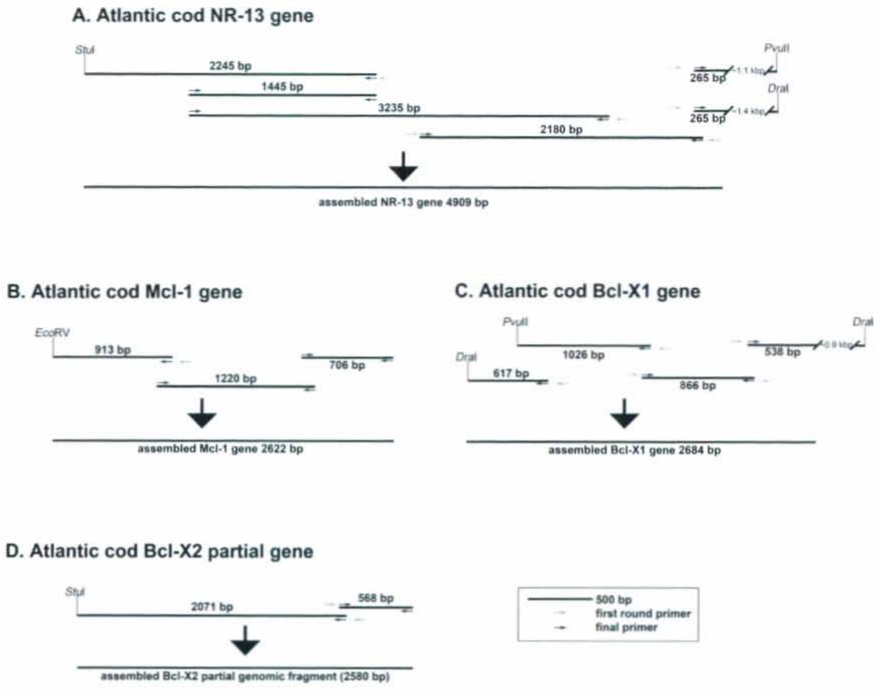
A. Atlantic cod NR-13 gene
StuI
2245 bp
1445 bp
3235 bp
2180 bp
PvuII
265 bp
~1.1 kbp
DraI
265 bp
~1.4 kbp
assembled NR-13 gene 4909 bp
B. Atlantic cod Mcl-1 gene
EcoRV
913 bp
706 bp
1220 bp
assembled Mcl-1 gene 2622 bp
C. Atlantic cod Bcl-X1 gene
PvuII
DraI
1026 bp
538 bp
~0.9 kbp
DraI
617 bp
866 bp
assembled Bcl-X1 gene 2684 bp
D. Atlantic cod Bcl-X2 partial gene
StuI
2071 bp
568 bp
assembled Bcl-X2 partial genomic fragment (2580 bp)
500 bp
first round primer
final primer

Figure 3-2. The genomic sequence and promoter region cloning strategies for Atlantic cod NR-13, Mcl-1, Bcl-X1, and Bcl-X2 using genome walking and genomic PCRs. For all panels, the grey arrows indicate gene specific primers (GSPs) used for the first round of PCR, while the black arrows indicate the GSPs used to generate to final PCR product used for sequencing. The sequences for all primers can be found in Table 3-2. For each PCR fragment amplified from a genome walker library, the site of restriction is labelled by the name of the restriction enzyme (see Materials and methods sections for details). For each gene, the length of the final compiled sequence is shown on the bottom of each panel. (A) Cloning and compilation of the Atlantic cod NR-13 gene. The assembly of the NR-13 gene is based on a total of five overlapping fragments generated using a combination of PCR (with genomic DNA template) and genome walking PCR. To obtain the NR-13 upstream genomic region, reverse primers (NR-13_R2, NR-13_R1) were paired with AP1 and AP2 (see Materials and methods for details) for nested genome walking PCRs, and a product amplified from the *Stu*I library was sequenced. The 5'-genomic fragment was generated by nested PCRs using the following primer combinations: NR-13_F1/NR-13_R4 and NR-13_F1/NR-13_R3. Nested PCRs were also performed to obtain the 3'-genomic fragment using primer combinations NR-13_F2/NR-13_R6 and NR-13_F3/NR-13_R5. The 3'-genome walking PCRs were performed using the following primer combinations: NR-13_F4/AP1 and NR-13_F5/AP2, and PCR products amplified from the *Dra*I library and the *Pvu*II library were sequenced. Genomic PCR using primer combination NR-13_F1/NR-13_R1 was also performed to confirm the presence of the first intron. (B) Cloning and compilation of the Atlantic cod Mcl-1 gene. The assembly of the Mcl-1 gene is based on three overlapping

fragments generated using a combination of PCR (with genomic DNA template) and genome walking PCR. Primer combinations Mcl-1_R2/AP1 and Mcl-1_R1AP2 were used for nested genome walking PCRs, and a PCR product amplified from the *Eco*RV library was sequenced. In order to obtain the other two genomic DNA fragments, two genomic PCRs were performed using the following primer combinations: Mcl-1_F1/Mcl-1R6 and Mcl-1_F5/Mcl-1_R7. (C) Cloning and compilation of the Atlantic cod Bcl-X1 gene. The assembly of the Bcl-X1 gene is based on four overlapping fragments generated using a combination of PCR (with genomic DNA template) and genome walking PCR. Using primer combinations Bcl-X1_R4/AP1 and Bcl-X_R3/AP2, a PCR product was obtained from the *Pvu*II library. Bcl-X1_R2 and Bcl-X1_R1 were used for a second round of 5'-genome walking, in which a PCR product amplified from the *Dra*I library was sequenced. Nested genomic PCRs were performed using primer combinations Bcl-X1_F1/Bcl-X1_R6, Bcl-X1_F2/Bcl-X1_R5 to obtain the genomic sequence. The 3'-genome walking PCRs using primer combinations Bcl-X1_F3/AP1 and Bcl-X1_F4/AP2 were performed to generate a PCR product amplified from the *Dra*I library. (D) Cloning and compilation of the Atlantic cod Bcl-X2 gene. Two overlapping genomic sequences were obtained and assembled for the Bcl-X2 gene. Nested 5'-genome walking PCRs were performed using nested reverse primers Bcl-X2_R2 and Bcl-X2_R1 to produce a PCR product using the StuI library as template. Nested genomic PCRs were performed using the following primer combinations: Bcl-X2_F1/Bcl-X2_R3 and Bcl-X2_F2/Bcl-X2_R3.

Figure 3-3

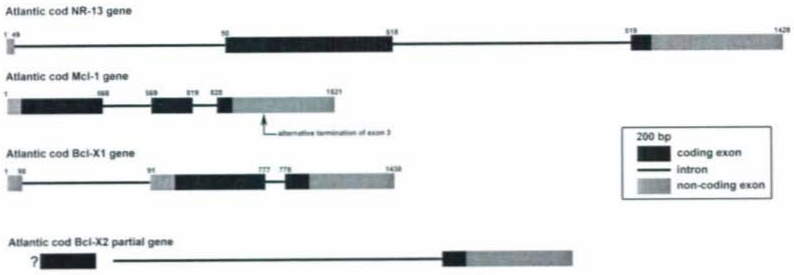
Atlantic cod NR-13 gene
1 49
50
518
519
1428
Atlantic cod Mcl-1 gene
1
568
569
619
620
1621
alternative termination of exon 3
Atlantic cod Bcl-X1 gene
1 90
91
777
778
1438
200 bp
coding exon
intron
non-coding exon
Atlantic cod Bcl-X2 partial gene
?

Figure 3-3. Schematic representation of gene organisations for Atlantic cod NR-13, Mcl-1, Bcl-X1, and Bcl-X2. Exons are shown as boxes, while horizontal lines represent introns. The coding region within an exon is shown in black while the non-coding region of an exon is shown in grey. The numbering at the start and the end of each exon indicates the location with respect to the mRNA, with 1 being the transcription start. The gene organisation for Atlantic cod Bcl-X2 has not been fully determined. The genomic sequences are available in NCBI GenBank under the accession numbers: NR-13 (GQ380491), Mcl-1 (GQ387050), and Bcl-X1 (GQ387051).

Figure 3-4

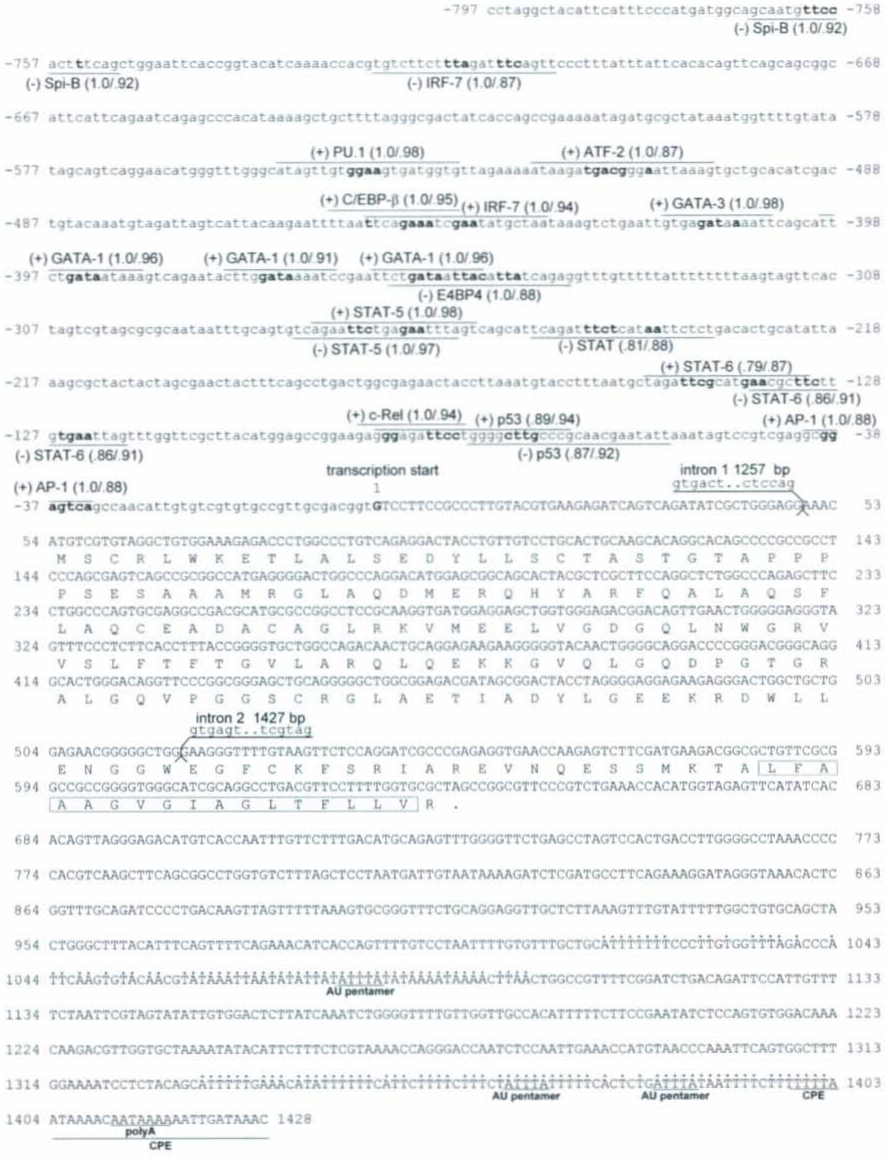
(-) Spi-B (1.0/.92)
(-) Spi-B (1.0/.92)
(-) IRF-7 (1.0/.87)
(+) PU.1 (1.0/.98)
(+) ATF-2 (1.0/.87)
(+) C/EBP-β (1.0/.95)
(+) IRF-7 (1.0/.94)
(+) GATA-3 (1.0/.98)
(+) GATA-1 (1.0/.96)
(+) GATA-1 (1.0/.91)
(+) GATA-1 (1.0/.96)
(-) E4BP4 (1.0/.88)
(+) STAT-5 (1.0/.98)
(-) STAT-5 (1.0/.97)
(-) STAT (.81/.88)
(+) STAT-6 (.79/.87)
(-) STAT-6 (.86/.91)
(+) c-Rel (1.0/.94)
(+) p53 (.89/.94)
(+) AP-1 (1.0/.88)
(-) STAT-6 (.86/.91)
(-) p53 (.87/.92)
transcription start
intron 1 1257 bp
(+) AP-1 (1.0/.88)
intron 2 1427 bp
AU pentamer
AU pentamer
AU pentamer
CPE
polyA
CPE

Figure 3-4. Nucleotide and inferred amino acid sequences of the Atlantic cod NR-13 gene (accession no. GQ380491). The cDNA sequence is shown in upper case letters, while the introns and the promoter regions are shown in lower case letters. The numbers on both sides mark the location with respect to the transcription start (bolded and labelled), and negative numbers are used to mark the nucleotides 5'of the transcription start. The locations and sizes of the introns are indicated, while the intronic sequences are not shown to save space. The putative amino acid sequence is shown below the ORF. The transmembrane (TM) domain, predicted using PSORTII (see Web Site References), is boxed. Using MatInspector Professional 8.0 (see Web Site References), selected (See Materials and methods for selection criteria) putative transcription factor binding sites identified on the positive (5'-3') and negative (3'-5') strands are indicated with lines above and below the nucleotides, respectively. Numbers in parentheses reflect MatInspector Professional values (see Web Site References) for matrix and core (i.e. the most conserved positions in the matrix), where 1 is a perfect match. Nucleotides that appear in a position where the matrix exhibits a high conservation profile [conservation index (ci)-value > 60, see (13) for ci calculation] are bolded. The putative RNA regulatory motifs are underlined and labelled by name. The possible AT rich regions neighbouring the AU pentamers or AU nonamers are denoted by dotted AT nucleotides.

Figure 3-5

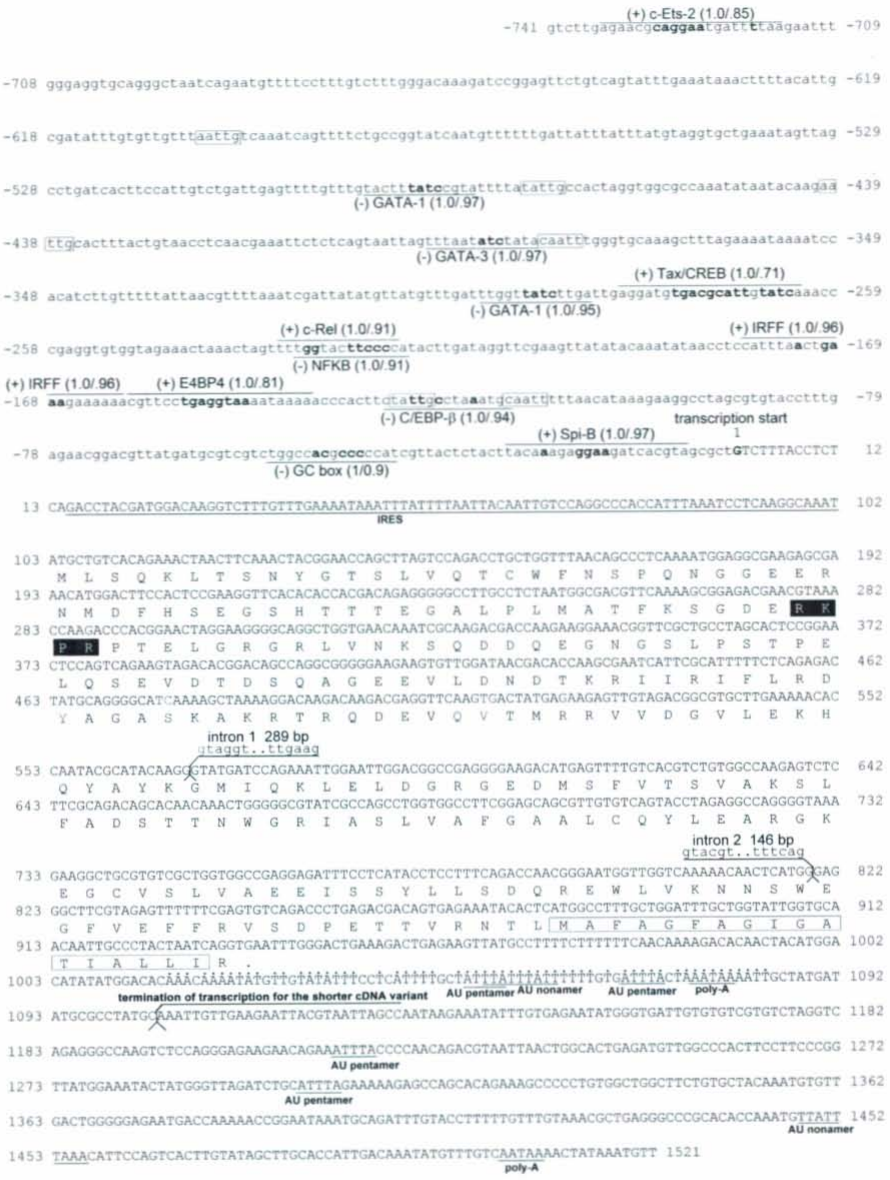
(+) c-Ets-2 (1.0/.85)
(-) GATA-1 (1.0/.97)
(-) GATA-3 (1.0/.97)
(+) Tax/CREB (1.0/.71)
(-) GATA-1 (1.0/.95)
(+) c-Rel (1.0/.91)
(+) IRFF (1.0/.96)
(-) NFKB (1.0/.91)
(+) IRFF (1.0/.96)
(+) E4BP4 (1.0/.81)
(-) C/EBP-β (1.0/.94)
transcription start
(+) Spi-B (1.0/.97)
(-) GC box (1/0.9)
IRES
intron 1 289 bp
gtaggt..ttgaag
intron 2 146 bp
gtacgt..tttcag
termination of transcription for the shorter cDNA variant
AU pentamer
AU nonamer
AU pentamer
poly-A
AU pentamer
AU pentamer
AU nonamer
poly-A

Figure 3-5. Nucleotide and inferred amino acid sequence of the Atlantic cod Mcl-1 gene. The cDNA sequences are shown as upper case letters, while the introns and the promoter regions are shown as lower case letters. The numbers on both sides mark the location with respect to the transcription start (bolded and labelled), and negative numbers are used to mark the nucleotides upstream of the transcription start. The location and size of the introns are indicated, while the sequence can be found in the NCBI database (accession no. GQ387050). The putative TM domain and NLS predicted using PSORTII (see Web Site References) are indicated by clear and black boxes, respectively. Selected (See Materials and methods for selection criteria) putative transcription factor binding sites are indicated in the same way as in Figure 3-4 (See texts for Figure 3-4 and Materials and Methods for details). The putative p53 binding site consisted of 3 quarter sites are noted by a grey box with arrows showing the orientation of each of the quarter sites (see Materials and Methods for details). The putative GM-CSF (granulocyte-macrophage colony-stimulating factor) binding motifs (a repeated CAATW required for promoter activity) are boxed. The putative RNA regulatory motifs are underlined and labelled by name. The AT rich regions neighbouring the putative RNA instability motifs are denoted by dotted AT nucleotides.

Figure 3-6

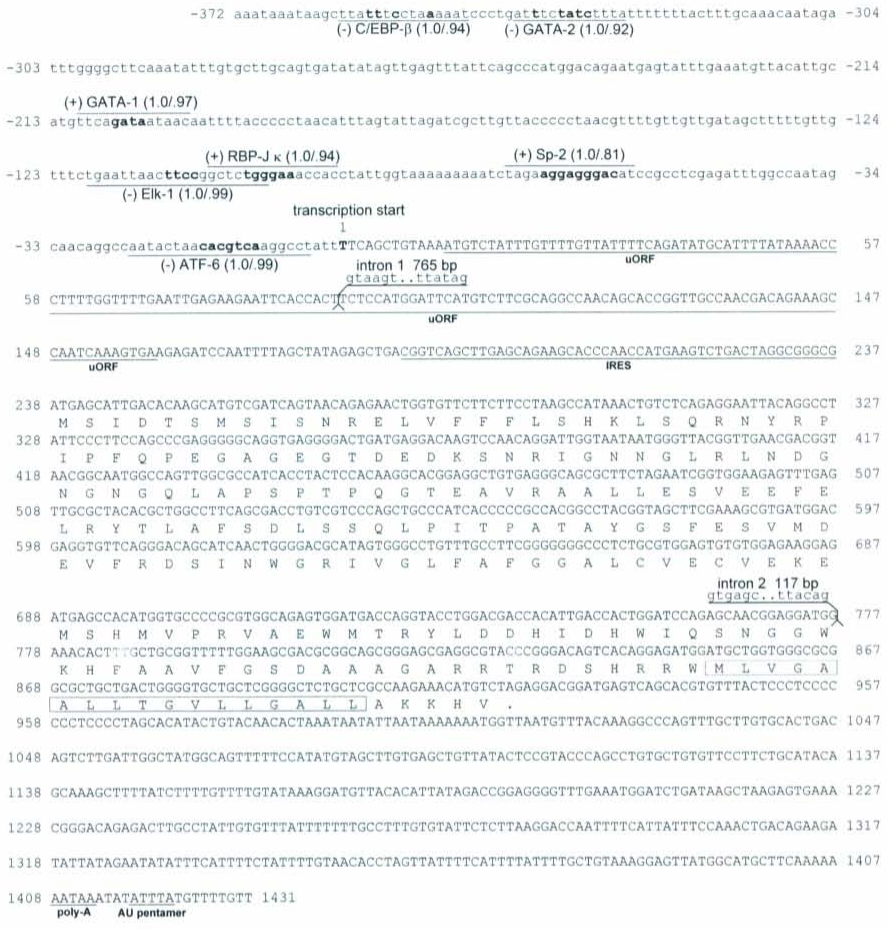
(-) C/EBP-β (1.0/.94)
(-) GATA-2 (1.0/.92)
(+) GATA-1 (1.0/.97)
(+) RBP-J κ (1.0/.94)
(+) Sp-2 (1.0/.81)
(-) Elk-1 (1.0/.99)
transcription start
(-) ATF-6 (1.0/.99)
intron 1 765 bp
gtaagt..ttatag
uORF
uORF
uORF
IRES
intron 2 117 bp
gtgagc..ttacag
poly-A
AU pentamer

Figure 3-6. Nucleotide and inferred amino acid sequence of the Atlantic cod Bcl-X1 gene. The cDNA sequences are shown as upper case letters, while the introns and the promoter regions are shown as lower case letters. The numbers on both sides mark the location with respect to the transcription start (bolded and labelled), and negative numbers are used to mark the nucleotides upstream of the transcription start. The location and size of the introns are indicated, while the sequence can be found in the NCBI database (accession no. GQ387051). The putative amino acid sequence is shown under the ORF, and the TM domain predicted using PSORTII is boxed. Selected (See Materials and Methods for selection criteria) putative transcription factor binding sites are indicated in the same way as in Figure 3-4 (See texts for Figure 3-4 and Materials and Methods for details). The putative RNA regulatory motifs are underlined and labelled by name.

Figure 3-7

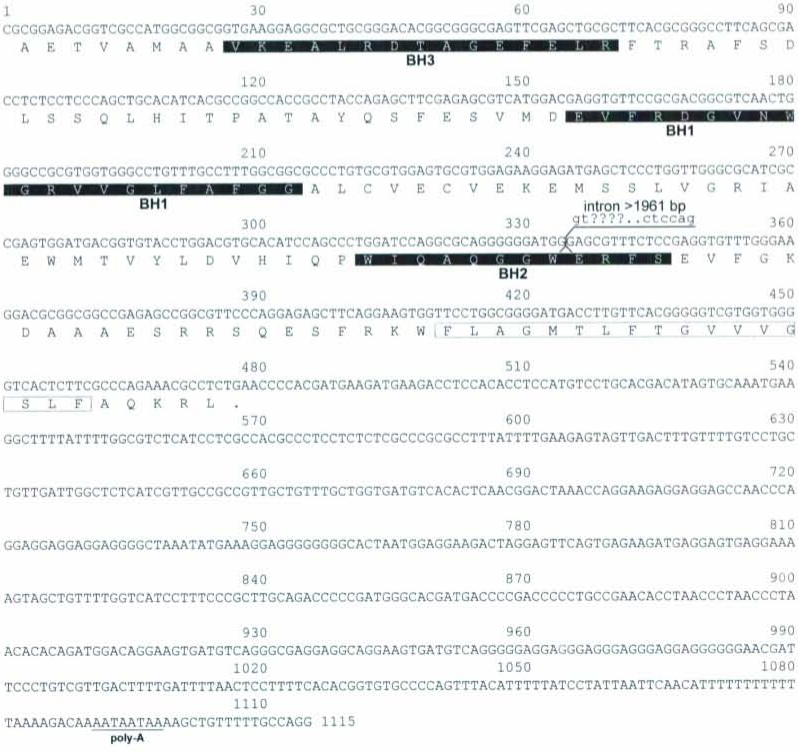

Figure 3-7. Partial nucleotide and inferred amino acid sequence of the Atlantic cod Bcl-X2 gene. The cDNA sequences are shown as upper case letters, while the intronic sequence is shown as lower case letters. The numbers on top of the sequences mark the location with respect to the start of the partial cDNA sequence. The location of the intron is indicated, while its size is still unknown. Sequences can be found in the NCBI nucleotide database (accession no. GQ387052 and GQ387053 for partial genomic and partial cDNA sequences, respectively). The putative amino acid sequence is shown below the ORF. The BH domains, predicted based on PROSITE consensus patterns (see Materials and methods for details), are shaded in black. The TM domain, predicted using PSORTII, is boxed. The putative RNA regulatory motifs are underlined and labelled by name.

Figure 3-8

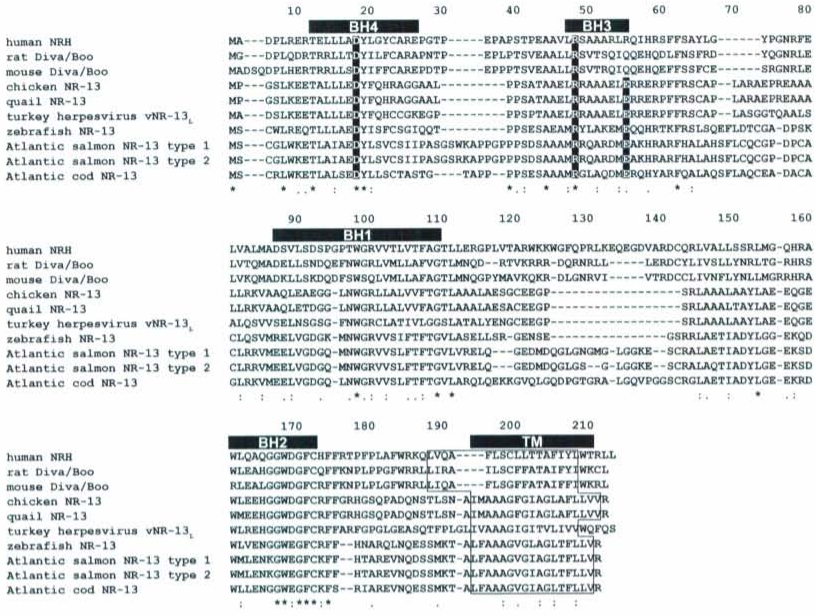
10 20 30 40 50 60 70 80
BH4
BH3
human NRH
rat Diva/Boo
mouse Diva/Boo
chicken NR-13
quail NR-13
turkey herpesvirus vNR-13L
zebrafish NR-13
Atlantic salmon NR-13 type 1
Atlantic salmon NR-13 type 2
Atlantic cod NR-13
90 100 110 120 130 140 150 160
BH1
170 180 190 200 210
BH2
TM

Figure 3-8. Multiple alignment of putative NR-13 amino acid sequence of Atlantic cod (*Gadus morhua*) with putative orthologous sequences from other species retrieved from the NCBI protein database (see Tables 3-3 and 3-4). Asterisks (*) are used to denote identical residues; conservative substitutions (as defined by CLUSTALX) are denoted by colons (:); semi-conservative substitutions (as defined by CLUSTALX) are denoted by periods (.). Individual sequences are designated by the common name of the species followed by the gene name (see Table 3-4 for naming information). The GenBank accession numbers for all sequences are listed in Table 3-3. The conserved BH and TM domains are indicated above the alignment (see Table 3-4 for detailed explanation). The aspartate (D), arginine (R), and glutamic acid (E) residues that are important in the BH domain interaction of chicken NR-13 are highlighted in black (see Results section for details).

Figure 3-9

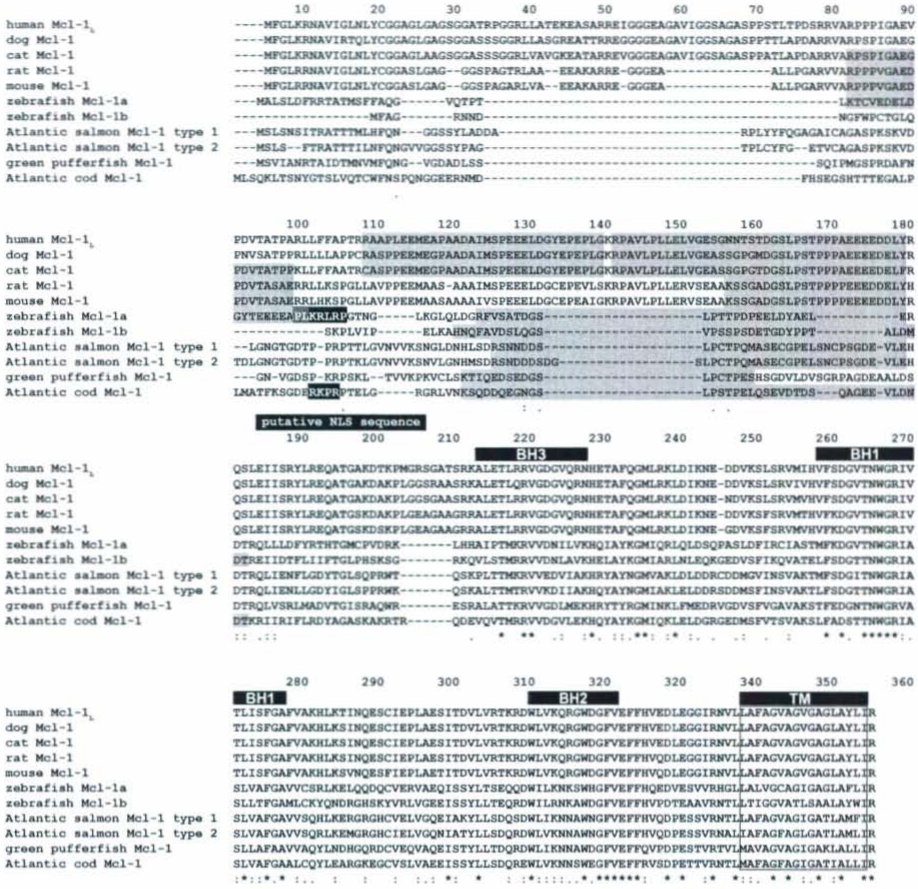
human Mcl-1L
dog Mcl-1
cat Mcl-1
rat Mcl-1
mouse Mcl-1
zebrafish Mcl-1a
zebrafish Mcl-1b
Atlantic salmon Mcl-1 type 1
Atlantic salmon Mcl-1 type 2
green pufferfish Mcl-1
Atlantic cod Mcl-1
putative NLS sequence
BH3
BH1
BH1
BH2
TM

Figure 3-9. Multiple alignment of putative Mcl-1 amino acid sequence of Atlantic cod (*Gadus morhua*) with putative orthologous sequences from other species retrieved from the NCBI protein database (see Tables 3-3 and 3-5). The NLSs predicted using PSORTII for zebrafish Mcl-1a and cod Mcl-1 are highlighted in black (see Results section for details). Asterisks (*) are used to denote identical residues; conservative substitutions (as defined by CLUSTALX) are denoted by colons (:); semi-conservative substitutions (as defined by CLUSTALX) are denoted by periods (.). The predicted PEST regions are highlighted in grey for each of the amino acid sequences (see Materials and Methods section for PEST region prediction). Individual sequences are designated by the common name of the species followed by the gene name (see Table 3-5 for naming information). The conserved BH and TM domains are indicated above the alignment (see Table 3-5 for detailed explanation).The GenBank accession numbers for sequences are listed in Table 3-3.

Figure 3-10

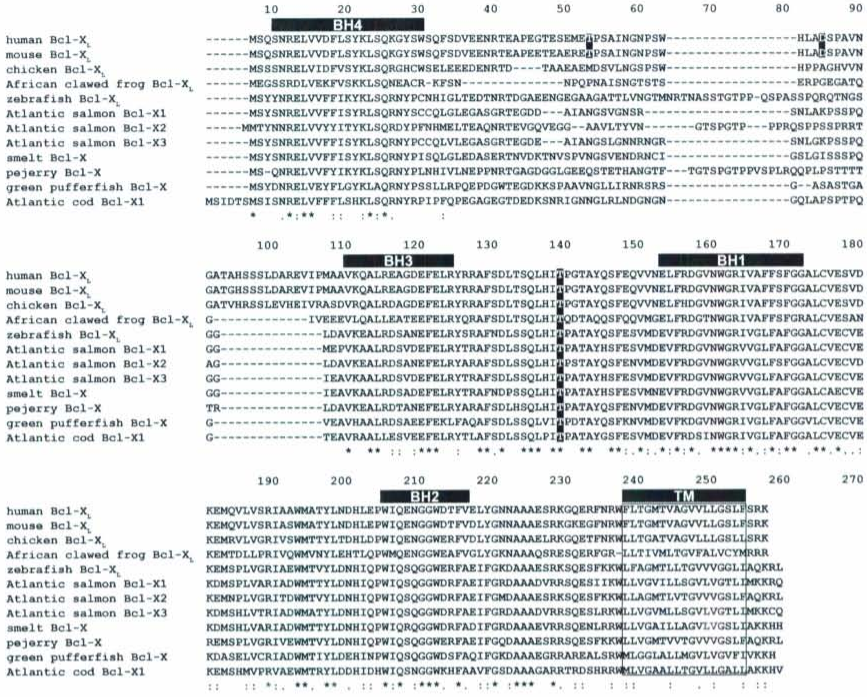
BH4
BH3
BH1
BH2
TM
human Bcl-X
mouse Bcl-X
chicken Bcl-X
African clawed frog Bcl-X
zebrafish Bcl-X
Atlantic salmon Bcl-X1
Atlantic salmon Bcl-X2
Atlantic salmon Bcl-X3
smelt Bcl-X
pejerry Bcl-X
green pufferfish Bcl-X
Atlantic cod Bcl-X1

Figure 3-10. Multiple alignment of putative Bcl-X1 amino acid sequence of Atlantic cod (*Gadus morhua*) with putative orthologous sequences from other species retrieved from the NCBI protein database (see Tables 3-3 and 3-6). Asterisks (*) are used to denote identical residues; conservative substitutions (as defined by CLUSTALX) are denoted by colons (:); semi-conservative substitutions (as defined by CLUSTALX) are denoted by periods (.). Individual sequences are designated by the common name of the species followed by the gene name (see Table 3-3 for naming). The GenBank accession numbers for all sequences are listed in Table 3-3. The conserved BH and TM domains are indicated above the alignment (see Table 3-6 for detailed explanation). The aspartate (D) and threonine (T) residues that are important in post-translational modification of human Bcl-X_L are highlighted in black (See Results section for details).

Figure 3-11

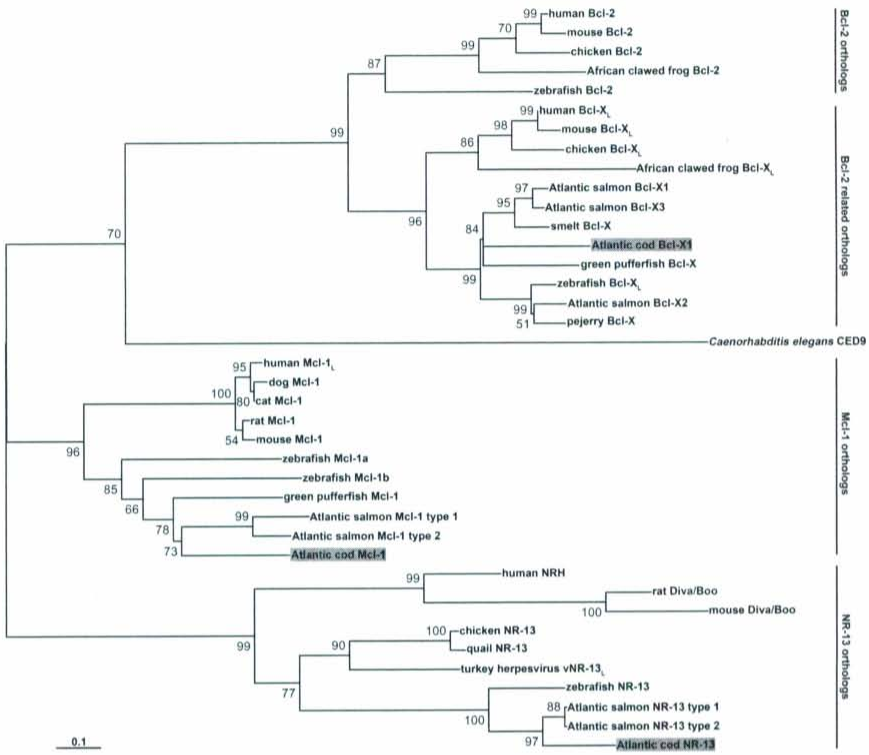
human Bcl-2
mouse Bcl-2
chicken Bcl-2
African clawed frog Bcl-2
zebrafish Bcl-2
human Bcl-X$_L$
mouse Bcl-X$_L$
chicken Bcl-X$_L$
African clawed frog Bcl-X$_L$
Atlantic salmon Bcl-X1
Atlantic salmon Bcl-X3
smelt Bcl-X
Atlantic cod Bcl-X1
green pufferfish Bcl-X
zebrafish Bcl-X$_L$
Atlantic salmon Bcl-X2
pejerry Bcl-X
Caenorhabditis elegans CED9
human Mcl-1$_L$
dog Mcl-1
cat Mcl-1
rat Mcl-1
mouse Mcl-1
zebrafish Mcl-1a
zebrafish Mcl-1b
green pufferfish Mcl-1
Atlantic salmon Mcl-1 type 1
Atlantic salmon Mcl-1 type 2
Atlantic cod Mcl-1
human NRH
rat Diva/Boo
mouse Diva/Boo
chicken NR-13
quail NR-13
turkey herpesvirus vNR-13$_L$
zebrafish NR-13
Atlantic salmon NR-13 type 1
Atlantic salmon NR-13 type 2
Atlantic cod NR-13
0.1
Bcl-2 orthologs
Bcl-2 related orthologs
Mcl-1 orthologs
NR-13 orthologs

Figure 3-11. Phylogenetic analysis of Atlantic cod NR-13, Mcl-1, and Bcl-X1. The Atlantic cod putative proteins (NR-13, Mcl-1, and Bcl-X1) were aligned with representative anti-apoptotic Bcl-2 sub-family members from other species using MEGA4 (67). Based on the multiple sequence alignment, an unrooted phylogenetic tree was constructed by the neighbour-joining method and was bootstrapped 10,000 times. The consensus tree was plotted with more than 50% of all replicates supporting any partitioning, and the bootstrap values (percentages) are marked at the branch points. The GenBank accession numbers for all sequences are listed in Table 3-3.

Figure 3-12

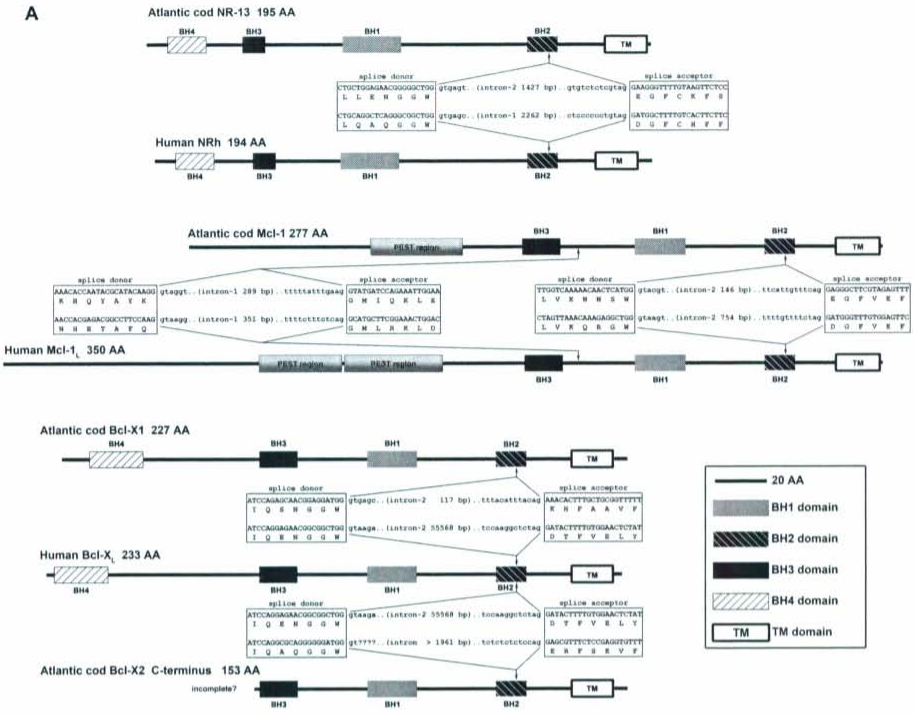
A
Atlantic cod NR-13 195 AA
BH4
BH3
BH1
BH2
TM
splice donor
splice acceptor
Human NRh 194 AA
Atlantic cod Mcl-1 277 AA
PEST region
Human Mcl-1L 350 AA
Atlantic cod Bcl-X1 227 AA
Human Bcl-XL 233 AA
Atlantic cod Bcl-X2 C-terminus 153 AA
incomplete?
20 AA
BH1 domain
BH2 domain
BH3 domain
BH4 domain
TM domain
B
BH2 signature consensus pattern
human NRH
chicken NR-13
zebrafish NR-13
Atlantic cod NR-13
human Mcl-1L
mouse Mcl-1
zebrafish Mcl-1a
zebrafish Mcl-1b
Atlantic cod Mcl-1
human Bcl-XL
mouse Bcl-XL
chicken Bcl-XL
zebra fish Bcl-XL
Atlantic cod Bcl-X1
Atlantic cod Bcl-X2
human Bcl-2
mouse Bcl-2
zebrafish Bcl-2
human Bcl-2-A1
mouse Bcl-2-A1
chicken Bcl-2-A1
human Bcl-w
mouse Bcl-w
C. elegans CED-9
mismatch with the BH2 consensus pattern
conserved intron/exon boundary

Figure 3-12. Comparison of domain organisations and intron/exon boundaries of anti-apoptotic sub-family members. (A) Diagram comparison of the domain organisations of predicted Atlantic cod NR-13, Mcl-1, Bcl-X1, and Bcl-X2 proteins and intron/exon boundaries with respect to the location of amino acid residues in putative human orthologues. The protein domains are derived from multiple alignments (see Figures 3-8, 3-9, and 3-10 for sequence alignments), and the criteria for domain definition can be found in the Materials and Methods section and Tables 3-4, 3-5, and 3-6. The intron/exon boundaries within the coding regions are compared between human and Atlantic cod putative orthologues, and the nucleotides that translate to 7 adjacent amino acid residues are shown and boxed. In order to identify of intron/exon boundaries, relevant human cDNA sequences (see Table 3-3 for GenBank accession numbers) were used for BLASTn against the human genome (NCBI database). The size of the Bcl-X2 intron is unknown beyond the fact it is greater than 1961 bp (see Results for details). (B) Anti-apoptotic Bcl-2 sub-family genes contain intron/exon boundaries at a conserved location in the BH2 domains of the predicted proteins. The BH2 consensus pattern is based on PROSITE signature sequence (accession no. PS01258). The mismatches with the BH2 consensus pattern are shaded in grey. The BH2 sequences were retrieved from representative members of the Anti-apoptotic Bcl-2 sub-family. In order to identify of intron/exon boundaries, cDNA sequences encoding the anti-apoptotic Bcl-2 sub-family proteins were used for BLASTn against appropriate genomes. The GenBank accession numbers for all amino acid and nucleotide sequences are listed in Table 3-3.

Figure 3-13

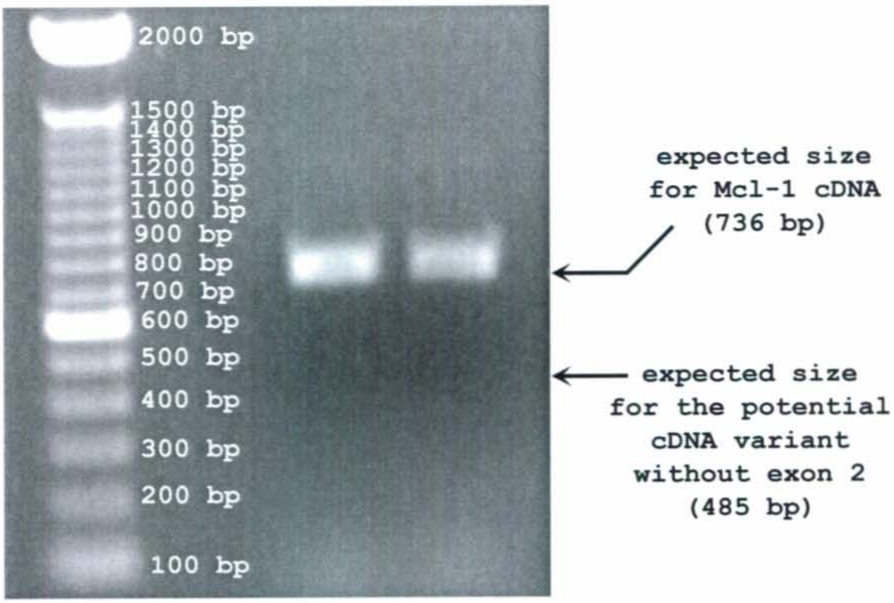
2000 bp
1500 bp
1400 bp
1300 bp
1200 bp
1100 bp
1000 bp
900 bp
800 bp
700 bp
600 bp
500 bp
400 bp
300 bp
200 bp
100 bp
expected size
for Mcl-1 cDNA
(736 bp)
expected size
for the potential
cDNA variant
without exon 2
(485 bp)

Figure 3-13. The analysis of Atlantic cod Mcl-1 cDNA PCR products by agarose gel electrophoresis. The Mcl-1 specific primer pairs flanking exon 2, used for nested PCRs, were Mcl-1_F1/Mcl-1R7 and Mcl-1_F2/Mcl-1_R6 (see Table 3-2 for primer sequences). RACE-ready cDNA derived from total RNA extracted from cod spleen was used as the PCR template (see Materials and Methods for details). The cDNA PCR reactions were run in duplicate, represented by two lanes. The Invitrogen 100 bp DNA ladder was used as a size marker, and the size of each band is marked on the right. Based on the compiled Mcl-1 cDNA (Figure 3-5), the expected size for the product containing exon 2 is calculated as 736 bp. By subtracting the size of exon 2 (251 bp) from 736 bp, the expected size for a potential product excluding exon 2 is calculated as 485 bp. The presence of a single band around 700-800 bp indicates absence of the transcript (Mcl-1_S) containing only exon 1 and 3 in the template used. Therefore, at least in spleen tissue, it is likely that skipping of the second exon does not occur in the transcription of the Atlantic cod Mcl-1 gene.

Figure 3-14

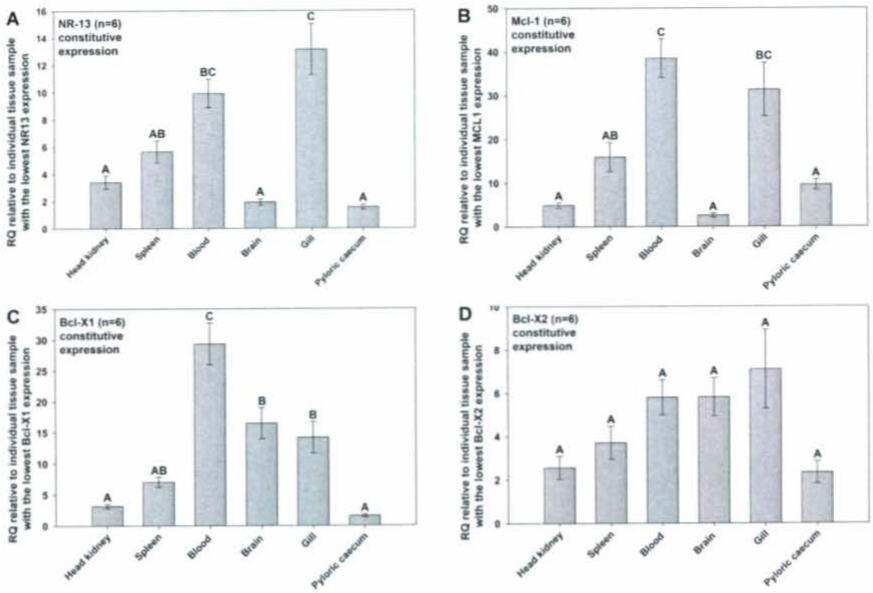
A
NR-13 (n=6)
constitutive
expression
RQ relative to individual tissue sample
with the lowest NR13 expression
A
AB
BC
A
C
A
Head kidney
Spleen
Blood
Brain
Gill
Pyloric caecum
B
Mcl-1 (n=6)
constitutive
expression
RQ relative to individual tissue sample
with the lowest MCL1 expression
A
AB
C
A
BC
A
Head kidney
Spleen
Blood
Brain
Gill
Pyloric caecum
C
Bcl-X1 (n=6)
constitutive
expression
RQ relative to individual tissue sample
with the lowest Bcl-X1 expression
A
AB
C
B
B
A
Head kidney
Spleen
Blood
Brain
Gill
Pyloric caecum
D
Bcl-X2 (n=6)
constitutive
expression
RQ relative to individual tissue sample
with the lowest Bcl-X2 expression
A
A
A
A
A
A
Head kidney
Spleen
Blood
Brain
Gill
Pyloric caecum

Figure 3-14. QPCR analyses of NR-13, Mcl-1, Bcl-X1, and Bcl-X2 constitutive gene expression across tissues. Gene expression data are presented as means (± standard error). RQ: relative quantity normalized to 18S ribosomal RNA and calibrated to the individual sample with the lowest gene of interest expression. Within each gene of interest study, identical letters indicate no significant difference ($p > 0.01$) between the tissues.

Figure 3-15

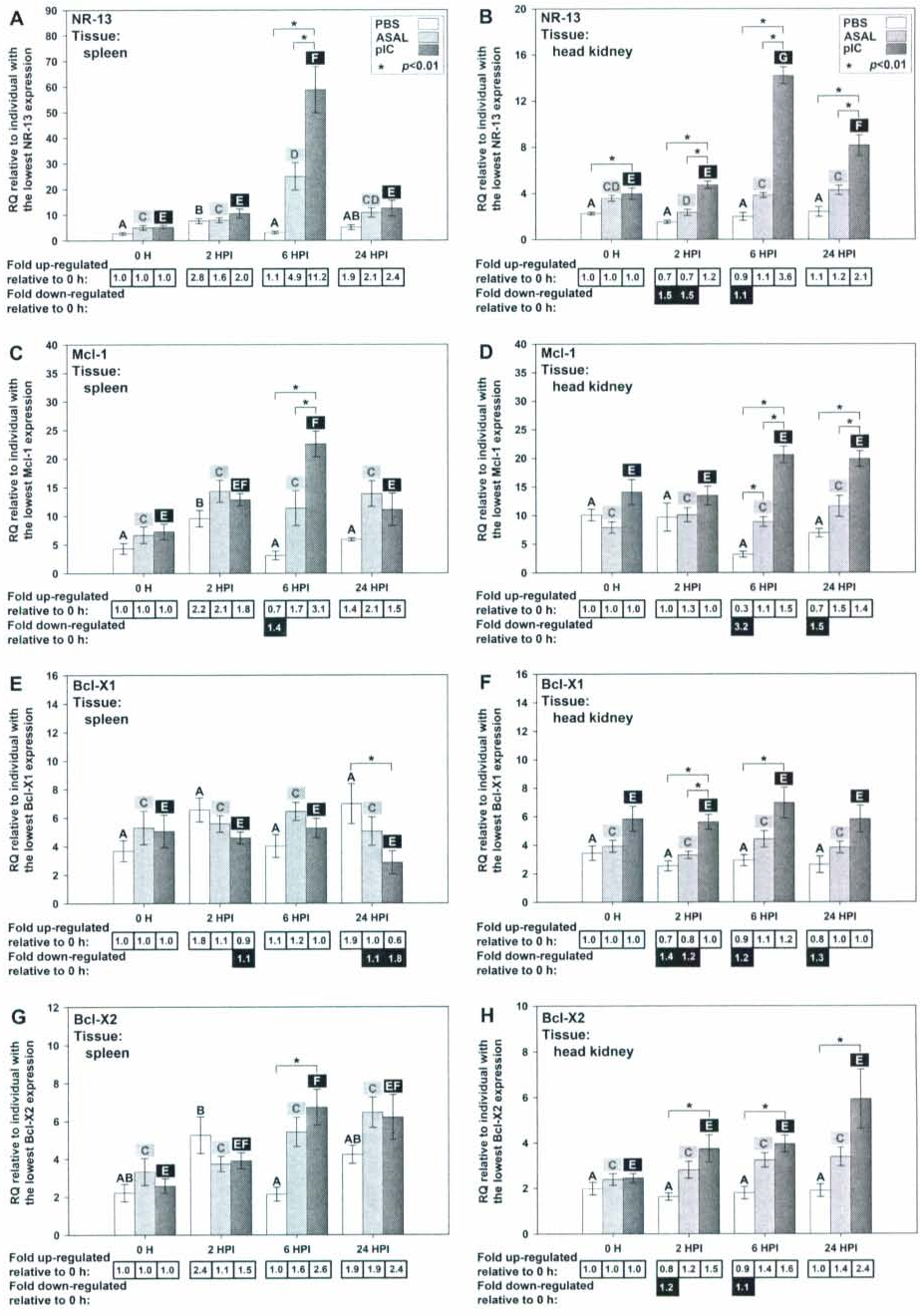
A
NR-13
Tissue:
spleen
PBS
ASAL
pIC
* p<0.01
RQ relative to individual with the lowest NR-13 expression
Fold up-regulated relative to 0 h:
Fold down-regulated relative to 0 h:
0 H
2 HPI
6 HPI
24 HPI
1.0 1.0 1.0
2.8 1.6 2.0
1.1 4.9 11.2
1.9 2.1 2.4
B
NR-13
Tissue:
head kidney
1.0 1.0 1.0
0.7 0.7 1.2
1.5 1.5
0.9 1.1 3.6
1.1
1.1 1.2 2.1
C
Mcl-1
Tissue:
spleen
RQ relative to individual with the lowest Mcl-1 expression
1.0 1.0 1.0
2.2 2.1 1.8
0.7 1.7 3.1
1.4
1.4 2.1 1.5
D
Mcl-1
Tissue:
head kidney
1.0 1.0 1.0
1.0 1.3 1.0
0.3 1.1 1.5
3.2
0.7 1.5 1.4
1.5
E
Bcl-X1
Tissue:
spleen
RQ relative to individual with the lowest Bcl-X1 expression
1.0 1.0 1.0
1.8 1.1 0.9
1.1
1.1 1.2 1.0
1.9 1.0 0.6
1.1 1.8
F
Bcl-X1
Tissue:
head kidney
1.0 1.0 1.0
0.7 0.8 1.0
1.4 1.2
0.9 1.1 1.2
1.2
0.8 1.0 1.0
1.3
G
Bcl-X2
Tissue:
spleen
RQ relative to individual with the lowest Bcl-X2 expression
1.0 1.0 1.0
2.4 1.1 1.5
1.0 1.6 2.6
1.9 1.9 2.4
H
Bcl-X2
Tissue:
head kidney
1.0 1.0 1.0
0.8 1.2 1.5
1.2
0.9 1.4 1.6
1.1
1.0 1.4 2.4

Figure 3-15. QPCR analyses of NR-13, Mcl-1, Bcl-X1, and Bcl-X2 gene expression following immune stimulation. Gene expression data are presented as means (± standard error). RQ: relative quantity normalized to 18S ribosomal RNA and calibrated to the individual with the lowest gene of interest expression. Within each gene of interest study, identical letters indicate no significant difference ($p > 0.01$) between the groups at the different time points post-injection. [Letters with no shading are for saline injected control (PBS) data; letters that are shaded grey and black are for ASAL and pIC gene expression data, respectively.] The asterisks and associated brackets identify significant ($p \leq 0.01$) differences between any two of the three treatment groups at a particular time point. For each condition and time point (e.g. pIC, 24 h), fold up-regulation was calculated as (average RQ) / (average RQ for the appropriate 0 h control group); fold down-regulation, where appropriate, was calculated as the inverse of fold up-regulation.

Figure 3-16

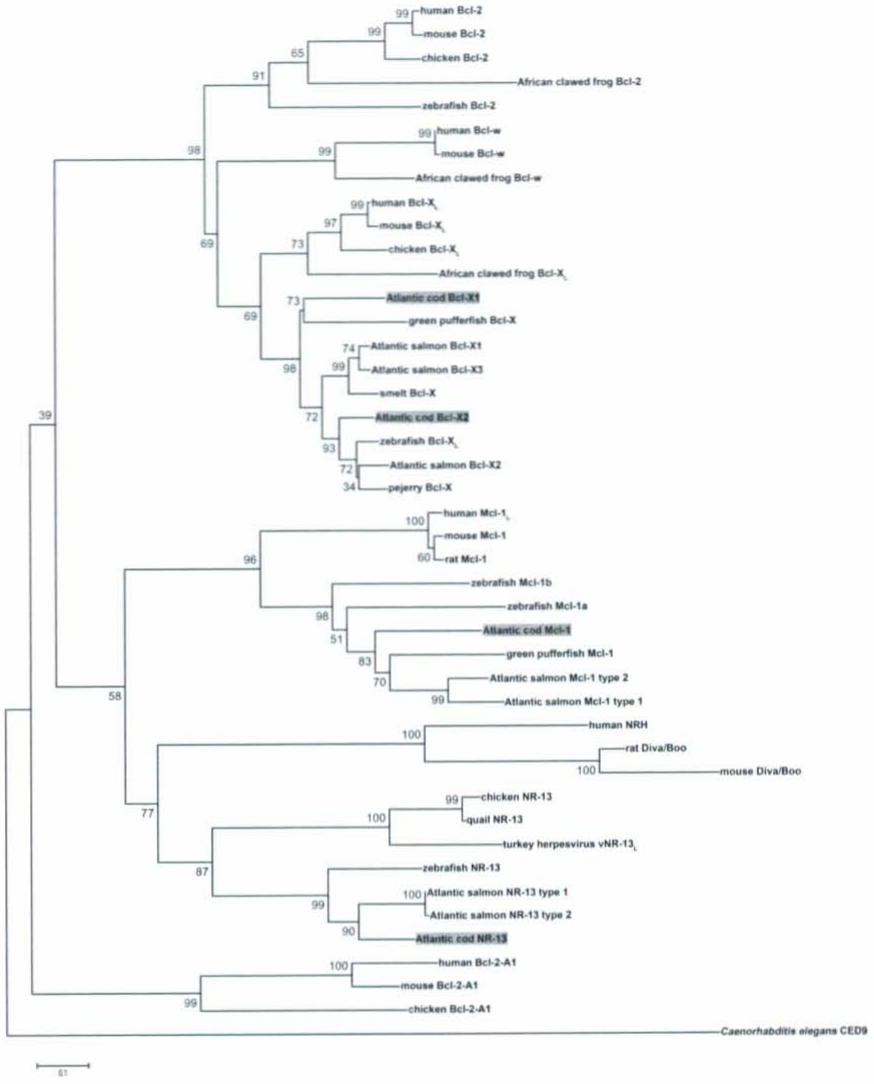
human Bcl-2
mouse Bcl-2
chicken Bcl-2
African clawed frog Bcl-2
zebrafish Bcl-2
human Bcl-w
mouse Bcl-w
African clawed frog Bcl-w
human Bcl-X$_L$
mouse Bcl-X$_L$
chicken Bcl-X$_L$
African clawed frog Bcl-X$_L$
Atlantic cod Bcl-X1
green pufferfish Bcl-X
Atlantic salmon Bcl-X1
Atlantic salmon Bcl-X3
smelt Bcl-X
Atlantic cod Bcl-X2
zebrafish Bcl-X$_L$
Atlantic salmon Bcl-X2
pejerry Bcl-X
human Mcl-1$_L$
mouse Mcl-1
rat Mcl-1
zebrafish Mcl-1b
zebrafish Mcl-1a
Atlantic cod Mcl-1
green pufferfish Mcl-1
Atlantic salmon Mcl-1 type 2
Atlantic salmon Mcl-1 type 1
human NRH
rat Diva/Boo
mouse Diva/Boo
chicken NR-13
quail NR-13
turkey herpesvirus vNR-13$_L$
zebrafish NR-13
Atlantic salmon NR-13 type 1
Atlantic salmon NR-13 type 2
Atlantic cod NR-13
human Bcl-2-A1
mouse Bcl-2-A1
chicken Bcl-2-A1
Caenorhabditis elegans CED9
0.1

Figure 3-16. Phylogenetic analysis of Atlantic cod NR-13, Mcl-1, Bcl-X1 and Bcl-X2 based on partial predicted amino acid sequences. The Atlantic cod putative proteins (NR-13, Mcl-1, Bcl-X1, and Bcl-X2) were aligned with representative anti-apoptotic Bcl-2 sub-family members from other species using MEGA (67). Since the predicted protein sequence for Bcl-X2 appeared to be missing the N-terminus (20-30 residues based on BLASTx analysis, see the Results section for details), this region of the N-terminus of the sequence alignment was truncated for all aligned sequences for construction of the phylogenetic tree to avoid any bias against Bcl-X2. Based on the partial multiple sequence alignment, an unrooted phylogenetic tree was constructed by the neighbour-joining method and was bootstrapped 10,000 times. The consensus tree was plotted with most of the replicates supporting any partitioning, and the bootstrap values (percentages) are marked at the branch points. The GenBank accession numbers for all sequences are listed in Table 3-3.

CHAPTER 4 : General conclusions

4.1 Major findings

The study of Atlantic cod immune tissue responses using functional genomics approaches has allowed the identification and characterization of genes and molecular pathways involved in cod immune responses to pathogens. The major findings and outcomes of this research are listed below:

- Identification of differentially expressed genes in spleen and head kidney tissues of Atlantic cod challenged with intraperitoneal injections of formalin-killed, atypical *Aeromonas salmonicida* by analyzing 4154 expressed sequence tags (ESTs) generated from four reciprocal suppression subtractive hybridization (SSH) cDNA libraries.
- Identification and QPCR-based expression analysis of the following Atlantic cod transcripts as up-regulated by formalin-killed, atypical *A. salmonicida*: interleukin 1β (IL1β), interleukin 8 (IL8), a small inducible cytokine (SCYA), interferon regulatory factor 1 (IRF1), ferritin heavy subunit (FTH), cathelicidin, and hepcidin.
- Identification, acquisition, and assembly of the full length cDNA sequences for the following Atlantic cod genes: IRF1, NR-13, Mcl-1, and Bcl-X1 using RACE, cloning, and sequencing.

- Characterization of the gene structures and promoter regions for NR-13, Mcl-1, and Bcl-X1 using a combination of PCR (using genomic DNA as template) and genome walking PCR.
- Identification of two differentially expressed Bcl-X paralogues in Atlantic cod (Bcl-X1 and Bcl-X2).
- Identification of Atlantic cod NR-13, Mcl-1, and Bcl-X2 as immune-relevant transcripts that are involved in response to polyriboinosinic polyribocytidylic acid (a viral mimic).

4.2 Future research

Using functional genomics approaches, this research has lead to the identification and characterization of many immune-relevant genes in Atlantic cod, and the expression of several of these transcripts has been studied in detail. However, further studies (e.g. listed below) are needed to fully understand immune responses of Atlantic cod at the molecular level.

- Along with other studies, the identification of ASAL-responsive Atlantic cod transcripts in this study contributed to the CGP EST database and 20,000-gene (20K) Atlantic cod microarray platform. Using this platform, the transcriptomic responses of Atlantic cod immune-relevant tissues to different immunogenic stimuli (e.g. following intraperitoneal injection with a bacterial antigen) will be studied in the future.

- Although the change in mRNA expression level of a given gene during Atlantic cod immune responses suggests that the gene is immune-relevant, processes operating at translational and post-translational levels may affect expression and activity at the protein level. In order to study how immune-relevant Atlantic cod gene (e.g. Mcl-1) expression influences the protein levels, recombinant protein or antigenic peptides may be used to generate specific antibodies (e.g. polyclonal and/or monoclonal antibodies against cod immune-relevant proteins). These antibodies should be developed, and will be important for future cod immune studies involving techniques such as western blotting and immunohistochemistry.
- The definitive roles of genes in the immune response can be further studied by functional characterization of the proteins encoded by these genes. Future research using recombinant protein technology should be conducted to functionally characterize immune-relevant genes such as cytokines and anti-microbial peptides (AMPs). For example, the chemotactic effects of cod chemokines on fish cells can be studied using chemotactic assays, and the anti-bacterial activity of AMPs can be studied using bactericidal assays. An important candidate for such studies would be a small inducible cytokine (SCYA), the mRNA expression of which was upregulated following stimulation with both bacterial and viral antigens. Important candidate proteins for such studies include cathelicidin and hepcidin, which have been identified and characterized at transcript level.

- The transcription control mechanisms for many immune-relevant genes can be studied to explain their expression patterns following immune responses. The cloning and analysis of the promoters of target genes (e.g. NR-13, Mcl-1, and Bcl-X1 in this research) mark the first step in understanding the transcription regulatory mechanisms of these genes. However, further study is needed to verify the putative transcription factor binding sites identified using bioinformatics tools. For example, to determine if STAT-5 binds to the Atlantic cod NR-13 promoter, recombinant STAT-5 transcription factor and cloned NR-13 promoter can be used for DNA footprinting and gel eletromobility shift assays.

- Functional characterization of apoptotic regulators belonging to the Bcl-2 family can be achieved through modification of gene expression in a fish cell line (e.g. GL-av cells) or a fish embryo as these proteins function at the intracellular level. Taking NR-13 as an example, in order to access its role as an apoptotic inhibitor, a cell line (GL-av) can be transformed with NR-13 expression vectors (experimental) or empty vectors (control) (co-expression of green fluorescent protein may be needed for detection), following which the cells can be exposed to a pro-apoptotic stimulus (e.g. LPS, pIC, and UV) to determine if overexpression of NR-13 can rescue cells from apoptosis. If embryos were to be used as the experimental model, the overexpression of cod NR-13 could be achieved in cod embryo by micro-injection of *de novo* synthesized NR-13 mRNA; conversely, the endogenous NR-13 expression

can be "knocked down" by micro-injection of antisense morpholino oligonucleotides that inhibit the translation of NR-13 mRNA. The phenotype generated from such manipulations can then be studied using functional genomics tools (e.g. microarray and QPCR) and observation techniques (e.g. microscopy).

Appendix. List of the presentations delivered.

May 6th 2008 Conference of the Canadian Society of Zoologists, Halifax, NS

Identification and analysis of differentially expressed genes in the immune tissues of Atlantic cod (Gadus morhua) challenged with formalin-killed atypical Aeromonas salmonicida