Thesis
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Macrophage Activating Factor (MAF) in rainbow trout (*Oncorhynchus mykiss*) - biological activity and molecular source

By

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Declaration

I, Rubina Qasour Sharif, hereby declare that this thesis has been composed by myself. It is a record of my own work and that it has not been accepted in partial or complete fulfilment of any other degree or qualification.

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List of abbreviations

aa	amino acids
AMV	avian myeloblastosis virus
ANOVA	analysis of variance
BCIP	5-bromo-4-chloro-3-indlyl phosphate
BMPs	bone morphogenetic proteins
cDNA	complementary deoxyribonucleic acid
CEF	chick epithelial fibroblasts
CFS	cell-free supernatant
CHSE	chinook salmon embryo
CL	chemiluminescence
CLP	chemiluminescent probe
CMI	cell-mediated immunity
Con A	concanavalin A
CRP	C-reactive protein
CRγC	cytokine receptor γ chain
CSF	colony stimulating factor
CSFIR	colony-stimulating factor-1 receptor
CTF	chemotactic factor
DEPC	diethylpyrocarbonate
df	dilution factor
dH_2O	distilled water
DIG	digoxigenin
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
dsRNA	double stranded ribonucleic acid
DTT	dithiotreitol
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
EPO	eosinophil peroxidase
EST	expressed sequence tag
EtBr	ethidium bromide
FGF	fibroblast growth factor

GALT	gut-associated lymphoid tissue
GMCSF	granulocyte macrophage colony stimulating factor
HBSS	Hank's balanced salt solution
HI-FBS	heat inactivated foetal bovine serum
hIL-1	human interleukin-1
H_2O_2	hydrogen peroxide
hTGF	human transforming growth factor
hv	photon
ICE	IL-1β converting enzyme
ICSAT	interferon Consensus Sequence binding protein for activated T cells
ICSBP	interferon consensus sequence-binding protein
IFN	interferon
IFNGR	interferon gamma receptor
IGIF	IFN-γ inducing factor
IGF-I	insulin-like growth factor-I
IHNV	infectious haematopoietic necrosis virus
IL	interleukin
IL-2	interleukin-2
IPNV	infectious pancreatic necrosis virus
IPTG	isopropyl-β-D-thiogalactopyranoside
IRF	the interferon regulatory factors
ISAV	infectious salmon anaemia virus
ISGF3γ	interferon-γ stimulated gene factor 3
JAKs	Janus tyrosine kinases
kDa	kilodalton
L-15	leibovitz-15 culture medium
LAK	lymphokine-activated killer
LB	luria-bertani
LPS	lipopolysaccharide
LSC	liquid scintillation counting
LSIRF	lymphocyte-specific interferon regulatory factor
LTβ	lymphotoxin β
mAb	monoclonal antibody
MAF	macrophage activating factor
MCP	macrophage chemoattractant protein
2ME	2- mercaptoethanol

MIF	migration inhibition factor
MIP	macrophage inflammatory protein
MHC	major histocompatability complex
MMLV-RT	Moloney murine leukaemia virus reverse transcriptase
MPO	myeloprexidase
mRNA	messenger ribonucleic acid
mw	molecular weight
NADPH	nicotinamide adenine dinucleotide phosphatase
NBT	nitroblue tetrazolium
NCC	non-specific cytotoxic cells
NDV	newcastle disease virus
NK	natural killer cell
NO	nitric oxide
O_2^-	superoxide anion radical
OD	optical density
ORF	open reading frame
PBL	peripheral blood lymphocytes
PCR	polymerase chain reaction
PDGFRβ	platelet derived growth factor β receptor
PHA	phytohaemagglutinin
PMA	13-phorbol myristate acetate
PMNs	polymorphonuclear neutrophils
PMNL	polymorphonuclear leucocyte
RANTES	regulated on activated, normal T-cell expressed and secreted
ATP	adenosine triphosphate
RBC	red blood cells
RNA	ribonucleic acid
RLU	relative light units
rRNA	ribosomal RNA
RNS	reactive nitrogen species
ROI	reactive oxygen intermediates
ROS	reactive oxygen species
rt	room temperature
RT	reverse transcriptase
RTG	rainbow trout gonad
RT-PCR	reverse trancriptase polymerase chain reaction
SDS	sodium dodecylsulphate

SHK-1	salmon head kidney
SI	stimulation indices
sIg^-	surface immunoglobulin negative cells
sIg^+	surface immunoglobulin positive cells
SOCS	suppressors of cytokine signalling
ss	single stranded
SSH	suppression subtraction hybridisation
STAT	signal transducers and activators of transcription
TCR	T cell receptor
TGEV	transmissible gastroenteritis coronavirus
TGF	transforming growth factor
Th 1/2	T-cell-helper type 1/2
T_{Ann}	annealing temperature
T_m,	melting temperature
TNF	tumour necrosis factor
TPA	12-O-tetradecanoylphorbol-13-acetate TCR
tRNA	transfer RNA
UV	ultraviolet
VHSV	viral haemorrhagic septicaemia virus
v/v	volume by volume
w/v	weight by volume
X-gal	5-bromo-4-chloro-3-β-D-galactoside
γ-IP	γ-interferon inducible protein
zfIFN	zebrafish interferon

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List of fish species

Common name used in the thesis	Scientific name
Carp (common)	*Cyprinus carpio*
Carp (ginbuna crucian)	*Carasius auratus langsdorfii*
Catfish (north african)	*Clarias gariepinus*
Catfish (channel)	*Ictalurus punctatus*
Catshark (Spotted)	*Scyliorhinus canicula*
Dogfish	*Triakis scyllia*
Eel (European)	*Anguilla anguilla*
Eel (Japanese)	*Anguilla japonica*
Flatfish / Flounder (Japanese)	*Paralichthys olivaceus*
Goldfish	*Carassius auratus*
Plaice	*Pleuronectes platessa*
Puffer fish	*Fugu rubripes*
Salmon (Atlantic)	*Salmo salar*
Salmon (chinook)	*Oncorhynchus tshawytscha*
Salmon (coho)	*Oncorhynchus kisutch*
Seabass (European)	*Dicentrarchus labrax*
Seabass (striped)	*Morone saxatilis*
Seabass (hybrid-striped)	*Morone chrysops*
Sea bream (gilthead)	*Sparus aurata*
Shark	*Triakis scyllia*
Sturgeon (Siberian)	*Acipenser bari*
Trout (rainbow)	*Oncorhynchus mykiss*
Trout (brook)	*Salvelinus fontinalis*
Turbot	*Scophthalmus maximus*
Yellowtail	*Seriola quinqueradiata*
Zebrafish	*Danio rerio*

Abstract

This study investigated the biological activity of a macrophage activating factor (MAF) produced by activated lymphocytes from the rainbow trout (*Oncorhynchus mykiss*) and attempts to discover its molecular source.

Peripheral blood lymphocytes were shown to release factors with MAF activity following incubation with a variety of stimulants and were subsequently shown to activate macrophages using at least two different methods, the nitroblue tetrazolium (NBT) colourimetric assay and the luminol-dependent chemiluminescent assay. The latter technique detected an immediate response which decayed over a 40 minute period on the addition of cell-free supernatants from activated lymphocytes to macrophages.

A number of molecular approaches, including degenerate PCR primer amplification, DNA cross-hybridisation and cDNA library screening were used in this study to try to isolate any cytokine genes from *Oncorhynchus mykiss.* As a control β-actin cDNA was successfully amplified from *Oncorhynchus mykiss* using primers based on the salmon sequence. The *Oncorhynchus mykiss* orthologue of IFN-γ was initially targeted. However, although a PCR product of the appropriate size was amplified using degenerate primers based on mammalian and avian IFN-γ sequences, the sequence was not related to IFN-γ or any other known *Oncorhynchus mykiss* sequence. A similar strategy was used to try and amplify the *Oncorhynchus mykiss* orthologue of mammalian IL-15. Again despite amplification of a DNA fragment of approximately the correct size there appeared to be no relationship between it and the known IL-15 sequences.

As an alternative strategy a cDNA library from stimulated peripheral blood lymphocytes (PBLs) was constructed and screened using cDNA probes derived from stimulated and non-stimulated PBLs in order to detect mRNAs which might have been upregulated as a result of *in vitro* stimulation. A number of positive clones were obtained from the differential screening of the library including cDNAs showing similarity to other unidentified fish sequences as well as to a

number of proteins predicted to be involved in regulation of cell proliferation, neocorticogenesis and embryo development. Additionally, the library was also screened using ovine cytokine cDNA probes, although no positively hybridising clones were obtained. The ovine IFN-γ gene was also used to probe genomic DNA from *Oncorhynchus mykiss*, but unlike previous studies with human IFN-β gene no hybridisation between the ovine IFN-γ gene and *Oncorhynchus mykiss* DNA was observed.

This investigation highlights the potential difficulties of using various molecular strategies such as DNA cross-hybridisation or PCR techniques for the cloning of fish cytokine sequences. Consequently, future strategies for cloning fish cytokine genes may require targeting the biological activity through expression libraries.

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Introduction

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1.1 General Introduction

The aquaculture industry has expanded significantly over the past two decades. However, the threat of infectious diseases continues to be a major problem to successful fish husbandry due to the high intensity culture of fish. Difficulties associated with antibiotic resistance and the lack of effective chemotherapy for the control of viral diseases has led scientists to focus on fish immunology.

In mammals cytokines have been shown to regulate immune activity and form an integral part of the immune response to pathogens. As a result of this, therapies based on the use of cytokines as adjuvants, or on the stimulation of their production, have been assessed as a means of controlling infectious diseases. Relatively little is known about the fish immune system by comparison to that of mammals and the existence of cytokines in fish has only recently been established. However from the literature that has emerged over the last few decades it would appear that the fish immune system, including the role of cytokines, is likely to be similar to that found in mammals. As a result, attempts to clone and express immune effector molecules from fish such as the cytokines have increased greatly. It is hoped that identification and isolation of these molecules from fish will lead to an improved health status and reduce economic losses within the aquaculture industry.

1.2 The Fish Immune system

The immune system of fish is similar to higher vertebrates and consists of innate defences and adaptive defences. Leucocytes present in lymphoid tissues or circulating blood can be divided into phagocytes and cytotoxic cells which affect the innate responses, and lymphocytes which effect the adaptive responses (Secombes, 1994).

The major lymphoid tissues of fish include the thymus, kidney and spleen (Rowley *et al.*, 1988). As in higher vertebrates the thymus of fish plays a significant role in the development of immunity within individuals and normally involutes with age (Chilmonczyk, 1992). In adult fish, the kidney is the most important lymphoid tissue and is subdivided into the

head kidney (pronephros) and middle kidney (mesonephros). Both regions exhibit haemopoietic activity, producing red and white blood cells although the greatest production is in the head kidney, which loses its renal function (Ellis and De Sousa 1974; Zapata, 1979, 1981). In some species, for example elasmobranchs, the spleen can also be divided into erythroid (red pulp) and lymphoid (white pulp) regions. However, in teleosts it is typically an unorganised tissue. Scattered foci of leucocytes also exist in mucosal sites such as the skin, gut and gills but again no organised lymphoid structures exist (Secombes, 1994). Evidence supports the presence of T-like and B-like lymphocyte populations and of antigen-presenting cells all of which have been shown to be involved in the fish immune response to infection (Zapata and Cooper, 1990).

1.2.1 Innate Immunity

In addition to the physical barriers such as epithelial surfaces and mucus, both cellular and humoral responses are involved in the mediation of fish innate defences. The innate system is the first line of defence against microbial infection in vertebrates and is mediated by an array of cells and antimicrobial proteins, glycoproteins and peptides in tissues and body fluids. It is triggered by the recognition of such molecules as fungal cell wall β-glucans, bacterial lipopolysaccharide (LPS) and peptidoglycan, bacterial DNA and viral double stranded RNA. In fish the innate defence system has been found to be similar to that in higher vertebrates and includes the presence of phagocytic and non-specific cytotoxic cells and the production of molecules in the blood and mucus such as lysozyme and complement which directly lyse bacteria, anti-proteases (α_2-macroglobulin, α_1-proteinase inhibitor), transferrin and Type I interferon (IFN-α,-β) which inhibit bacterial and viral replication respectively (Alexander and Ingram, 1992; Dalmo *et al.*, 1997). As in mammals the innate defence can be down-regulated by stress (Anderson, 1992; Robertsen *et al.*, 1994, Raa, 1996, Robertsen, 1999).

Phagocytes have important pro-inflammatory, accessory and effector activities in innate immune responses. Phagocytosis and subsequent killing of internalised bacteria by both macrophages (monocytes) and polymorphonuclear leucocytes are important immunological

events in the prevention of disease (Bly and Clem, 1992). In mammalian systems, the mechanisms that lead to the clearance of pathogenic bacteria are well defined. However, phagocytosis of bacteria and various foreign particles by macrophages and neutrophils has also been reported for a range of fish species (MacArthur and Fletcher, 1985). Antibodies, complement and C-reactive protein (CRP) have all been described in fish and can act as opsonins to increase uptake of bacteria by phagocytic cells (Finco-Kent and Thune, 1987; Ainsworth and Dexiang, 1990; Alexander and Ingram 1992; Manson *et al.*, 1992). The resulting production of reactive oxygen species (ROS) and nitrogen species (RNS) within the phagocytic cells leads to the intracellular killing of bacteria (Secombes, 1998). The production of toxic oxidative burst products has been assessed in rainbow trout Onchorynchus *mykiss* macrophages (Sharp *et al.*, 1991) and channel catfish *Ictalurus punctatus* neutrophils (Dexiang and Ainsworth, 1991) amongst others. In particular, superoxide anion and hydrogen peroxide have been studied and their production blocked by specific respiratory burst inhibitors (Sharp *et al.*, 1991). Chemiluminescence has also been used as an indicator of an intracellular oxidative burst (Scott and Klesius 1981; Waterstrat *et al.*, 1991).

Non-specific cytotoxic cells (NCC) that lyse target cells by necrotic and apoptotic mechanisms have been described in fish (Greenlee *et al.*, 1991). Evans and Jaso-Friedmann (1992) have suggested that these cells are the equivalent of natural killer (NK) cells and can lyse a variety of target cells including tumour cell lines and protozoan parasites. The NCC antigen receptor is a vimentin-like molecule which recognises a ~40 kDa molecule on the target cells (Harris *et al.*, 1992). A monoclonal antibody to this antigen receptor has been shown to inhibit mammalian NK cell activity, suggesting it is evolutionarily conserved (Harris *et al.*, 1992).

1.2.2 Adaptive immunity

Adaptive immunity in fish is mediated by two populations of lymphocytes: B-like cells, responsible for antibody secretion, and T-like cells which direct cell killing and regulate immune responses. Indirect evidence for the presence of these two populations exists in fish, that is they have been shown to produce antibodies, reject grafts, and secrete lymphokines

(Rowley *et al.*, 1988; Secombes, 1991), however antibodies to the cell markers which distinguish these phenotypes in mammals are as yet unavailable in the fish. There are many studies describing the specific antibody response of fish to a variety of pathogens, but this review will concentrate on the specific cell-mediated response to infection.

In general, T-like lymphocytes are described as surface immunoglobulin negative (sIg^-) cells whereas the B-like cells are considered to be sIg^+ (Sizemore *et al.*, 1984; Miller *et al.*, 1987; Thuvander *et al.*, 1990; Navarro *et al.*, 1993). Several efforts have been made to generate reagents to distinguish the T-like cell population from other cell phenotypes in the fish, but so far have been largely unsuccessful. However, in the channel catfish (*Ictalurus punctatus*), one antibody has been found which reacts with cells that have T-cell functions, but it also reacts with neutrophils and thrombocytes. A second antibody has been described that reacts with the majority of thymocytes and does not recognise B cells, granulocytes, thrombocytes, macrophages or erythrocytes (Miller *et al.*, 1987, Passer *et al.*, 1997). Similarly, in Seabass *Dicentrarchus labrax*, a monoclonal antibody mAb DLT15, which reacts with the majority of cells in the thymus and T-like cells in the periphery, has also been characterised (Scapigliati *et al.*, 1995). $DLT15^+$ cells have also been found in the gut-associated lymphoid tissue GALT; (Picchietti *et al.*, 1997). Gene cloning studies have identified a number of T cell receptor (TCR) sequences in several teleost species, including rainbow trout (*Oncorynchus mykiss*), Atlantic salmon (*Salmo salar*) and channel catfish (Partula *et al.*, 1994, 1995; Hordvick *et al.*, 1996; Wilson *et al.*, 1998). Despite this, no TCR-specific mAbs have been generated, although techniques such as *in situ* hybridisation with the TCR sequences as probes have been used to detect fish T cells in different haemopoietic organs. Finally, the division of the T-cell population into specific subsets is yet to be established in fish as again there are no definitive T-cell surface markers for the teleost equivalents to mammalian CD4 and CD8 molecules.

In mammals, antigen-specific cell-mediated immunity is primarily a function of the T-helper (CD4+) and T-cytotoxic (CD8+) cell subsets. Indirect evidence for the existence of both of these cell types in fish has been demonstrated in that T-like cells have been reported to have an important accessory function in the activation of both B cells (Clem *et al.*, 1991; Kaattari,

1992) and macrophages. Similarly rejection of allogeneic scale and skin grafts in fish has been studied extensively (Stet and Egberts, 1991). Invasion of the graft with lymphocytes and the establishment of immunological memory accompany the rejection, suggesting it is an event mediated by lymphocytes similar to mammalian cytotoxic T cells (Nakanishi *et al.*, 1999). Although, from a functional perspective the presence of allo- and virus- specific cytotoxic T cells in teleosts has been reviewed (Nakanishi *et al.*, 2002), the mechanisms of recognition and killing and the precise nature of cells involved is less well defined.

Graft rejection does not occur within clonal lines of fish (Komen *et al.*, 1991) and is delayed within serologically typed second generation gynogenetic siblings (Stet *et al.*, 1990). Also, it has been demonstrated that allogeneic macrophages cannot effectively present thymus-dependent antigens for induction of antibody production and that homologous (but not heterologous) alloantisera block this presentation by autologous cells for the same reason (Vallejo *et al.*, 1992a). This data suggests histocompatibility genes and their products influence the immune system of fish, and that fish T cells will probably respond to antigens in association with the major histocompatability complex (MHC) molecules as with mammals.

The existence of MHC genes in fish has been demonstrated in a number of species, including rainbow trout, Atlantic salmon, carp (*Cyprinus carpio)*, channel catfish (*Ictalurus punctatus*), shark (*Triakis scyllia*), striped bass (*Morone saxatilis*) and zebrafish (*Danio rerio*). Both class I and class II genes have been sequenced (Juul-Madsen *et al.*, 1992; Okamura *et al.*, 1993, Ono *et al.*, 1993a; Okamura *et al.*, 1997; Hordvick *et al.*, 1993; Hardee *et al.*, 1995; Van Erp *et al.*, 1996a,b; Takeutchi *et al.*, 1995; Grimholt *et al.*, 2000) as well as the β_2 - microglobulin gene (Ono *et al.*, 1993b; Dixon *et al.*, 1993). A number of groups have reported extensive polymorphism within the fish MHC (Ono *et al.*, 1992, 1993a; Sultmann *et al.*, 1993; Grimholt *et al.*, 1994; Miller and Withler, 1996). However, the exact number of both MHC class I and class II loci, and the number of alleles contained within these loci, has not been established, although in zebrafish, it has been shown that the MHC class I and class II loci are located on different chromosomes (Postlethwait *et al.*, 1994; Bingulac-Popovic *et al.*, 1997). Linkage analysis in Atlantic salmon indicated the two classes are either located far apart on one

chromosome or are situated on different chromosomes (Grimholt and Lie, 1998). Analysis of the MHC gene sequences has revealed many sequence similarities to those in higher vertebrates, reflecting both functional and structural requirements (reviewed by Kaufman *et al.*, 1994). For example, disulphide bridges, glycosylation sites and salt bridges found in higher vertebrate MHC molecules are also present in the fish cDNA sequences. In addition potential CD4/CD8 binding domains have been reported (Stet *et al.*, 1996; Rode, 1997), providing more indirect evidence of the MHC restricted T cell sub populations.

Investigation of MHC class II β-gene expression has been assessed in both lymphoid cells and organs of fish. In carp, relatively higher levels of expression have been found in peripheral blood leucocytes, thymocytes and head kidney leucocytes than in gut leucocytes and splenocytes (Rodrigues *et al.*, 1995). High expression of the class II β-gene was also found by northern blot analysis in the gills of a variety of salmon species (Grimholt *et al.*, 1994). The regulation of MHC class II gene expression has been studied (Mach *et al.*, 1996). Whilst constitutive expression on B cells and macrophages occurs, many soluble factors have been shown to either up-regulate or down-regulate this expression (Glimcher and Kara, 1992). For example, up-regulation of MHC class II β-chain expression on trout head kidney macrophages has been shown to occur using a trout MHC class II β polyclonal antiserum (Van Lierop *et al.*, 1998), as a result of incubating the macrophages with tumour necrosis factor (TNFα) and bacterial lipopolysaccharide LPS (Knight *et al.*, 1998). Although, the expression was modulated by the synergistic action of the cytokine and bacterial product, tumour necrosis factor TNFα alone had little effect. This is similar to previous studies of other fish leucocyte functions, where TNFα has little effect unless in combination with a second signal (Hardie *et al.*, 1994; Jang *et al.*, 1995a). Down regulation of both MHC class I and II mRNA expression has also been demonstrated using a macrophage-like cell line derived from the head kidney of Atlantic salmon, following stimulation with human recombinant insulin-like growth factor-I (IGF-I). A decline in both MHC class I and II mRNA expression, using IGF-I, was seen in SHK-1 (salmon head kidney) cells (Koppang *et al.*, 1999), as with mammalian cells (Trojan *et al.*, 1996;

Shevelev *et al.*, 1997). Whereas, infection of SHK-1 cells with infectious pancreatic necrosis (IPNV) and infectious salmon anaemia virus (ISAV), produced initial increase in levels of mRNA expression followed by a decline in expression (Koppang *et al.*, 1999).

As mentioned previously, T cell receptor genes have been described in rainbow trout (Partula *et al.*, 1994, 1995) and are very similar to the TCR α and β genes of higher vertebrates. Genes similar to the mammalian γ and δ isoforms have been described in cartilaginous species (Rast and Litman, 1994; Rast *et al.*, 1995, 1997). Cell-mediated responses are also characterised by the production of cytokines by T cells following stimulation with mitogens or specific antigen. The biological activities of many such cytokines have been described in fish (Secombes, 1991), and will be discussed in more detail later. Some of the activities described so far include those analogous to mammalian macrophage activating factor (MAF) / γ-interferon (Graham and Secombes, 1990a,b), interleukin-2 (IL-2) (Grondel and Harmsen, 1984), migration inhibition factor (MIF) (Smith *et al.*, 1980; Mckinney *et al.*, 1981) and chemotactic factor (CTF) (Bridges and Manning, 1991). More recently a number of cytokine genes/cDNAs have been described. These will also be discussed in more detail later.

One of the most important differences between specific cell-mediated immune responses (CMI) in fish compared with mammals is the temperature dependence of fish T cell responses (Clem *et al.*, 1991; Bly and Clem, 1992). Low temperatures inhibit proliferation of fish T cells to both mitogens (and allogeneic cells). Also, the inhibition of helper activity, which B cells require to respond to thymus dependant antigens occurs. Following cell activation, the inability of T cells to desaturate stearic acid to oleic acid in the plasma membrane, possibly through an effect on membrane viscosity had been implicated in the reduction of signalling T-cell functions at lower temperatures (Bly and Clem, 1992). However, it now seems unlikely that low temperature immunosuppression results from this inability of T cells to adapt and instead minor changes in T cell plasma membrane are thought to be involved (Clem *et al.*, 1991).

If cytokine release from T cells is inhibited at low temperatures in fish, then in addition to the potential problems for signal transduction at low temperature there may be a lack of critical growth factors for lymphocyte responses (Hardie *et al.*, 1995).

1.3 General macrophage functions

In vertebrates, cell types displaying phagocytic activity are well-characterised and are broadly classified as granulocytes and monocytes/macrophages (Rowley *et al.*, 1988), and in higher vertebrates macrophages in particular are multi-functional. In teleost fish, phagocyte cell types are less well characterised, although it is known that macrophages, monocytes and neutrophils (PMNs) in blood, tissues and inflammatory exudates are phagocytic (McKinney *et al.*, 1977; Braun-Nesje *et al.*, 1981; Thuvander *et al.*, 1987; Secombes and Fletcher, 1992; Pedrera *et al.*, 1993; Lamas and Ellis, 1994a, b; Brattgjerd and Evensen, 1996; Dalmo *et al.*, 1997).

Macrophages play an important role in both the innate and adaptive immune responses in fish. Apart from being migratory and actively phagocytic (Secombes and Fletcher, 1992), macrophages generate a number of oxygen dependent and independent microbiocidal molecules such as ROS and RNS (Secombes and Fletcher, 1992; Neumann *et al.*, 1995). Additionally, they secrete soluble mediators (important in inflammatory events) such as cytokines (Zelikoff *et al.*, 1990; Clem *et al.*, 1991; Jang *et al.*, 1995a) and eicosanoids (Pettit *et al.*, 1991a,b; Rowley *et al.*, 1995). Macrophages act as accessory cells for lymphocyte responses and are involved in the processing and presentation of antigens (Vallejo *et al.*, 1992a,b).

The role of macrophages as accessory cells in the immune responses of higher vertebrates is well-established (Unanue *et al.*, 1984). In studies on the proliferative responses of fish to Concanavalin A (Con A), monocytes/macrophages or factors released by them have been shown to be necessary for the activation of lymphocytes (Smith and Braun-Nesje, 1982; Sizemore *et al.*, 1984; Clem *et al.*, 1985). The dependence upon accessory cells for the optimal production of MAF was shown by Graham and Secombes (1988), in studies on the cellular requirements for lymphokine secretion by rainbow trout leucocytes. Some MAF activity has

been detected in supernatants produced by stimulated lymphocytes in the absence of macrophages.

1.3.1 The priming and activation of macrophages

Primed macrophages are usually considered to be cells that have some increased functions, for example phagocytosis or chemotaxis, but have not yet acquired a heightened killing activity. In comparison, activated macrophages are defined as cells having an increased ability to kill micro-organisms (Nathan, 1986). The molecular basis for the priming of macrophages could include modification of the cell in a number of ways. However, an increase in the number and affinity of plasma membrane receptors does not appear to explain priming on its own. Changes in the transduction events responsible for stimulus-response coupling might lead to a more efficient stimulation or function of the enzyme responsible for respiratory burst. Thus it may well be that priming can be explained partly by a modification of the respiratory burst enzyme such that it binds its substrate nicotinamide adenine dinucleotide phosphatase (NADPH) more efficiently. This could be achieved either by an increase in the amount of enzyme or by an increase in its efficiency, an increase in an enzyme cofactor, or a reduction of an enzyme regulator (Johnston and Kitagawa, 1985).

In mammals, IFN-γ has been shown to be a primary agent in both priming and activation of macrophages and neutrophils early in infections with pathogenic bacteria (Byrne and Turco 1988). Recombinant IFN-γ (rIFN-γ) has been shown to enhance the functional activities of human, bovine and porcine neutrophil and chicken heterophils (Steinbeck *et al.*, 1986; Canning and Roth 1989; Berton and Cassatella 1992; Coe *et al.*, 1993; Semani *et al.*, 1993; Kabbur *et al.*, 1995; Kogut *et al.*, 2001). Meyer *et al.*, (1991) investigated the priming effects of IFN-γ *in vitro*, on the ability of human alveolar macrophages and monocytes to release superoxide anions in response to exposure to phorbol myristate actetae (PMA). They also determined whether simultaneous exposure of these cell types to IFN-γ and LPS enhanced or suppressed superoxide anion generation. Their results demonstrated that simultaneous

exposure to lower concentrations of LPS had an additive effect with IFN-γ in priming alveolar macrophages for an increased respiratory burst. In fish it is recognised that macrophages can be activated by a soluble factor derived from lymphocytes. This soluble factor has both acid and heat labile properties characteristic of mammalian IFN-γ, and therefore it is thought that the major macrophage activating factor in fish could be the equivalent of mammalian IFN-γ.

It has been demonstrated that several specific cell proteins undergo phosphorylation when macrophages are exposed to PMA (Vaux and Gordon 1982, Andrews and Babior 1983, Babior *et al.*, 1984a,b, Kiyotaki and Bloom 1984, White *et al.*, 1984). The phosphorylation of proteins by protein kinases acting synergistically with increased concentrations of intra-cellular Ca^{2+} may act to mediate the respiratory response.

1.3.2 Macrophage respiratory burst activity

The respiratory burst reaction, catalysed by a plasma membrane-bound NADPH oxidase, is characterised by the reduction of molecular oxygen to the superoxide anion (O_2^-) radical (reviewed by Seifert and Gunter, 1991). These highly reactive oxygen products possess microbiocidal activities and are responsible for the intracellular killing of engulfed bacteria. An increased oxygen uptake together with the production of oxygen free radicals has been demonstrated in salmonid leucocytes and macrophages (Nagelkerke *et al.*, 1990). The kinetics of oxygen consumption by Japanese eel (*Anguilla japonica*) PMNs during respiratory burst has also been shown to be similar to the pattern seen in mammalian PMNs.

In mammals, the capacity to respond to stimulation with enhanced respiratory burst can be induced *in vitro* by incubating cultured macrophages (or blood monocytes) with: soluble stimulants such as ConA, PMA (Graham and Secombes, 1988), bacterial endotoxin, bacterial cell wall component muramyl dipeptide (Pabst *et al.*, 1982; Pabst and Johnston, 1980), proteolytic enzymes (Johnston *et al.*, 1981; Speer *et al.*, 1984), sodium periodate (Tsunawaki and Nathan, 1984), supernatants from stimulated lymphocytes (reviewed by Nathan *et al.*, 1983) and IFN-γ (Murray *et al.*, 1983; Nathan *et al.*, 1983). In addition, macrophages are activated *in*

vivo by bacterial products or by other molecules generated during an inflammatory response (Johnston and Kitagawa, 1985). Similarly teleost phagocytes in blood, haemopoietic organs and the peritoneal cavity have been reported to synthesise a variety of ROS upon stimulation with different particulate and soluble stimulants (Scott and Klessius, 1981; Higson and Jones, 1984; Chung and Secombes, 1988; Secombes *et al.*, 1988; Zelikoff *et al.*, 1991; Anderson *et al.*, 1992). In addition, evidence exists that trout phagocytes produce these ROS, including the glucose dependent production of superoxide anion (O_2^-), and the presence of a membrane bound NADPH oxidase enzyme (Higson and Jones, 1984; Secombes, *et al.*, 1988; Nagelkerke *et al.*, 1990).

It has been established that in fish, the production of oxygen species is not sensitive to mitochondrial inhibition (Secombes *et al.,* 1988). Studies on the stimulation of rainbow trout phagocytes by Nagelkerke *et al.,* (1990) in the presence of sodium azide, demonstrated that respiratory burst is independent of the mitochondrial cytochrome system, agreeing with studies on mammalian phagocytes (Karnovsky 1962; Babior 1978, 1984a, b). Following membrane stimulation, rainbow trout phagocytes have a markedly increased oxygen uptake, which is dependent upon the presence of glucose. In the absence of glucose, superoxide anion production by trout macrophages is completely inhibited (Secombes *et al.*, 1988).

Macrophage activation can be assessed by measuring respiratory burst activity via a number of *in vitro* functional assays. The generation of microbiocidal products is useful for monitoring whether macrophages are activated, since they contribute directly to any increased killing. Individual ROS released following macrophage stimulation, such as O_2^- (Secombes *et al.*, 1988) and hydrogen peroxide (H_2O_2) (Chung and Secombes, 1988) can be detected. The reduction of ferricytochrome C (Secombes, 1990) and nitroblue tetrazolium can be used to measure the production of the superoxide anion radical O_2, whereas the horseradish peroxidase-dependent oxidation of phenol red $^-$ measures H_2O_2 (Chung and Secombes, 1988).

Chemiluminescence analysis also provides a sensitive measure of phagocytic function (Anderson and Brendzel, 1978; Easmon *et al.*, 1980; Nelson *et al.*, 1977), and the substrate Luminol can be used as a chemiluminescence probe for the measurement of respiratory burst

metabolism following macrophage stimulation. The low amounts of measurable light produced in inherent chemiluminescence, requires the need for an amplification compound such as luminol, a cyclic hydrazide, which enhances the magnitude of the light produced during chemiluminescence (Nagelkerke *et al.*, 1990; Vazzana *et al.*, 2003).

1.4 Regulation of macrophage activity in fish

Macrophage activity in fish can be modulated by exogenous molecules and such signals can act synergistically and antagonistically to achieve up or down regulation of macrophage function. Modulation of respiratory burst of head kidney macrophages isolated from rainbow trout was observed following treatment with several biologically active substances (Novoa *et al.*, 1996a,b). Studies have shown synergistic effects of MAF-containing supernatants with LPS and TNF-α on macrophage respiratory burst activity (Hardie *et al.*, 1994; Jang *et al.*, 1995a), nitric oxide (NO) production (Neumann *et al.*, 1995) and production of macrophage-derived MAF (Jang *et al.*, 1995b). Potential interaction of both stimulatory (cytokines, LPS, glucans) and inhibitory (prostaglandins) molecules on respiratory burst activity *in vitro* was examined. Of the stimulants used, MAF-containing supernatants were most stimulatory. Addition of LPS, glucans and TNF-α simultaneously with MAF supernatants increased respiratory burst activity further and the inhibitory effect of prostaglandin on macrophage function could be overcome by co-incubation or pre-treatment with stimulatory molecules.

In comparison little is known about the interactions between suppressive agents on fish macrophages, or between suppressive and stimulatory factors. Precedents exist for interactions of suppressive agents on other fish leucocytes, as with the interaction seen between testosterone and cortisol in reducing antibody-secreting cell numbers (Slater and Schreck, 1993). Similarly, in mammals TGF-β, can down-regulate macrophage activity (Adams and Hamilton, 1992; Alleva *et al.*, 1995).

It is well established that fish T-cell responses are particularly temperature sensitive (Bly and Clem, 1992; Clem *et al.*, 1984; Miller and Clem, 1984). The effect of temperature on

macrophage activation and the production of MAF by rainbow trout leucocytes have been investigated. It is generally accepted that the production of MAF is temperature dependent both *in vivo* and *in vitro* and bacteriocidal activities are severely impaired at low temperatures (Scott *et al.*, 1985; Ainsworth *et al.*, 1991; Hardie *et al.*, 1995). MAF production in rainbow trout leucocytes is inhibited at non-permissive temperatures (temperature below optimal but within physiological range) which produces immunosuppression. Thus, it is the T cell that is the temperature sensitive cell; the macrophage function itself remains intact at low temperatures when stimulated with MAF (Hardie *et al.*, 1995).

1.5 Cytokines

Interactions between cells of the immune system are characterised by the release of regulatory soluble factors known as cytokines which act as signalling molecules within the immune system (Callard and Gearing, 1994; Thomson, 1994). Their constitutive production is usually low, and their synthesis is triggered as a result of new gene transcription following cell stimulation. Since transcription is typically transient and cytokine mRNA short-lived, cytokine secretion is a brief self-limiting event (Secombes *et al.*, 1996). Cytokines normally act locally within the tissues and the range of their influence is usually restricted to cells within the immediate vicinity of the cytokine-producing cell. Cytokines normally act in a paracrine or autocrine fashion (Hamblin, 1993) and therefore act as local chemical mediators, although in some instances, they can function in an endocrine manner and reach target cells via the blood stream. Also, they can display pleiotropic effects: inducing different responses in different targets by acting on target cells via high affinity specific receptors (Secombes *et al.*, 1996).

Cytokines can act in both a stimulatory or inhibitory manner, leading to an altered biological response, as a result of influencing the expression of other cytokines. Cytokines often act synergistically (Vilcek and Le, 1994) with other cytokines and pathogen-derived molecules, for example LPS, and therefore it is unlikely for a cell to encounter a single cytokine *in vivo.* Synergy can result when two signals are required for stimulation of a target cell or when contact

with the first signal / cytokine induces receptor expression for the second signal (Hamblin, 1993). Although less well documented, there are also examples of antagonistic effects between cytokines. For example, transforming growth factor TGFβ_1 down regulates MAF initiated reactions such as macrophage respiratory burst activity. Type I interferons (IFNα and IFNβ) and growth factors (Fibroblast growth factor FGF, epidermal growth factor EGF, and platelet derived growth factor PDGF) can act as mutual antagonists. Growth factors reverse antimitotic effects of IFNs and in turn IFNs can block growth factor induced entry of cells into the S phase growth (De Maeyer and De Maeyer Guignard, 1988).

Conventially cytokines have been divided into a number of different families, for example interferons (IFN), interleukins (IL), chemokines, colony stimulating factors (CSF), and TNF. However, due to increased structural knowledge available regarding cytokines (Sprang and Bazan, 1993), a revision of this classification was required based primarily on the helical class (α/β), type of protein folding of the cytokine, and receptor type. Using structural criterion, several hormones including insulin, prolactin and growth hormone display an obvious association with cytokines. Classification of cytokines on a structural basis has led to the natural grouping of receptor molecules they bind. So far, at least three receptor families have been identified (Miyajima *et al.*, 1992; Foxwell and Barrett, 1993) namely Type I cytokine receptors, including the receptors for hemopoietic growth factors and IL-2 – IL-7, Type II receptors which include those for IFNα, IFN β and IFNγ, and Type IV or Immunoglobulin-like receptors.

Fish produce a number of cytokine like soluble products, of which most have been identified in biological assays on the basis of their functional similarity to mammalian cytokine activities, whilst others have been detected either through their antigenic cross-reactivity with mammalian cytokines or on the basis of biological cross-reactivity on cytokine-dependent mammalian cell lines. Indirect evidence for TNFα-like, TGFβ_1-like and IL-1-like factors in rainbow trout has been provided by biological and antigenic cross-reactivity (Zelikoff *et al.*, 1990; Ahne, 1993, 1994; Jang *et al.*, 1994).

Jang *et al.*, (1995a) demonstrated that macrophage respiratory burst, mitogen-induced lymphoproliferation and neutrophil migration in the trout were exacerbated by incubation with human TNFα, and all activities were reduced by either co-incubation with antibodies against human TNF-α or prior incubation of cells with antibodies against human TNF receptor 1. Respiratory burst activity of rainbow trout macrophages was also shown to be increased by incubation with supernatants derived from activated trout macrophages (Jang *et al.*, 1995b), suggesting the presence of a TNF-α like factor in these supernatants. Similar studies have been carried out with human hTGFβ_1, showing a dual effect on respiratory burst activity depending on the concentration employed (Jang *et al.*, 1994).

Indirect evidence also suggests inducible production of an IL-1-like factor by common carp *Cyprinus carpio*, macrophages and neutrophils *in vitro*. Supernatants from these cells induced proliferation of an IL-1-dependent mammalian T-cell line, which was suppressed by co-incubation with antibodies against hIL-1α and hIL-1β (Verburg van Kemende *et al.*, 1995, Weyts *et al.*, 1997). Two different monocyte-derived IL-1-like proteins been identified in channel catfish (Ellsaesser and Clem, 1994).

1.5.1 Macrophage activating factor (MAF)

Approaches to isolate/identify MAF in fish have involved looking for functional activity. MAF production in fish has mainly been demonstrated by stimulation with mitogens, although some reports also report MAF production as a result of stimulation of fish leucocytes with specific antigens. Graham and Secombes (1988) described the production of a macrophage activating factor in rainbow trout leucocytes incubated in the presence of the T cell mitogen, Concanavalin A. They found that the cell-free supernatants were capable of activating fish macrophages *in vitro*, as shown by their ability to increase the release of ROS and bacteriocidal activity of rainbow trout macrophages in culture.

The isolation and purification of the molecule responsible for the macrophage activating activity has however proved difficult. Nevertheless it appears that the MAF is a T cell product

with a molecular weight of approximately 19 kDa and is temperature and pH sensitive, properties similar to mammalian IFN-γ (Graham and Secombes, 1990b). Based on this, it has been suggested that it may well correspond to mammalian IFN-γ.

Marsden *et al.*, (1994) investigated antigen-induced release of MAF from rainbow trout leucocytes to establish whether detection of a variety of lymphocytes responses after immunisation could be used to measure the success of a vaccination system, especially if such responses corresponded to disease resistance. The aim of their study was to confirm the production of MAF in response to specific antigens following immunisation against *Aeromonas salmonicida* and investigate the kinetics of this response in relation to antibody and proliferation responses. Their results demonstrated that MAF can be released *in vitro* in response to specific antigens and following incubation with supernatants derived using the highest dose of antigen (to stimulate MAF release from primed cells), the relative increase in macrophage respiratory burst activity was similar to that found when using mitogens. Also, the release of MAF following immunisation with *A. salmonicida* peaked approximately 4-5 weeks post immunisation and correlated with peak antibody titres.

1.5.2 Interferons (IFNs)

The interferons are glycosylated proteins of approximately 20kDa, which represent a large family of soluble cytokines with biological and antiviral activity, acting via specific receptors (Gresser, 1997). The IFNs have been implicated in cell proliferation and differentiation, as well as in the suppression of some forms of cancer (Chawla-Sarkar *et al.*, 2001; Kang *et al.*, 2002).

IFNs are divided into two groups, a group consisting of IFN-α,-β,-ω,-δ,-κ,-τ, and a group consisting of IFN-γ (Sen and Lengyel, 1992). Genes for IFN-α/β/ω/δ/κ/τ group have been cloned from a variety of mammals, including humans (Lawn *et al.*, 1981a,b), mice (Shaw *et al.*, 1983; Daugherty *et al.*, 1984), pigs (Lefevre and La Bounardiere *et al.*, 1986) and several avian species such as ducks (Schultz *et al.*, 1995), chickens (Sekellick *et al.*, 1994) and turkeys (Suresh *et al.*, 1995).

IFN-α and IFN-β are glycoproteins with molecular weights of between 16 and 26kDa. IFN-α is very polymorphic with nine types in humans, whereas IFN-β appears to be of a single type (Yano, 1996a,b). IFN-α and IFN-β, due to their many similarities are grouped together as type I IFN. They share approximately 30% amino acid (a.a) sequence homology, are acid stable and act via a single receptor. The 45% nucleotide homology detected between cloned human IFN-α and IFN-β suggests that they originated from a common ancestral gene (Taniguchi *et al.*, 1980; Miyata *et al.*, 1985). IFN-α belongs to a multigene family, whereas a single gene encodes IFN-β. IFN-α and IFN-β genes do not contain introns. Both IFNs are produced by all cell types, are induced in cells infected with virus and exhibit a broad range of antiviral activity. Hybridisation studies by Wilson *et al.*, (1983), using the human interferon-β gene as a probe against fish genomic DNA suggested that fish too are likely to possess an IFN-β gene although to date the gene has not been isolated.

IFN-γ is unrelated to IFN-α and IFN-β and is termed a Type II IFN. IFN-γ is a glycoprotein with a molecular weight (mw) similar to IFN-α and IFN-β (Yip *et al.*, 1982) but it shares less than 10% a.a. sequence homology. IFN-γ is unstable at pH below 4 and at high temp. (> 56°C) and acts via a separate receptor (Epstein, 1984; Langer and Pestka, 1988). It differs from Type I IFN in its spectrum of biological activities which include cell regulation, cell differentiation and intercellular communication (Trinchieri and Perussia, 1985). In addition to its antiviral activity, IFN-γ can activate macrophages (Schultz and Kleinschmidt 1983), augment B-cell responses (Leibson *et al.*, 1984), enhance T-cell responses (Frasca *et al.*, 1985) and induce expression of both class I and class II MHC molecules on a variety of cell types (reviewed by De Maeyer and De Maeyer-Guignard, 1988).

IFN-γ is produced by a restricted set of immune cells (T cells and NK cells), in response to immune and / or inflammatory stimuli and functions to stimulate the development and actions of immune effector cells. One of the most important consequences of IFN-γ secretion is the activation of macrophages, this is achieved through the induction of ROS and nitrogen oxide (NO) which activate a variety of anti-bacterial, anti-tumour and anti-viral responses (Billiau,

1996a,b). So far no IFN-γ genes have been identified in fish, although as mentioned previously circumstantial evidence suggests that the macrophage activating factor produced by fish lymphocytes may be the equivalent molecule.

IFN-γ receptors are expressed on nearly all cell types, with possible exceptions of mature erythrocytes, and display strict species specificity in their ability to bind IFN-γ (Farrar and Shreiber, 1993). Binding of IFN-γ to its receptor on the surface of a cell results in the activation of signal transducers and activators of transcription mediated through Janus tyrosine kinases (JAKs) at the cell membrane. IFN-γ signalling is dependent on five distinct proteins: type 1 integral membrane proteins IFNGR1 and IFNGR2 (subunits of IFN-γ receptor) and JAK1, JAK2 and signal transducers and activators of transcription STAT1 (Schindler and Darnell, 1995; Bach *et al.*, 1997). The signalling pathway results in the release of signal transducers and transcription factors and their migration to the nucleus, where they can induce or suppress the expression of many different genes (Stark *et al.*, 1998). The IFN-induced family of DNA-binding transcription factors include the interferon regulatory factors: IRF1, IRF2, IRF3, interferon-γ stimulated gene factor 3 (ISGF3γ), IFN consensus sequence-binding protein (ICSBP) and IRF4, a transcription factor expressed only in lymphocytes also known as consensus sequence-binding protein in adult T-cell leukemia cell line or activated T cells: Interferon Consensus Sequence binding protein for Activated T cells (ICSAT), and lymphocyte-specific interferon regulatory factor (LSIRF).

Proteins of the IRF family such as IRF2 (Harada *et al.*, 1989), ICSBP (Nelson *et al.*, 1993) and ICSAT (Yamagata *et al.*, 1996), bind to interferon-stimulated regulatory elements and negatively regulate the expression of the associated genes.

IFN-γ can up-regulate the expression of MHC class I proteins, an activity it shares with IFN-α and IFN-β, and thereby promote the development of $CD8^+$ T-cell responses (Boehm *et al.*, 1997). This expression is known to be driven by the transcription factor IRF1 (Reis *et al.*, 1992; Chang *et al.*, 1992). Interferons also play an important role in antigen processing by regulating the expression of many proteins required to generate antigenic peptides. IFN-γ

modifies the activity of proteasomes (multi-subunit enzyme complex responsible for the generation of all peptides that bind to MHC class I proteins), by modulating the expression of both enzymatic and non-enzymatic components (York and Rock, 1996; Boehm *et al.,* 1997). Thus, interferons enhance immunogenicity by increasing the quantity and repertoire of peptides displayed in association with MHC class I proteins.

IFN-γ has an important effect on Th1 cell development. *In vitro,* antibody-mediated neutralisation of IFN-γ greatly reduces the development of Th1 cells and augments the development of Th2 cells (Hsieh *et al.*, 1993). IFN-γ facilitates Th1 production by enhancing the synthesis of IL-12, which drives developing CD4+ T cells to become Th1 cells (Hsieh *et al.*, 1993; Trinchieri, 1995). In addition, IFN-γ blocks the development of Th2 cells by inhibiting production of IL-4, required for Th2-cell proliferation (Szabo *et al.*, 1995) and thereby preventing proliferation (Gajewski and Fitch, 1988).

Interferons play complex and sometimes conflicting roles in regulating humoral immunity. They exert their effects either indirectly, by regulating the development of specific T helper cell subsets, or directly at the level of B cells. In the latter case, interferons are predominantly responsible for regulating three specialised B-cell functions: development and proliferation, immunoglobulin secretion and immunoglobulin heavy-chain switching (Stark *et al.*, 1998).

Processes that negatively regulate IFN-γ signalling are now being defined. For example, in certain cells such as T cells, IFN-γ can induce desensitisation by down-regulating the expression of the interferon-γ receptor signal-transducing chain IFNGR2 mRNA and protein (Bach *et al.*, 1995; Pernis *et al.*, 1995). Recently a family of proteins known as suppressors of cytokine signalling (SOCS) have also been shown to affect IFN-γ signalling (Greenhalgh and Hilton, 2001). These proteins are induced by IFN-γ and other cytokines and regulate IFN-γ signal transduction in a classic negative feedback mechanism, by binding to and inhibiting activated JAKs. In addition, they combine such direct inhibitory interactions on the cytokine

receptors and signalling proteins with a mechanism of targeting associated proteins for degradation (Alexander, 2002).

Most of the current literature on interferon synthesis has focussed on human and murine systems, although a few studies have addressed the issue of whether lymphocytes from lower vertebrates produce interferon. There are no reports of cytokines with antiviral activity in amphibian or reptile species. IFN like activity involving cytokines showing antiviral or tumouricidal activity have been demonstrated in birds. For example, avian reoviruses induce IFN-like activity in chick epithelial fibroblasts (CEF) *in vitro* and *in vivo* (Ellis *et al.*, 1983). Subsequently, a gene encoding chicken IFN was cloned and expressed from a primary chick embryo cDNA library (Sekellick *et al.*, 1994). In addition, IFN genes from several other avian species such as ducks (Schultz *et al.*, 1995), and turkeys (Suresh *et al.*, 1995) have been identified.

Anti-viral activity in fish cells has been reported (Gravell and Marlsberger, 1965; Oie and Loh, 1971; De Sena and Rio, 1975; Graham and Secombes, 1990b). Previous studies in fish have reported the production of IFN-like activities *in vivo* and *in vitro* in response to viral infection (De Sena and Rio, 1975; De Kinkelin *et al.*, 1982) and of cytokines in general in response to mitogen stimulation *in vitro* (Smith and Braun-Nesje, 1982, Graham and Secombes, 1988). Interferon-like anti-viral activity was shown in the serum of rainbow trout after experimental infection with several pathogenic salmonid viruses: viral haemorrhagic septicaemia virus (VHSV) (De Kinkelin and Dorson, 1973), infectious haematopoietic necrosis virus (IHNV) (De Kinkelin and Le Berre, 1974), and IPNV (Dorson *et al.*, 1992). In addition anti-viral activity has been demonstrated in rainbow trout leucocytes in response to stimulation *in vitro* with both live and inactivated VHSV (Gaillard *et al.*, 1989). A similar activity was found in salmonids after exposure to the interferon-β inducer poly I: C, a synthetic ds RNA (Eaton, 1990). Studies by Snegaroff (1993) described the ability of various viruses to stimulate interferon-like activities in leucocytes from rainbow trout kidney *in vitro* and also tested its protective action *in vivo* with VHSV infected trout. The best inducer found was Newcastle disease virus (NDV), followed by two coronaviruses (porcine transmissible gastroenteritis

coronavirus (TGEV) and a bovine coronavirus). Interferon was also produced by leucocytes stimulated with glutaraldehyde-fixed cells infected with VHSV, but the titre was much lower. None of the individual proteins responsible for the IFN-like anti-viral activities have been isolated. However the various activities obtained provide evidence for the existence of both Type I and Type II IFNs.

1.5.3 Interferon induced genes

The production of IFN and subsequent receptor signalling events leads to the transcription of a variety of other genes. It is these IFN-induced genes which mediate both the anti-viral effects and cell regulatory effects of IFN. Genes corresponding to several IFN-induced genes in mammals have been cloned and sequenced in fish and include: Mx (Leong *et al.*, 1998), Vig-1 (Boudinot *et al.*, 1999), Vig-2 (Boudinot *et al.*, 2001) and IRF (Yabu *et al.*, 1998; Richardson *et al.*, 2001). The latter, a member of a family of DNA-binding transcription factors is implicated in the regulation of IFN production and cell growth (Eisenbeis *et al.*, 1995; Matsuyama *et al.*, 1995; Yamagata *et al.*, 1996). The existence of such genes in the fish is further evidence for the likely existence of IFN in fish.

1.5.4 Interferon related cytokines

Regulation of IFN-γ synthesis is one of the most stringently controlled processes of an immune response. Its production is essentially restricted to activated $CD4^+$ T helper cells, $CD8^+$ cytotoxic T cells and NK cells (Billian *et al.*, 1996). For each cell type, IFN-γ secretion is further restricted by the availability of IFN-γ inducing cytokines such as interleukin (IL)-12 and TNFα, which arise from accessory cells following activation (Billiau, 1996a,b). Apart from IL-12, TNFα and IL-2 which directly induce the expression of IFN-γ (Billiau 1996a,b; Locksley, 1993), IL-18 has been added to the short list of IFN-γ regulators (Ushio *et al.*, 1996). In addition, IL-15 has been shown to activate peripheral blood lymphocytes (PBLs; Grabstein *et*

al., 1994; Giri *et al.*, 1994, 1995a,b) and to stimulate the production of IFN-γ (Calarota *et al.*, 2003; Strengell *et al.*, 2003).

IL-18 was originally known as IFN-γ inducing factor (IGIF) and is a pro-inflammatory cytokine which is a potent inducer of IFN-γ production by T cells (Okamura *et al.*, 1995, Micallef *et al.*, 1996) and NK cells (Tsutsui *et al.*, 1996). IL-18 is structurally similar to IL-1β and as such is initially synthesised as a biologically inactive precursor molecule, requiring the IL-1β converting enzyme (ICE) for cleavage into an active mature molecule. The activity of mature IL-18 is also closely related to that of IL-1, (Dinarello, 1999) with profound effects on T-cell activation.

Either independently or in synergy with IL-12, the effects of IL-18 through its induction of IFN-γ can lead to a rapid activation of the monocyte/macrophage system resulting in up regulation of these cells innate immune capabilities (Billiau, 1996a,b). IL-18 is induced by stressful stimuli (bacterial or neurogenic signals) (Okamura *et al.*, 1995; Conti *et al.*, 1997), and it has been proposed that a stress-induced release of the cytokine can lead to a further cycle of IFN-γ/IL-18 production. Following initial IL-18 induced IFN-γ production, newly secreted IFN-γ can stimulate macrophages/monocytes to increase their ICE activity. In the presence of continued IL-18, increased ICE activity probably results in more processed IL-18, which leads to more lymphocyte IFN-γ production and subsequent macrophage ICE activity (Suda *et al.*, 1993). Therefore, the IFN-γ inducing factor not only promotes IFN-γ synthesis but also possibly participates in its overall activities (Figure 1.1). IL-18 has been cloned in mice from partial a.a. sequences deduced from the purified proteins (Okamura *et al.*, 1995) and in humans (Ushio *et al.*, 1996). More recently IL-18 was cloned from the chicken, but this is the only non-mammalian vertebrate to date known to possess this cytokine (Schneider *et al.*, 2001).

IL-15, formerly known as T-cell growth factor in recognition of its ability to induce the proliferation of T cells (Grabstein *et al.*, 1994; Giri *et al.*, 1994, 1995a,b), is a novel cytokine that shares many biological properties with IL-2. However it lacks any obvious amino acid homology with IL-2. IL-15 has been shown to be a chemoattractant for human blood T

lymphocytes (Wilkinson and Liew, 1995) and able to induce lymphokine-activated killer (LAK) activity in NK cells, as well as inducing the generation of cytolytic effector cells. High affinity cell surface receptors for IL-15 have been detected on a variety of T cells and B cells, as well as non-lymphoid cells (Giri *et al.*, 1994, 1995a,b).

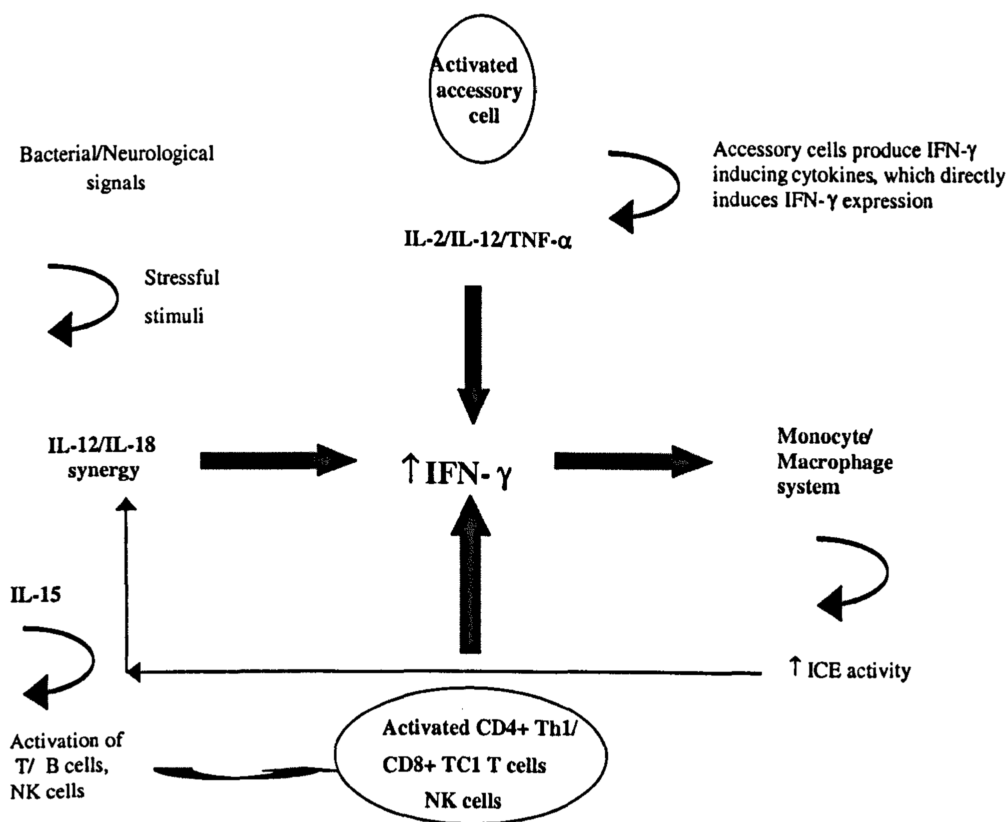

Figure 1.1 Regulation of IFN-γ synthesis and the role of IFN-γ regulators IL-18/IL-15

1.6 The cloning of fish cytokine genes

Although the biological activities of fish cytokines are known, only recently has there been progress with cloning the genes. A number of fish cytokine genes have been sequenced in recent years, and include amongst others, Interleukin-1β (Secombes *et al.*, 1997, 1998; Fujiki *et al.*, 1998), transforming growth factor-β (Hardie *et al.*, 1998), fibroblast growth factor (Kiefer *et al.*, 1996; Hata *et al.*, 1997) and several chemokines (Dixon *et al.*, 1997; Fujiki *et al.*, 1998; Najakshin *et al.*, 1998). A complete list of the fish cytokine genes/cDNAs which have been cloned from a variety of different species is given in Table 5.4. The most common approach taken has been PCR based homology cloning, which has been successful for the cloning of cytokines from several teleost species including rainbow trout, Atlantic salmon and plaice (Laing *et al.*, 2000, 2001; Subramaniam *et al.*, 2001). The fish cDNAs were successfully amplified using the PCR with primers based on highly conserved regions of the known mammalian sequences.

1.6.1 Interleukin-1

In mammals, IL-1 is the prototype pro-inflammatory cytokine (Dinarello, 1997) and exists in two forms, IL-1α and IL-1β, which are indistinguishable on the basis of their biological effects. They are produced as precursor molecules but only the IL-1α precursor is biologically active. The crystal structures of the mature peptides of IL-1α and IL-1β, together with the IL-1 receptor antagonist, consist of 12 β-sheets forming 6 hairpins (Nicola, 1994).

IL-1β has been sequenced in rainbow trout (Zou *et al.*, 1998) and carp (Fujiki *et al.*, 1998), but as yet the existence of a fish equivalent to IL-1α or the IL-1 receptor antagonist has yet to be established. Isolation of the trout and carp IL-1β sequences involved different approaches. In trout, primers designed against conserved regions of known mammalian IL-1β amino acid sequences were used in the (PCR) polymerase chain reaction (using trout macrophage cDNA) to amplify the IL-1β cDNA. With carp, a subtractive cDNA library was generated from stimulated peritoneal cells, followed by random sequencing of individual clones.

Both trout and carp sequences share approximately 41-48% nucleotide homology (28-31% a.a. identities) to various mammalian IL-1β cDNAs. However, they are also only 47% similar to each other at the nucleotide level (36% a.a. identity), showing that even within teleosts there can be considerable divergence between individual species of fish (Secombes, *et al.*, 1999a,b). The trout and the carp genes are predicted to encode proteins of 260 and 276 a.a. respectively, a size similar to the mammalian proteins, suggesting that they too are produced as precursor molecules. The trout gene is not constitutively expressed in leucocytes but expression can be induced in both head kidney leucocytes and macrophages, by stimulation with phytohaemagglutinin PHA or LPS (Zou *et al.*, 1998). The full-length sequence of a second IL-1β gene (IL-$1\beta_2$) in rainbow trout has also been obtained (Pleguezuelos *et al.*, 2000). The predicted 254 amino acid sequence of the second IL-1β gene has 82% identity to the first gene, 45% identity to carp IL-1β, and 40% identity to human IL-1β. Expression studies performed by reverse transcriptase-polymerase chain reaction (RT-PCR) using primers specific for the IL-$1\beta_2$ transcript revealed a clear dose-dependent induction of this gene in cultured trout leukocytes by stimulation with LPS.

1.6.2 Transforming growth factor β

TGF-β is a pleiotropic cytokine involved in proliferation and differentiation of cells, tissue remodelling, wound repair and is expressed in a wide range of cells and tissues. Three isoforms of TGF-β exist in mammals (TGF-β_{1-3}) and birds (TGF-β_{2-4}) and two in frogs (TGF-β_2 and TGF-β_5) (Burt and Law, 1994). TGF-β belongs to a superfamily of structurally related proteins including activins and inhibins, growth differentiation factors and bone morphogenetic proteins (BMPs) (Burt and Law, 1994). Their conserved cysteine residues show a characteristic 'cysteine knot' crystal structure (Nicola, 1994). The mature TGF-β peptide is a potent differentiation modulator and immunosuppressive agent within the immune system, and is able to down-regulate the expression of many cytokines and cytokine induced effects, for example the deactivation of macrophages (Derynck, 1994).

Fish macrophages not only possess receptors that are cross-reactive to mammalian TGF-β_1, but bioassay experiments also suggested the existence of TGF-β in fish. For example, it has been shown that mammalian (bovine) TGF-β suppresses the activation of trout macrophages and thereby inhibits respiratory burst activity in a dose-dependent way, following their activation by MAF (Jang *et al.*, 1994). A full-length cDNA, probably corresponding to TGF-β_1, has now been sequenced in rainbow trout. It is predicted to encode a 382 a.a precursor molecule containing a 20 a.a. signal peptide, a tetrabasic cleavage site, downstream of which is a 112 a.a. region thought to correspond to the mature functionally active peptide (Secombes *et al.*, 1999a). This mature peptide contains the TGF-β superfamily motif as with other known forms of TGF-β. The trout TGF-β has highest homology (68%) to mammalian TGF-β_1 and *Xenopus* TGF-β_5 (62.5%) suggesting that *Xenopus* TGF -β_5 may also be related to an ancestral form of TGF-β_1 (Secombes *et al.*, 1998).

Other members of the TGF-β superfamily have been cloned in fish, including the bone morphogenetic proteins (BMPs) and activins. Three BMPs have been cloned in zebrafish, encoding proteins of 386, 400 and 411 a.a. (Martinez-Barbera *et al.*, 1997), and giving rise to mature peptides of 112, 113 and 115 a.a. in length that have ~85-92% a.a. identity with their mouse counterparts (BMP2 and BMP4). Activins modulate secretion of follicle stimulating hormone in addition to mesoderm inducing and erythroid differentiation activity. Two activin genes βA and βB have also been cloned in goldfish (Ge *et al.*, 1993). Both activin genes show high homology to their mammalian counterparts with 78% and 94% a.a. homology to human βA and βB genes respectively.

1.6.3 Tumour necrosis factor

In mammals, TNF-α is a pro-inflammatory cytokine produced by monocytes/macrophages in response to antigen exposure. In studies reviewed below, it has been shown that TNF-α plays an essential role in the inflammatory response to pathogen invasion and in immunological regulation, apoptosis and lipid metabolism.

Several components of the TNF family have been cloned and characterised in fish. A TNFα gene was discovered as part of EST studies in the Japanese flounder *Paralichthys olivaceus,* (Hirono *et al.*, 2000) and is the first non-mammalian TNF sequence to be reported. The flounder sequence shows 29-31% a.a. identity with human TNF molecules: TNFα, TNFβ, LT (lymphotoxin) and LTβ. The gene has also been sequenced in several other fish species, including rainbow trout *O. mykiss* (Laing *et al.*, 2001a), brook trout *Salvelinus fontinalis* (Bobe and Goetz, 2000) and channel catfish *Ictalurus punctatus* (Zou *et al.*, 2001). In rainbow trout, two TNFα genes have been discovered (Zou *et al.*, 2002) which share 95% a.a. identity. In addition, a TNF receptor ligand and a TNF decoy receptor were cloned and expressed in brook trout (Bobe and Goetz, 2000b). The similarity between catfish TNF and other fish TNF homologues was markedly higher (range 30.9–45.7%). Furthermore, except for the expected high similarity between the trout and carp TNF-1 and TNF-2 sequences (92.5 and 78.2%, respectively), the percent similarity in pairwise comparisons among fish TNF sequences (range 35.8–64.7%) was lower than among mammals. Thus, within the six mammalian species examined, the percent similarity between pairwise combinations of TNF genes ranged from 69% (cow vs. rat) to 93% (rat vs. mouse) and from 70% (mouse vs. human) to 96% (rat vs. mouse) among TNF genes. While the number of species examined was relatively small, it appears that sequence divergence among fish TNF is greater than that seen among mammalian TNF proteins. The greater divergence in fish vs. mammalian TNF may be a reflection of the longer (compared to mammals) separation times of extant fish species. In keeping with this observation, carp, which are more closely related taxonomically to catfish than trout and flounder, show higher levels of amino acid similarity (47.5%) within the TNF gene than do trout and flounder (36 and 39%, respectively).

1.6.4 Fibroblast growth factor

Fibroblast growth factors (FGFs) are a family of 15-31kDa, heparin binding proteins, involved in cell growth, differentiation, angiogenesis and tissue repair (Nicola, 1994). The crystal

structure of FGF is similar to IL-1 and consists of 12 β-sheets, forming a β-trefoil. Some members of the family lack a signal peptide and the mechanism of their secretion is unknown.

In mammals, there are nine members, sharing 30-70% a.a. identity. Five FGFs have been sequenced in birds and six in amphibians (Secombes, 1998). In fish, an FGF cDNA has been cloned and sequenced in rainbow trout (Hata *et al.*, 1997) and zebrafish (Kiefer *et al.*, 1996). In trout, the gene codes for a protein of 155 a.a (~17kDa) and has 70% a.a identity with mammalian FGF-2. A recombinant trout FGF produced from bacteria binds tightly to heparin-sepharose and promotes fibroblast proliferation and blastemal growth (Hata *et al.*, 1998), which is typical of known vertebrate FGFs. The zebrafish FGF is composed of approximately 155 a.a, shares 78% identity with the analogous region of *Xenopus laevis* FGF3 and 72% identity with the product of the more distantly related human gene. However, the N-and C-terminal domains of zebrafish FGF3 are very different from those of other known homologues.

1.6.5 Chemokines

Chemokines are a low molecular weight group of structurally related cytokines able to attract leucocytes (Wuyts *et al.*, 1998). They are characterised by the presence of four conserved cysteine residues, important in determining the secondary structure (Nicola, 1994). Depending on whether the first two cysteines are separated by an amino acid they are classified either as C-C (or β) or C-X-C (or α) subfamily. C-C chemokines can attract and activate a wide range of leucocytes but not neutrophils, which are targeted by C-X-C chemokines. Within the two subfamilies the sequence similarity is quite variable, for example ranging from 23-88% a.a identity for C-X-C group. A gene (9E3/CEF4) with homology to C-X-C chemokines sharing 51% homology with IL-8 and 45% homology to a growth related protein GRO -α, has been sequenced in chickens (Martins-Green and Hanafusa, 1997).

A number of chemokine genes have been sequenced in fish. These include a C-C chemokine in rainbow trout (Dixon *et al.*, 1997) and carp (Fujiki *et al.*, 1998) and a C-X-C chemokine in lamprey (Najakshin *et al.*, 1998). The trout CK-1 gene has 46% nucleotide identity and 65% a.a.

similarity to mammalian C-C chemokines, in particular with the C6-β chemokine subfamily. The C-X-C chemokine IL-8, which activates neutrophils and is up-regulated by LPS has also been discovered in both rainbow trout and Japanese flounder and demonstrates 48-51% nucleotide identity with known mammalian IL-8 genes (Laing *et al.*, 2002). Chemokine receptors CXCR4 (CXC receptor family) and CCR7 (CC receptor family) have also recently been cloned and sequenced in rainbow trout (Daniels *et al.*, 1999) and carp (Fujiki *et al.*, 1999).

Two TNF receptors have been cloned in expressed sequence tag (EST) studies in Japanese flounder (Nam *et al.*, 2000). Receptors for platelet derived growth factor β (PDGFRβ) and colony stimulating factor-1 (CSFIR) in Puffer fish *Fugu rubripes,* have also been sequenced from genomic DNA (How *et al.*, 1996). In addition, the common cytokine receptor γ chain (CRγC) has been cloned in trout (Wang and Secombes, 2001).

1.7 Project aims and rationale

The aim of this study was to examine the biological source of a macrophage activating factor produced by leucocytes from rainbow trout and to try and determine the identity of this molecule. In addition, a number of different molecular strategies such as homology cloning and hybridisation studies were taken to try and isolate cytokine cDNAs from the trout.

The thesis is divided into several experimental chapters addressing different aspects of the research:

Develop T cell and macrophage culture methods for the establishment of a reproducible source of macrophage activating factor. Assessment of cell-free supernatants derived from stimulated lymphocytes to determine their capacity to activate macrophages *in vitro*. (Chapter 3) Homology cloning approach to isolate fish cytokines, using PCR amplification with degenerate primers designed against highly conserved regions of mammalian and avian cytokine sequences. (Chapter 4)

Screening of a stimulated leucocyte cDNA library using a number of hybridisation methods, including the use of ovine cytokine probes and the differential screening with probes derived from stimulated and non-stimulated lymphocytes, to isolate genes up or down regulated as a result of lymphocyte stimulation. (Chapter 5)

CHAPTER 2

General materials and methods

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General materials and methods

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2.1 Chemicals and reagents

All chemicals and reagents used were supplied from Sigma Chemicals, England unless otherwise stated.

2.2 Fish

Rainbow trout (*O.mykiss*) were purchased from a local fish farm (Penicuik Trout Farm, Valleyfield Road, Penicuik) and transported by road in a 54.5 litre tank to the Moredun Research Institute. The average weight and length of fish ranged between 600-900g and 36-43cm respectively. Upon arrival fish were immediately transferred to a 20 litre holding container and anaesthetised with $40mgL^{-1}$ benzocaine in methanol.

After anaesthesia, fish were killed with a sharp blow to the head using a blunt instrument and bled via the caudal vein using a 10ml vacutainer and 20G needle (Becton Dickinson, Plymouth). Approximately 10ml of blood per fish were collected in vacutainer tubes, containing 100μl heparin ($10Unitsml^{-1}$) to prevent clotting.

2.3 Isolation and culture of lymphocytes from peripheral blood and head kidney

Lymphocytes were prepared as described by Secombes (1987). Peripheral blood and head kidney cell suspensions were diluted in L-15 medium with 10% heat inactivated foetal bovine serum (10% HI FBS), layered onto 51% (v/v) Percoll and centrifuged at 900xg for 35-45 min at 18°C. Alternatively, lymphocytes derived from peripheral blood were isolated from the buffy coat, formed following centrifugation of heparinised blood at 600xg for 10 min prior to density centrifugation. The resulting lymphocyte enriched band of cells was collected from the interface of the L-15 medium and 51% Percoll and washed twice in L-15 medium by centrifugation at 600xg for 10 min. The final pellet was resuspended in L-15 medium (0.1% v/v HI FBS) and the cell concentration adjusted to 2×10^6 or 1×10^7 viable cells ml^{-1}. An estimation of a viable cell count was obtained as described in Section 2.5.1. Lymphocytes were incubated for 24/48 h in L-15 medium, in the presence of appropriate mitogens, in $25cm^2$ tissue culture flasks or 96-well microtitre plates.

2.4 Isolation and culture of head kidney macrophages

Head kidney macrophages were prepared from rainbow trout, as described by Brauwn-Nesje *et al* (1981) and modified by Secombes (1990). Cell suspensions were enriched for macrophages using discontinuous Percoll density gradient centrifugation, followed by further purification utilising the inherent property of macrophages to adhere to plastic.

Immediately after fish were anaesthetised, sacrificed and bled as described (Section 2.2), the head kidney was dissected using aseptic techniques. A single cell suspension in homogenising medium (L-15 medium containing $10Uml^{-1}$ heparin, $100Uml^{-1}$ penicillin, $0.1mgml^{-1}$ streptomycin) was obtained by disrupting the head kidney tissue through a 100µm nylon gauze. The disrupted cell suspension was layered onto a 34% / 51% Percoll (Pharmacia) density gradient.

Percoll density gradients were freshly prepared, using two solutions of different density; the bottom 51% (v/v) Percoll solution ($1.080gl^{-1}$) and the top 34% (v/v) Percoll solution ($1.070gl^{-1}$). The gradients were prepared by carefully layering the 34% Percoll solution on top of the 51% Percoll solution, without disturbing the interface between the two solutions.

Density gradients were centrifuged at 900xg for 35-45 mins at 18°C and the band of cells at the interface between the two different densities collected. Cells were then washed twice in attachment medium (L-15 medium containing 0.1% HI FBS) by centrifugation at 600xg for 10 min at 18°C and then resuspended in L-15 medium. An estimation of viable cell concentration was obtained using 0.4% (w/v) trypan blue dye (Section 2.5.1).

The cell concentration was adjusted to 2×10^7 viable cells ml^{-1} and 100µl of cell suspension was dispensed into triplicate wells of a 96 well sterile flat-bottom microtitre plate (Nunc, UK). Macrophages were allowed to adhere for 2-3 h at 18-20°C. Unattached cells were removed by gentle washing with L-15 medium (0.1%HI FBS). The attachment medium was replaced with $100\mu l well^{-1}$ of growth culture medium (L-15 medium containing 10% v/v heat inactivated HI FBS) and the monolayer of cells incubated for 24 – 48 h prior to use.

2.5.1 Lymphocyte and macrophage viable cell counts

Estimation of the number of viable lymphocyte and macrophage cells isolated from both head kidney and peripheral blood was made using 0.4% (w/v) trypan blue dye and a Neubauer haemocytometer (Hawksley, England). Live cells exclude the blue dye and appear clear whilst dead cells stain blue. The average number of cells per large square (mm^2) of the haemocytometer was estimated under phase contrast, using an inverted microscope (Leica, Portugal). The numbers of cells ml-1 of the original suspension were calculated using the following equation:

Viable cells ml^{-1} = cell count x df x 10^4

df (dilution factor)

10^4 (factor to adjust for the volume between the coverslip and haemocytometer chamber)

2.5.2 Estimation of adherent macrophages

Estimation of adherent macrophages was made as described by Secombes (1990). Unattached cells were removed from the microplate by gentle pipetting, and the macrophage cultures washed with L-15 medium (0.1% v/v HI FBS). To allow the release of nuclei from the attached macrophages, 50µl macrophage lysis buffer (0.1M citric acid, 1% (v/v) Tween 20, 0.05% (w/v) crystal violet) was added per well. The number of macrophage nuclei per ml of lysis buffer was estimated using the Neubauer haemocytometer at a magnification of >x 600. The number of nuclei, and consequently original attached macrophages per 50 µl lysis buffer and hence per well was determined by dividing the count per ml by 20:

$$\text{No. of macrophages / } \mu\text{l lysis buffer} = \frac{\text{nuclei x df x } 10^4}{20}$$

2.6 Preparation of MAF containing supernatants from stimulated lymphocytes

Supernatants from stimulated and non-stimulated lymphocytes were prepared using a modification of the method of Secombes (1987), for the preparation of MAF from rainbow trout leucocytes.

Lymphocytes were isolated from peripheral blood (Section 2.3) and seeded at 2×10^6 / 1×10^7 cells ml^{-1} in $25cm^2$ tissue culture flasks. A range of mitogens including PMA, Con A and LPS were used to induce lymphocyte stimulation. Lymphocytes were incubated at 20°C in L-15 medium (10%v/v HI FBS) containing mitogens at a range of concentrations. After several h the cells had adhered slightly to the culture flasks thus enabling the removal of supernatants containing mitogen without disturbing the monolayer. To remove any residual mitogen, the cultures were resuspended in PBS and centrifuged at 600xg for 10 min and the supernatants discarded. The cell pellets were then resuspended in fresh L-15 medium (10% v/v HI FBS) and cultured for a further 24/48h, after which supernatants were collected and stored at -70°C. Flasks of lymphocytes grown without stimulation by the mitogens, were incubated at 20°C for 24/48 h before collection of control supernatants.

2.7 Assessment of lymphocyte proliferation

The capacity of T and B lymphocytes, derived from peripheral blood, to be stimulated either by mitogens or cell free supernatants was determined by a lymphocyte stimulation assay.

To triplicate wells of a 96-well sterile flat-bottomed microplate, 20μl volumes of L-15 Medium (10%v/v HI FBS) were dispensed. In addition, 20μl of the following stimulants: ConA, PMA, PHA, Pokeweed, LPS at a range of concentrations were added separately to appropriate wells. 2×10^5 lymphocytes (Section 2.3) in 200μl of the prepared lymphocyte suspension were added to all wells containing either control medium or stimulants, followed by incubation at 20°C for 3 - 4 days. Radiolabelled ^{3}H-thymidine was diluted to 0.74 Bqμl^{-1} in L-15 medium and 50μl added to each well and the plate incubated for approximately 18 h prior to scintillation counting. The cells were harvested with a semi-automatic multiple harvester,

transferred onto glass fibre filters and washed in distilled water. Radioactivity of the filters was counted in a liquid scintillation counter.

Results were expressed as either 'mean counts per min' (cpm) or as stimulation indices (SI):

$$SI = \frac{\text{mean cpm stimulated cultures}}{\text{mean cpm non-stimulated cultures (control)}}$$

2.8 Assessment of respiratory burst activity of macrophages

Lymphocyte supernatants were assayed for their capacity to induce respiratory burst activity in macrophages. This was measured in two ways, either spectrophotometrically measuring the reduction of nitroblue tetrazolium (NBT) to formazan (Pick and Mizel, 1981; Rook *et al*, 1985; Chung and Secombes, 1988), or using a luminometer to measure reduction of the substrate Luminol (aminophthalic acid hydrazide).

2.8.1 Reduction of NBT

Respiratory burst can be determined by the addition of NBT to the macrophage suspension. The yellow NBT reacts with the superoxide anions generated by the burst and turns into the blue-black formazan:

$$NBT + O_2^- \longrightarrow formazan + O_2$$

Precipitation of the formazan can be visualised and measured spectrophotometrically.

Supernatants from both stimulated and non-stimulated lymphocytes were diluted in L-15 medium (10%v/v HI FBS). Triplicate 200µl samples of supernatants, each at the following dilutions: neat, 1/10, 1/100 were added to 1x $10^7 ml^{-1}$ macrophage monolayers prepared previously (Section 2.4) and incubated at 20°C for 48 h.

The macrophage cultures were washed twice with L-15 medium to remove unattached cells. With the exception of the macrophage controls, 100µl NBT ($1mgml^{-1}$)/ PMA ($1\mu gml^{-1}$) were added per well and the plate incubated at 20°C for 30-40 min. The supernatant medium was removed and the reduction reaction stopped by fixing the cells with 100µl methanol for 5-10 min. After washing the macrophages with 70% (v/v) methanol to remove any non-reduced

NBT the wells were air-dried. The reaction products were dissolved in 120µl 2M KOH and 140µl dimethyl sulphoxide (DMSO).

The optical density (OD) at a wavelength of 620nm was determined using an enzyme-linked immusorbent assay (ELISA) plate reader (Dynatech MR5000). The number of macrophages per well was estimated from the triplicate control wells, containing macrophage cells incubated with L-15 medium alone. The macrophages were lysed with lysis buffer for 2 min and the released nuclei counted (Section 2.5.2).

The results were expressed as 'Macrophage activity' and adjusted to absorbance per 10^5 cells. The mean OD for each triplicate culture was calculated and divided by the number of cells per well to obtain the OD per 10^5 cells.

$$OD_{620} / 2x\ 10^5 \text{ cells} = \frac{\text{mean OD per triplicate culture x 100}}{\text{no. of cells / well}}$$

2.8.2 Reduction of Luminol

The macrophage activation assay with luminol, was used as an alternative method of assessing respiratory burst metabolism following macrophage stimulation. Chemiluminescence is an energy product of phagocyte oxygenation activity, and the chemiluminesecent probe (CLP) luminol allows continuous monitoring of early oxygenation activity (Allen, 1977) Figure 2.1-2.2.

Viable macrophages suspended in Ca^{2+}/Mg^{2+}- free, indicator-free, hank's balanced salt solution (HBSS) at $2x10^6$ cells ml^{-1} (1.2×10^6 cells $well^{-1}$) were added 100µl $well^{-1}$ to triplicate wells of a white, opaque 96 well microplate (Dynex Microlite). Equal volumes of luminol (200µM) in Ca^{2+}/Mg^{2+}-, indicator free HBSS, followed by cell free supernatant from stimulated PBL were added. PMA (5µgml^{-1} HBSS) was used as a positive control to stimulate respiratory burst. HBSS and L-15 medium were included as negative controls.

Chemiluminescence was monitered for 40 min at 20°C, using a luminometer (TR717, PE Biosystems). The results were expressed as relative light units per second (RLU sec^{-1}), and

the area below the response curve (integral) was determined in triplicate samples. Kinetic measurements were analysed using the 'WinGlow' 1.24 software program.

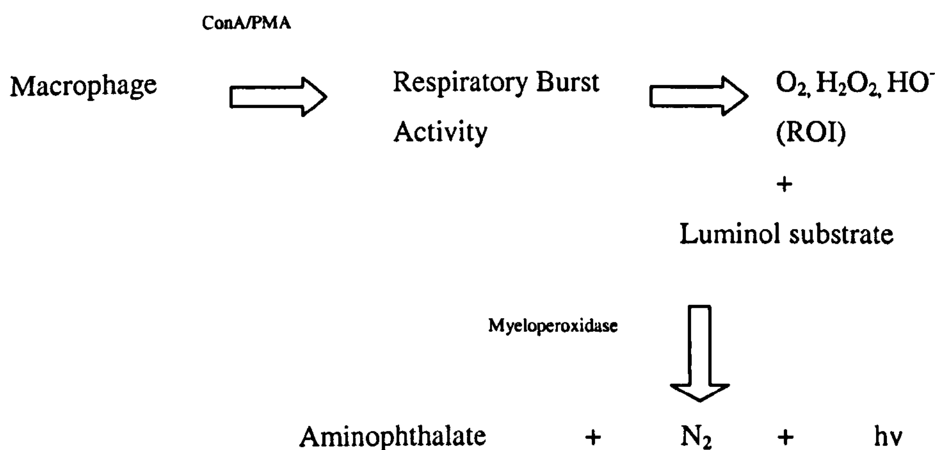

Figure 2.1 Schematic diagram representing the respiratory burst activity of macrophages using Luminol as substrate. Macrophages respond to stimuli by 'activating respiratory burst' metabolism. A proportion of the oxygenating agents generated yield electronically excited products of which a number of excited molecules relax by photon emission.

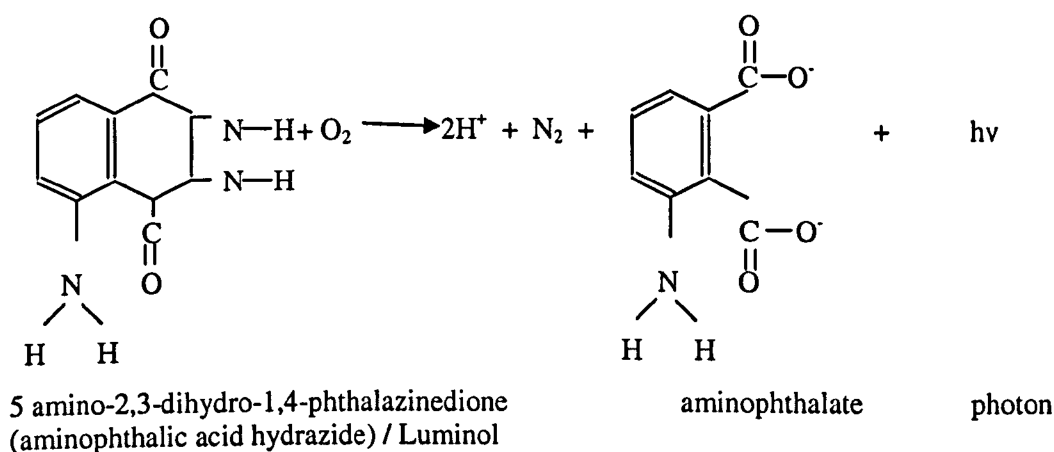

Figure 2.2 Dioxygenation of luminol to aminophthalate.

The overall reaction responsible for luminol chemiluminescence is an oxidation. Luminol reacts with O_2 to yield aminophthalate and a photon.

2.9 cDNA library synthesis using pCMV mammalian plasmid expression vector system

A cDNA library was prepared from RNA isolated from stimulated peripheral blood lymphocytes, using the pCMV-Script^{R} XR cDNA library construction kit (Stratagene). This kit is designed for generating directional libraries in the pCMV-script mammalian expression, such that it can be screened by functional assay in mammalian cells or with a DNA probe in *E.coli* cells.

The pCMV-Script mammalian expression vector is derived from a high copy-number ColE1 based plasmid and is designed to allow protein expression in mammalian systems. The presence of the kanamycin-resistance gene allows for prokaryotic selection. The vector is predigested with *Eco*RI and *Xho*I restriction enzymes and allows the finished cDNA to be inserted into the vector in a sense orientation (*Eco*RI and *Xho*I). The cloning region of the pCMV-vector does not possess an ATG initiation codon and therefore only clones containing their own ATG initiation codon will be expressed.

cDNA synthesis of the first-strand is initiated in the presence of nucleotides and buffer, when reverse transcriptase locates a template and a primer. In this instance the template is messenger RNA (mRNA) and the primer is a 50-base oligonucleotide sequence:

5′- GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAGTTTTTTTTTTTTTTTTTT-3′

"GAGA" Sequence   *Xho* I   Poly (dT)

The oligonucleotide sequence design features an 18 - base poly(dT) sequence with a "GAGA" sequence to protect the *Xho* I restriction enzyme recognition site. The poly (dT) region binds to the 3'Poly (A) region of the mRNA template, allowing priming of the first strand cDNA synthesis by Maloney murine leukemia virus reverse transcriptase (MMLV-RT).

For the first strand synthesis reaction, the nucleotide mixture contains normal dATP, dGTP, dTTP in addition to the analogue 5-methyl dCTP. Consequently, the completed first strand will possess a methyl group on each cytosine base, which functions to protect the cDNA from restriction enzymes used in subsequent cloning steps.

RNase H is used to introduce nicks into the RNA bound to the first strand cDNA and these act as priming sites for DNA polymerase I, which 'nick-translates' them into second-strand cDNA. The resulting double stranded cDNAs possess uneven termini which are blunted with *Pfu* DNA polymerase, prior to ligation with *Eco*R I adapters. The *Eco*R I adapters are composed of complementary oligonucleotides, with an *Eco*R I cohesive end.

Release of the *Eco*R I adapter and residual linker-primer from the 3' end of the cDNA is performed by *Xho* I digestion (Figure 2.3). The resulting fragments are separated on a drip column containing Sepharose CL-2B gel filtration medium. The size fractionated cDNA is then precipitated and ligated to the pCMV-Script vector. Since most *E.coli* strains digest DNA containing 5'-methyl dCTP, it is necessary to initially transform XL10-Gold cells ($McrA^-$ $McrB^-$ strain) with the ligated DNA, to obtain a high yield. After which the DNA can be grown on $McrA^+$ $McrB^+$ strains eg XL1-Blue strain, as it is no longer hemimethylated.

2.10 cDNA synthesis

The pCMV-Script XR cDNA library construction kit (Stratagene) is optimised for 5μg of poly $(A)^+$ RNA (prepared from total RNA isolated from stimulated lymphocytes, Sections 2.17-2.18). All non-enzymatic first and second strand components were thawed and briefly vortexed before being placed on ice.

2.10.1 First strand cDNA synthesis

The following reagents were added in order to an RNase-free tube: 5μl 10x first-strand buffer, 3μl first-strand methyl nucleotide mixture, 2.8ng linker-primer (1.4μg ml^{-1}).

12.5μl diethylpyrocarbonate (DEPC) -treated water, 40U RNase block ribonuclease inhibitor. The reaction was gently mixed and 24.5μl of poly $(A)^+$RNA added. For the control, the above annealing reaction was used with 5μg of test RNA and 12.5μl DEPC-treated water. The primer was allowed to anneal to the template for 10 min at room temperature (rt) before the addition of 50U MMLV-RT, to give a final reaction volume of 50μl. The sample was mixed gently and centrifuged. 5μl of this first-strand synthesis reaction was transferred to a tube

containing 0.5μl [α-^{32}P]dCTP (800Ci mmo^{-1}l) and this constituted the first strand synthesis control reaction. Both reactions were incubated at 37°C for 1hr: the non-radioactive first strand synthesis reaction was placed on ice and the radioactive control reaction kept at -20°C.

2.10.2 Second strand cDNA synthesis

The following components were added in order to the 45μl non radioactive first strand synthesis reaction on ice: 1x second strand buffer, 6μl second strand deoxyribonucleotide (dNTP) mixture, 114μl sterile water, 2μl [α-^{32}P]dCTP (800Ci mmol^{-1}). To this second strand synthesis reaction 3U of RNase H and 99U DNA polymerase I enzymes were added. The reaction was gently mixed, centrifuged and incubated at 16°C for 2.5 h. After the second strand synthesis, the reaction was placed on ice immediately. The low incubation temperature was critical in preventing formation of unclonable hairpin structures.

To the second strand synthesis reaction 23μl blunting dNTP mix and 10U cloned *Pfu* DNA polymerase were gently mixed and centrifuged followed by incubation at 72°C for 30 min. 200μl phenol-chloroform [1:1(v/v)] was added and mixed by vortexing. The reaction was centrifuged for 2 min at rt and the upper aqueous layer containing the cDNA transferred to a fresh tube, carefully avoiding removal of any interphase. An equal volume of chloroform was added and mixed. The cDNA was precipitated overnight at -20°C, with 0.1 x vol 3M sodium acetate and 2 x vol 96-98% (v/v) ethanol, followed by centrifugation at maximum speed for 60 min at 4°C. The supernatant was discarded and the cDNA pellet gently washed with 500μl 70% (v/v) ethanol and air-dried. The pellet was resuspended in 9μl *Eco*R I adapters and incubated at 4°C for at least 30 min. 1μl of this second strand synthesis reaction was transferred to a fresh tube and this represented the second strand synthesis control reaction.

2.10.3 Resolving cDNA on alkaline agarose and non-denaturing acrylamide gels

cDNAs can be resolved by electrophoresis on an alkaline agarose gel to determine their size range. The first and second strand cDNA synthesis reactions prepared in Section 2.10 including controls were resolved on a 1% (w/v) alkaline agarose gel (1-3kb cDNA size range). Due to the

low buffering capacity of these gels the reactions were electrophoresed in 1x alkaline buffer (5M NaOH, 0.5M EDTA) and run at 5 Vcm^{-1} for approximately 3.5 h. The gel was dried using a gel dryer and exposed to x-ray film over night at -70°C.

Alternatively, the size fractionated cDNAs prepared in Section 2.11 were electrophoresed at 10-20 Vcm^{-1} for 1 hr on a 5% (w/v) non-denaturing acrylamide gel and exposed to x-ray film over night at –70°C.

2.10.4 Ligation of *Eco*R I adapters

The following kit components were added to the blunted cDNA and *Eco*R I adapters: 1x ligase buffer, 1μl 10mM rATP and 4U T4 DNA ligase and incubated overnight at 8°C. The ligase was heat inactivated at 70°C for 30 min, after which the reaction was centrifuged for 2 seconds before being cooled at rt for 5 min.

2.10.5 Phosphorylation of *Eco*R I ends

The *Eco*R I adapter ends were phosphorylated by the addition of: 1x ligase buffer, 2μl 10mM rATP, 6μl sterile water and 10U T4 polynucleotide kinase and incubated for 30 min at 37°C. The kinase was heat inactivated for 30 min at 70°C, after which the reaction was centrifuged for 2 seconds before being cooled at rt for 5 min.

2.10.6 *Xho* I digestion

The cDNA was digested with *Xho* I restriction endonuclease with the addition of 28μl *Xho* I buffer and 120U *Xho* I, followed by incubation for 1.5 h at 37°C. 5μl of 10 x STE buffer (1M NaCl, 200mM Tris-HCL pH 7.5, 100mM EDTA) and 12μl 96-98% (v/v) ethanol were added and the cDNA precipitated overnight at -20°C.

Following precipitation, the cDNA was centrifuged for 60 min at 4°C, and the pellet dried completely before resuspension in 14μl 1x STE buffer. 3.5μl of the column loading dye was added to the sample, prior to size fractionation.

2.11 cDNA size fractionation

The cDNA was size fractionated using a drip column containing sepharose CL-2B gel filtration medium assembled according to the manufacturer's instructions. The column was washed with 10ml of 1x STE buffer, ensuring a steady flow was maintained and preventing the column drying out. When approximately 50μl of STE buffer remained above the surface of the resin, the cDNA sample was loaded gently onto the column bed. Once the sample entered the sepharose CL-2B gel filtration medium, 3ml of 1 x STE buffer were added to the reservoir.

In order to gauge sample elution from the column, the progress of the dye was monitored. As the cDNA sample eluted through the column, the dye gradually migrated through the resin. A minimum of 12 fractions, each containing 3 drops (~100μl) were collected. To ensure the cDNA had been successfully eluted, the fractions were monitored for the presence of radioactivity and collected until the unincorporated nucleotides were eluted.

Before process of the fractions and recovery of the size-fractionated cDNA, approximately a tenth of each fraction was saved for analysis by alkaline agarose gel electrophoresis (Section 2.10.3): to assess the effectiveness of the size fractionation and determine which fractions would be used for ligation.

2.12 Processing cDNA fractions

To recover the size selected cDNA, the fractions collected from the drip column were extracted with phenol-chloroform and precipitated with ethanol to remove contaminating proteins, carried over from previous steps. To ensure the cDNA had been recovered, the level of radioactivity present in the pellet was monitored.

The pellet was carefully washed with 200μl 80% (v/v) ethanol, centrifuged for 2 min at rt and air-dried for a maximum of ~ 5 min. Recovery of the cDNA was verified by radioactive monitoring.

2.13 Quantification of DNA: Ethidium Bromide Plate Assay

DNA can be quantified by u.v visualisation on ethidium bromide (EtBr) agarose plates [0.8 %(w/v) agarose/Tris-acetate media (0.04M Tris-acetate, 0.001M EDTA)/EtBr ($10mgml^{-1}$)], using a DNA sample of known concentration as a standard.

Several dilutions of a DNA sample of known concentration (1Kb DNA ladder) were prepared in 100mM EDTA ranging from 10-200 $ng\mu l^{-1}$. 0.5μl of each standard was spotted on the surface of 100mm Petri dishes containing the above agarose. Immediately, 0.5μl of the cDNA was spotted adjacent to the standards, and the samples absorbed into the plate for 10-15 min at rt. The spotted sample of unknown concentration was then compared with the standards and quantified by examination under u.v. transillumination.

2.14 Generation of inserts

2.14.1 Ligations and transformations

Five individual ligations were prepared using 10ng cDNA/ 30ng vector, including a LacZ test insert control (2.25.1). Individual transformation reactions were prepared with XL-10 Gold ultracompetent *E.coli* cells (Stratagene) with each ligation reaction, including a pUC18 plasmid control. Following heat pulse, and incubation at 42°C with NZY^+ broth (NZ amine/ casein hydrolysate, yeast extract, NaCl, supplemented with the following: 1M $MgCl_2$, 1M $MgSO_4$, 20% w/v glucose) for 1 hr with shaking at 225-250 rpm, the transformation reactions were pooled (2.27.1). This resulting pool constituted the primary library and was stored at 4°C, ready for amplification as soon as possible.

In order to determine the total number of primary transformants the pooled transformations were plated out in 1μl, 10 and 50μl volumes onto LB-kanamycin (X-gal/IPTG) agar selection plates (Section 2.28.2). The ampicillin - resistant test insert transformation was plated onto LB-ampicillin ($100\mu gml^{-1}$) agar plates: (NaCl, tryptone, yeast extract, agar: adjusted to pH 7 with 5 N NAOH and diluted to 1litre with deionised water).

2.14.2 Verifying insert percentage and size

Individual colonies were examined to determine the percentage of plasmids with inserts, and the average insert size, by restriction analysis of individually prepared plasmid DNA. Plasmids DNA of single, random clones from the primary library were purified from *E.coli* (50μgml^{-1} kanamycin) and digested with *Eco*R I and *Xho* I restriction enzymes (Section 2.20). Restriction profiles were obtained by resolving the digested DNA on a 1% agarose / TBE gel.

2.15 Amplification of the pCMV-Script cDNA Library

Amplification of the primary library was necessary to obtain a large and stable quantity of clones. However, in order to avoid under representation of slow growing clones it was important to only perform one round of amplification. The library was amplified in semi-solid suspension allowing for three-dimensional, uniform colony growth.

The plasmid library was amplified in 500ml bottles of 2x Luria-Bertani (LB) agarose using a semi-solid amplification method. Each bottle could accommodate ~$5x10^5$ primary cfu. On a heated stirring plate, 0.135g SeaPrep^R agarose was added to 45ml of 2 x LB and heated until the agarose was in solution and cooled to 37°C for 1 hr. Kanamycin (50μgml^{-1}) and $3x10^4$ cfu/bottle of primary library was added and stirred for several min. The library was incubated for 1 hr in an ice bath at 0°C followed by incubation at 30°C for 40-45 min, since incubation at the latter temperature reduces under-representation of slower growing colonies. The library was centrifuged at 10,000xg for 20 min at rt and the pellet resuspended in 4.5ml of 12.5% 2 x LB-glycerol: 2x LB broth, 100% v/v glycerol). For the estimation of the library titre and further characterisation, 50μl of the library was removed and the remainder stored at -70°C.

2.16 Estimation of the amplified library titre

The total numbers of transformants in the amplified library were determined by preparation of 10-fold serial dilutions with 50μl of amplified library diluted in 450μl 2xLB glycerol medium. A number of dilutions of the library: 50μl of $10^{-5,}$ 10^{-6} and 10^{-7} were plated out onto LB agar /

$50\mu g ml^{-1}$ kanamycin (Section 2.27.2), and the number of colonies counted over a portion of the plate. Amplification of the primary library should result in a 10^3 fold increase of total number of transformants.

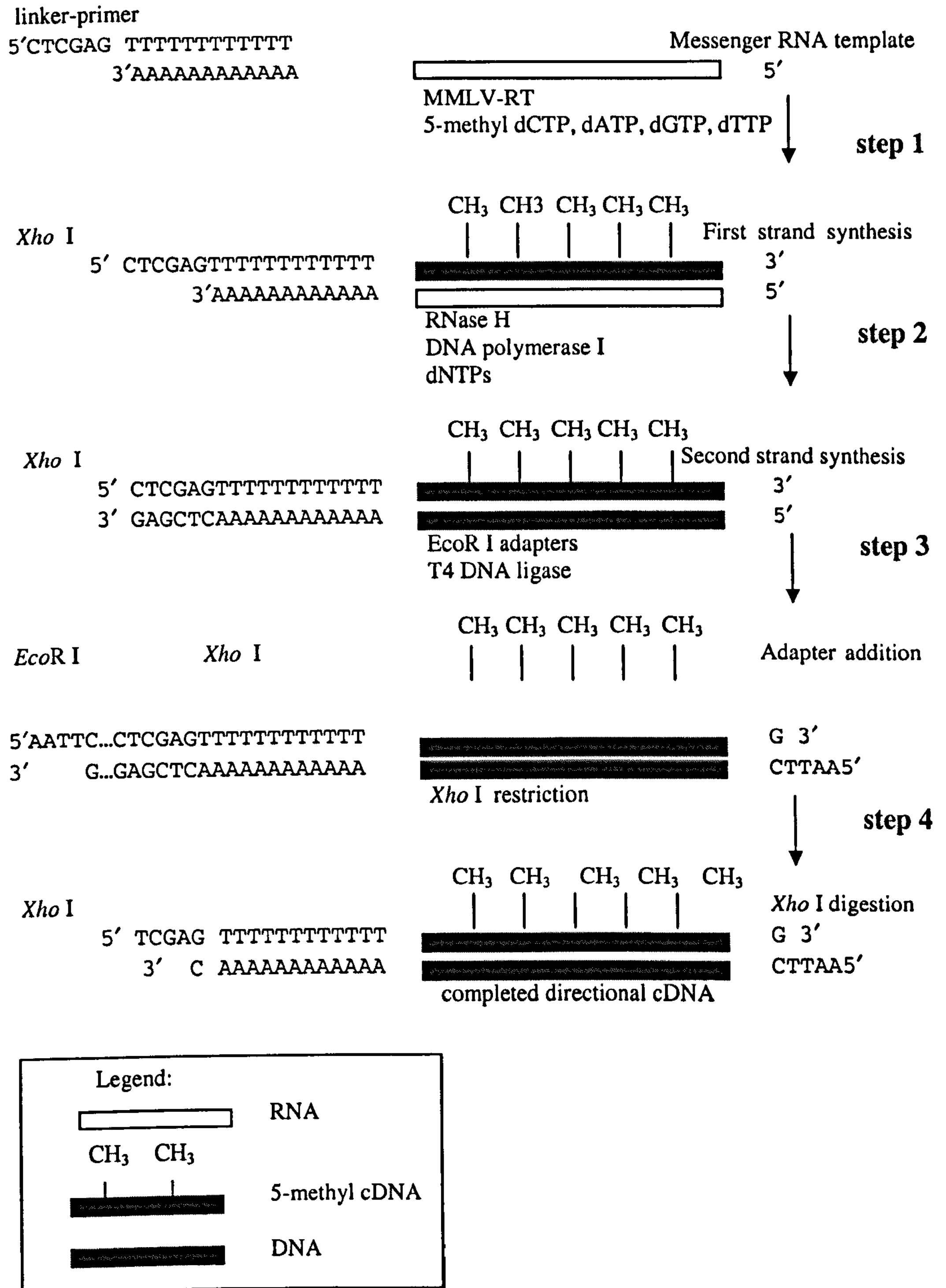

Figure 2.3: Flow chart of cDNA synthesis

During the first-strand synthesis an oligo (dT) linker-primer containing the *Xho* I restriction site anneals to the messenger RNA, which is reverse transcribed using MMLV-RT and 5-methyl dCTP. RNA fragments are 'nick translated' by DNA polymerase I during second strand synthesis, resulting in double stranded cDNA. The blunt ends of the cDNA fragments are ligated with *EcoR* I adapters and subsequent digestion with Xho I, releases the *EcoR* I adapters and residual primer-linker from the 3' end of the cDNA.

2.17 Isolation of total RNA from tissues/cells

The guanidinium thiocyanate-phenol/chloroform extraction method (Short and Sorge, 1992) was used to isolate total RNA.

To achieve a good quality preparation of undegraded RNA, it is essential to inhibit the activity of RNases during cell lysis by the use of RNase inhibitors. Cells can be disrupted and RNases inhibited simultaneously by the use of strong denaturants such as guanidinium thiocyanate and β-mercaptoethanol and detergents such as sodium lauroyl sarcosine. If the cell lysate is then mixed with phenol / chloroform under acidic conditions, protein, genomic DNA and membrane lipids will partition to the organic phase, whilst RNA will partition to the aqueous phase.

RNA was isolated from a range of tissues and lymphocytes. Liver, kidney and spleen tissue were ground to a fine powder using a pestle and mortar with liquid nitrogen and homogenised in the following extraction buffer: 4M guanidine isothiocyanate, 250mM sodium citrate pH7, 10 % (w/v) sodium lauroyl sarcosine, 14.2M β-mercaptoethanol. For 1g of tissue or 10^8 cells, 5ml extraction buffer was used. Peripheral blood lymphocytes grown in suspension were harvested by centrifugation at 12000 rpm and RNA extracted immediately or disrupted with RNA extraction buffer and stored at -20°C for later extraction.

A tenth of the extraction buffer volume of 3M sodium acetate (pH 5.2) was added. The low pH of the acid causes neutralisation, resulting in the precipitation of most of the protein and genomic DNA. An equal volume (with respect to the total aqueous volume) of phenol, followed by a fifth volume chloroform, was added and the solution shaken vigorously for 10 min. It was then left on ice for 10 min and the aqueous and organic phases separated by centrifugation at 12000 rpm for 20 min at rt. The aqueous phase was carefully removed to a fresh tube and the RNA precipitated by addition of an equal volume of isopropanol. The solution was mixed and left at -20°C for at least 1 hr, followed by centrifugation at 12000 rpm for 20 min.

Depending on the clarity of the preparation, i.e. presence of protein / DNA contamination after the isopropanol precipitation, the procedure may be repeated from the addition of sodium acetate onwards.

Once the supernatant had been discarded, the resulting pellet was washed with 70% (v/v) ethanol and allowed to air dry for a few min. The RNA pellet was resuspended in 200μl in TE buffer (10mM Tris.Cl pH 8, 1mM EDTA pH 8) / 10^8 starting cells.

The RNA extracts were electrophoresed at 100V for 30 – 60 min, using 1% (w/v) agarose gel prepared in MOPS/EDTA buffer (0.2M MOPS [3-(N-morpholino) propanesulfonic acid], 50mM sodium acetate, 10mM EDTA). The agarose was dissolved using a microwave and once cooled below 50°C, 2ml of 37% formaldehyde was added and the gel allowed to set. RNA extracts were diluted 1/5 with RNA loading buffer (0.75ml formamide, 0.15ml 10xMOPS buffer, 0.24ml formaldehyde, 0.1ml glycerol, 0.1ml of 0.5% (w/v) bromophenol blue, 0.01ml ethidium bromide (10mgml^{-1}) and 0.1ml sterile water) and heated to 65°C for 10 min followed by chilling on ice before loading.

2.18 Nucleic acid quantification

The concentration of RNA can be determined by measurement of ultraviolet (UV) absorbance spectrophotometrically at OD_{260} of a diluted sample RNA, using the following conversion factor: A_{260} of 1 corresponds to 40μg RNA ml^{-1}. UV absorbance was also used to check the purity of the RNA preparations. For a pure sample of RNA the ratio of absorbance at 260nm and 280nm (A_{260}/A_{280}) is approximately 2.

The concentration of DNA samples was quantified by UV absorbance at A_{260}, at which wavelength an absorbance of 1.0 corresponds to 50μg of ds DNA ml^{-1}. With a pure sample of DNA the ratio of the absorbances at (A_{260}/A_{280}) is approximately 1.8.

2.19 Isolation of PolyA$^+$ mRNA

The quantity and expression pattern of polyA$^+$ RNA in cells and tissues varies with cell type and developmental stage. Total cellular RNA is composed mainly of ribosomal RNA (rRNA) and

transfer RNA (tRNA), with mRNA accounting for only 1-5%. Some mRNAs constitute less than 0.01% of the mRNA pool. Enrichment of such low level messages is essential for cDNA library construction where isolation of pure, intact mRNA is crucial for characterising mRNA molecules.

Purification of polyA$^+$ mRNA from total RNA was performed with the Oligotex mRNA mini kit using the spin column protocol (Qiagen) as per the manufacturer's instructions. The procedure takes advantage of most eukaryotic mRNAs ending in a polyA tail of 20-250 nucleotides, as opposed to rRNA and tRNA which are non- polyadenylated.

PolyA$^+$ mRNA is purified by hybridisation of the polyA tail to an oligo-dT oligomer coupled to a solid-phase matrix. The tRNA and rRNA species do not bind to the oligo-dT and are washed from the column. As hybridisation requires high salt conditions the polyA$^+$ mRNA is readily dissociated by lowering the ionic strength and destabilising the dT-dA hybrids.

The concentration and purity of total RNA was determined spectrophotometrically (Section 2.18) and approximately 100μg of total RNA suspended in 500μl with RNase-free water. An equal volume of binding buffer and 55μl oligotex suspension were added and mixed. (The maximum theoretical binding capacity is 600mgμl^{-1} mRNA oligotex suspension). The sample was incubated for 3 min at 70°C to disrupt the secondary structure of the RNA and hybridised with the oligotex particle at room temperature for 10 min. The oligotex-mRNA complex was centrifuged for 2 min at 13,000x g and the supernatant discarded. The oligotex-mRNA pellet was resuspended in 400μl wash buffer, transferred to a spin column and centrifuged for 1 min at maximum speed. The column was washed with 400μl wash buffer as above and the PolyA$^+$ mRNA eluted by resuspension of the resin with 40μl hot (70°C) elution buffer, followed by centrifugation for 1 min. The PolyA$^+$ mRNA was quantified by UV absorption at A_{260} and stored at -20°C until required.

2.20 Purification of Plasmid DNA

Methods used for the purification of plasmid DNA exploit the small size and covalently closed circular nature of the plasmid DNA. Bacteria are recovered by centrifugation and lysed by a number of methods, including treatment with nonionic/ionic detergents, organic solvents, alkali or heat. These treatments cause the host bacterial chromosomal DNA to denature by disrupting base pairing. However, strands of closed circular plasmid DNA remain intertwined.

Plasmid DNA was purified from *E. coli* using an adaptation of the alkaline lysis methods of Birnboim and Doly (1979), and Ish-Horowicz and Burke (1981). A 10ml culture of *E.coli* inoculated in LB medium containing 50μgml^{-1} ampicillin at 37°C overnight, was harvested by centrifugation at 3000rpm for 10 min. Cells were resuspended thoroughly in 200µl alkaline lysis buffer I (50mM glucose, 10mM EDTA pH 8, 25 mM Tris.HCl pH 8), followed by incubation on ice for 5 min, after which 400µl of alkaline lysis buffer II (0.2M NaOH, 1%SDS) was added and mixed by inversion. This was incubated on ice for 5 min after which 300µl of alkaline lysis buffer III (3M sodium acetate pH 4.8/5.2) was added and mixed by vortexing for 15 seconds and incubated for 30 min on ice. The cellular debris was pelleted by centrifugation at 13000 rpm for 10 min. To 0.7ml supernatant transferred to a fresh tube, 0.6 volume of isopropanol was added and left on ice for at least 10 min. The precipitated nucleic acids were centrifuged and the supernatant discarded. The nucleic acid pellet was washed with 1ml 70% (v/v) ethanol and re-centrifuged. The supernantant was discarded and the pellet allowed to air dry for a few min, prior to resuspension in 50µl TE buffer (10mM Tris.Cl, 1mM EDTA pH8) containing RNase A (0.1mgml^{-1}).

Alternatively plasmid DNA was purified using the spin miniprep kit protocol (Qiagen) as per the manufacturer's instructions. The procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt (Vogelstein and Gillespie 1979).

The protocol allows the purification of up to 20µg of high-copy plasmid DNA from 10ml overnight cultures of *E.coli* in LB medium. The pelleted bacterial cells were resuspended

in 250μl of buffer P1, an equal volume of buffer P2 was added and the contents mixed by inversion. To prevent shearing of the genomic DNA mixing by vortexing was avoided. After the addition of 350μl of buffer N3 the solution was mixed by inversion and centrifuged at 13,000 rpm for 10 min. The supernatant was decanted to a silica spin column and centrifuged for 1 min. Following salt removal by washing with 750μl of buffer PE, the DNA was eluted in either 50μl of buffer EB (10mM Tris.Cl, pH 8.5), TE (10mM Tris.Cl, 1mM EDTA, pH 8.0) or water (pH 7-8). The purified plasmid DNA is quantified by UV absorbance at A_{260} (Section 2.18).

2.21 Purification of DNA from TBE agarose gels

The GeneClean II protocol (Bio101) was used to purify DNA from agarose gel slices as per the manufacturer's instructions. The method is based on the principle of DNA binding to silica in high salt concentrations and elution in low salt (Vogelstein and Gillespie, 1979). Different sizes of DNA remain bound to the silica under various salt, pH and wash conditions. Maximum binding efficiency of smaller DNA fragments (200-500bp) can be achieved by lowering the pH of NaI to 6-6.5 by the addition of 10% v/v acetic acid or TBE modifier™ (kit component consisting of a mixture of concentrated salts for removing DNA, and is used to overcome the inhibitory effect of TBE and other borate-containing buffers on the binding of DNA to silica).

The DNA to be purified was excised from an ethidium bromide-stained agarose gel and weighed. The agarose was dissolved by incubation at 45-55°C for 5 min with 0.5 volumes of TBE modifier and 4.5 volumes of 6M NaI. To allow binding of the DNA to the silica matrix 5μl of resuspended silica was added, followed by vortexing and incubation at rt for 5 min. The amount of silica required is based on the amount of DNA and volume of NaI solution. 1μl of silica matrix binds 1-2μg DNA. The silica-bound DNA is pelleted by centrifugation at 13,000 rpm and the supernatant discarded. The resulting pellet was washed 3 times with 700μl NEW Wash (kit component containing NaCl, Tris, EDTA, 96-98% v/v ethanol) and the pellet air-dried for 5-10 min to remove any residual ethanol which could interfere with downstream reactions. The DNA was eluted from the silica by resuspending the pellet in 10μl water or TE,

pelleting the matrix and collecting the supernatant. Approximately 80% of the bound DNA is eluted, though a second elution can recover an additional 10-20%.

2.22 Purification of cDNA using Phenol-chloroform extraction

The cDNA fractions prepared (Section 2.11) using the pCMV-Script XR cDNA Library Construction Kit were extracted with phenol-chloroform and precipitated with ethanol to recover the size selected cDNA. Phenol-chloroform extraction was necessary to remove contaminating proteins, in particular kinases carried over from previous steps since they often retain activity following heat treatment.

An equal volume of phenol-chloroform [1:1(v/v)] was added to the fractions collected from the drip column, followed by mixing by vortexing and centrifugation at 13,000 rpm for 2 min at rt. The upper aqueous layer was transferred to a fresh tube and an equal volume of chloroform added. After mixing by vortexing and centrifugation for 2 min at rt the upper aqueous layer was transferred to a fresh tube. To each extracted sample, 96-98% (v/v) ethanol equal to twice the individual sample volume was added, followed by precipitation overnight at -20°C. The samples were pooled at this stage and centrifuged at 13,000 rpm for 60 min at 4°C and the supernatant transferred to a fresh tube. To ensure the cDNA had been recovered, the level of radioactivity present in the pellet was monitored.

The pellet was carefully washed with 200μl 80% (v/v) ethanol and centrifuged for 2 min at rt. The ethanol was discarded and the cDNA pellet air-dried for approximately 5 min. To confirm the cDNA had been recovered, the numbers of counts per second (cps) were detected by radioactive monitoring.

2.23 cDNA amplification by RT-PCR of mRNA

Coupled Reverse Transcription and PCR amplification (RT-PCR) is used to establish relative levels of mRNA species in cells/tissues. RT-PCR allows the amplification of DNA generated by reverse transcription of mRNA. Since RNA cannot be used as a template for PCR, the Reverse Transcriptase (RT) uses an RNA transcript to generate DNA that can be subsequently amplified

by PCR. RT catalyses the first step of RT-PCR, the reverse transcription of RNA into single-stranded cDNA.

This first strand synthesis can be primed using anchored oligo-dT primers, random hexamers or sequence-specific primers. The oligo-dT primers are designed to anneal at the mRNA / Poly A junction end of the mRNA, and so transcription is primed at the beginning of the portion of the mRNA of interest, rather than an arbitrary point within the poly-A tail. By avoiding unnecessary transcription through the polyA tail, an increased cDNA yield and specificity of PCR products are achieved compared with other first strand synthesis primers. Random hexamer primers anneal non-specifically along the whole length of the RNA target and are used when problems of secondary structure are encountered in the target DNA. Sequence-specific primers are used when the target sequence is known, often resulting in lower cDNA yields.

The reaction mixture for the first strand synthesis was prepared with the following components: 1x Avian Myeloblastosis Virus (AMV) RT Reaction Buffer (250mM Tris. HCl, 40mM $MgCl_2$, 150mM KCl, 5mM dithiothreithol pH 8.5), dNTPs each at 2mM, Oligo dT (20-200 pmols) or random hexamer primers (20-50 pmols) or specific internal primers (10-15pmols), $1\mu g$ total celluar RNA or 100ng Poly $(A)^+$ RNA, suspended in a volume of $49\mu l$ with sterile RNase-free distilled water. The reaction mixture was heated to 65°C for 10 min, cooled on ice and briefly centrifuged, to remove secondary structure. 25U AMV RT was added and the reaction incubated at 42°C for 1.5 h. The resultant cDNA was stored at -20°C.

2.24 Polymerase Chain Reaction (PCR)

Selective amplification of a specific sequence of DNA by the polymerase chain reaction (PCR) is performed by the thermostable Taq DNA polymerase (Chien *et al.*, 1976). Two oligonucleotides are used as primers for a series of synthetic reactions catalysed by DNA polymerase.

Template DNA is initially denatured, by heating in the presence of an excess of primers and the 4 dNTPs. The reaction mixture is cooled to allow annealing of the primers to the

template DNA sequence, after which the annealed primers are extended with DNA polymerase. The cycle of extension, annealing and DNA synthesis is repeated and the products from the initial round of amplification serve as templates for the next cycle. Consequently each successive cycle results in the doubling of the amount of DNA product. The exponential amplification of target sequence is not an unlimited process. The Taq DNA polymerase becomes limiting after 25-30 cycles of amplification (amplification level of 10^6).

The PCR reaction mixture contained the following components in order: 1x DNA buffer, 25mM $MgCl_2$, dNTPs each at 2mM and 2.5U Taq DNA polymerase. 0.1-0.5μm ($100pmol\mu l^{-1}$) of each forward and reverse primer (MWG-Biotech UK Ltd, Milton Keynes) and various amounts of template DNA / cDNA (0.001-1ng) were added and sterile water, to give a final reaction volume of 25μl.

PCR amplification was performed with a number of different oligonucleotide primers at a range of annealing temperatures (Table 4.3), using a thermal cycler MultiBlock System (Hybaid Ltd). Amplification conditions for denaturation, annealing and polymerisation were as follows: first cycle: denaturation at 95°C for 5 min, subsequent 35 cycles: denaturation at 95°C for 45 seconds, primer annealing between 37°C-55°C for 45 seconds, elongation at 72°C for 1 min, final cycle: denaturation at 95°C for 1 min, primer annealing at 37°C-55°C for 1 min, followed by primer polymerisation at 72°C for 8 min (Table 4.3). Amplified PCR fragments were electrophoresed using 2% agarose gel.

2.25 Cloning of PCR products using pGEM-Teasy Vector

PCR products were cloned using the pGEM-Teasy Vector System 1 (Promega), which allows blue/white selection of recombinants. The vector has *Eco*R I and *Not* I recognition sites flanking the insertion site, allowing the removal of inserts by a single restriction digest after cloning. Purified PCR products are ligated to the linearized vector, using T4 DNA Ligase and competent JM109 *E.coli* cells transformed (Section 2.29); followed by selection of white colonies on ampicillin / IPTG / X-gal plates (Section 2.28.2).

2.25.1 Ligation of PCR products using the pGEM-Teasy Vector

Ligation reactions with 10ng PCR product and 50ng vector (1:5 molar ratio) were set up using the following: 1x Rapid Ligation Buffer (60μM Tris-HCL pH 7.8, 20mM $MgCl_2$, 20mM DTT, 2mM ATP, 10% (v/v) polyethylene glycol), 50ng pGEM-Teasy Vector, 10ng PCR product, 3U T4 DNA ligase and deionised water to give a final volume of 10 μl. The reactions were mixed and incubated at 4°C overnight. A positive control ligation reaction using 8ng control insert DNA was included to determine the ligation efficiency. In addition, a background control ligation was set up with 50ng of vector with out insert, to determine the number of background colonies.

2.25.2 Transformations using pGEMT-easy vector ligation reactions

LB/ampicillin/IPTG/X-Gal plates were prepared for each ligation reaction (Section 2.25.1) and for determining transformation efficiency. Ligation reactions were briefly centrifuged and 2μl of each reaction were added to a sterile 1.5ml prolypropylene tube (Falcon) and placed on ice. To determine the transformation efficiency of the *E.coli* (JM109) competent cells, 0.1ng uncut plasmid was used for the transformation reaction. To each reaction 50μl of thawed competent JM109 cells were added, mixed gently and incubated on ice for 30 min. The cells were heat-shocked for 2 min at 42°C and immediately placed on ice for a further 2 min. An optional step involving the incubation of transformed cells with SOC medium (2g Bacto-tryptone, 0.5g Bacto-yeast extract, supplemented with 1M NaCl, 1M KCl, 2M Mg^{2+}stock, 2M glucose) may be included. In which case, to the cells transformed with ligation reactions and the cells transformed with uncut plasmid, 900μl SOC medium was added and the reactions incubated at 37°C for 1.5-2 h. 100μl of each transformation culture was plated onto duplicate LB/ampicillin/IPTG/X-gal plates and incubated at 37°C overnight (16-24 h).

The transformation efficiency of the competent cells was determined by transformation with uncut plasmid and calculation of cfu/μg DNA:

$$\frac{\text{cfu on control plate}}{\text{ng vector plated}} \times \text{final dilution} = \text{cfu/}\mu\text{g DNA}$$

2.26 Cloning of cDNA using the pCMV- Script plasmid vector

2.26.1 Ligation of cDNA into pCMV- Script plasmid vector

Purified size fractionated cDNA (Section 2.22) was ligated into the pCMV-Script Vector using pCMV-Script XR cDNA library construction kit (Stratagene). A pilot ligation and transformation for each sample was performed to establish the ligation efficiency of the test insert and cDNA with the pCMV-Script vector. The ligation and transformation reactions were then scaled up and optimized to achieve a primary library consisting of a target number of transformants.

A control ligation to ligate the test insert into the pCMV-Script vector was set up with the following: 30ng pCMV-Script vector, 10ng LacZ test insert, 1x ligase buffer, 1x 10mM rATP (pH 7.5), and 1.5μl water. 2U T4 DNA ligase was added. For the sample ligation the following components were added: ~10ng resuspended cDNA, 0.5μl 1x ligase buffer, 0.5μl 10mM rATP (pH 7.5), 1.0μl pCMV-Script vector ($30ng\mu l^{-1}$) and 1.5μl water to give a final volume of 4.5μl. To this 2U T4 DNA ligase was added. Both the control and sample ligations were incubated for 2 days at 4°C.

2.27.1 Transformation using pCMV- Script Vector ligation reactions

XL-10 Gold ultra competent *E.coli* (Stratagene) were transformed using pCMV script vector. To an aliqout of 100μl of ice-thawed XL10-Gold ultracompetent cells, 4μl of the β-ME mix was added and gently mixed by swirling, followed by incubation on ice for 10 min. The entire ligation reaction was added to the cells and gently mixed as above. For the control, 1μl of the ligation was added to 100μl cells and 0.1ng of pUC18 plasmid diluted 1/10 in water was used as a transformation control. All 3 reactions were incubated on ice for 30 min and heat pulsed at 42°C for 30 seconds, followed by incubation on ice for 2 min. The duration and temperature of the heat pulse was critical for obtaining the highest efficiencies. To each tube 0.9ml of preheated (42°C) NZY^{+} broth (10g NZ amine /casein hydrolysate, 5g yeast extract, 5g NaCl,

supplemented with 1M $MgCl_2$, 1M $MgSO_4$ and 2M filter-sterilised glucose solution) was added and the reactions incubated at 37°C for 1 hr with shaking at 225-250 rpm.

2.27.2 Determination of number of transformants

For plating quantities of the ligations; 1µl, 10µl and 50µl of each 1ml pilot transformation was plated out onto LB-kanamycin (X-gal/IPTG) agar plates (Section 2.28.2). In addition 1µl, 10µl and 50µl of the ampicillin-resistant test insert transformation was plated out on LB-ampicillin agar plates.

The numbers of resistant colonies were determined as follows:

colonies / xµl x 1000µl = total cfu.

2.28.1 Preparation of LB agar ampicillin /kanamycin plates

To prepare antibiotic plates, 500ml LB agar was melted using a microwave at low power for 20 min. Appropriate antibiotics (ampicillin at 100µgml^{-1}, kanamycin at 50µgml^{-1}) were added after the agar was cooled to below 55°C. Approximately 25 ml of LB agar was poured into 100mm petri dishes and allowed to set at room temperature. The plates were dried for 10-15 min and stored at 4°C in the dark for up to 1 month.

2.28.2 Preparation of Ampicillin/ IPTG/X-Gal plates

LB agar plates with ampicillin prepared in Section 2.28.1 were supplemented with 100µl IPTG (100µM in H_2O) and 20µl X-Gal (50mgml^{-1} in dimethylformamide). The mixture was spread over the surface of the agar plate and left for 30 min at 37°C before use.

2.29 Preparation of competent *E.coli* (JM109) cells

JM109 cells are maintained on M9 minimal medium plates (42mM Na_2HPO_4, 22mM KH_2PO_4, 0.85mM NaCl, 18mM NH_4Cl, 1M $MgSO_4$, 1M $CaCl_2$, 20% (w/v) glucose, 1M Thiamine HCl) supplemented with thiamine HCl, which selects for the presence of the F' episome carrying the lacZ gene required for the blue/white screening process. The following procedure developed by Hanahan (1983), was used to produce competent *E.coli*.

E.coli was streaked on M9 minimal plates and incubated at 37°C overnight. A single colony was picked and cultured overnight in liquid broth (10g Bacto tryptone, 5g Bacto yeast extract, 0.17M Sodium chloride, pH 7), with shaking at 37°C. From the overnight culture, 10ml of cells were added to 500ml LB and incubated with shaking at 37°C, until an OD_{600} of approximately 0.9 was reached. The bacteria were harvested by centrifugation at 5000rpm for 15 min at 4°C (using a Beckman J2-21 centrifuge and JA-14 rotor), and the supernatant discarded. Cells were resuspended in 250ml ice-cold sterile 100mM $CaCl_2$, placed on ice for 20 min and centrifuged as above. Following resuspension in a further 20ml ice-cold 100mM $CaCl_2$, glycerol was added to a final concentration of 10% and the cells gently mixed. Aliquots of 0.5ml of competent cells were stored at -70°C.

The transformation efficiency of the JM109 *E.coli* cells was determined using $10ng\mu l^{-1}$ pUC 18 supercoiled plasmid as described in Section 2.27.2.

2.30 Preparation of ^{32}P α-dCTP labelled cDNA probes

Radioactive cDNA probes complementary to single stranded RNA were generated by reverse transcription of entire mRNA populations using oligo (dT) or short oligonucleotides of random sequence as primers. Oligo (dT) primers are used with RNAs possessing poly (A) tracts and subsequently generate probes which contain mostly sequences derived from 3' terminus of the mRNAs.

Radioactive cDNA is generated by the incorporation of α-^{32}P-dNTP during the reverse transcriptase step of cDNA synthesis using an RNA dependent DNA polymerase.

The following constituents were added to a centrifuge tube: 1x AMV reaction buffer (Roche Diagnostics Ltd, Sussex) 1x dNTP mix containing dATP, dGTP, dTTP (each at 2mM), 0.5µg Oligo (dT) or random hexamer primer, 0.5µg RNA and the volume adjusted to 44µl with sterile water. The mixture was vortexed and heated to 65°C for 10 min to allow annealing of the primers to the RNA. 1.85 MBq Redivue α-^{32}P-dCTP (Amersham Pharmacia biotech, UK) was

added to the mixture followed by 25U AMV Reverse Transcriptase and the solution mixed and incubated at 42°C for several h.

2.31 Radioactive labelling of DNA probes with random hexanucleotides

DNA probes were labelled using the Rediprime™II random labelling system (Amersham Pharmacia Biotech). The use of random sequence hexanucleotides to prime DNA synthesis on denatured template DNA at a number of sites along its length was introduced by Feinberg and Vogelstein (1983, 1984). The primer-template complex is a substrate for the klenow fragment of DNA polymerase I, with the radioactive DNA being produced by the incorporation of radiolabelled nucleotides in newly synthesised strand of DNA.

The rediprime system allows labelling of very small quantities of DNA (<25ng) to a high specific activity of 1.9×10^9 dpmμg^{-1}, using α-^{32}P-dCTP. The DNA to be labelled was diluted to 45µl in TE buffer (10mM Tris HCL, 1mM EDTA pH8.0) and denatured by heating at 95-100°C for 5 min. The DNA was snap cooled on ice for 5 min, briefly centrifuged and added to the labelling reaction containing: buffered solution of dATP, dGTP, dTTP, exonuclease free Klenow enzyme and random primers. To the reaction mixture 1.85MBq of Redivue α-^{32}P - dCTP was added, mixed by pipetting and incubated at 37°C for at least 10 min.

2.32 Non-radioactive labelling of DNA probes with digoxigenin 11-dUTP

As an alternative to radioactive-labelling DNA can be labelled with the non-radioactive substrate digoxigenin-11-dUTP, using the DIG-Nick translation mix (Roche) and following the manufacturer's instructions.

The nick translation method (Rigby *et al.*, 1977) is based on the ability of DNase I to introduce nicks randomly distributed into the double stranded DNA, at low enzyme concentrations in the presence of Mg^{2+}. 5'-3' exonuclease activity of *E.coli* DNA polymerase I, produces single stranded nicks and eliminates stretches of single stranded DNA (ssDNA). The degraded DNA is then replaced with labelled dNTPs by the 5'-3' polymerase activity. Labelled fragments obtained in the standard labelling reaction, range from 200-500 nucleotides.

For the labelling reaction, 1µg template DNA was added to 4µl of DIG-Nick Translation Mix containing DNA polymerase I, DNase I, digoxigenin (DIG)-11-dUTP, dATP, dCTP, dGTP, dTTP and reaction buffer concentrate in 50% glycerol. The volume was adjusted to 20µl, and the reaction mixed, briefly centrifuged and incubated at 15°C for 90 min. The reaction was chilled to 0°C and stopped with the addition of 1µl 0.5M EDTA (pH8.0) and heating to 65°C for 10 min.

2.33 Detection of DIG-11-dUTP labelled nucleic acids

DNA labelled with DIG–11-dUTP can be detected using an antibody against digoxygenin (Anti-Digoxygenin, Fab fragments conjugated to alkaline phosphatase), and the colorimetric detection reagents nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl (BCIP). Following hybridisation and post-hybridisation washes, the membrane was equilibrated in washing buffer (100mM maleic acid, 150mM NaCl, pH 7.5, 3% (v/v) Tween 20) for 1 min and then blocked in 1%w/v DIG Blocking Reagent (Roche) dissolved in Maleic acid buffer (0.1M maleic acid, 0.15M NaCl, pH 7.5, 3% (v/v) Tween 20) for 30 min. The membrane was incubated at rt for 30 min with 150Uml^{-1} anti-DIG- AP conjugate (Anti-Digoxygenin -AP, Fab fragments conjugated to alkaline phosphatase) diluted 1/5000 in blocking buffer. To remove non-specifically bound and unbound antibody conjugate, the membrane was washed twice with washing buffer for 15 min, followed by equilibration in detection buffer (100mM Tris.HCl, 100mM NaCl, pH 9.5) for 2 min. The substrate mixture of 45µl NBT and 35µl BCIP, (Roche) was added to 10ml of detection buffer and the filter incubated in the dark at rt for 10 min or until bands become visible. The colour reaction was stopped by washing the membrane in distilled water or TE Buffer (10mM Tris, 1mM EDTA pH 8.0) and air-dried.

2.34 Nucleic Acid Hybridisation

2.34.1 Southern hybridisation

The transfer of DNA from gels to nitocellulose filters or nylon membranes by capillary transfer was described by Southern (1975). The DNA is exposed to weak acid resulting in

partial depurination, followed by alkaline denaturation with a strong base and subsequent neutralisation. Hydrolysis of the phosphodiester backbone occurs at depurinated sites, resulting in smaller DNA fragments (~1kb) which can be transferred efficiently.

The DNA to be blotted was electrophoresed in 1% agarose gels in TBE buffer (1M Tris, 10mM EDTA, 1M Boric acid, ethidium bromide ($0.01\mu gml^{-1}$). If the fragments of interest were larger than 15Kb, transfer was improved by depurinating the DNA prior to denaturation. In these instances the gel was washed with 0.25M HCl for 15 min at rt and rinsed with water. Following this the DNA was denatured by submerging the gel in denaturation solution (0.5N NaOH, 1.5M NaCl) for 2 x 15 min, followed by neutralisation with 1M NH_4 acetate and 0.02M NaOH for 2x15 min. Membrane filters for the southern transfer were prepared according to manufacturer's instructions and the DNA blotted overnight by capillary action. The DNA was fixed to the membrane by exposure to UV irradiation (300nm) for 1 min.

DNA was on occasion transferred simultaneously and rapidly from an agarose gel to two membranes as described by Smith and Summers (1980). This procedure was useful when target DNA fragments were to be screened with more than one probe. After fixing the DNA to the membrane, the membrane was prehybridised. Pre-hybridisation prepares the membrane for probe hybridisation by blocking non-specific nucleic acid binding sites on it, resulting in a lowered background. Filters were pre-hybridised in 5ml pre-hybridisation buffer (Rapid-hybaid buffer, Amersham Pharmacia Biotech, UK) for several h at 65°C. Double stranded DNA probes were boiled for 10 min to denature the DNA and immediately chilled on ice.

The DIG 11-dUTP labelled or α-^{32}P-dCTP labelled probes were diluted at optimum concentrations ($5\text{-}25ngml^{-1}$) in hybridisation buffer and incubated with the membrane overnight at 65°C. The diluted probes could be stored at -20°C and reused by denaturing at 95°C for 10 min prior to hybridisation.

Following hybridisation unbound probe was removed by washing the filter twice with 2xSSC / 0.1% (w/v) SDS for 5min at rt and then twice with 0.2xSSC / 0.1% (w/v) SDS at 65°C

for 15 min. Once washed, the filter was sandwiched between two layers of "Saran Wrap" and subjected to autoradiography or antibody detection as appropriate.

2.34.2 Northern hybridisation

The transfer of RNA from gels to nylon membranes is similar to those used for the transfer of DNA. Following electrophoresis of RNA using MOPS/EDTA buffer (Section 2.17), the gel was washed with 10 x SSC transfer buffer (NaCl, Na citrate adjusted to pH7) for 2 x 10 min with shaking. The denaturing and neutralising steps are not necessary, as the RNA is denatured in the gel.

Membrane filters for the northern transfer were prepared according to manufacturer's instructions. The RNA was blotted by capillary action onto a nylon membrane (Hybond N, Amersham) using 10 x SSC as the transfer buffer, either for several h or over night. The transferred RNA was fixed to the membrane by UV-cross linking at 300nm for 1 min. Blots were rinsed in 2 x SSC before hybridisation.

Conditions for prehybridisation, hybridisation and post-hybridisation washes were essentially the same as those used for DNA except that prehybridisation and hybridisation was carried out using northern blot prehybridisation buffer (5x SSPE, 5x Denhardt's reagent, 50% formamide, 0.5% SDS) at 42°C. To remove unbound probe, the filter was washed twice with 2xSSC / 0.1% (w/v) SDS for 5min at 42°C and then twice with 0.2xSSC / 0.1% (w/v) SDS at 42°C for 15 min.

2.35 Colony Hybridisation

Colony hybridisation as described by Grunstein and Hogness (1975) allows the screening of bacterial libraries for specific DNA sequences. The bacterial colonies are transferred to a nylon membrane. Alkaline treatment lyses the colonies and the denatured DNA is immobilised on the membrane. A digoxigenin labelled probe was used and hybrids were detected with the colorimetric immunoassay using the chromogenic substrates NBT/BCIP as before.

Bacterial colonies were replicated from the surface of agar plates onto nitrocellulose filters, to screen large numbers of colonies by hybridisation.

The bacteria adhering to the filter can be lysed immediately or the filter can be placed colony side up on the surface of a fresh LB agar plate with antibiotics for a few h at 37°C to allow the colonies to regenerate.

Replica filters were prepared when colony hybridisation with several probes was performed. The original filter was placed colony side up on the surface of a fresh LB agar plate containing the appropriate antibiotic. A second dry filter was carefully placed on top of the template membrane and its orientation marked to allow realignment to the original. The replica filter was carefully peeled off and depending on the efficiency of the transfer; the filters were incubated at 37°C until colonies were obtained.

Master colony filters and replicas were stored at -70°C. Hybond N^+ membrane moistened with LB agar containing antibiotics and 15% v/v glycerol were placed on top of the colony filter. Filters were sandwiched between two layers of 3MM paper and sealed in plastic bags and stored at -70°C.

The method for colony lysis, based on the original procedure of Grunstein and Hogness (1975), describes the release of DNA from bacterial colonies and it's binding to nitrocellulose filters *in situ*.

The filters were placed colony side up on a pool of 10% w/v SDS for 3 min. Filters were then placed, in turn, onto a pool of; denaturing solution (0.5N NaOH, 1.5M NaCl) for 5 min, and neutralising solution (1.5M NaCl, 0.5M Tris.Cl pH 7.4) for 2x 5 min. The membranes were treated with 2xSSC solution for 2x 5 min and air-dried for 30 min at rt. Bacterial debris was removed by submerging the filters in 2xSSC and wiping the surface with a paper towel. This reduces background hybridisation without lowering the intensity of the signal. Filters were air-dried as before and the DNA fixed to the membrane by exposure to UV illumination (300nm) for 1 min.

CHAPTER 3

Investigating the biological source of macrophage activating factor (MAF)

CHAPTER 3

Investigating the biological source of macrophage activating factor (MAF)

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3.1 Introduction

Respiratory burst is an innate response of macrophages to exposure to a wide range of external stimuli. These include particulate stimulants, for example zymosan, bacterial species including fish pathogens *Aeromonas salmonicida* (Sharp and Secombes, 1992) and *Vibrio anguillarum* (Stave *et al.,* 1985), parasite extracts (Whyte *et al.*, 1989) and soluble stimulants such as PMA, Con A, LPS, PHA and pokeweed mitogen (Chung and Secombes, 1988; Tillit *et al.*, 1988; Zelikoff *et al.,* 1991). This alteration in oxidative metabolism is enhanced by the priming of macrophages via immunologic stimuli such as cytokines (Babior, 1984a,b).

Graham and Secombes (1988), using an *in vitro* functional assay based on the reduction of NBT showed that mitogen stimulated *O. mykiss* head kidney and blood leucocytes were capable of secreting a soluble factor capable of activating macrophages (MAF). In mammals, a macrophage activating factor (MAF) has been identified showing antiviral and macrophage activating properties, sensitive to both acid pH and temperature, parameters which are known to affect IFN-γ. Although IFN-γ or genes encoding IFN-γ have not yet been identified in fish, it has been suggested that *O. mykiss* MAF might be equivalent to mammalian IFN-γ (Graham and Secombes, 1990b) based on similarities with the mammalian cytokine (Nathan *et al.*, 1983). The factor produced by mitogen-stimulated fish lymphocytes was both heat and pH labile and supernatants containing the factor protected a trout cell line challenged with infectious pancreatic necrosis virus (Graham and Secombes, 1990b). Isolation of MAF from these supernatants has proved difficult, but it appears to be a T cell product (Graham and Secombes, 1990a) with a molecular weight of approximately 19 kDa. However, attempts to isolate cDNA clones corresponding to the mRNA encoding MAF have so far failed.

The main aim of this particular study was the development of T cell and macrophage culture methods, to establish a reproducible source of MAF from *O. mykiss* lymphocytes. Lymphocyte stimulation assays were performed to optimise culture conditions and to evaluate the responsiveness of T cell lymphocytes to the following mitogens: PMA, Con A, LPS, PHA and pokeweed, the latter stimulates B-cells but in a T-cell dependent manner. Cell-free

supernatants from the stimulated T-cells were then assayed for their ability to stimulate respiratory burst activity in *O. mykiss* macrophages *in vitro*, as assessed by the reduction of NBT and Luminol.

3.2 Materials and Methods

To establish MAF production, *O. mykiss* leucocytes derived from peripheral blood were cultured *in vitro* with T cell mitogens. Macrophage activation by supernatants from these stimulated cultures was determined by the reduction of either NBT or the chemiluminescence substrate Luminol, following incubation of the macrophages with the supernatants.

3.2.1 Production of cell-free supernatants from stimulated lymphocytes

Cell suspensions were prepared from *O. mykiss* lymphocytes derived from either head kidney or peripheral blood, according to Secombes (1990). Briefly, the cell suspension diluted in L-15 (5ml) was layered onto a 51% Percoll (Amersham) density gradient and the leucocytes carefully isolated from the medium/Percoll interface as described in Section 2.3.

Viable cells were adjusted to 2.0×10^6 cells ml^{-1}, and aliquots of the cell suspensions were added to $25cm^2$ tissue culture flasks. ConA/PMA, diluted to appropriate concentrations ($20\mu gml^{-1}$ and $5ngml^{-1}$ respectively), were added and cells containing L-15 instead of mitogen were included as controls. Leucocytes were stimulated for 3 h, washed with fresh L-15 and incubated at 20°C for 48 h, after which supernatants were collected by centrifugation at 600 xg for 10 min.

3.2.2 Lymphocyte stimulation assay (LSA) with a range of mitogens

A number of mitogens and soluble stimulants were assessed for their ability to activate *O. mykiss* peripheral blood lymphocytes (PBL) *in vitro*. Lymphocytes isolated from peripheral blood, were prepared and cultured as described in Section 2.3 and stimulated with the following: Concanavalin A (Con A), Phorbol-myristate acetate (PMA), Phytohaemagglutinin (PHA), Pokeweed and Lipopolysaccaride(LPS). Mitogens were diluted at 1, 5, 10 and $20\mu gml^{-1}$ in L-15 medium. Leibowitz L-15 medium or lymphocytes grown without mitogen in the medium were included as controls. After culturing the lymphocytes in the presence of the

mitogens for 3-4 days, the extent of stimulation was assessed by measuring the incorporation of ^{3}H-thymidine into the cells after further 18 h incubation.

The cells were harvested with a semi-automatic multiple harvester (Micromate ™ 196 Packard), transferred onto glass fibre filters (Packard) and washed in distilled water. The amount of ^{3}H-thymidine incorporated by the cells was assessed using liquid scintillation counting. Each mitogen was assessed in triplicate and results expressed as mean 'counts per minute'.

3.2.3 Respiratory burst activity of head kidney macrophages assessed by reduction of NBT

Macrophage intracellular respiratory burst activity was measured by the reduction of NBT (Pick and Mizel, 1981; Rook *et al.*, 1985) as described in Section 2.8.1. MAF activity of CFS from stimulated lymphocytes was assessed by incubating macrophage monolayers ($1 \times 10^{7} ml^{-1}$) in the presence of PMA ($1 \mu g ml^{-1}$), with either supernatants from control cells or lymphocytes stimulated from 0 to 48 h. Supernatants were used undiluted or as dilutions of 1:10 and 1:100 in L-15 medium. The assay was developed as described in Section 2.8.1 and results were expressed as mean OD (A_{630nm}) values per 1×10^{5} cells ± SD for triplicate samples.

To determine the optimum period of lymphocyte stimulation to produce MAF activity in the cultures, CFS from lymphocytes stimulated with mitogen for 0-80 h were also assessed. Supernatants were assayed undiluted and at 1:10 and 1:100 dilutions in L-15 medium, as above.

3.2.4 Respiratory burst activity of head kidney macrophages assessed by reduction of Luminol

MAF activity of CFS derived from stimulated lymphocytes was assessed by monitoring the chemiluminescence produced by isolated head kidney macrophages using the substrate Luminol, as described in Section 2.8.2.

Macrophages isolated from the head kidney of *O. mykiss* were prepared and cultured as described in Section 2.4. Luminol was added to triplicate samples of an equal suspension of macrophages (1.2×10^{6}cells/well) and CFS from either 24/48 h mitogen-stimulated lymphocytes.

The corresponding CFS from non-stimulated lymphocytes derived from the same source were also assessed. PMA (5μgml^{-1} HBSS) was used as a positive control to stimulate respiratory burst, and indicator-free HBSS and L-15 medium were included as negative controls. Chemiluminescence was monitored for approximately 40 mins using a luminometer (TR717, PE Biosystems). The results were expressed as relative light units (RLU/Sec) and kinetic measurements analysed with the 'Winglow' 1.24 software program.

3.2.5 Statistical analysis

Statistical tests were performed using Minitab V13. Results from each experiment were analysed by one-way ANOVA with an accepted significance level of $P<0.05$ and Tukey's multiple comparison tests with a family error rate of 0.05. One way - ANOVA was chosen as it allowed the differences among means with equal sample size to be examined using multiple comparisons (Appendices 3.1-3.7).

In addition, a set of residual plots were generated for analysis. Normality probability plots of the residuals (observed-predicted values) and plots of residual versus fits were used to assess if residuals were random / normally distributed and to examine best fit (Appendix 3.8). Normality of variance was confirmed before any parametric test was applied. In instances where this failed, logarithmic transformations (log10) of data were applied to normalise variability within each treatment (Appendices 3.4 -3.7).

3.3 Results

3.3.1 Activation of lymphocytes with a variety of stimulants *in vitro*

Lymphocytes were stimulated using either LPS, PHA, Con A, PMA or Pokeweed mitogen. All of the mitogens tested, with the exception of PMA, were able to stimulate the lymphocytes to proliferate to a greater or lesser extent. The degree of stimulation varied with concentration of the mitogen. The results expressed as mean cpm ± standard error of the ^{3}H-thymidine incorporated are presented in Table 3.1. Optimal stimulation of lymphocytes was obtained with 10μgml^{-1} LPS, 1μgml^{-1} PHA, 20μgml^{-1} Pokeweed and 20μgml^{-1} Con A. When mitogens were compared, LPS produced consistently higher SI values than any of the other mitogens at all concentrations tested (Figure 3.1).

Table 3.1 Incorporation of ^{3}H-thymidine by peripheral blood lymphocytes following stimulation *in vitro* with LPS, PHA, Con A, Pokeweed and PMA

	Mitogen (μg ml^{-1})			
	1	**5**	**10**	**20**
LPS	638.7 ± 103.9*	874.7 ± 215.9	1048.0 ± 32.7	1038.0 ± 60.6
PHA	388.0 ± 44.3	327.6 ± 48.9	257.6 ± 3.4	153.3 ± 16.9
Con A	64.3 ± 18.8	299.3 ± 88.8	351.7 ± 55.4	388.0 ± 56.2
Pokeweed	311.3 ± 22.3	342.7 ± 15.3	329.3 ± 13.4	474.0 ± 21.8
PMA	92.0 ± 6.7	33.7 ± 6.9	20.3 ± 0.9	33.0 ± 11.5
Cells only	128.7 ± 18.8	132.3 ± 49.9	92.7 ± 29.4	65.0 ± 9.0
L-15 Medium only	27.7 ± 0.9	17.7 ± 2.8	14.6 ± 2.9	26.0 ± 4.6

Stimulation of peripheral blood lymphocytes (2x10^6/ml) *in vitro* with LPS, PHA, Con A, Pokeweed and PMA (1-20μgml^{-1}) was assessed by the incorporation of ^{3}H-thymidine. The amount of ^{3}H-thymidine incorporated after 18h in culture is shown. *Results are expressed as mean cpm ± standard error. The different concentrations of mitogens were tested on cells from the same fish and the data are from a single experiment representative of at least two similar experiments. Non-stimulated lymphocytes and L-15 medium are included as controls.

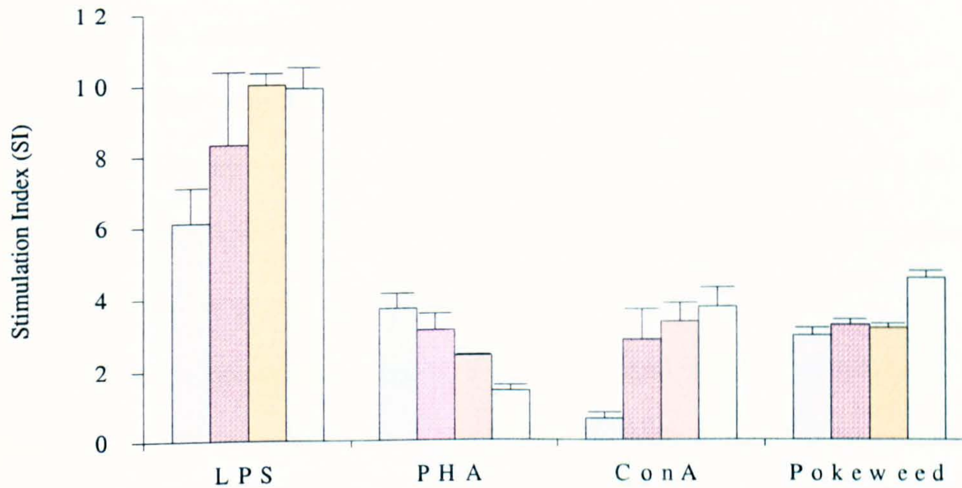

Figure 3.1 Stimulation of lymphocytes *in vitro* with LPS, PHA, Con A and Pokeweed at a range of different concentrations: 1 ■ 5 ■ 10 ■ and 20 ■ μgml^{-1}.

Cellular proliferation is expressed as Stimulation Indices (SI) ± standard error. SI = mean cpm stimulated cultures / mean cpm non-stimulated cultures. Results are from a single experiment representative of two experiments.

Lymphocyte stimulation with PHA decreased with increasing concentration of mitogen. In comparison, both Con A and Pokeweed demonstrated a slight increase in lymphocyte stimulation with increasing mitogen concentration.

Statistical analysis was performed with these SI values using a one-way ANOVA test ($P<0.05$) and Tukey's pairwise comparisons with a family error rate of 0.05. The analysis confirmed a significant difference in responses between LPS stimulated lymphocytes and all other treatments (Appendix 3.1).

At the concentration range tested (ie 1-20μgml^{-1}) PMA proved to be ineffective at stimulating PBLs. Instead, SI values of <1 were obtained, indicating a lack of cell proliferation and suggesting that PMA concentrations above 1μgml^{-1} may be toxic to the cells, resulting in their death. A further assay was performed to optimise lymphocyte stimulation using PMA, where cells were cultured in the presence of a lower range of PMA concentrations (0.005-5μgml^{-1}), the results of which are presented in Table 3.2 and Figure 3.2.

Table 3.2 Incorporation of ^{3}H thymidine by peripheral blood lymphocytes following *in vitro* stimulation with PMA.

Mean counts per minute (cpm)				
PMA (μgml^{-1})	**0.005**	**0.01**	**0.02**	**0.04**
	555.0 ± 69.0*	963.0 ± 45.2	1070.7 ± 47.8	1165.0 ± 194.2
	0.08	**0.10**	**0.25**	**0.50**
	1164.0 ± 33.6	1380.5 ± 68.5	1389.0 ± 15.6	1406.3 ± 204.9
	0.75	**1.00**	**2.50**	**5.00**
	1346.0 ± 215.1	1467.0 ± 80.3	1059.3 ± 23.6	33.3 ± 1.2
Cells only	307.7 ± 58.7	287.7 ± 23.6	301.7 ± 22.4	306.3 ± 42.5
Medium only	15.6 ± 4.7	49.6 ± 9.9	25.6 ± 9.5	28.6 ± 4.1

Stimulation of Peripheral blood lymphocytes ($2x10^{6}ml^{-1}$) *in vitro* with PMA (0.005-5μgml^{-1}) was assessed by the incorporation of ^{3}H-thymidine. The amount of ^{3}H-thymidine incorporated after 18h in culture is shown. *Results are expressed as mean cpm ± standard error. The different concentrations of stimulant were tested on cells from the same fish and the data are from a single experiment representative of at least two similar experiments. Non-stimulated lymphocytes and L-15 medium are included as controls.

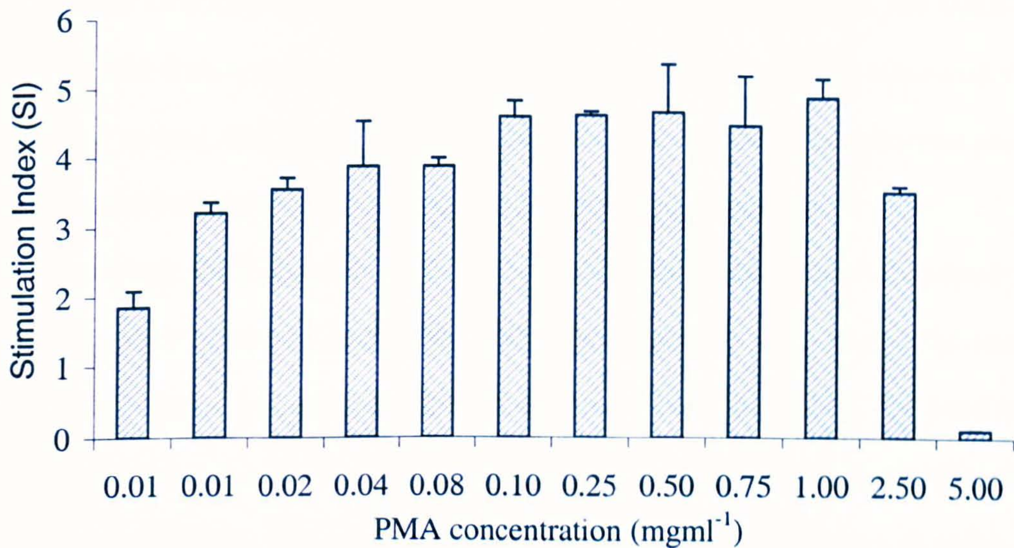

Figure 3.2 Optimisation of lymphocyte stimulation *in vitro* with PMA.

The cellular proliferative responses from Table 3.2 expressed as Stimulation Indices (SI) ± standard error. SI = mean cpm stimulated cultures/ mean cpm non-stimulated control cultures. Peripheral blood lymphocytes ($2x10^6ml^{-1}$) stimulated *in vitro* with PMA ($0.005\text{-}5\mu gml^{-1}$) were grown in the presence of ^{3}H-thymidine.

In this subsequent assay results indicate a gradual increase in the proliferative response of blood leucocytes to PMA with concentrations between $10ngml^{-1}$ - $2.5\mu gml^{-1}$, with the optimum dose being $1\mu gml^{-1}$ (Figure 3.2). At $5\mu gml^{-1}$ PMA no stimulation was observed.

3.3.2. Reduction of NBT by *O. mykiss* head kidney macrophages

Evidence of macrophage activation by the supernatants of *O. mykiss* lymphocytes cultured in the presence of mitogens was demonstrated in the present study. PMA ($5ngml^{-1}$) was used as a co-stimulant in addition to Con A ($10\mu gml^{-1}$), to increase the production of MAF.

The length of incubation of the lymphocytes with the mitogens is critical to obtain macrophage activation activity in the supernatant. The reduction of NBT by head kidney macrophages in the presence of PMA ($1\mu gml^{-1}$), with control supernatants (derived from non-stimulated lymphocytes) or supernatants from lymphocytes stimulated for between 0 and 48 h is shown in Figure 3.3. No significant difference was observed in macrophage activation after

incubating with CFS from controls. However, stimulation of MAF production was significantly higher with CFS from lymphocytes stimulated for 48 h compared with non-stimulated control cultures. The optimal dilution of 1:10 of supernatants from stimulated lymphocytes produced the maximum stimulation for NBT reduction by head kidney macrophages.

A one-way ANOVA test ($P<0.05$) and Tukey's pairwise comparison with a family error rate of 0.05, were performed using mean OD values from the NBT assay. The statistical analysis demonstrated a significant difference in the reduction of NBT by head kidney macrophages incubated with CFS from lymphocytes stimulated for 48 h (* diluted 1:10) compared with CFS from unstimulated lymphocytes. No significant differences in means of the responses between the remaining individual treatments were observed (Appendix 3.3).

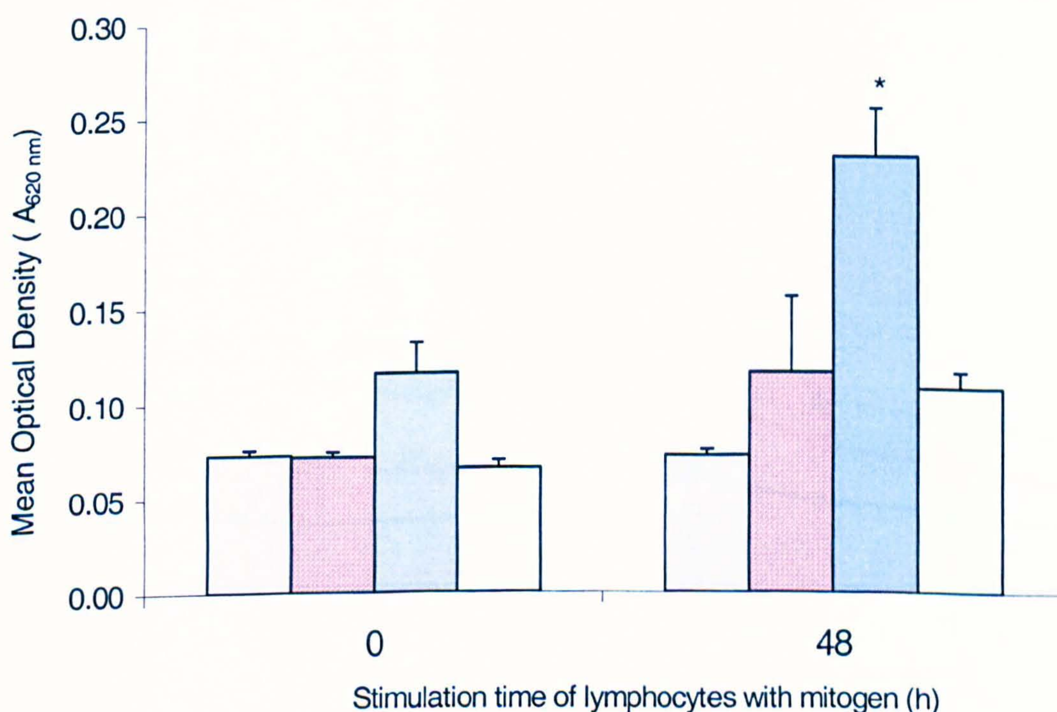

Figure 3.3 Reduction of NBT by head kidney macrophages, following incubation in the presence of PMA (1µgml^{-1}), with supernatants from non-stimulated lymphocytes ■ or supernatants from lymphocytes undiluted ■ ; 1:10 ■ ; 1:100 ■ for 0 or 48 h.

Results are expressed as means of triplicate readings per 10^5 cells ± standard error and are representative of two experiments. * Significant difference in reduction of NBT by head kidney macrophages achieved for 1:10 diluted CFS from lymphocytes stimulated for 48 h.

3.3.3 Determining the optimal length of lymphocyte stimulation to produce MAF, as assessed by NBT reduction assay

Results from the initial assay (Figure 3.3), with supernatants from lymphocytes stimulated with mitogen compared with unstimulated lymphocytes demonstrated macrophage activating activity by the reduction of NBT. These findings are shown in Figure 3.4, in which the optimal time for lymphocyte stimulation to produce MAF was assessed using a NBT assay.

Macrophages incubated with CFS from 48 h cultures produced the greatest levels of respiratory burst activity, with significantly higher NBT reduction by these head kidney macrophages than macrophages cultured with CFS from 24 and 72 h cultures. Supernatants diluted 1:10 in L-15 produced the greatest reduction of NBT.

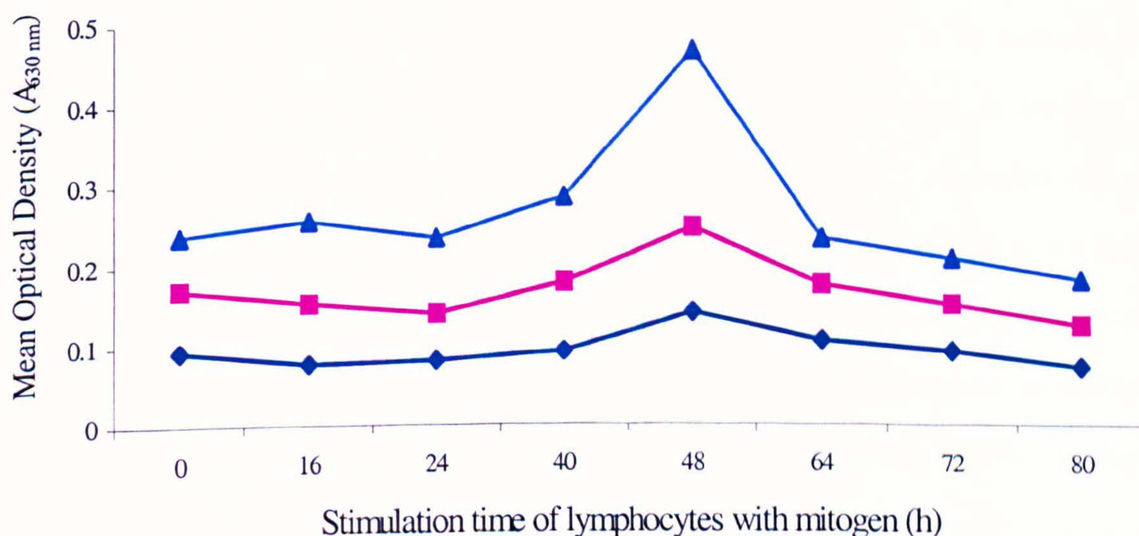

Figure 3.4 Reduction of NBT by head kidney macrophages in the presence of PMA ($1\mu g ml^{-1}$) following incubation with supernatants from lymphocytes stimulated for varying times with Con A. The supernatants were derived from 0-80 h Con A / PMA ($20\mu g/5ng\ ml^{-1}$) stimulated lymphocytes and assayed using: undiluted◆ 1:10 ▲ 1:100 ■ dilutions in L-15 medium. Results are expressed as means of triplicate readings per 10^5 cells and are representative of two experiments.

3.3.4. Respiratory burst activity of head kidney macrophages as assessed by reduction of Luminol

A chemiluminescence-based assay was used *in vitro* to investigate the Luminol-dependent chemiluminescent response attained when *O. mykiss* head kidney macrophages, were incubated with CFS derived from mitogen-stimulated lymphocytes. A number of experiments were performed to optimise the assay conditions necessary to assess the respiratory burst of head kidney macrophages using Luminol, and to examine factors which influence the level of chemiluminescence. The influence of serum, pH indicator present in the medium (phenol red) and macrophage viability on chemiluminescence output was assessed. The data presented for each set of conditions/factors are from a single experiment and the chemiluminescent responses are representative of several replicate experiments.

3.3.4.1 The effect of Phenol red indicator on Luminol-dependent chemiluminescence

The effect of phenol red indicator on the chemiluminescent response produced as a result of oxidative burst by *O. mykiss* macrophages was investigated. The media in all instances were identical apart from the presence or absence of phenol red indicator. A reduction in chemiluminescence was observed with both CFS from LPS ($10\mu g ml^{-1}$) stimulated PBL and corresponding CFS from non-stimulated PBL (Figure 3.5). It was demonstrated by the results that although the presence of the indicator may reduce the chemiluminescence output measured, the reduction was not sufficient to mask the overall response seen in the activated macrophages. Phenol red, similarly to the effect seen in intact and lysed red blood cells, absorbs a portion of the emitted light and thus reduces the overall light detected.

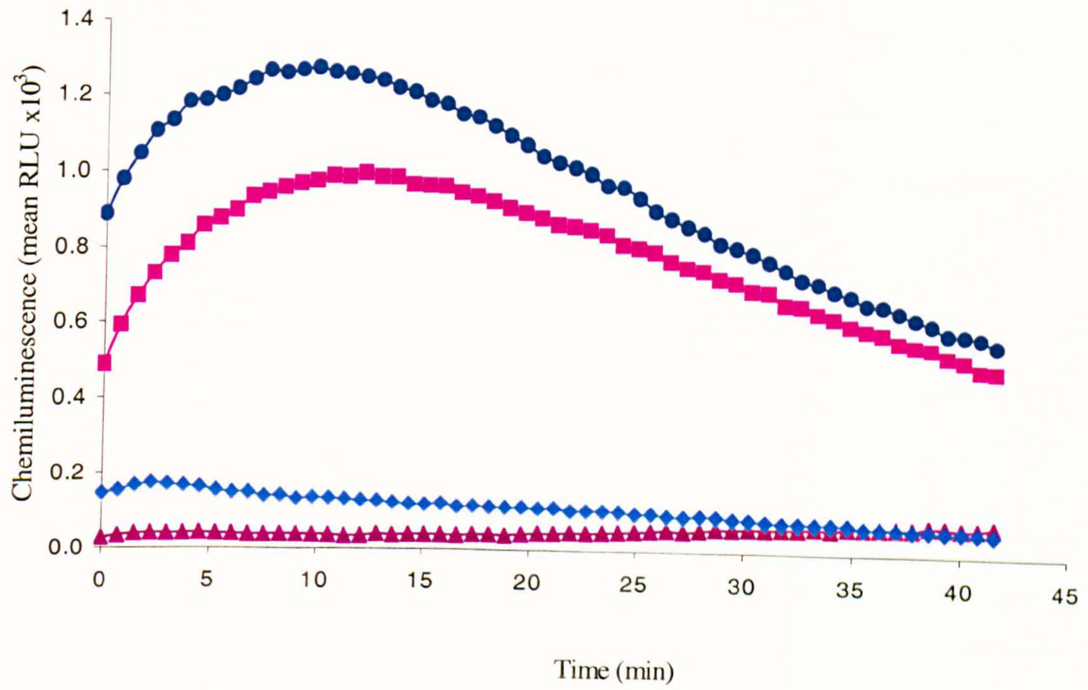

Figure 3.5 Effect of phenol red indicator on the chemiluminescent response of *O. mykiss* head kidney macrophages. Macrophages ($2x10^6 ml^{-1}$) were incubated with: CFS from PBL stimulated with LPS ($10\mu g ml^{-1}$) in L-15 medium containing indicator ■, CFS from PBL stimulated with LPS ($10\mu g ml^{-1}$) in L-15 medium without indicator ●, with CFS from non-stimulated cultures in L-15 medium containing indicator ◆ and with non-stimulated PBL cultures without indicator ▲ . The results are expressed as means of triplicate readings per 10^6 cells.

3.3.4.2 The effect of serum on Luminol-dependent chemiluminescence

The presence of serum in L-15 medium of CFS was assayed for its effect on the chemiluminescence signal (Figure 3.6). A significant reduction was apparent in the chemiluminescence output when measuring CFS without serum compared with CFS containing serum. A strong response with a sharp peak at 10^4 relative light units (RLU), followed by a gradual decline in chemiluminescence was seen with CFS containing serum, whereas cells without serum produced a relatively poor response. Macrophages with L-15 medium alone elicited a baseline chemiluminescent response as expected.

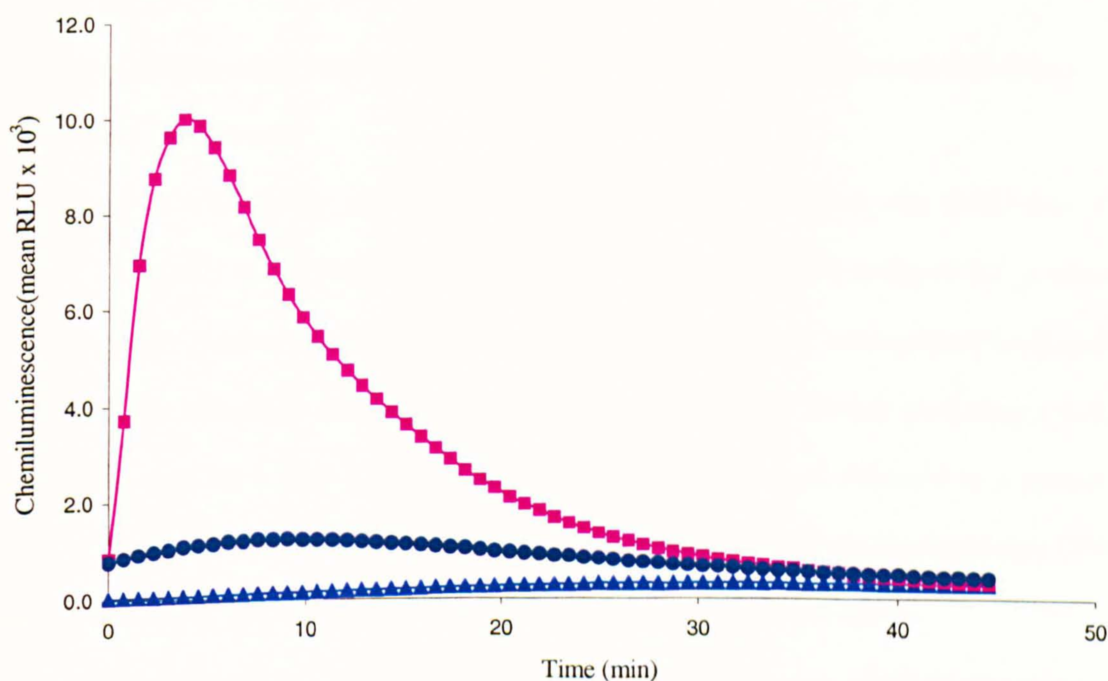

Figure 3.6 Effect of serum on the chemilululminescent response of *O. mykiss* head kidney macrophages. Macrophages ($2x10^6 ml^{-1}$) were incubated with L-15 medium ▲ , CFS from PMA ($1\mu gml^{-1}$) stimulated lymphocytes in L-15 medium containing FBS (10%) ■ and with CFS from PMA ($1\mu gml^{-1}$) stimulated lymphocytes in L-15 medium without FBS ● . The results are expressed as means of triplicate reading per 10^6 cells.

3.3.4.3 The effect of macrophage viability on Luminol-dependent chemiluminescence

The effect of macrophage viability on chemiluminescence production was assessed. A reduction in total chemiluminescent output by a third, was demonstrated using macrophages incubated overnight at +4°C in Hank's balanced salt solution (HBSS) compared to fresh macrophages from the same source (results not shown). Phagocytes maintained in incomplete media or buffered salt solutions exhibit a rapid decline in activity. This is in contrast to *I. punctatus* phagocytes which maintain a high level of activity over a prolonged period of time, as assessed after incubation for 10 h at 20°C in complete HBSS (Scott and Klesius, 1981).

3.3.4.4 Chemiluminescent response of head kidney macrophages to CFS from PMA/Con A-stimulated lymphocytes

The response of head kidney macrophages to CFS from PBL stimulated with PMA/ Con A ($5ngml^{-1}$, $250\mu gml^{-1}$) was investigated, the results of which are presented in Figure 3.7. A clear difference in the level of chemiluminescence was observed between macrophages incubated with CFS from stimulated and non-stimulated PBL cultures. The former producing a bell shaped curve reaching a peak 10-12 min after the addition of Luminol, followed by a gradual decline in response. In comparison, baseline values were obtained with the corresponding CFS from non-stimulated PBL and macrophages incubated with L-15 medium alone.

CFS from stimulated PBL elicited a chemiluminescent response, which increased more rapidly, reaching a higher mean peak of 1.8×10^3 RLU with Fish A and 1.6×10^3 RLU with Fish B. The response seen with Fish A was also sustained for a longer period of time than that elicited by fish B. Although a similar kinetics profile was obtained for both fish, differences confirmed the general variations observed between individual fish.

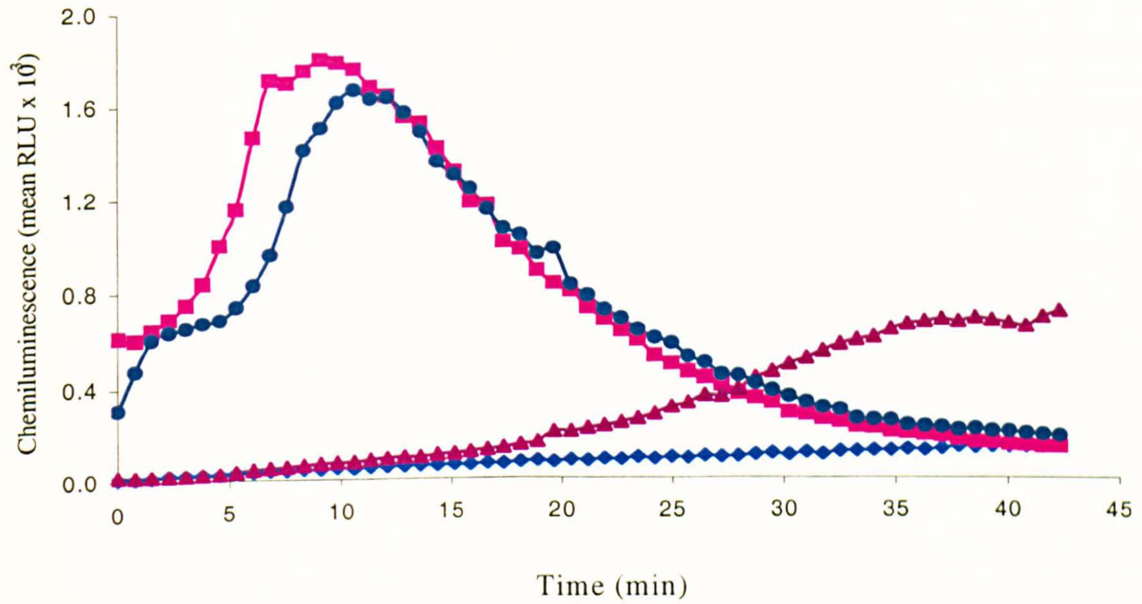

Figure 3.7 Chemiluminescent response of *O. mykiss* head kidney macrophages to CFS from PMA/Con A-stimulated lymphocytes. Macrophages ($2x10^6 ml^{-1}$) derived from two fish designated A and B were incubated with CFS from PBL stimulated with PMA/Con A ($5ngml^{-1}$, $250\mu gml^{-1}$) in L-15 medium (A ●, B ■) and with CFS from non-stimulated PBL cultures in L-15 medium (A ▲, B◆). The results are expressed as means of triplicate reading per 10^6 cells.

3.3.4.5 Chemiluminescent response of head kidney macrophages to CFS from PMA-stimulated lymphocytes

The chemiluminescent response using head kidney macrophages with cell-free supernatants from PBLs stimulated with PMA (1μgml^{-1}) was assessed (Figure 3.8). Supernatants from stimulated PBL elicited a chemiluminescent response, reaching a plateau within the first 5 - 10 minutes of the cycle time and continuing for the monitoring period. The plateau effect could be due to the macrophages becoming saturated and not able to respond further, thus achieving their maximum response. In comparison, low baseline results were obtained with CFS derived from non-stimulated PBL and L-15 medium alone.

3.3.4.6 Chemiluminescent response of head kidney macrophages to CFS from LPS-stimulated lymphocytes

The chemiluminescent response using head kidney macrophages with supernatants from PBLs stimulated with LPS (10μgml^{-1}) was assessed (Figure 3.9). Supernatants from stimulated PBL elicited a chemiluminescent response, reaching a peak of 1.2 x10^3 (mean RLU) after 10 min, followed by a gradual decline. In comparison, a much lower chemiluminescent response was obtained with CFS derived from non-stimulated PBL reaching a peak of 0.2 x10^3 (mean RLU), and baseline response with L-15 medium alone.

The effect of LPS as a soluble stimulant to directly potentiate respiratory burst in *O. mykiss* macrophages was assessed, by performing a titration using the mitogen at a concentration range of 10-250μgml^{-1} (results not shown). When using LPS, an immediate rapid chemiluminescent response (15-23.5 x 10^3 RLU) was evident which decayed down to background level in less than 10 min. At lower concentrations, the initial magnitude of the response was reduced, although the time course of the reaction remained unaltered.

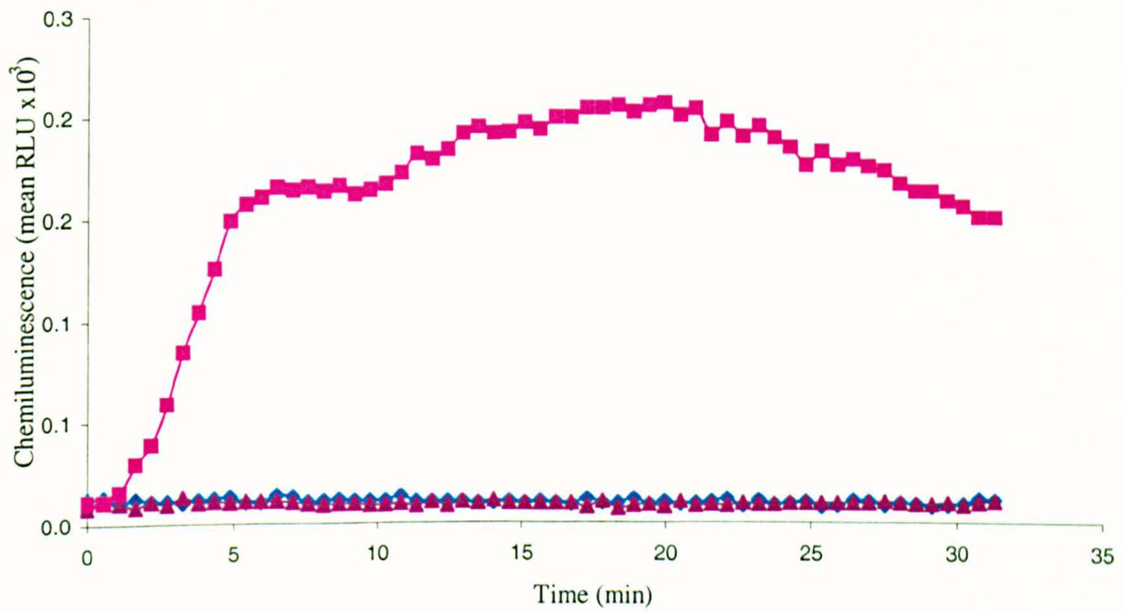

Figure 3.8 Chemiluminescent response of *O. mykiss* head kidney macrophages to CFS from PMA-stimulated lymphocytes. Macrophages (2 x$10^6$$ml^{-1}$) were incubated with L-15 medium alone ◆ CFS from PBL stimulated with PMA (1μgml^{-1}) in L-15 medium ■ and with CFS from non-stimulated PBL cultures ▲. The results are expressed as means of triplicate reading per 10^6 cells.

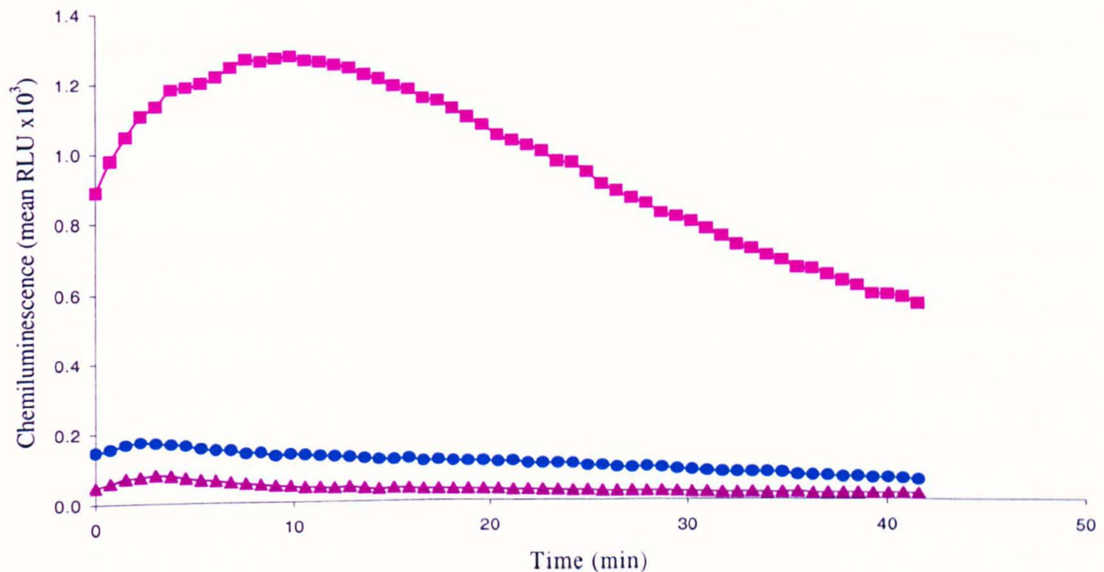

Figure 3.9 Chemiluminescent response using *O. mykiss* head kidney macrophages to CFS from LPS-stimulated lymphocytes. Macrophages ($2x10^6ml^{-1}$) were incubated with L-15 medium alone ▲, CFS from PBL stimulated with LPS ($10\mu gml^{-1}$) in L-15 medium ■ and with CFS from non-stimulated with PBL cultures ●. The results are expressed as means of triplicate reading per 10^6 cells.

To overcome the problem of variability of macrophage responses between each experiment, the results of a single experiment are shown (where macrophages from the same source were used) and are representative of the general trend observed. In addition, all the different CFS were tested on macrophages from the same fish, to allow direct comparisons to be made.

Statistical analysis was performed on all data using a one-way ANOVA test ($P<0.05$) and subsequent Tukey's pairwise comparisons with a family error rate of 0.05. The analysis demonstrated a significant difference in the chemiluminescent response variation between means of the individual treatments within each experiment (Appendices 3.4-3.7).

3.4 Discussion

Cellular immune reactivity of lymphocytes *in vitro* can be evaluated using leucocyte microtitration assays (Galeotti *et al.*, 1996). Peripheral blood lymphocytes of *O. mykiss* have been previously reported to be stimulated by mitogens (Warr and Simon, 1983; Tillit *et al.*, 1988; Reitan and Thuvander, 1991), which provoke lymphoblastic transformation of T cells (PHA and Con A) and B cells (LPS) in higher vertebrates (Janossy and Greaves, 1971). It was shown in the present study that leucocytes from peripheral blood of *O. mykiss* responded to a variety of mitogens and soluble stimulants: LPS, PHA, Con A, PMA and Pokeweed. The optimal stimulation conditions reflected the mitogen dose used in the assay. LPS is a good stimulator of lymphocytes from lymphoid organs and generally gives higher SI values than PHA over a wide dose range. LPS gave the highest stimulation of all the mitogens used in this study and was comparable to results previously found for *O. mykiss* (Reitan and Thuvander, 1991), Atlantic salmon (Smith and Braun-Nesje, 1982), and sea bass (Galeotti *et al.*, 1996). This is in contrast to the response observed with lymphocytes from mammals (mice and humans), which respond only weakly to low levels of LPS whilst higher levels are toxic. It has been suggested the apparent differences in LPS sensitivity, between fish and mammals, might be related to the fact that mononuclear cells from non-mammalian species lack a specific LPS binding protein (Roeder *et al.*, 1989).

It was observed that PBL from *O. mykiss* were stimulated less by Con A than by LPS, similar to the results obtained by Etlinger *et al.*, (1976). However, studies by Chilmonczyk (1978) on *O. mykiss* PBL stimulation by mitogens *in vitro*, demonstrated the dose range of optimal concentrations to be broader for PHA (1-50μgml^{-1}) than Con A (1-10μgml^{-1}), and stimulation with LPS was only obtained at a dose of 1μgml^{-1}. These variations are probably due to differences in the experimental conditions.

The lower cell proliferation obtained with Con A compared with LPS may be explained by the fact that Con A is known to activate T cell suppressor activity. Thus, demonstrating that the degree of lymphocyte activation can also be influenced by cell regulators present in the

culture (Stites, 1987). Several reports have described interactions between serum component(s) and mitogen responding cells resulting in either suppression or activation of lymphocytes (Etlinger *et al.*, 1976), by altering the specificity of a selective mitogen.

Typical dose-response and kinetic experiments in the past have involved collating cultures of lymphocytes from several fish, due to limitations in the yields of cells from any single fish. However, data presented in these studies for each set of test conditions are derived from the cells of a single fish. The responses are representative of the activity found from several repetitions of the same experiment, although on occasion lymphocytes from an individual fish failed to respond or responded very poorly (results not shown).

This variation in the level of stimulation found amongst fish may be in part caused by individual differences between fish and variation within fish species. Reduced sensitivity maybe species specific, since the efficacy of the different mitogens varies amongst the different fish species (Sahai, 1981; Reitan and Thuvander, 1991; Hamers, 1994).

The results of this study demonstrate that supernatants of lymphocytes from *O. mykiss* stimulated with mitogens release a soluble factor(s) capable of activating trout macrophages *in vitro*, as shown by their ability to produce respiratory burst activity measured by the reduction of NBT (Section 3.3.2). These observations are in accordance with activities shown both *in vivo* and *in vitro*, in response to T-lymphocyte mitogens (Smith and Braun-Nesje, 1982; Graham and Secombes, 1990b) and antigens such as *A. salmonicida* (Francis and Ellis, 1994; Marsden *et al.*, 1994). Also confirming findings of Graham and Secombes (1988), who showed that stimulation of MAF release was significantly higher when PMA was used as a co-stimulant with Con A, similar to that seen for mammalian MAF production.

The NBT time-course assay assessed the capacity of cell-free supernatants derived from lymphocytes stimulated for different times (0-80 h), to initiate respiratory burst in *O. mykiss* macrophages. The assay demonstrates that optimal macrophage-activating activity of the supernatants was obtained with cultures taken from lymphocytes 48 h after stimulation with PMA/Con A. On the other hand, CFS derived from lymphocytes stimulated with mitogen for less than or more than 48 h induced a lower respiratory burst activity in macrophages. Results

from the present study are in accordance with studies by Graham and Secombes (1988), who have shown that supernatants from lymphocytes incubated for 48 h had consistently increased levels of activity compared to those from 24 h cultures. In addition, the optimum dilution of 1:10 is similar to 1: 8 obtained by Graham and Secombes (1988), using 2-fold dilutions of supernatants.

An inhibitory effect on macrophage activity has also been seen with leukocyte supernatants containing MAF when used at sub-optimal concentrations (Graham and Secombes, 1990a). These supernatants probably contained less MAF activity than the 48 h supernatants, since they were less stimulatory to head kidney macrophages. Little difference was observed in responsiveness to MAF using supernatants from >48 h stimulated lymphocytes compared with the 48 h cultures. At high concentrations of lymphokine-containing supernatants, a suppressive effect on NBT reduction was sometimes observed (Graham and Secombes, 1988). This could be due to a variety of factors being present in the supernatant, some of which are suppressive.

The NBT reduction assay has been widely accepted as the traditional method of assessing respiratory burst activity in both mammalian and fish macrophages. However, from personal observations during the course of these investigations, the assay has proved highly variable and difficult to repeat. This lack of consistency led to efforts of seeking an alternative method for assessing macrophage activity.

Luminol dependent chemiluminescence analysis is a reliable and efficient method of measuring respiratory burst with advantages over liquid scintilation counting (LSC) measurements. However, reliability of chemiluminescence as a measure of macrophage respiratory burst depends mainly on the preparation and maintenance of cell suspensions. Thus, a number of factors which may influence chemiluminescence output were investigated, including the composition of the medium, particularly the presence of phenol red indicator and serum.

The presence of erythrocytes or haemoglobin is an important factor shown to influence chemiluminescence (Nelson *et al.*, 1977; Anderson and Brendzel, 1978; Anderson and Amirault, 1979; Easmon *et al.*, 1980) and can be a major limitation. Isolation of phagocytically

active cells from the peripheral blood on a Percoll gradient can produce an erythrocyte-free preparation (Scott and Klesius, 1981). In the present study macrophages were isolated from the head kidney to reduce any effects of contaminating RBC.

When assessing Luminol dependent chemiluminescence, a critical feature of the medium is the presence of phenol red indicator, which markedly diminishes the response as observed by Nelson *et al.*, (1977) and Easmon *et al.*, (1980). As with erythrocytes, phenol red indicator consistently suppresses the light detected when using Luminol due to the overlap of its absorption spectrum with a significant portion of the chemiluminescence emission spectrum.

The presence of serum in the medium was assessed for its effect on Luminol dependent chemiluminescence. The results demonstrated the addition of protein to the system increased chemiluminescence output, although this response was minimal at the lower concentration of serum (1%). This may possibly be due to the presence of components within the serum which are cross reactive with the macrophages, leading to a direct increase in their activation. Studies by Easmon *et al.* (1980), when using human PBLs (5×10^5) with opsonized zymosan demonstrated the presence of FCS influenced the chemiluminescence output. However, their observations showed a reduction in chemiluminescence from 18 to 12 (mV) over FCS concentrations ranging from 0.3% - 6%.

The effect of cell viability was also assessed in the present study (results not shown). A reduction in total chemiluminescence output by a third was observed, when using macrophages incubated overnight at +4°C in HBSS compared to fresh macrophages. These findings are in accordance with studies by Easmon *et al.*, (1980) where a loss of 40% cell viability and 30% reduction in chemiluminescence was observed, when using cell suspensions (human PBLs in HBSS) maintained in balanced solutions after 4 h.

Chemiluminescent responses from *O. mykiss* head kidney macrophages to CFS from lymphocytes stimulated with various mitogens were recorded. The results indicate a range in ability of lymphocytes to initiate a chemiluminescent response, and hence activate macrophages. Different kinetic profiles were obtained using CFS from lymphocytes stimulated with PMA/Con A, PMA alone and LPS. The optimum concentration and magnitude of the

chemiluminescent response being characteristic of the specific mitogen used to produce MAF. For example, the chemiluminescent response of *O. mykiss* head kidney macrophages to CFS from PBL stimulated with PMA ($1\mu gml^{-1}$) produced a plateau effect maintained throughout the assay, which may be due to residual PMA stimulating macrophages and the target cells becoming saturated and unable to respond further. However, a bell-shaped curve was observed when using CFS from PMA/Con A co-stimulated lymphocytes to initiate a chemiluminescent response. A very rapid and intense response which was not sustained but fell rapidly was apparent. In comparison CFS from LPS ($10\mu gml^{-1}$) stimulated lymphocytes, produced a more rapid response which reached a peak that gradually declined.

The magnitude and the rate of chemiluminescence produced from *O. mykiss* cells are dependent on a number of factors. The respective kinetic profiles obtained from the various chemiluminescent responses are probably correlated to the amount of MAF present in the lymphocyte supernatants.

When using LPS as a direct stimulator of respiratory burst in *O. mykiss* macrophages, an immediate rapid chemiluminescent response was produced, which decayed down to background level (results not shown). At lower concentrations, the initial magnitude of the response was reduced, although the time course of the reaction remained unaltered. These results are comparable to decay curves observed by Lawrence *et al.*, (1982), when examining the Luminol-dependent respiratory burst of human neutrophils. Although LPS like PMA can prime macrophages for an enhanced respiratory burst (Pabst and Johnston, 1980; Drath, 1986; Kaku *et al.*, 1983), low concentrations of LPS have been reported to suppress the enhanced respiratory burst by IFN-γ primed murine peritoneal macrophages (Ding and Nathan, 1987).

The kinetic profile of the macrophage responses produced with CFS from stimulated lymphocytes differed in comparison to the chemiluminescent response attained with PMA or LPS as direct stimulators of respiratory burst. With the latter, a rapid response initiated within the first few minutes produced a significant peak, whereas the chemiluminescent responses achieved with CFS from stimulated PBL gave a delayed and more gradual response sustained

over a longer period of time, as expected of a biological response. Thus, confirming the responses are not due to a sudden exposure to the Luminol substrate but a genuine response as a result of biological stimulation.

Interestingly, although LPS produced the highest lymphocyte stimulation of PBLs in earlier studies (Section 3.3.1), the corresponding CFS from these stimulated lymphocytes did not however result in the greatest activation of macrophages. Instead, highest chemiluminescent response was attained using CFS from PBLs co-stimulated with PMA/Con A.

From the bioassay studies, it was established that *O. mykiss* lymphocytes cultured in the presence of mitogens were sufficiently stimulated to produce factors capable of activating macrophages as demonstrated by their respiratory burst activity. In addition, chemiluminescence measurement using Luminol has been shown to be a more reliable assay in comparison to the traditional NBT assay, in the assessment of respiratory burst by trout head kidney macrophages.

Despite finding that Con A/PMA gave the highest production of MAF, it was decided to stimulate lymphocytes with all the mitogens tested to allow a greater possibility of isolating cytokine genes. Having achieved a reproducible source of MAF, RNA from these stimulated lymphocytes was subsequently used as source material for both PCR amplification and construction of a cDNA expression library in the molecular studies performed in subsequent chapters.

CHAPTER 4

PCR amplification approach for the identification of cytokine cDNAs/genes

CHAPTER 4

PCR amplification approach for the identification of cytokine cDNAs/genes

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4.1 Introduction

Research on fish cytokine activity has focused primarily on Rainbow trout (*O.mykiss*) with the majority of cytokines being identified in biological assays on the basis of their functional similarity to mammalian cytokines. Many phagocyte functions are modulated by cytokines but although cytokine-like biological activity has been detected in fish it is only recently that molecular techniques have been used to identify some of the cytokines themselves. Progress in the cloning of fish cytokine genes, their receptors and several homologues of chemokines has been made but only a few such genes have been described to date (Table 4.1).

In recent years several fish cytokine genes have been cloned amongst others including those molecules encoding the homologue of mammalian IL-1β (Zou *et al.*, 1999a,b; Scapigliati *et al.*, 2001), TNF-α (Hirono *et al.*, 2000; Laing *et al.*, 2001a) and TGF-β (Hardie *et al.*,1998, Laing *et al.*, 2000). Several homologues of chemokines, including IL-8 and γ-IFN inducible protein (γ-IP) have also been identified (Laing *et al.*, 2002).

A number of molecular based approaches have been adopted in the search to identify fish cytokine genes; PCR amplification, screening with homologous probes and EST analysis. This chapter will focus on the PCR approach to cloning of fish cytokine cDNAs. Nucleotide or protein sequences conserved over a range of species can be used in the design of common primers to amplify a particular gene fragment by PCR, and thereby allow rapid cloning of known genes from different species. PCR amplification using degenerate primers based on homologous sequences has proved successful in the cloning of cytokines cDNAs in the trout.

For example, IL-1β cDNA has been amplified from a number of different fish species using degenerate primers, based on evolutionarily conserved regions of the molecule, to amplify the cDNA from cells likely to secrete IL-1β. These include the IL-1β from Rainbow trout, the first non-mammalian IL-β sequence to be cloned, (Zou *et al.*, 1999a,b), carp (Fujiki *et al.*, 2000) and seabass (Scapigliati *et al.*, 2001). Similarly, a trout TGF-β sequence was obtained using degenerate primers based on conserved motifs within the mature peptide of the different mammalian TGF-βs (Laing *et al.*, 1999a). Degenerate primers based on TGF-β genes, have also

been used in a PCR to probe plaice genomic DNA for TGF-β (Laing *et al.*, 2000). A number of different isoforms of TGF-β have been isolated in different fish species (Sumathy *et al.*, 1997; Hardie *et al.*, 1998; Daniels *et al.*, 1997 and Harms *et al.*, 1997), including TGFβ3 in trout, sturgeon and eel using PCR (Laing *et al.*, 1999a).

Once a cytokine has been cloned in one species of fish PCR primers based on that sequence have been successfully used to isolate the corresponding sequence from other fish species. For example, PCR with primers specific for the recently published TNFα sequence of Japanese flounder (Hirono *et al.*, 2000), were used to isolate full length cDNA sequence of trout TNFα from a PHA-stimulated leucocyte cDNA library. In this study, a PCR approach was used in an attempt to isolate the Rainbow trout (*O. mykiss*) cDNAs corresponding to a number of mammalian cytokines, with particular emphasis on those corresponding to IFN-γ, IL-15 and IL-18. RNA isolated from activated lymphocytes (Chapter 3) was used as the template for cDNA production since in mammals the majority of cytokines are not expected to be expressed constitutively. Initial work concentrated on attempting to amplify a cDNA corresponding to IFN-γ as the molecule most likely to be the source of the MAF activity produced by the activated lymphocytes. IFN-γ cDNAs have been isolated from a number of species and therefore oligonucleotides corresponding to regions of conserved sequence between these species were used as primers for the PCR. Similarly primers based on regions of the IL-15 and IL-18 molecules conserved across a number of species were also used in PCR reactions. IL-18 is a potent inducer of IFN-γ production by T cells (Okamura *et al.*, 1995; Micallef *et al.*, 1996). It is also known as IFN-γ inducing factor and is a pro-inflammatory cytokine, closely related to IL-1 (Dinarello, 1999) with profound effects on T-cell activation. IL-15 is a cytokine with biological activities similar to IL-2 and has been shown to activate PBLs and stimulate the proliferation of T-cells (Grabstein *et al.*, 1994; Giri *et al.*, 1994, 1995a,b).

Table 4.1 *O. mykiss* cytokine sequences identified to date and comparisons with known mammalian, vertebrate and fish cytokines.

Cytokine	%Amino Acid homology	% Nucleotide homology	Species homology	Accession number
IL-1β (AJ004821)	49 - 56	43 - 49	Mammalian IL-1β (260 aa translated molecule)	*
	42 - 45		Mammalian IL-1α	*
	71	70	Plaice (237 bp partial sequence)	AJ010640
	57	49	Carp	AB010701
	59 - 62	58	Seabass (nt homology to 168-234 partial sequence)	AJ269472
TGF-β_1 (X99303)	68		Human β_1	BC022242
	53		Human β_2	BC011170
	59		Human β_3	NM003239
	62		Chicken β_4 (specific to birds)	M31160
	62		Xenopus β_5 (specific to amphibians)	J05180
TNF-α (AJ277604)	38-41		Mammalian TNFα	*
	35-39		Mammalian TNFβ	*
IL-8 (AJ279069)	35	50	Human	NM000584
	38		Chicken isoform K60 peptide	NM205018
	56	66	Flounder	AF216646
	31	47	Lamprey (CXC chemokine)	AJ231072
	17	35	Zebra fish (SCYBA peptide)	AF279919
	18	47	Rainbow trout CK-1 (CC chemokine)	AF093802
CK-1 (AF093802)	33-36		Mammalian C6-β chemokine	*
Chemokine Receptors (AJ003159)	81	67	Human CXC-R4	AF005058
	59	56	Human CC-R7	NM001838

Chemokine, their receptors and other related molecules are also presented. * Accession numbers for mammalian cytokine sequences are absent, as results are expressed as a range and are compiled from a number of different mammalian species.

4.2 Materials and Methods

RT-PCR experiments with degenerate primers designed and based on a range of mammalian and vertebrate cytokines, were performed with RNA extracted from mitogen (PMA/Con A) stimulated head kidney cells and peripheral blood lymphocytes of *O. mykiss*.

4.2.1 Isolation of total RNA and conversion to cDNA

Total RNA was isolated from either activated lymphocytes or kidney and spleen tissues of individual fish using guanidinium thiocyanate - phenol/chloroform extraction method (Section 2.17).

PolyA$^+$ RNA was purified from total cellular RNA, using an oligotex ™ mRNA kit (Qiagen) as per manufacturers instructions (Section 2.19). The cDNA was synthesised from 1μg total RNA or 100ng mRNA (polyA$^+$ RNA) using AMV reverse transcriptase (Roche), at 42°C for 1.5 hrs with oligo (dT) or random hexamer primers (Life Technologies) and used as template for PCR.

4.2.2 Design of PCR primers

IFN-γ homologues from 21 species were aligned at both the amino acid and nucleotide level using Clustal W (Version 1.74), to highlight conserved regions of sequence. In addition, a codon usage table was constructed to check for bias towards particular codons found in salmon sequences. The table was created from 16 salmon gene sequences from a GenBank database search of approximately 300 sequences (Appendix 4.1). The multiple alignments were used to determine which regions of the IFN-γ sequence would be used to design the degenerate PCR primers and the codon frequency table was used to ensure that any bias present in salmonid DNA was reflected in the primer sequences. Similar approaches were employed for the design of both the IL-15 and IL-18 cytokine primers. To verify that the RT-PCR was working primers specific for β-actin and aldolase B cDNA were used. The trout sequences for these molecules were not available and so the primers were based on the corresponding salmon sequences.

4.2.3 PCR amplification and cloning of PCR products using pGEMT-Easy Vector

PCR amplification was performed using the cDNA prepared in Section 4.2.1 as template and the various sets of degenerate forward (F) and reverse (R) primers (Table 4.2) designed against conserved regions of known mammalian cytokine sequences (Section 2.24).

PCR reactions were conducted using a thermal cycler MultiBlock System (Hybaid), following cycling protocols specific for each set of primers (Table 4.3.). Resultant PCR products were visualised on 2% (w/v) agarose gels containing ethidium bromide ($100ngml^{-1}$) using 100 bp ladder (Promega) and 1kb DNA ladder (MBI) as size markers. Amplified PCR products were ligated into the linearized PCR cloning vector pGEMT-Easy (Section 2.25.1) using T4 DNA Ligase. Following transformation of competent JM109 *E. coli* cells, recombinants were identified using blue/white colour selection on ampicillin /IPTG/X-Gal plates (Section 2.25.2). DNA clones were digested with *Eco* RI and/or *Not* I and were analysed on a 2% metaphor® agarose gel.

4.2.4 Sequence analysis

Plasmid DNA from several independent clones was recovered using a QIAprep spin Miniprep kit (Qiagen). Sequence reactions were performed on double stranded plasmid DNA (25-$500ng/\mu g^{-1}$) with T7 and SP6 vector specific primers and run on an ABI 377 fluorescent DNA automated sequencer (PE Biosystems) using "Big-Dye" Terminator chemistry (courtesy of the Functional Genomics Unit).

The nucleotide and amino acid sequence data generated were compared with entries in the GenBank and Swissprot databases respectively, using the FASTA (Pearson and Lipman, 1988) and BLAST (Altschul *et al.*, 1990) alignment search programmes. Results were presented as percentage identity to sequences in the databases.

Direct comparison between the trout cDNA sequences and potential homologous sequences from the databases were also performed using the GAP alignment program within the Wisconsin Genetics Computer Group Sequence Analysis Software Package (Version 10.0).

Table 4.2 Degenerate oligonucleotide primer against multiple alignments used for the amplification of the following *O. mykiss* cytokine genes: IFN-γ, IL-15, IL-18.

Primer: Forward (F) Reverse (R)	5′→3′ Sequence	T_{Ann} (°C)	Length (nt)
IFN-γ (F)	CARATXGTYTCYTTYTAC	48.0	18
IFN-γ (R)	CTGRCTYCTYTTCCGCTT	54.0	18
IL-15 (F)	CCWAARACAGARGCMAA	49.2	17
IL-15 (R)	TCYTCACAYTCYTTGCA	49.2	17
IL-18 (F)	ARTRACATCATMTTYTT	40.7	17
IL-18 (R)	TTYTTYAAAATKARTTT	35.9	17
*B-actin (F)	CACTGGTTGTTGACAACGGA	57.3	20
*B-actin (R)	GATCTTCATCAGGTAGTCTG	55.3	20
*Aldolase B (F)	AGACGCTGTACCAAATGTCG	57.3	20
*Aldolase B (R)	AGACATGGTGGTCGTTCAGA	57.3	20

Wherever possible, primer pairs were designed to have similar T_{Ann} values. These were estimated using the following formula:

T_{Ann} = 2°C x (no. of A and T residues) + 4°C x (no. of G and C residues)

In some instances, optimal annealing temperatures may differ slightly from calculated values.

*Control PCR primers derived from *Salmo salar* (Atlantic salmon) β-actin mRNA and aldolase B mRNA sequences were used for the amplification of house keeping genes.

Nucleotide ambiguity codes include:

K	=	G/T	W	=	A/T
M	=	A/C	X	=	A/C/G/T
R	=	A/G	Y	=	C/T

Table 4.3 Gradient and standard PCR cycling protocols for IFN-γ, IL-15, IL-18 cytokine primers and control β-actin and aldolase B primers.

	Number of cycles	PCR conditions			
		Gradient		Standard	
IFN-γ					
	1	95 °C	5 min	95 °C	5 min
	36	95 °C	30 s	95 °C	30 s
		37-52	30 s	45 °C	30 s
		72 °C	1 min	72 °C	1 min
	1	72 °C	5 min	72 °C	5 min
IL-15 / IL-18					
	1	95 °C	5 min		
	36	95 °C	30 s		
		35-50	30 s		
		72 °C	30 s		
	1	72 °C	5 min		
Aldolase B					
	1	95 °C	5 min		
	36	95°C	30 s		
		43 -58	30 s		
		72 °C	30 s		
	1	72 °C	5 min		
β-actin					
	1	95 °C	5 min		
	30	95 °C	30 s		
		43-58	30 s		
		72 °C	30 s		
	1	72 °C	5 min		

4.3 Results

4.3.1 Control PCR primers

In order to verify that the RT-PCR was effective on each occasion, it was necessary to include a positive control. This normally consists of including primers corresponding to a specific mRNA considered to be expressed universally in each cell. To find such 'house-keeping' genes as positive controls for the PCR amplification, EMBL and GenBank database searches were performed for all trout (*O.mykiss*) and salmon (*S.salar*) sequences, using the LOOKUP / FASTA programme (GCG, Version 10). Two universally abundant proteins were selected: *Salmo salar* β actin mRNA, a cytoskeleton protein, and aldolase B mRNA, an enzyme involved in glycolysis. The β-actin control primers selected were based on an internal region of the complete salmon β-actin cDNA sequence (accession number AF012125), such that a fragment of approximately 550 bp would be amplified from the trout cDNA. Similarly, the aldolase B primers were designed from the salmon aldolase B cDNA sequence (accession number AF067796), such that a PCR product of approximately 420 bp would be expected.

PCR amplification conditions for both salmon β-actin and aldolase B were optimised by varying annealing temperatures (T_{Ann}) and $MgCl_2$ concentrations, using standard PCR cycling protocols (Table 4.3). Amplified PCR fragments of the sizes expected for both β-actin (550bp) and aldolase B (400bp) were obtained from the cDNA derived from both kidney and spleen (Figure 4.1). The strongest amplification was achieved using an annealing temperature of 52°C.

4.3.2 Cloning and subsequent sequence analysis of β-actin PCR products

Amplified PCR products generated using the β-actin primers were cloned into the PCR cloning vector (pGEM-TEasy) and plasmid DNA prepared. Purified plasmid DNA from two independent clones was digested with *Eco*R1 restriction enzyme to verify that the ~550 bp fragment had been cloned (Figure 4.2). Two fragments which correspond to the vector (~ 3000 bp) and a 575 bp insert are highlighted.

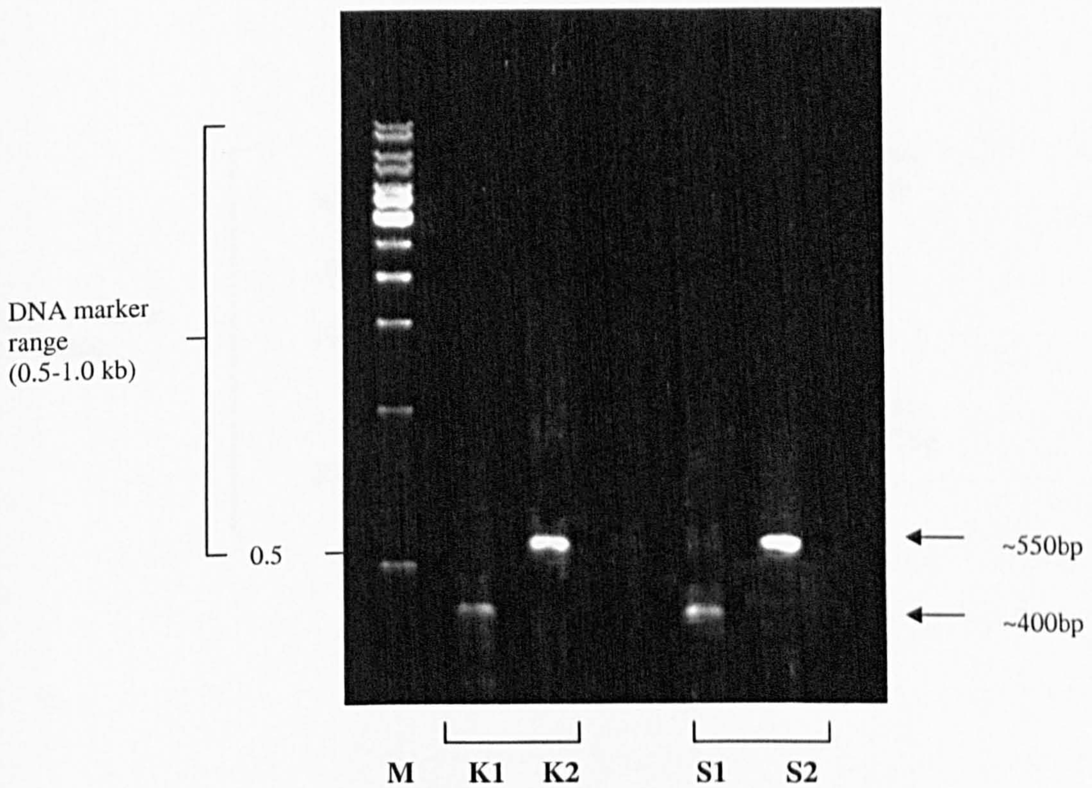

Figure 4.1 PCR amplification products of rainbow trout kidney and spleen cDNA with primers designed against salmon aldolase B and β-actin.

Amplification products of approximately 400 bp and 550 bp which correlate with aldolase B and β-actin are shown using rainbow trout kidney cDNA (K1, K2) and spleen cDNA (S1, S2). A 1Kb DNA ladder (MBI) with fragments ranging from 0.5-10 kb was used as a DNA marker (M).

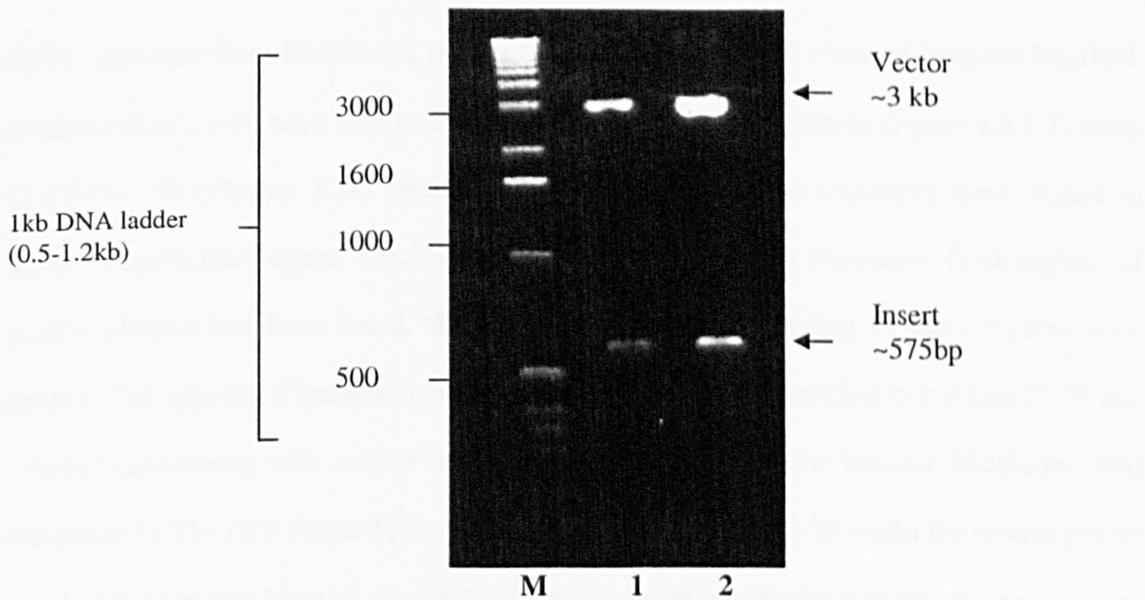

Figure 4.2 Restriction enzyme digestion of cloned rainbow trout β-actin DNA with *Eco*RI

Purified β-actin DNA: clone **#1**, clone **#2** digested with *Eco*R1 and electrophoresed on a 1% agarose gel. Two fragments which correspond to the vector (~ 3000 bp) and a 575 bp purified β-actin insert are presented. The insert size is calculated as follows: 557 + 18 bp (Two *Eco*R1 flanking insertion sites at positions 70 and 52 within the multiple cloning site of pGEMT-Easy vector). A 1kb DNA ladder (500-1200 bp) was included as a DNA marker (**M**).

The sequence generated from the cloned PCR amplification product was used to search the GenBank nucleotide database and was found to correspond to β-actin cDNA. A pairwise alignment of the nucleotide sequence with the salmon β-actin cDNA sequence is presented in Figure 4.3. The trout sequence was 557 bp in length and was found to be 98 % identical to the salmon β-actin cDNA sequence, with only the occasional single base variation/difference between the two fish sequences. A pairwise comparison with the human β-actin sequence demonstrated that the trout and human sequences were 87% identical.

4.3.3 The design of IFN-γ degenerate primers

IFN-γ sequences from 18 different species (mammalian and avian) obtained from the GenBank database (Table 4.4), were used to create a multiple sequence alignment (Figure 4.4-4.7) using CLUSTAL W (Version 1.74). Initially the predicted amino acid sequences were aligned to check for particular regions which were conserved across all the sequences. Once regions of protein identity had been found, the DNA sequences corresponding to these regions were aligned. Two regions of conserved amino acids were found corresponding to residues 73-78 and 156-161 (numbering with respect to the human sequence, with the initiator Methionine (M) numbered 1). The PCR forward primer was derived from region 73-78 whilst the reverse primer was based on region 156-161. Ambiguities were included in the primer sequences according to the nucleotide alignments or if the codon usage table suggested a particular bias (Figure 4.8). If the trout sequence was similar to the sequences used to design the primers a fragment of approximately 260 bp would be amplified.

Initial PCR reactions using cDNA from mitogen stimulated lymphocytes and degenerate primers designed against IFN-γ sequences produced an amplification product of approximately 280 bp. The PCR product was amplified using a gradient PCR protocol (Table 4.3) with a range of annealing temperatures (37-52°C). Although, a gradient PCR was used to optimise conditions for the amplification of a PCR product, subsequent PCR conditions used a

standard PCR protocol with an annealing temperature of 45°C to verify that a fragment of ~280 bp was reproducibly produced.

```
                  *        20         *        40         *
Bactin-PCR : CACTGGTTGTTGACAACGGATCCGGTATGTGCAAAGCCGGATTCGCCGGAGATGAC :  56
Bactin-tro : CACTGGTTGTTGACAACGGATCCGGTATGTGCAAAGCCGGATTCGCCGGAGATGAC :  56
Bactin-sal : CACTGGTTGTTGACAACGGATCCGGTATGTGCAAAGCCGGGTTCGCTGGAGATGAC :  56

              60         *        80         *       100         *
Bactin-PCR : GCGCCTCGGGCTGTCTTCCCCTCCATCGTCGGGCGTCCCAGGCATCAGGGAGTGAT : 112
Bactin-tro : GCGCCTCGGGCTGTCTTCCCCTCCATCGTCGGGCGTCCCAGGCATCAGGGAGTGAT : 112
Bactin-sal : GCGCCTCGGGCTGTCTTCCCCTCCATCGTCGGTCGTCCCAGGCATCAGGGAGTGAT : 112

                  120         *       140         *       160
Bactin-PCR : GGTTGGATGGGCCAGAAAGACAGCTACGTGGGAGACGAGGCTCAGAGCAAGAGGG : 168
Bactin-tro : GGTTGGATGGGCCAGAAAGACAGCTACGTGGGAGACGAGGCTCAGAGCAAGAGGG : 168
Bactin-sal : GGTTGGATGGGCCAGAAGGACAGCTACGTTGGAGACGAGGCTCAGAGCAAGAGGG : 168

              *       180         *       200         *       220
Bactin-PCR : GCATCCTGACCCTGAAGTACCCCATTGAGCATGGCATCGTCACCAACTGGGACGAC : 224
Bactin-tro : GCATCCTGACCCTGAAGTACCCCATTGAGCATGGCATCGTCACCAACTGGGACGAC : 224
Bactin-sal : GTATCCTGACTCTGAAGTACCCCATCGAGCACGGCATCGTCACCAACTGGGACGAC : 224

                  *       240         *       260         *       280
Bactin-PCR : ATGGAGAAGATCTGGCATCACACCTTCTACAACGAGCTGAGGGTGGCTCCAGAGGA : 280
Bactin-tro : ATGGAGAAGATCTGGCATCACACCTTCTACAACGAGCTGAGGGTGGCTCCAGAGGA : 280
Bactin-sal : ATGGAGAAGATCTGGCATCACACCTTCTACAACGAGCTGAGAGTGGCACCAGAGGA : 280

                  *       300         *       320         *
Bactin-PCR : GCACCCCGTCCTGCTCACAGAGGCCCCCCTCAACCCCAAAGCCAACAGGGAGAAGA : 336
Bactin-tro : GCACCCCGTCCTGCTCACAGAGGCCCCCCTCAACCCCAAAGCCAACAGGGAGAAGA : 336
Bactin-sal : GCACCCAGTCCTGCTCACTGAGGCCCCACTCAACCCCAAAGCCAACAGGGAGAAGA : 336

              340         *       360         *       380         *
Bactin-PCR : TGACCCAGATCATGTTTGAGACCTTCAACACCCCTGCCATGTACGTGGCCATCCAG : 392
Bactin-tro : TGACCCAGATCATGTTTGAGACCTTCAACACCCCTGCCATGTACGTGGCCATCCAG : 392
Bactin-sal : TGACCCAGATCATGTTTGAGACCTTCAACACCCCCGCCATGTACGTGGCCATCCAG : 392

                  400         *       420         *       440
Bactin-PCR : GCCGTGTTGTCCCTGTACGCCTCTGGCCGTACCACCGGTATCGTCATGGACTCCGG : 448
Bactin-tro : GCCGTGTTGTCCCTGTACGCCTCTGGCCGTACCACCGGTATCGTCATGGACTCCGG : 448
Bactin-sal : GCAGTGTTGTCCCTGTACGCCTCTGGCCGTACCACCGGTATCGTCATGGACTCCGG : 448

              *       460         *       480         *       500
Bactin-PCR : TGACGGCGTGACCCACACAGTACCCATCTACGAGGGCTACGCTCTGCCCCACGCCA : 504
Bactin-tro : TGACGGCGTGACCCACACAGTACCCATCTACGAGGGCTACGCTCTGCCCCACGCCA : 504
Bactin-sal : TGACGGCGTGACCCACACAGTACCCATCTACGAGGGTTACGCTCTGCCCCACGCCA : 504

                  *       520         *       540         *
Bactin-PCR : TCCTGCGTCTGGATCTGGCCGGCCGCGACCTCACAGACTACCTGATGAAGATC : 557
Bactin-tro : TCCTGCGTCTGGATCTGGCCGGCCGCGACCTCACAGACTACCTGATGAAGATC : 557
Bactin-sal : TCCTGCGTCTTGATCTGGCCGGACGCGACCTCACAGACTACCTGATGAAGATC : 557
```

Figure 4.3 Nucleotide sequence alignment of trout and salmon β-actin sequences.

The actin sequence derived from PCR amplification of trout cDNA using salmon based actin primers, is compared with the rainbow trout and salmon β-actin (Accession numbers: AJ438158 and AF012125). The PCR derived sequence demonstrates 100% identity with the rainbow trout sequence and 98% identity with the salmon sequence over a stretch of 557bp sequence.

Table 4.4 Mammalian and avian mRNA sequences employed in the design of degenerate, cytokine primers for RT-PCR.

Common Name	Latin Name	Accession Number		
		IFN-γ	IL-15	IL-18
Cat	*Felis catus*	D30619	AF108148 (precursor)	AB046211
Cattle	*Bos taurus*	M29867	Q28028 (precursor)	AF124789 (precursor)
Deer (Red)	*Cervus elaphus*	L07502		
Dog	*Canis familiaris*	AF126247		Y11133
Dolphin (marine)	*Tursiops truncatus*	AB022044		
Horse	*Equus caballus*	D28520		Y11131
Human	*Homo sapiens*	M29383	NM000585 (partial cds)	
Mouse	*Mus musculus*	XM125899	NM008357 (partial cds)	
Pig	*Sus scrofa*		Q95253 (protein)	AF191088
Rat	*Rattus norvegicus*		AF015719	
Woodchuck	*Marmota monax*	Y14138		
Sheep	*Ovis aries*	X52640	Q9XSJ6 (protein)	AJ401033 (gene)
African green monkey	*Cercopithecus aethiops*		NM008357	
Mangabey (red-crowned)	*Cercocebus torquatus*	L26025		
Macaque (crab-eating)	*Macaca fascicularis*	D89985	AB000555 (precursor)	
Macaque (rhesus monkey)	*Macaca mulatta*	L26024	U19843	AF303732
Macaque (pig-tailed)	*Macaca nemestrina*	L26026		
Chicken	*Gallus gallus*	AF424744	AF139097	AJ277865 (gene)
Duck	*Anas platyrhynchos*	AJ012254		AF336122 (partial cds)
Guinea fowl	*Numida meleagris*	AJ001263		
Japanese quail	*Coturnix japonica*	AJ001678 (gene)		

Consensus	I	I	Q	S	Q	I	V	S	F	Y	
Cat	I	I	Q	S	Q	I	V	S	F	Y	
Cattle	I	I	Q	S	Q	I	V	S	F	Y	
Sheep	I	I	Q	S	Q	I	V	S	F	Y	
Deer	I	I	Q	S	Q	I	V	S	F	Y	
Dog	I	I	Q	S	Q	I	V	S	F	Y	
Dolphin	I	I	Q	S	Q	I	V	S	F	Y	
Horse	I	I	Q	S	Q	I	V	S	F	Y	
Human	I	M	Q	S	Q	I	V	S	F	Y	
Mangabey	I	M	Q	S	Q	I	V	S	F	Y	
Macaque (crab-eating)	I	M	Q	S	Q	I	V	S	F	Y	
Macaque (rhesus)	I	M	Q	S	Q	I	V	S	F	Y	
Macaque (pig-tailed)	I	M	Q	S	Q	I	V	S	F	Y	
Mouse	I	L	Q	S	Q	I	I	S	F	Y	
Woodchuck	V	I	Q	S	Q	V	V	S	F	Y	
Duck	I	I	L	S	Q	I	V	T	L	Y	
Guinea fowl	I	I	L	S	Q	I	V	S	M	Y	
Quail	I	I	L	S	Q	I	V	S	M	Y	
Chicken	I	I	L	S	Q	I	V	S	M	Y	
71				*	*					*	80

Figure 4.4 Multiple amino acid alignments of known mammalian and avian IFN-γ sequences.
Design of the forward IFN-γ primer was based on the stretch of highly conserved amino acids QIVSFY (residues 75 -80), showing highest identity. The consensus sequence is highlighted in bold and identical (*) residues, identified by the CLUSTAL programme are indicated. Residues non-identical to the consensus sequence are shaded.

Consensus	**S**	**N**	**L**	**R**	**K**	**R**	**K**	**R**	**S**	**Q**
Cat	S	N	L	R	K	R	K	R	S	Q
Cattle	S	N	L	R	K	R	K	R	S	Q
Sheep	S	S	L	R	K	R	K	R	S	Q
Deer	S	N	L	I	K	R	K	R	S	Q
Dog	S	N	L	R	K	R	K	R	S	Q
Dolphin	S	N	L	R	K	R	K	R	S	Q
Horse	A	N	L	R	K	R	K	R	S	Q
Human	A	K	T	G	K	R	K	R	S	Q
Mangabey	A	K	I	G	K	R	K	R	S	Q
Macaque (crab-eating)	A	K	I	G	K	R	K	R	S	Q
Macaque (rhesus)	A	K	I	G	K	R	K	R	S	Q
Macaque (pig-tailed)	A	K	I	G	K	R	K	R	S	Q
Mouse	S	S	L	R	K	R	K	R	S	R
Woodchuck	S	S	L	R	K	R	K	R	S	Q
Duck	S	S	S	-	K	R	K	R	S	Q
Guinea fowl	P	P	L	-	K	R	K	R	N	Q
Quail	P	P	S	-	K	R	K	R	S	Q
Chicken	P	P	F	-	K	R	K	R	S	Q
152					*	*	*	*		161

Figure 4.5 Multiple amino acid alignments of known mammalian and avian IFN-γ sequences.
Design of the IFN-γ reverse primer was based on the stretch of highly conserved amino acids KRKRSQ (residues 156 - 161) showing highest identity. The consensus sequence is highlighted in bold and identical (*) residues, identified by the CLUSTAL programme are indicated. Residues non-identical to the consensus sequence are shaded.

Consensus	**A T T**	**C A G**	**A G C**	**C A A**	**A T T**	**G T C**	**T C C**	**T T C**	**T A C**	**T T C**
Cat	A T T	C A A	A G C	C A A	A T T	G T C	T C C	T T C	T A C	C T G
Cattle	A T T	C A G	A G C	C A A	A T T	G T C	T C C	T T C	T A C	T T C
Sheep	A T T	C A G	A G C	C A A	A T T	G T C	T C C	T T C	T A C	T T C
Deer	A T T	C A G	A G C	C A A	A T T	G T C	T C C	T T C	T A C	T T C
Dog	A T T	C A G	A G C	C A A	A T T	G T C	T C T	T T C	T A C	T T G
Dolphin	A T T	C A G	A G C	C A A	A T A	G T C	T C C	T T C	T A C	T T C
Horse	A T T	C A G	A G C	C A A	A T C	G T C	T C C	T T C	T A C	T T C
Human	A T G	C A G	A G C	C A A	A T T	G T C	T C C	T T T	T A C	T T C
Mangabey	A T G	C A G	A G C	C A A	A T T	G T C	T C C	T T T	T A C	T T C
Macaque (crab-eating)	A T G	C A G	A G C	C A A	A T T	G T C	T C C	T T T	T A C	T T C
Macaque (rhesus)	A T G	C A G	A G C	C A A	A T T	G T C	T C C	T T T	T A C	T T C
Macaque (pig-tailed)	A T G	C A G	A G C	C A A	A T T	G T C	T C C	T T T	T A C	T T C
Mouse	C T G	C A G	A G C	C A G	A T T	A T C	T C T	T T C	T A C	C T C
Woodchuck	A T C	C A G	A G C	C A A	G T T	G T C	T C T	T T C	T A C	T T C
Chicken	A T A	C T G	A G C	C A G	A T T	G T T	T C G	A T G	T A C	T T G
Duck	A T A	C T G	A G C	C A G	A T T	G T T	A C C	C T G	T A C	T T G
Guinea fowl	A T A	C T G	A G C	C A G	A T T	G T T	T C A	A T G	T A C	T T G
Japanese Quail	A T A	C T G	A G C	C A G	A T T	G T T	T C A	A T G	T A C	T T G
205 – 234		*	* * *	* *	*		*	*	* * *	*

Figure 4.6 Multiple nucleotide alignments of known mammalian and avian IFN-γ sequences.
Design of the forward IFN-γ primer was based on the following stretches of highly conserved sequence (nucleotides 214 - 231), showing highest identity. The consensus sequence is highlighted in bold and identical (*) nucleotides, identified by the CLUSTAL programme are indicated. Regions non-identical to the consensus sequence are shaded.

Consensus	**T C T**	**A A C**	**C T C**	**A G A**	**A A G**	**C G G**	**A A A**	**A G G**	**A G T**	**C A G**
Cat	T C T	A A C	C T G	A G G	A A G	C G G	A A A	A G G	A G C	C A G
Cattle	T C T	A A C	C T C	A G A	A A G	C G G	A A G	A G A	A G T	C A G
Sheep	T C T	A A C	C T C	A G A	A A G	C G G	A A G	A G A	A G T	C A G
Deer	T C T	A A C	C T C	A T A	A A G	C G G	A A G	A G A	A G T	C A G
Dog	T C C	A A C	C T A	A G G	A A G	C G G	A A A	A G G	A G T	C A G
Dolphin	T C T	A A T	C T C	A G A	A A G	C G G	A G G	A G A	A G T	C A G
Goat	T C T	A A C	C T C	A G A	A A G	C G G	A A G	A G A	A G T	C A G
Horse	G C T	A A C	C T G	A G G	A A G	C G G	A A G	A G G	A G T	C A G
Human	G C T	A A A	A C A	G G G	A A G	C G A	A A A	A G G	A G T	C A G
Mangabey	G C T	A A A	A T A	G G G	A A G	C G A	A A A	A G G	A G T	C A G
Macaque (crab-eating)	G C T	A A A	A T A	G G G	A A G	C G A	A A A	A G G	A G T	C A G
Macaque (rhesus)	G C T	A A A	A T A	G G G	A A G	C G A	A A A	A G G	A G T	C A G
Macaque (pig-tailed)	G C T	A A A	A T A	G G A	A A G	C G A	A A A	A G G	A G T	C A G
Mouse	T C C	A G C	C T C	A G G	A A G	C G G	A A A	A G G	A G T	C G -
Woodchuck	T C T	A C C	C T A	A G G	A A G	C G A	A A A	A G G	A G T	C A G
Chicken	T C C	G A G	T T T	C A A	A A G	- - G	A A A	A G G	A G C	C A G
Duck	T T C	G A C	T T C	C A A	A A G	- - G	A A A	A G G	A G C	C A G
Turkey	T C C	A A G	T T C	C A A	A A G	- - G	A A A	A G G	A G C	C A T
Guinea fowl	T C C	G A G	T C T	C A A	A A G	- - G	A A A	A G G	A A C	C A G
Pheasant	T C C	G A G	T T C	C A A	A A G	- - G	A A A	A G G	A G T	C A G
Quail	T C C	G A G	T T C	T A A	A A G	- - G	A A A	A G G	A G C	C A G
452					* * *		*	* *	*	* **483**

Figure 4.7 Multiple nucleotide alignments of known mammalian and avian IFN-γ sequences.
Design of the IFN-γ reverse primer was based on the following stretches of highly conserved sequence (nucleotide 464 - 483), showing highest identity. The consensus sequence is highlighted in bold and identical (*) nucleotides, identified by the CLUSTAL programme are indicated. Regions non-identical to the consensus sequence are shaded.

IFN-γ (1) Forward primer

Amino acid		**Q**	**I**	**V**	**S**	**F**	**Y**	
Nucleotide	5′	CAG	/ATG	/GTG	/AGG	/TTT	/TAC	3′
Primer sequence	5′	CAR	/ATX	/GTY	/TCY	/TTY	/TAC	3′

IFN-γ (1) Reverse primer

Amino acid		**K**	**R**	**K**	**R**	**S**	**Q**	
Nucleotide	5′	AAG	/AGG	/AAG	/AGG	/AGG	/CAG	3′
Antisense strand	3′	TTC	/GCC	/TTT	/TCT	/TCG	/GTC	5′
Primer sequence	5′	CTG	/RCT	/YCT	/YTT	/CCG	/CTT	3′

IL-15 Forward primer

Amino acid		**P**	**K**	**T**	**E**	**A**	**N**	
Nucleotide	5′	CCT	/AAA	/ACA	/GAA	/GCC	/AA	3′
Primer sequence	5′	CCW	/AAR	/ACA	/GAR	/GCM	/AA	3′

IL-15 Reverse primer

Amino acid		**C**	**K**	**E**	**C**	**E**	**E**	
Nucleotide	5′	TGC	/AAA	/GAA	/TGT	/GAG	/GAA	3′
Primer sequence	5′	TCY/	TCA	/CAY	/TCY	/TTG	/CA	3′

IL-18 Forward primer

Amino acid		**S**	**D/N**	**I**	**I**	**F**	**F**	
Nucleotide	5′	AAT	/GAC	/ATC	/ATA	/TTC	/TTT	3′
Primer sequence	5′	ART	/RAC	/ATC	/ATM	/TTY	/TT	3′

IL-18 Reverse primer

Amino acid		**K**	**L**	**I**	**L**	**K**	**K**	
Nucleotide	5′	AAA	/CTC	/ATT	/TTG	/AAA	/AAA	3′
Primer sequence	5′	TTY	/TTY	/AAA	/ATK	/ART	/TT	3′

Nucleotide ambiguity codes:

K	=	G/T	W	=	A/T
M	=	A/C	X	=	A/C/G/T
R	=	A/G	Y	=	C/T

Figure 4.8 Design of IFN-γ, IL-15 and IL-18 degenerate primers using both multiple sequence alignments of a range of species, and a codon usage frequency table specific for fish. Nucleotides selected from the codon frequency table are presented in bold whilst nucleotide ambiguity codes are underlined.

4.3.4 Cloning and subsequent sequence analysis of IFN-γ PCR products

The PCR products resulting from the use of the IFN-γ primers, were purified with QIAprep spin Miniprep kit (Qiagen) and cloned into a PCR cloning vector pGEM-TEasy (Promega). Plasmid DNA was prepared from five independent clones and was digested with a) *Not* I and b) *Eco* RI restriction enzymes and analysed on a 2% metaphor® agarose gel. Digestion with *Not* I produced a single restriction fragment of approximately 300 bp whereas, digestion with *Eco* RI enzyme generated two bands of approximately 120 and 170 bp (Figure 4.9). This suggested that each of the clones contained an identical insert and contained an internal *Eco* RI restriction site. Clones #1 and #3 were selected for sequencing.

The sequence of each clone was identical. The data was analysed to verify that the PCR amplification product contained sequence corresponding to both a forward and a reverse IFN-γ primer (Figure 4.10).

TGGCTTCTTTTTCGCTTCCTGGTGCCCCTCCACGTCCTTTTTCTCTCCATTGCTGTTGCTGCAG

GGAGAGTGGTTTCGTGCAATATCTGGCATAGTGACCGGGAATTCCGCAGTTATACCACTGGTAG

TTTCCCCCTCTGTATGGTCCAGTGTAGGGGCCTCGTTGAGCCCGTTGCTGTTGCAGGATATACC

ACTGTGGGGGAGGGTAATGGGGTGGTGGGGCTGCCTGCTGTTGGATCATCT**GAGAGACAATTTG**

GCTC

Figure 4.10 Nucleotide sequence of PCR fragment amplified using degenerate IFN-γ primers
Nucleotide sequence of the amplified fragment obtained from initial PCR amplification using IFN-γ degenerate primers, followed by automated sequencing using vector specific SP6 primers. Both IFN-γ forward (5'gagagacaatctggctc 3') and reverse primer (5' tggcttctttttcgctt 3') sequences are presented in bold.

The nucleotide sequence data was compared with entries in the Genbank database using the Fasta3 search programme. The results of the top 20 DNA sequences matching with the query DNA sequence are presented in Appendix 4.2. Unfortunately the DNA sequence did not appear to correspond to any known IFN-γ sequence, however to verify that this was the case the DNA nucleotide sequence was translated into 6 reading frames, all of which were analysed for

the presence of an open reading frame (Figure 4.11). Reading frames that did not contain stop codons were used to quiz the Swissprot protein database in search of homologous sequences.

The BLAST P programme, which compares a protein query to a protein database was used for the protein homology search to generate sequences with best scores (E-value) for each ORF (Figure 4.12). The Z-scores and percentage identities indicate the similarity between sequences. Once again however none of the ORFs showed any significant homology to any of the known IFN-γ sequences. Furthermore, none of the ORFs appeared to correspond to any known protein sequences in the database.

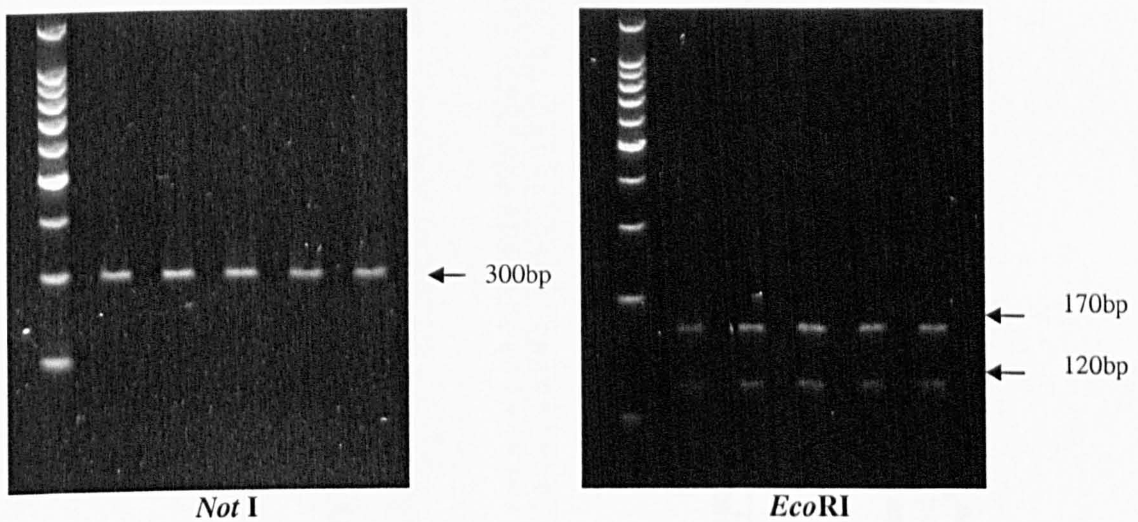

Figure 4.9 Restriction enzyme digestion of cloned PCR products amplified using IFN-γ primers.

Plasmid DNA clones: #1-5 (from left to right following DNA markers) digested with a) *Not*I and b) *Eco*RI restriction enzymes and analysed on a 2% metaphor® agarose gel. A 100bp DNA ladder (Promega) is included as a size marker.

a) *Not*I digestion generates a single fragment of approximately 300 bp.

b) *Eco*RI digestion generates 2 distinct bands of approximately 120 and 170 bp.

Figure 4.11 Protein maps representing open reading frames (ORFs) derived from IFN-γ PCR product.
Upper strand open reading frames 1,2 and 3 represent forward sequence (red) and lower strand frames 4,5 and 6 represent reverse sequence (green). The translated protein sequence for the ORF generated (MapDraw™ V 5.03) is displayed using single-letter standard genetic codes.

ORF	Sequence	Accession number	Z-Score	E Value	% Identity
ORF #1	Hypothetical protein (*Burkholderia fungoram*)	NZ_AAAC01000292	30.4	5.2	36
	Hypothetical protein XP_164074 (*Mus musculus*)	XM_164074	30.0	7.0	34
ORF #2	Hypothetical membrane protein (*Corynebacterium*) Glutamicum ATCC	AP005280	33.7	1.5	48
	MRNA-binding protein CSP41 precursor (*Spinacia oleracea*)	U49442	33.7	1.5	40
ORF #4	Myosin heavy chain Myr 8 (*Homo sapiens*)	XM_170134	36.3	0.24	53
	Unknown (*Streptococcus agalactiae*)	AL766849	35	0.59	57
ORF #5	Hypothetical protein (*Homo sapiens*)	AL832323	37.4	0.047	38
	Gag polyprotein (Simian immunodeficiency virus)	AL832323	37.4	0.047	38
ORF #6	Probable 60s ribosomal protein L19 (*Caenorhabditis elegans*)	NM_058980	30.4	4.9	32
	Amiloride-sensitive sodium channel type I delta polypeptide (*Homo sapiens*)	NM_002978	29.6	8.7	28

Figure 4.12 Protein homology searches performed using Blast P programme with open reading frames derived from the IFN-γ PCR product
Open reading frames coding throughout the entire sequence of the amplified PCR product were chosen for further analysis using the Blast P programme which compares a protein query to a protein database. Protein sequences with best scores for each ORF are presented and the overall similarity between sequences is indicated by the Z-scores and % identities. An expected E value is calculated, which gives the expected numbers of random alignments with Z-scores greater than or equal to the value observed.

Consensus	**G**	**C**	**I**	**S**	**A**	**G**	**L**	**P**	**K**	**T**	**E**	**A**	**N**	
Cat	S	C	I	N	A	G	L	P	K	T	E	A	N	
Cattle	G	C	I	S	A	S	L	P	K	T	E	A	N	
African green monkey	G	C	F	S	A	G	L	P	K	T	E	A	N	
Human	G	C	F	S	A	G	L	P	K	T	E	A	N	
Rhesus monkey	G	C	F	S	A	G	L	P	K	T	E	A	N	
Macaque (crab-eating)	G	C	F	S	A	G	L	P	K	T	E	A	N	
Mouse	G	C	V	S	A	G	L	P	K	T	E	A	N	
Pig	G	C	I	S	A	G	L	P	K	T	E	A	T	
Sheep	G	C	I	S	A	G	L	P	K	T	E	A	N	
Chicken	-	C	A	Y	V	-	-	P	K	T	E	A	N	
58		*						*	*	*	*	*		**70**

Consensus	**C T C**	**C C T**	**A A A**	**A C A**	**G A A**	**G C C**	**A A C**	**T G G**	**C T G**	
Cat	C T T	C C T	A A A	A C A	G A G	G C A	A A C	T G G	C A G	
Cattle	C T T	C C C	A A A	A C A	G A A	G C A	A A C	T G G	C A G	
Sheep	C T T	C C C	A A A	A C A	G A A	G C A	A A C	T G G	C A G	
Pig	C T T	C C T	A A A	A C A	G A A	G C A	A C C	T G G	C A G	
African green monkey	C T C	C C T	A A A	A C A	G A A	G C C	A A C	T G G	G T G	
Macaque (crab-eating)	C T C	C C T	A A A	A C A	G A A	G C C	A A C	T G G	G T G	
Rhesus monkey	C T C	C C T	A A A	A C A	G A A	G C C	A A C	T G G	G T G	
Mouse	C T C	C C T	A A A	A C A	G A G	G C C	A A C	T G G	A T A	
Rat	C T C	C C T	A A A	A C A	G A G	G C C	A A C	T G G	A T A	
Chicken	G T A	C C A	A A G	A C A	G A A	G C A	A A T	C A -	C T G	
184	*	* *	* *	* * *	* *	* *	*			**210**

Figure 4.13 Multiple nucleotide and amino acid alignments of known mammalian and avian IL-15 sequences. Design of the IL-15 forward primer was based on the following stretches of highly conserved sequence: nucleotides 187 - 203 / residues 65 - 70, showing highest identity. The consensus sequence is highlighted in bold and identical (*) residues, identified by the CLUSTAL programme are indicated. Regions non-identical to the consensus sequence are shaded.

Consensus	**E**	**S**	**G**	**C**	**K**	**E**	**C**	**E**	**E**	**L**	**E**	**E**	**K**	**N**	
Cat	E	T	G	C	K	E	C	E	E	L	E	E	K	N	
Cattle	E	L	G	C	K	E	C	E	E	L	E	E	K	S	
African green monkey	E	S	G	C	K	E	C	E	E	L	E	E	K	N	
Human	E	S	G	C	K	E	C	E	E	L	E	E	K	N	
Rhesus monkey	E	S	G	C	K	E	C	E	E	L	E	E	K	N	
Macaque (crab-eating)	E	S	G	C	K	E	C	E	E	L	E	E	K	N	
Mouse	E	S	G	C	K	E	C	E	E	L	E	E	K	T	
Pig	E	S	G	C	K	E	C	E	E	L	E	E	K	N	
Sheep	*E*	*L*	*G*	*C*	*K*	*E*	*C*	*E*	*E*	*L*	*E*	*K*	*K*	*S*	
Chicken	*S*	*K*	*K*	*C*	*K*	*E*	*C*	*E*	*E*	*Y*	*E*	*E*	*K*	*N*	
	159			*	*	*	*	*	*		*		*		**173**

Consensus	**T G C**	**A A A**	**G A A**	**T G T**	**G A G**	**G A A**	**C T G**	**G A G**	**G A A**	**A A A**	
Cat	T G C	A A A	G A A	T G T	G A G	G A A	C T G	G A G	G A A	A A G	
Cattle	T G C	A A A	G A A	T G T	G A G	G A A	C T G	G A G	G A A	A A A	
Sheep	T G C	A A A	G A A	T G T	G A G	G A A	C T G	G A G	A A A	A A A	
Pig	T G C	A A A	G A A	T G T	G A G	G A G	C T G	G A G	G A A	A A A	
African green monkey	T G C	A A A	G A A	T G T	G A G	G A A	C T A	G A G	G A A	A A A	
Rhesus monkey	T G C	A A A	G A A	T G T	G A G	G A A	C T A	G A G	G A A	A A A	
Macaque (crab-eating)	T G C	A A A	G A A	T G T	G A G	G A A	C T A	G A G	G A A	A A A	
Mouse	T G C	A A G	G A A	T G T	G A G	G A G	C T G	G A G	G A G	A A A	
Rat	T G C	A A G	G A A	T G T	G A G	G A G	C T G	G A G	G A G	A G A	
Chicken	T G C	A A A	G A G	T G T	G A A	G A G	T A T	G A A	G A A	A A A	
470	* * *	* *	* *	* * *	* *	* *		* *	*	*	**499**

Figure 4.14 Multiple nucleotide and amino acid alignments of known mammalian and avian IL-15 sequences. The following stretches of highly conserved sequence: nucleotides 162 - 167 / residues 470 - 488 were used in the design of an IL-15 reverse primer. The consensus sequence is highlighted in bold and identical (*) residues, identified by the CLUSTAL programme are indicated. Regions non-identical to the consensus sequence are shaded.

Consensus	S	D	I	I	F	F	Q	R	S	V	P	G	H	
Cat	N	D	I	I	F	F	Q	R	S	V	P	G	H	
Dog	N	D	I	I	F	F	Q	R	S	V	P	G	H	
Cattle	S	D	I	I	F	F	Q	R	S	V	P	G	H	
Horse	N	D	I	I	F	F	Q	R	S	V	P	G	H	
Sheep	S	D	I	I	F	F	Q	R	S	V	P	G	H	
Pig	N	D	I	I	F	F	Q	R	S	V	P	G	H	
Rhesus monkey	S	D	I	I	F	F	Q	R	S	V	P	G	H	
Chicken	S	D	I	I	F	F	K	K	T	F	T	S	C	
Duck	S	D	I	I	F	F	K	K	T	F	T	S	Y	
153		*	*	*	*	*								165

Consensus	A	A	T	G	A	C	A	T	C	A	T	A	T	T	C	T	T	T	C	A	G	A	G	A	A	G	T	
Cat	A	A	T	G	A	C	A	T	C	A	T	A	T	T	C	C	T	T	C	A	G	A	G	A	A	G	T	
Dog	A	A	T	G	A	C	A	T	C	A	T	A	T	T	C	C	T	T	C	A	G	A	G	A	A	G	T	
Cattle	A	G	T	G	A	C	A	T	C	A	T	A	T	T	C	C	T	T	C	A	G	A	G	A	A	G	T	
Sheep	A	G	T	G	A	C	A	T	C	A	T	A	T	T	C	C	T	T	C	A	G	A	G	A	A	G	T	
Pig	A	A	T	G	A	C	A	T	C	A	T	A	T	T	C	C	T	T	C	A	G	A	G	A	A	G	T	
Horse	A	A	T	G	A	C	A	T	C	A	T	A	T	T	C	C	T	T	C	A	G	A	G	A	A	G	T	
Rhesus monkey	A	G	T	G	A	C	A	T	C	A	T	A	T	T	C	C	T	T	C	A	G	A	G	A	A	G	T	
Chicken	A	G	T	A	A	C	A	T	C	A	T	A	T	T	T	T	T	C	A	A	A	A	A	G	A	C	A	
Duck	A	G	C	A	A	C	A	T	C	A	T	C	T	T	C	T	T	C	A	A	A	A	A	G	A	C	A	
451	*				*	*	*	*	*	*	*		*	*		*	*			*		*			*			477

Figure 4.15 Multiple nucleotide and amino acid alignments of known mammalian and avian IL-18 sequences. The following stretches of highly conserved sequence: nucleotides 451 - 468 / residues 153 - 158 were used in the design of an IL-18 forward primer. The consensus sequence is highlighted in bold and identical (*) residues identified by the CLUSTAL programme, are indicated. Regions non-identical to the consensus sequence are shaded.

Consensus	**E**	**K**	**E**	**N**	**D**	**L**	**F**	**K**	**L**	**I**	**L**	**K**	**K**
Cat	E	K	E	K	D	L	F	K	L	I	L	K	K
Dog	K	K	E	N	D	L	F	K	L	I	L	K	D
Cattle	K	K	E	N	D	L	F	K	L	I	L	K	K
Horse	E	K	E	N	D	L	F	K	L	I	L	K	E
Sheep	K	K	E	N	D	L	F	K	L	I	L	K	R
Pig	K	K	E	N	D	L	F	K	L	I	L	K	E
Rhesus monkey	E	K	E	R	D	L	Y	K	L	I	L	K	K
Chicken	E	E	E	D	S	L	R	K	L	I	L	K	K
Duck	E	E	E	D	S	L	R	K	L	I	L	K	K
185			*			*		*	*	*	*	*	**197**

Consensus	**A C G**	**A C C**	**T T T**	**T C C**	**A A A**	**C T C**	**A T T**	**T T G**	**A A A**	**A A A**
Cat	A A G	A T C	T T T	T C C	A A A	C T C	A T T	T T G	A A A	A A A
Dog	A C G	A T C	T T T	T C C	A A A	C T C	A T T	T T G	A A A	G A C
Cattle	A T G	A C C	T T T	T C C	A A A	C T C	A T T	T T G	A A A	A A A
Sheep	A T G	A C C	T T T	T C C	A A A	C T C	A T T	T T G	A A A	A G A
Pig	A C G	A C C	T T T	T C C	A A A	C T C	A T T	T T G	A A A	G A A
Horse	A T G	A T C	T T T	T C C	A A A	C T C	A T T	T T G	A A A	G A A
Rhesus monkey	G A G	A C C	T T T	A T C	A A A	C T C	A T T	T T G	A A A	A A A
Chicken	A C T	C C T	T A A	G A C	A A A	C T A	A T T	T T A	A A G	A A A
Duck	A C T	C C T	T A A	G A C	A A A	T T A	A T T	T T A	A A G	A A A
557			*	*	* * *		* * *	* *	* *	**588**

Figure 4.16 Multiple nucleotide and amino acid alignments of known mammalian and avian IL-18 sequences. The following stretches of highly conserved sequence: nucleotides 568 - 588 / residues 192 - 197 were used in the design of an IL-18 reverse primer. The consensus sequence is highlighted in bold and identical (*) residues, identified by the CLUSTAL programme are indicated. Regions non-identical to the consensus sequence are shaded.

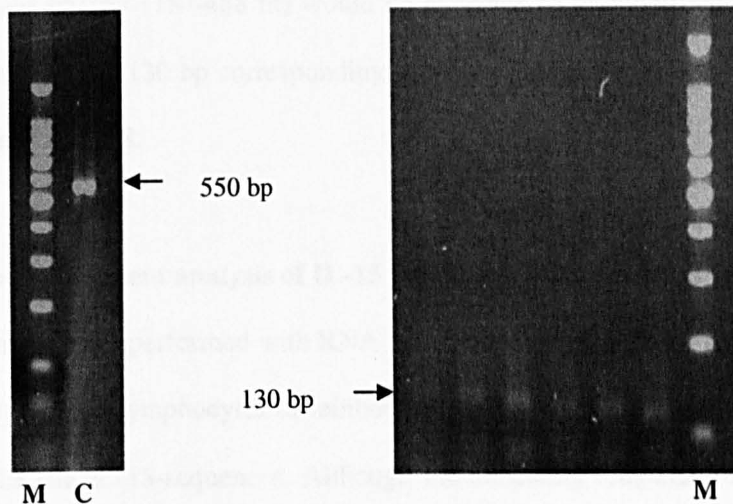

Figure 4.17 PCR amplification using IL-15 degenerate primers

A PCR amplification product of 130bp generated, using cDNA from mitogen stimulated lymphocytes and the following degenerate oligonucleotide primers designed against the IL-15 forward primer 5' cc(at)aa(ag)acaga(ag)gc(ac)aa 3' and 5' tc(ct)tcaca(ct)tc(ct)ttgca 3' reverse primer. Salmon β-actin primers were used as a PCR control to generate an approximately 550bp product (C). A 100bp DNA ladder (0.1-1.5kb) is included as a size marker (M).

4.3.5 The design of IL-15/IL-18 degenerate primers and subsequent RT-PCR

A similar approach as outlined previously for IFN-γ was used in the design of degenerate primers for both the IL-15 and IL-18 cytokines (Table 4.4). Oligonucleotides of either 15 or 18 bases in length were constructed, based on conserved regions of homology between IL-15 or IL-18 sequences from different species (Figures 4.13- 4.16). If the corresponding trout sequences were similar to the mammalian cytokines a fragment of approximately 300 bp corresponding to amino acid residues 65-167 (187-488 nt) would be expected to be amplified for IL-15, and a fragment of approximately 130 bp corresponding to amino acid residues 153-197 (451-588 nt) would be expected for IL-18.

4.3.6 Cloning and subsequent analysis of IL-15 and IL-18 PCR products

RT-PCR experiments were performed with RNA isolated from mitogen-stimulated head kidney cells and peripheral blood lymphocytes of rainbow trout using the degenerate primers designed for both the IL-15 and IL-18-sequences. Although the annealing temperatures for both IL-15 forward and reverse primers were identical, the IL-18 primers had a T_{Ann} value of 40.7 and 35.9 respectively. As a consequence, the PCR reactions were performed using a gradient PCR protocol with a range of annealing temperatures (35 - 50°C) as described in Table 4.3.

PCR products of approximately 130 bp were obtained for the IL-15 primers (Figure 4.17) but unfortunately, no amplification products were obtained using the IL-18 degenerate primers. Amplified fragments were cloned into a PCR cloning vector (pGEMT-Easy) and the resultant plasmid DNA clones were digested with *Eco* RI restriction enzyme. All the clones containing the IL-15 amplification product had an insert of approximately 130 bp. Two of these clones were selected for sequencing.

Although the amplified fragments obtained using IL-15 primers were not of the size anticipated, the sequences of the PCR products were determined. This was because there is always the possibility that the corresponding trout sequences may contain either deletions or

insertions of sequence in comparison to the mammalian sequences. The sequence of both IL-15 clones was identical. However analysis of it revealed that the forward degenerate primer was present at both ends of the PCR product. Therefore it was highly unlikely that the PCR product was in any way related to IL-15. Nevertheless the sequence was compared with entries in the EMBL database to try and identify if it was related to any known fish sequences. The result of the database searching found that the sequence was possibly related to a zebrafish (*D.rerio*) DNA sequence derived from genomic DNA (accession #AL929078). The trout sequence was 64% identical over 93 nucleotides with the zebrafish sequence (Figure 4.18). No function has been assigned to the zebrafish sequence.

```
                                                           120
IL15                                                 CCTAAGACAGAAGCCA
                                                      :: ::::::::   :
EM_OV:    GATTTGCTTTAGAAATAGAAATATTTTGCTATTATCTACT--TTTCTTAGACAGAAAATA
           90250     90260     90270     90280       90290     90300

          110       100       90        80          70        60
IL15      ACACGCACATAGGTAACACACACAGACAGCTTTACC--TCCTTAGCTAACCTGTGGGAGC
          :   :: :  :  ::  ::::::::: : ::::: :  :: ::: : :::: : :  :::
EM_OV:    AGCAGCTCTGAACTACGACACACAGAGACCTTTATCAATCTTTATCAAACCAGAGTAAGC
             90310     90320     90330     90340     90350     90360

             50        40        30        20        10
IL15      AGCCAAAACTACACCTCAGTGTCATGTTTGGCCTCTGTCTTTGG
           ::
EM_OV:    TGCTGCCATATATTATACTGCCTTTTATGGGTTTAGGTGAATAA
             90370     90380     90390     90400     90410
```

Figure 4.18 CLUSTAL generated nucleotide alignment comparing PCR sequence amplified using IL-15 degenerate primers with a Zebrafish *(D. rerio)* DNA sequence from the EMBL database (Accession #AL929078). The sequence derived from clone CH211-155I15 in linkage group 3, complete sequence demonstrates 64% identity over 93 (nt) region with the reverse complement sequence. Conservation of nucleotide identity is shown as (:) and both forward and reverse primer sequence are presented in bold.

4.3.7 Amplification of cytokine cDNA using ovine cytokine primers

PCR experiments were performed on trout cDNA using a number of paired ovine cytokine primers available in the laboratory (kindly provided by A. Wood): IL1β, IL2, IL3, IL4, IL5, IL6, IL8, GMCSF, IFN, LT, MCP, MIP, RANTES, TGFβ, TNF-α. The initial PCR comprised of 20 cycles with an annealing temperature of 37°C. No amplification products could be

visualised on an agarose gel from these initial reactions, but the an aliquot from each of the reactions was used in a second round of amplification involving 30 cycles at a higher annealing temperature of 50 °C. However, no amplification products could be detected for any of the primer pairs after this second PCR.

4.4 Discussion

The polymerase chain reaction has been used successfully to isolate the fish counterparts of a number of mammalian cytokines. It was decided to use the same approach to try and isolate the rainbow trout equivalents of mammalian IFN-γ, IL-15 and IL-18. In order to verify that the RT-PCR reactions were working on each occasion primers designed to amplify the trout β-actin cDNA were included as a positive control. The trout β-actin sequence was not known and so primers based on the salmon sequence were used. These were designed to amplify a 557 bp fragment of the salmon β-actin sequence. A fragment of a similar size was amplified from the trout cDNA and was subsequently cloned and sequenced. The trout sequence was found to be 98% identical to the salmon sequence. Recently, a rainbow trout sequence (Accession no. AJ438158) has appeared in the DNA databases and a pairwise comparison of it with the trout PCR amplification product revealed that the two sequences were 100% identical. Pairwise alignment of the trout β-actin sequence with the human β-actin cDNA sequence showed that they were 87% identical.

The degenerate IFN-γ primers were designed from regions of the cDNA sequence corresponding to amino acids that were highly conserved between 15 mammalian and 6 avian species. If the trout IFN-γ sequence was similar to these sequences then a PCR product of approximately 260 bp would be expected. A PCR product of approximately 280 bp was amplified using a standard PCR protocol and an annealing temperature of 45°C. This PCR product was cloned, but although analysis of the sequence confirmed that it contained both the forward and reverse degenerate primers, the sequence itself did not appear to be related to IFN-γ, or indeed to any sequences in the databases. Furthermore, none of the potential ORFs

contained within the sequence corresponded to any known protein sequences. It was concluded that this PCR product was not related to IFN-γ. The reasons for not amplifying a PCR product corresponding to IFN-γ could be many fold. It may be simply that the trout sequence is so divergent from the other sequences that have been published that the PCR approach using these specific primers would just not work. Alternatively it may be that the trout IFN-γ sequence was not present, or was present at very low levels, in the starting population of RNA. However in order to counteract this possibility RNA isolated from cells activated with a number of different stimulants was pooled prior to preparing the cDNA. Another possibility is that fish do not possess an IFN-γ gene. So far IFN-γ sequences have been found only in mammalian and avian species. Attempts by many groups to isolate fish IFN-γ have so far failed. Even within the EST cloning approaches taken for a number of fish species no IFN-γ related sequence has been reported. To counteract this however, groups have reported being able to detect anti-viral activity, which is both heat and acid labile, a property that is associated with IFN-γ. Similarly in this thesis and elsewhere a macrophage activating activity (MAF activity) which could be due to an IFN-γ-like molecule has been demonstrated. In addition recently the trout sequence corresponding to an IFN-γ-inducible gene (Laing *et al.*, 2001b) has been reported.

Having failed to amplify a PCR fragment corresponding to IFN-γ, attention was focussed on other cytokines, which possess activities linked to IFN-γ. Interleukin-15 is a T-cell growth factor which appears preferentially to stimulate the growth of the Th1 subset of helper T-cells. Classically Th1 cells are thought of as IFN-γ producing cells and it has been shown that administration of IL-15 directly leads to the increased production of IFN-γ (Biber et al., 2002; Liew, 2003). Similarly IL-18 is alternatively known as interferon gamma inducing factor (IGIF). It too stimulates the production of IFN-γ from both Th1 cells and natural killer (NK) cells. Mice that are deficient in IL-18 have reduced production of IFN-γ, despite normal levels of IL-12 another cytokine with IFN-γ-inducing properties (Dinarello, 1999).

Once again all the available IL-15 and IL-18 sequences were aligned to find regions of amino acids that were conserved across as many species as possible. If the corresponding trout sequences were similar to those published sequences, the primer pairs that were designed would

be expected to amplify fragments of approximately 300 bp and 130 bp corresponding to IL-15 and IL-18 respectively.

Amplification with the IL-15 primers reproducibly produced a PCR product of approximately 130 bp in length, much smaller than was expected. This was cloned and sequenced. However analysis of the sequence revealed that the same degenerate primer was present at both ends of the fragment and therefore it was highly unlikely that it would be related to IL-15. This was verified by using the sequence to search the DNA databases. The best match to the amplification product was a sequence derived from a region of the zebrafish genome for which no function has yet been assigned. A search of the protein databases with the potential ORFs contained within the amplification product sequence also failed to suggest any function. Amplification with the IL-18 primers failed to produce a PCR product.

Unlike the sequence derived from using the IFN-γ primers, that derived from using the IL-15 primers appeared at least to be related to fish sequences. However even here it is not clear whether or not they are really related to expressed sequences or could be derived from contaminating genomic DNA. The results with the three sets of primer pairs also illustrate the dangers of using degenerate primers. The more degenerate the primer the greater the chance of amplifying something which is not related to the original target. However equally, if the target sequence is highly related to the sequences that the primers were designed from then the correct fragment should be amplified with the degenerate primers. With the IL-15 primer pairs used, a PCR fragment of reproducible size was obtained on each occasion the PCR was performed, but none corresponded to the cytokines that were being sought. The reasons for this are unknown, but could be the same as discussed previously for the IFN-γ.

As a final attempt to amplify trout cytokine cDNAs RT-PCR was performed with a range of primer pairs specific for ovine cytokines. However as one might have expected no amplification products were obtained in this instance. It is likely that there is insufficient homology at the nucleotide level between these ovine cytokine primers and the corresponding trout sequences as no attempt was made to ensure that the regions covered by the primers corresponded to regions known to be conserved across different species.

Since the PCR approached proved to be unsuccessful it was decided to construct a cDNA library from lymphocytes activated with a variety of stimulants and to screen this in several ways to try and isolate trout cytokine genes.

CHAPTER 5

Differential screening and hybridisation studies of rainbow trout stimulated lymphocyte cDNA library

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5.4 Discussion **148**

5.1 Introduction

The existence of a number of fish cytokines have been inferred based on functional similarity to, or biological cross-reactivity with, mammalian cytokines. Apart from MAF, there is evidence in fish for IL-1, IL-2, type 1 and 2 interferons, macrophage migration inhibition factor (MIF), TNF-α and TGF-β. Several groups have demonstrated cytokine-like biological activity in cell-free supernates from stimulated lymphocytes (Secombes *et al.*, 1996, 1998), but have failed to purify the molecules responsible for such activities using conventional protein purification techniques (Secombes, 1994a; Secombes *et al.*, 1996). For example, secretion of MAF from activated fish lymphocytes has been reported, but the factors responsible have yet to be purified (Graham and Secombes, 1990a,b).

Molecular techniques have recently enabled the identification of some fish cytokine genes and have provided support for the biological data on cytokine activity (Secombes, 1999a). However, there are still very few fish cytokine genes that have been cloned (Zou *et al.*, 1999b). Although it is considered unlikely that fish will possess all the cytokine genes present in mammals, it will be interesting to determine which cytokines genes they do possess and whether or not they possess any unique genes or activities (Secombes, 1999a).

Cytokine genes are unlikely to be expressed constitutively in most instances, and therefore to increase the chances of cloning their cDNAs it is important to use a reliable means of inducing their expression. This is achieved either *in vitro* by mitogen stimulation or *in vivo* using bacterial pathogens (Marsden *et al.*, 1996a,b). The cytokine cDNAs that have been cloned were identified either by screening cDNA libraries with gene probes derived from mammalian cytokine sequences or by other molecular techniques such as the PCR, or EST analysis (Wittbrodt *et al.*, 1998). For example, the IL-1β gene has been cloned recently in rainbow trout using the PCR (Zou *et al.*, 1998) and in Carp by the EST approach (Fujiki *et al.*, 2000; Yin and Kwang, 2000), and has been discovered in Seabass using the trout sequences as a probe (Scapigliati *et al.*, 2001).

In the trout, primers designed against conserved regions of known mammalian IL-1β genes were used to amplify the fish gene (Zou *et al.*, 1998). Whereas, the EST analysis approach for the isolation of the Carp IL-1β involved the generation of a cDNA library to allow for enrichment of genes of interest by *in vivo* stimulation of peritoneal cells with sodium alginate. Individual clones were randomly sequenced and some of the fragments of interest used as probes, yielding full-length cDNAs homologous to mammalian interleukin-1β (Fujiki *et al.*, 1998).

In the present study, a cDNA library was constructed from mitogen-stimulated rainbow trout leucocytes in order to screen for cytokine genes. Initial development of T-cell culture methods focussed on the establishment of a reproducible source of MAF, (Chapter 3), but RNA was eventually isolated from lymphocytes activated with a variety of different mitogens and stimulants. This was in order to increase the possibility of cloning a variety of different cytokines. The library was screened initially with a range of ovine cytokine cDNA / gene probes. Differential screening was also performed using probes prepared from stimulated and non-stimulated leucocyte RNA. Clones isolated from the library were sequenced for further characterisation.

5.2 Methods

5.2.1 Southern hybridisation of trout genomic DNA

Southern blot analysis was performed on genomic DNA, isolated from rainbow trout spleen tissue using a DNA isolation kit (Qiagen). The genomic DNA (0.9μg) was digested with the following restriction enzymes: *Bam*HI, *Eco*RI, *Hind* III and *Pst*1 and electrophoresed on a 0.5% agarose gel. A lower percentage gel was used to obtain better separation of the high molecular weight bands. The blot was hybridised with ^{32}P labelled β-actin (PCR product) and ovine IFN-γ (gene) probes at 65°C for 4 h. Following stringency washes, the membrane was exposed to x-ray film for ~12 h.

5.2.2 Construction of a cDNA library with RNA isolated from stimulated rainbow trout leukocytes

Peripheral blood leucocytes were stimulated with a range of T cell mitogens in an attempt to activate the expression of cytokine genes. A cDNA library was prepared from RNA isolated from the peripheral blood lymphocytes stimulated with the mitogens (Section 2.9), using the pCMV-script® XR cDNA library construction kit (Stratagene, USA). This kit is designed for the generation of directional libraries in the plasmid based pCMV-script mammalian expression vector, such that it can be screened by functional assay using mammalian cells or with DNA probes in *E.coli* cells.

First the mRNA was purified from total RNA using an oligotex-dT ™ Kit (Qiagen), following manufacturers instructions (Section 2.19). Then, cDNA was synthesised from the polyA$^+$ RNA (1.68μg) template using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and DNA polymerase I. The cDNA was size fractionated (1.8-0.5kb) and ligated into the pCMV-Script vector used to transform competent *E.coli* XL10-Gold® cells. The initial library was titred and then amplified as described in Sections 2.15 and 2.16. To ensure slow-growing clones were not under-represented in the library, only one round of amplification was performed.

5.2.3 Screening of the cDNA library

The cDNA library was plated out at high density (10^5 colonies per 22 cm^2 plate) onto L-agar plates containing kanamycin (50μg/ml) and grown overnight at 37 °C. Bacterial colonies were transferred onto nitro-cellulose filters, in preparation for colony hybridisation. The filters were placed colony side up in 10% SDS for 3 min. They were then placed, in turn, into denaturing solution (0.5N NaoH, 1.5M NaCl) for 5 min, neutralising solution (1.5M NaCl, 0.5M Tris.Cl pH 7.4) for 2 x 5 min, 2 x SSC solution for 2 x 5 min and then air dried for 30 min at room temperature. Bacterial debris was removed by submerging the filters in 2 x SSC and wiping the surface with a paper towel. This reduces background hybridisation without lowering the intensity of the specific signal. Filters were air-dried as before and the DNA fixed to the membrane by exposure to UV illumination (302nm) for 1 min. Replica filters were prepared as appropriate for screening with different α ^{32}P-dCTP radiolabelled probes (Section 2.35). Filters were pre-hybridised in Rapid-Hyb™ (Amersham Life Sciences) for several hours at 65°C prior to addition of radio-labelled probes. Probes were incubated overnight at 65°C in Rapid-Hyb™ buffer. After hybridisation filters were washed with 2 x SSC / 0.1% (w/v) SDS for 5 min at rt and then twice with 0.2 x SSC / 0.1% (w/v) SDS at 65 °C for 15 min prior to autoradiography.

Positive colonies were selected and re-plated on L-agar plates (containing kanamycin) at a lower density. These colonies were then re-screened with the radio-labelled probe to ensure that the correct clone had been selected. Individual clones selected from this second round of screening were amplified and sequenced.

5.2.4 Preparation of probes

5.2.4.1 Ovine cytokine cDNA probes

Several ovine cytokine cDNAs were used to screen the cDNA library. The cytokine cDNAs were purified from plasmids, the DNA quantified, and combined into three groups (Table 5.1). The grouped cDNAs (25ng) were labelled with α-^{32}P dCTP by the random prime method using Rediprime II DNA labelling system (Amersham, Section 2.31), and denatured by boiling for 5 min prior to hybridisation.

Table 5.1 Ovine cytokines used to screen the *O. mykiss* stimulated leucocyte cDNA library.

Group 1	Group 2	Group 3
IL-1β	IL-5	RANTES
IL-2	GMCSF	SCF
IL-3	TNFα	VEGF
IL-4	MIP1α	γ-IFN
		TGFβ

The DNA probes were [α-^{32}P]dCTP-labelled using random primer labelling (Rediprime II DNA labelling system, Amersham).

5.2.4.2 Single-stranded cDNA probes

Single-stranded cDNA probes were generated from both total RNA and polyA$^+$ RNA isolated from stimulated PBL, non-stimulated PBL and whole liver using MMLV reverse transcriptase. First-strand cDNA synthesis was primed as before (Section 2.23) using either Oligo (dT), random hexamers (pdN$_6$) or the primer TAAAT. Multiple repeats of the known mRNA instability motif (TAAAT) are commonly found in the 3' non-coding region of inflammatory cytokine genes, and have been reported previously in fish IL-1β (Secombes *et al.*, 1999a). Synthesis of the single-stranded cDNA was allowed to proceed for 1.5 h at 42°C, but using α-^{32}P-dCTP in place of the unlabelled dCTP. Probes were denatured by boiling for 5 min prior to hybridisation.

5.2.5 Sequence analysis

Clones selected from the various screenings of the library were sequenced on an ABI automated sequencer, with universal forward and reverse primers. Nucleotide sequence data were compared with entries in the GenBank/EMBL databases using the FASTA search program. Amino acid sequences were compared with protein sequences in the protein databases (TrEMBL, SWISSPROT and PIR).

5.2.6 Northern blot analysis of cDNA clones from trout leucocyte cDNA library

Northern blot analysis was performed using cDNA clones (clones #5 and #144/3) isolated from the library as a result of the differential screening using probes corresponding to stimulated and non-stimulated leukocytes. Total RNA (0.3g/lane) from non-stimulated lymphocytes and lymphocytes stimulated with PMA (μgml^{-1}) was electrophoresed through a 1 % w/v agarose gel containing formaldehyde (Section 2.17), transferred to a nylon membrane and probed with the ^{32}P-labelled cDNA clones. The trout β-actin cDNA was used as a positive hybridisation control. Hybridisation was at 65°C overnight and was followed by 2 washes in 2 x SSC for 5 min at 42°C and 2 washes in 0.2 x SSC for 15 min at 42°C, prior to autoradiography.

5.3 Results

5.3.1 Analysis of genomic DNA by Southern Hybridisation

In order to assess whether or not a cross-hybridisation approach using probes derived from mammalian cytokine sequences was likely to be successful, trout genomic DNA was probed with an ovine γ-IFN gene probe. The trout β-actin cDNA (Section 4.3.2) was used as a positive hybridisation control. No specific hybridisation was detected using the ovine γ-IFN probe, whereas several hybridisation signals were obtained with the control β-actin probe (Figure 5.1).

5.3.2 Random isolation of cDNA clones from the stimulated leukocyte primary cDNA library

Before amplification of the cDNA library, eight clones were randomly selected to determine the percentage containing cDNA inserts. Restriction endonuclease analysis showed that each of the eight clones contained a differently sized insert ranging from just below 0.5 kb to approximately 1.2 kb in length. Three of these clones (C3, C7 and C8) were subsequently sequenced using the vector-specific T3 and T7 primers. A search of the Genbank/EMBL databases using the FASTA search algorithm identified each as being similar to known mammalian and fish sequences. The most significant matches to each of the clones are listed in Table 5.2.

Table 5.2 Representative clones isolated from the *O. mykiss* stimulated leucocyte cDNA library

Clone	Homologous Sequences	Accession Number	% nucleotide identity
C3	*O. mykiss* mRNA βglobin	D82926	92 in 396 nt (533)
	S. salar mRNA βglobin	X69958	91 in 422 nt (747)
C7	Human 60S ribosomal protein L30	BE015979	
	D.rerio (Zebrafish) adult retina/olfactory cDNA5'	AW232981	
C8	S. scrofa (pig) cDNA from EST study	AW787193	
	H. alosoides (goldeye teleost) 18S rRNA gene	X98840	85 in 191 nt (1775)

Sequences from the cDNA library were compared with those in GenBank/EMBL, using the FASTA search program. Sequences showing greatest homology at the nucleotide level with the clones derived from the library are presented. Clones were sequenced in both directions using T3 forward and T7 reverse vector specific primers.

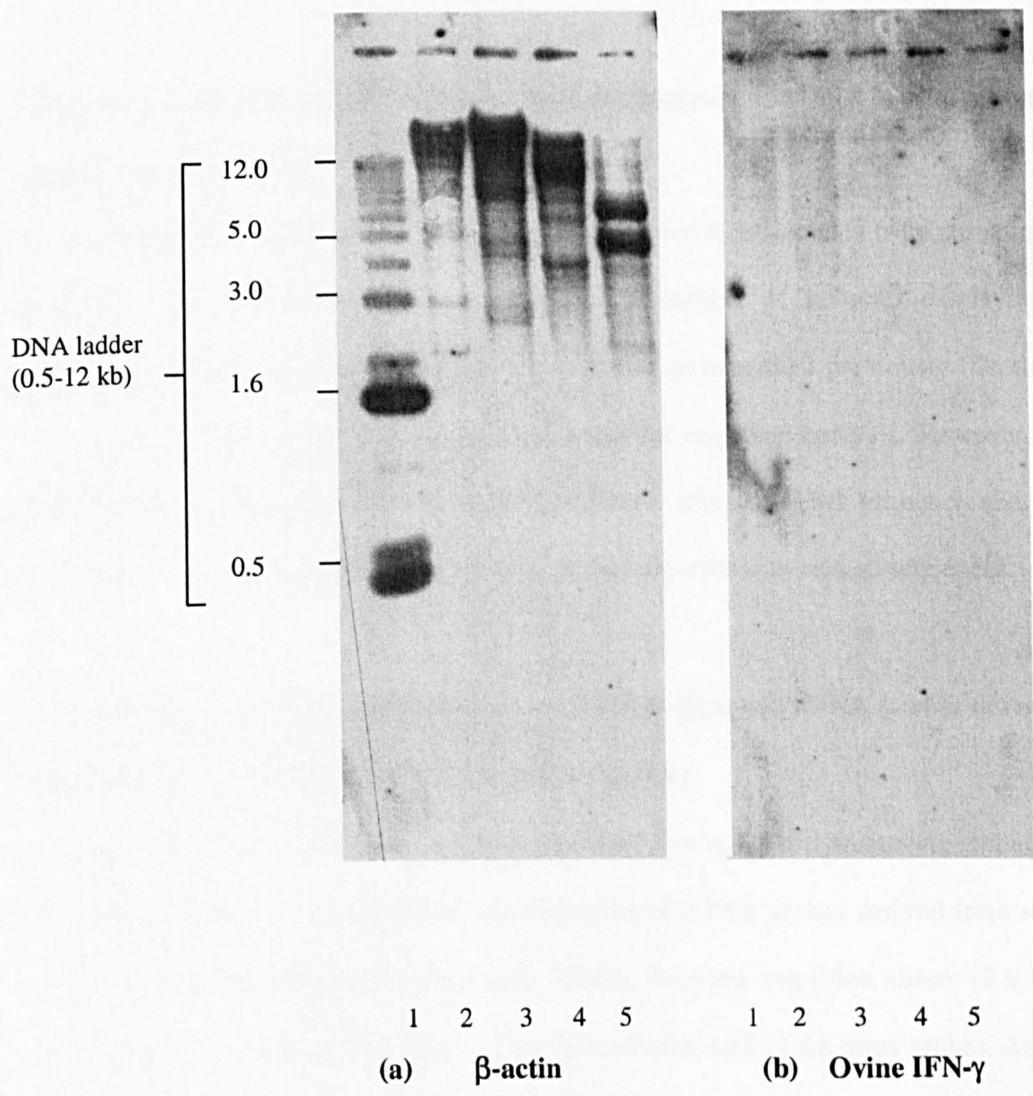


Figure 5.1 Southern blot analysis of *O. mykiss* genomic DNA.

Trout spleen genomic DNA digested with restriction enzymes and hybridised (0.9μg DNA/lane) with ^{32}P labelled trout (a) β-actin probe or (b) ovine IFN-γ probe. Lanes: (1) A 1kb BRL DNA ladder used to size linear double stranded DNA fragments ranging from 500bp to 12 kb; (2) *Bam* H1; (3) *Eco*R I; (4) *Hin*d III and (5) *Pst* I.

5.3.3 Screening of the rainbow trout leukocyte cDNA library with ovine cytokine probes

Despite not detecting a positive hybridisation signal between the ovine γ-IFN probe and the rainbow trout genomic DNA, it was still considered worthwhile to screen the cDNA library with a number of ovine cytokine cDNAs (Table 5.1). However, despite screening 4 x 10^5 clones from the amplified leukocyte cDNA library no positive clones were identified even when the filters were hybridised and washed at relatively low stringency.

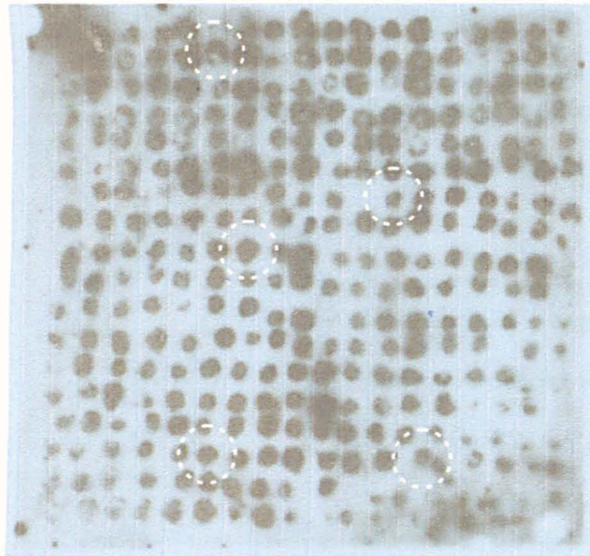
5.3.4 Screening of the trout cDNA library with single-stranded cDNA probes prepared using the primer TAAAT

Initial screening of the library with a ss-probe derived from RNA isolated from stimulated PBLs and primed with the primer TAAAT, produced a number of colonies displaying strong hybridisation signals. These positives were re-screened as described previously (Section 5.2.3) and 4 strong positives (nos. 2, 7, 8 and 9) selected for sequence analysis. However, FASTA searches of both the nucleotide and protein databases with the DNA sequence and potential amino acid sequences from these clones failed to find any cytokine-related sequences.

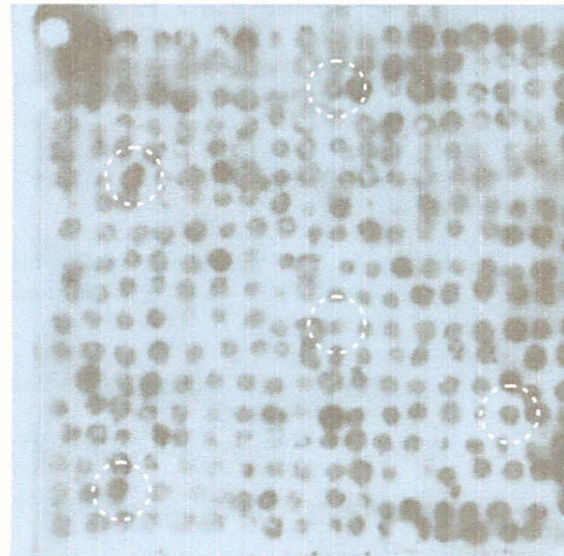
5.3.5 Screening of the trout cDNA library with single-stranded cDNA probes derived from stimulated PBLs, non-stimulated PBLs and whole liver

In order to identify genes either up or down regulated as a result of lymphocyte stimulation, the trout cDNA library was screened differentially using ss-cDNA probes derived from stimulated PBLs, non-stimulated PBLs and whole liver. Clones from the amplified library (2 x 10^3) were replica plated onto nitrocellulose filters and screened with each of the three probes. An example of replica filters is shown in Figure 5.2. The majority of the clones produced a positive hybridisation signal with both stimulated and non-stimulated PBL probes. However some hybridised more strongly with the stimulated PBL probe, whereas others hybridised more strongly with the probe from non-stimulated PBLs. Few clones hybridised strongly with the liver probe. Initially 460 clones were selected on the basis that they hybridised more strongly with one probe than with the others. These were re-plated on L-agar plates and the colonies re-

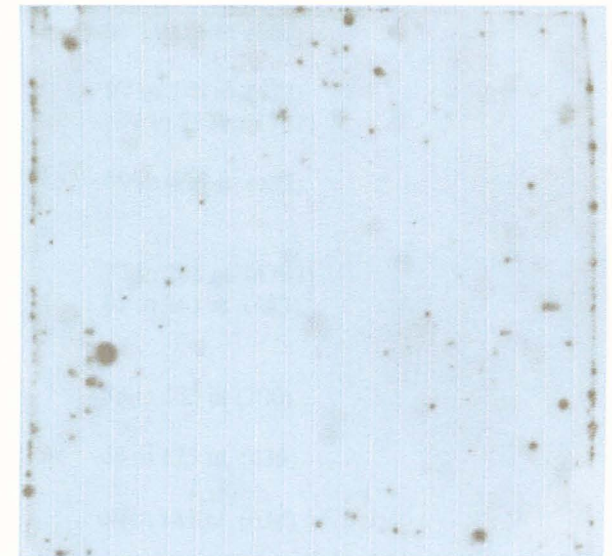
screened with the ss-cDNA probes. The cDNA clones demonstrating the greatest difference in hybridisation signal between the stimulated PBL and non-stimulated PBL probes were selected for sequencing. In total 24 clones were selected from this second screening. These were sequenced and found to represent 10 different gene sequences (Table 5.3). At the same time the purified plasmid DNA from each was re-probed with the ss-cDNA probes, derived from the stimulated and non-stimulated PBLs, to confirm that the insert sequences did indeed hybridise more strongly with one probe than with the other (Figure 5.3).



stimulated PBL



non-stimulated PBL



liver

Figure 5.2 An example of differential screening by colony hybridisation of the PBL expression library with: stimulated PBL (above), non-stimulated PBL (centre) and liver probes (right). Strong positives indicated with a white, dotted line.

Colonies were selected on the basis of strongest difference in hybridisation between the three probes. 23 positive colonies which hybridised strongly with the stimulated PBL probe and 7 positive colonies which hybridised strongly with non-stimulated probe were identified.

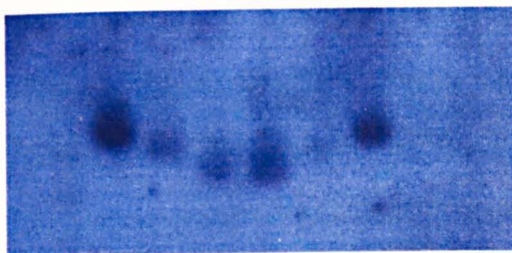
Table 5.3 Sequences from GenBank/EMBL databases, showing greatest homology to positive clones isolated from the *O. mykiss* stimulated leucocyte cDNA library.

Clone	Sequence Homology	Accession Number	E - value	%Nucleotide Identity
53/24	*O. mykiss* 18S ribosomal rRNA gene	AF243428	$6.3e^{94}$	96 in 354 nt (661)
57/32	*O. mykiss* β globin subunit mRNA	AY026061	$2.9e^{142}$	97 in 576 nt (622)
	S. salar mRNA β globin	X69958	$7.8e^{137}$	e96 in 577 nt (747)
59/43	*O. mykiss* mRNA for α globin	D88114	$9.1e^{120}$	99 in 466 nt (469)
144/3	*H. sapiens* mRNA for KIAA1068 protein (partial cds)	AB028991	$1.1e^{31}$	77 in 261 nt (4793)
	D. rerio C32 fin cDNA 3' similar to WP:F53A2.4 CE16096.mRNA sequence	BE605981	$8.2e^{38}$	82 in 261 nt (653)
5 (05/19)	*P. olivaceus* leucocyte cDNA, no assigned function	C82242	$2.7e^{19}$	83 in 133 nt (700)
48	*D. rerio* fin cDNA 5' similar to TR:O42585 fizzy-related protein	AI330687	0.04	60 in 175 nt (435)
	D. rerio gridded kidney cDNA 3' similar to TR:O75869 R33374_1 fizzy related protein	AW421025	0.13	60 in 181 nt (634)
46/19.2	*P. olivaceus* spleen cDNA clone HF1 mRNA	C23506	$4.3e^{42}$	66 in 468 nt (565)
07/7	*P. olivaceus* spleen cDNA clone HE8 (1) mRNA	C23502	$5.0e^{141}$	94 in 603 nt (636)
09/8	*P. olivaceus* spleen cDNA clone HH1 (1) mRNA	C23517	$6.6e^{34}$	67 in 405 nt (582)
20/2	*M. musculus* mRNA for NDAP7 gene Q99JA3	AB057593	$1.4e^{12}$	82 in 198 nt (2539)

Significance of the homology is represented by E-value and % identity at the nucleotide level. E-value represents the number of alignments with scores equal to (S) that would be expected by chance alone.

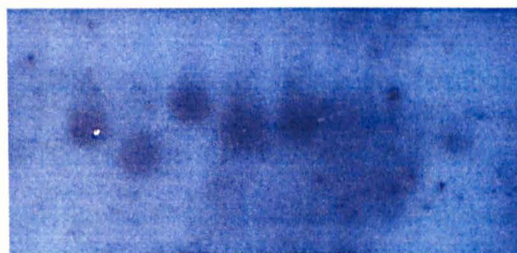
a) stimulated probe

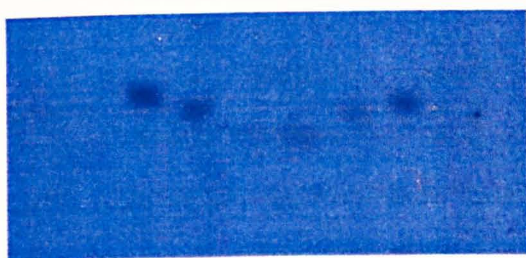
1 2 3 4 5 6



a) stimulated probe

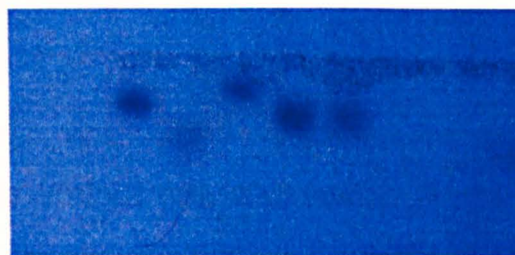
7 8 9 10 11 12 13





1 2 3 4 5 6

b) non-stimulated PBL probe



7 8 9 10 11 12 13

b) non-stimulated probe

Figure 5.3 Southern blot hybridisation of cDNA clones derived from the stimulated leucocyte trout library with $^{32}P\alpha$-dCTP labelled DNA probes

Plasmid DNA of positive cDNA clones digested with *Eco*RI and *Xho*I, denatured, separated by electrophoresis on a 0.7% agarose gel and blotted onto a nylon membrane. Hybridisation of cDNA clones was performed with a) stimulated PBL and b) non-stimulated PBL $^{32}P\alpha$-dCTP labelled DNA probes. A clear difference in hybridisation is apparent between the two probes. Strong hybridisation signals observed with cDNA clones using the stimulated PBL probe compared with the non-stimulated PBL probe, demonstrate the differential screening process.

5.3.6 Sequence analysis of positive clones isolated from differential screening

Of the 24 clones sequenced eight corresponded to β-globin, five to α-haemoglobin and four to 18S rRNA. However several were found to be similar to fish sequences derived from EST studies of various lymphoid tissues. These included, clone #5 which was 83% similar at the nucleotide level with a cDNA which had been isolated during an EST study of genes expressed in the leukocytes of a Japanese flounder, *Paralichthys olivaceus*, infected with Hirame rhabdovirus (Figure 5.4). It is thought that this cDNA may be similar to that encoding a ribosomal protein, L41 from the cat. However, neither our search of the nucleotide databases nor the protein databases, produced a positive match with the conceptual amino acid sequences from the three possible open reading frames (ORFs) present in clone #5.

```
          220       230       240       250       260       270
clone 5
          CAAAGGGCTTGGAGTGGGTGGAGAAAAACAATCACNGCAGTAGGGGAATGGGCCAGTCTA
                                       ::::: :: :::: :    ::::     ::
EM_EST    GTGTCAGTCGTATGGAGAGGCTCGGACGGGATCACAGCTGTAGCGATGAGGGC----TTA
            220       230       240       250       260           270

          280       290       300       310       320       330
clone 5   CTTGGACCTCTGCCTCATCTTTCGCCTTTTACGCTTCAGCCTGCGCATACGCTTCTTCCT
          ::::::::::::::::::::::: ::::::::::::::::::::::::::::::::::::
EM_EST    CTTGGACCTCTGCCTCATCTTTCTCCTTTTACGCTTCAGCCTGCGCATACGCTTCTTCCT
                280       290       300       310       320       330

          340       350       360       370         380
clone 5   CCACTTTGCTCTCATCTTGTGGAGATGTGTCTCTACAGC--GAAGCGAG
          :::::: ::::::::: :::::::: :  :::: : :::  :::: :::
EM_EST    CCACTTAGCTCTCATCGTGTGGAGAAGATTCTCGA-AGCGAGAAGGGAGACCTGGCCCAG
                340       350       360        370       380
```

Figure 5.4 Nucleotide sequence homology with EST encoding *P. olivaceus* leukocyte cDNA.
Clustal generated nucleotide alignment comparing clone #5 derived from the cDNA library with a Japanese flounder (*P. olivaceus*) EST from the GenBank/EMBL databases (Accession # C82242). The leucocyte cDNA sequence demonstrates 83% similarity over 133 (246-379 nt) region. Conservation of nucleotide similarity is shown as (:).

Three clones, #46/19.2, #07/7 and #09/8, were also found to be similar to Japanese flounder EST sequences derived from a survey of expressed genes in the liver and spleen (Inoue *et al.*, 1997, Appendices 5.1-5.3). No function has been ascribed to any of these ESTs. One clone, #48, was found to be related to two ESTs from zebra fish (*Danio rerio*), one derived from a study of expressed sequences in the kidney (EST: AW421025, Appendix 5.4) and one derived from sequences being expressed during the regeneration of fin tissue (EST: AI330687, Appendix 5.5). Both zebra fish ESTs have been suggested to correspond to that encoding fizzy-related protein from the African clawed frog (*Xenopus laevis*). However, the trout sequence is only 60% identical to both zebra fish sequences over approximately 180 nucleotides and so whether clone 48 corresponds to the fizzy-related protein or not is unknown.

Clone #144/3 appears to correspond to another zebra fish EST from a study of sequences expressed in fin tissue (EST: BE605981, Appendix 5.6). However, the best match in the FASTA analysis was with a human cDNA corresponding to a gene expressed primarily in brain tissue (Accession no. AB028991, Appendix 5.7). The function of the hypothetical protein encoded by this gene is unknown, but is probably related to that of a nuclear movement protein (NUD-1) from *Caenorhabditis elegans* which is required for germline proliferation and embryonic development.

The final cDNA clone appeared to correspond to a murine gene encoding a neuronal development associated protein (NDAP-7) identified during a study of transcripts expressed differentially in primary cultures of GABAergic neurons (Li *et al.*, 2002; Appendix 5.8).

5.3.7 Northern blot analysis of cDNA clones isolated from the stimulated leucocyte cDNA library

Clones 5 and 144/3 were labelled with ^{32}P-dCTP and used as probes in Northern blots of total RNA isolated from stimulated and non-stimulated PBLs. This was in order to try and verify that the cDNA clones did indeed correspond to mRNAs which were differentially expressed in lymphocytes according to their activation state. The trout β-actin cDNA was used as a control probe.

Strong hybridisation signals were observed with the β-actin probe for both RNAs from non-stimulated and stimulated lymphocytes (Figure 5.5). The signals were of equal intensity as anticipated, since β-actin (house-keeping gene) is expected to be constitutively expressed in both tissues and was not expected to be affected by mitogen stimulation. However, no hybridisation was observed with either of the probes (clones: #5, #144/3) isolated from the library.

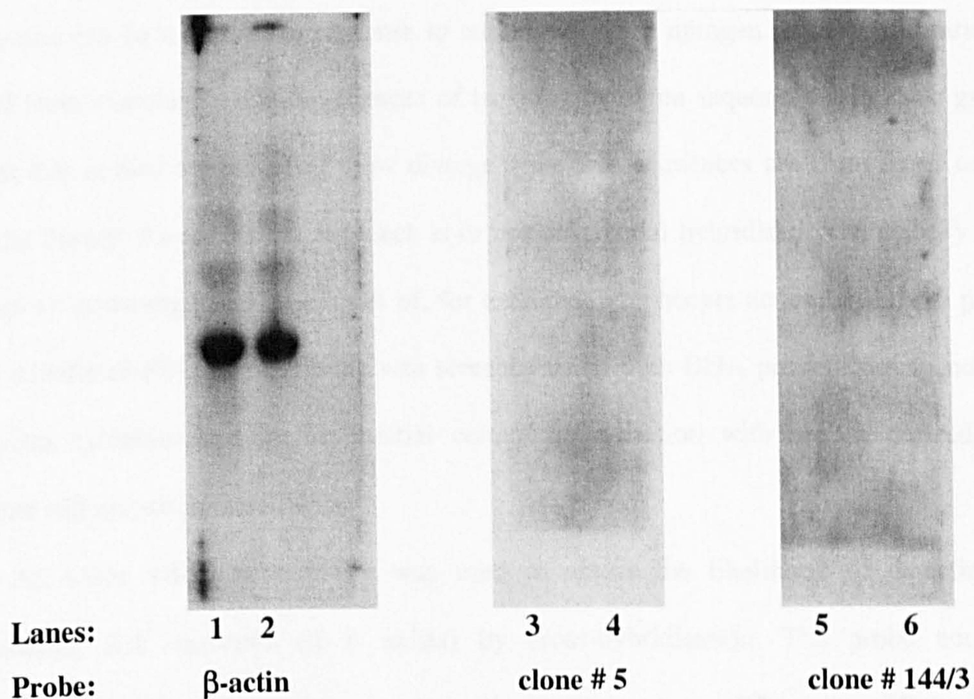


Figure 5.5 Northern blot analysis of positive cDNA clones from the mitogen stimulated leucocyte cDNA library (with ^{32}P α dCTP labelled probes)

RNA from non-stimulated and stimulated PBL was denatured, separated by electrophoresis on a 1% agarose gel and blotted onto a nylon membrane. Hybridisation of RNA from stimulated lymphocytes (Lanes 1,3,5) and non-stimulated lymphocytes (Lanes 2,4,6) was performed with positive clones selected from the leucocyte cDNA library: clone # 5 (Lanes 3-4), clone # 144/3 (Lanes 5-6) and β-actin control probes (1-2). Strong hybridisation signals with the β-actin probe are observed in lanes representing RNA from non-stimulated lymphocytes and stimulated lymphocytes.

5.4 Discussion

Many of the fish genes that play a regulatory role in the immune response, for example genes encoding MHC molecules and T-cell receptors, were identified by cross hybridisation and PCR techniques. However, these approaches have only been successful in the identification of a limited number of cytokines, in particular IL-1β, TNF, TGF-β and FGF (references for these cytokines are presented in Table 5.4). This may be due to the fact that most cytokine genes are transcribed transiently and at low levels (Fujiki *et al.*, 2003) or because the fish cytokines have diverged significantly from the mammalian sequences. Expression of cytokine genes in lymphocytes can be increased in response to stimulation with mitogen. If cDNA libraries are prepared from stimulated cells the chances of isolating cytokine sequences should be greater. However this is also dependent on how divergent the fish sequences are from those used to screen the library. An alternative approach is to use differential hybridisation to identify genes either up- or down-regulated as a result of, for example, lymphocyte activation. In the present study a stimulated PBL cDNA library was screened using both DNA probes corresponding to mammalian cytokines and by differential colony hybridisation with probes derived from stimulated and non-stimulated PBLs.

An ovine γ-IFN gene probe was used to assess the likelihood of detecting the corresponding fish sequence (if it exists) by cross-hybridisation. The probe contained approximately 7 kb of sequence covering the entire ovine γ-IFN gene. However, no hybridisation signal was detected on the Southern blot of trout genomic DNA suggesting that either there are no related sequences present in the fish genome or that the corresponding fish sequence has diverged such that it was not detectable under the hybridisation conditions used. The trout β-actin cDNA was used as a positive hybridisation control. Several bands were detected on the Southern blot. This is not unexpected since β-actin belongs to a multi-gene family whose members might be expected to cross-hybridise.

Table 5.4 Fish cytokine and receptor sequences identified using molecular approaches. *Chemokines IL-8 and γ-IP (γ- interferon inducible protein) are included.

Cytokine	Fish species	Reference
Allograft Inflammatory factor 1	Carp (*C.carpio*)	Fujiki et al 1999
Chemokine -1	Carp (*C.carpio*)	Fujiki et al 1999
CK-1 (CXC chemokine)	R. trout (*O.mykiss*)	Dixon *et al* 1998
IFN-like Protein	Flatfish (*P.olivaceus*)	Tamai *et al* 1993
NK cell enhancing factor	Carp (*C.carpio*)	Fujiki et al 1999
IL-1β	R. trout (*O.mykiss*)	Zou *et al* 1999, Pleguezuelos *et al* 2000
	Sea bass (*D.labrax*)	Scapigliati *et al* 2001
	Carp (*C.carpio*)	Fujiki *et al* 2000, Yin & Kwang 2000 Verburg -Van Kemenade *et al* 1995
	Gilthead Seabream (*S. auratus*)	Pelegrin *et al* 2001
	Turbot (*S. maximus*)	Low *et al.*, 2000
	Catshark (*S.canicula*)	Bird *et al* 2002
	Catfish (*I. punctatus*)	Ellsaesser & Clem 1994
	Goldfish (*C.auratus*)	Bird 2002
	Plaice (*P.platessa*)	Bird 2002
	Zebrafish (*D.rerio*)	Johnson 1998
IL-2	Flatfish (*P.olivaceus*)	Tamai *et al* 1992
*IL-8	R. trout (*O.mykiss*)	Laing *et al.*, 2001
*γ-IP	R. trout (*O.mykiss*)	Laing & Secombes 2001
IL-8	Halibut (*P.Olivaceus*)	Lee *et al* 2001b
	Dogfish (*T.scyllium*)	Inoue *et al.*, 2001
INOS	R. trout (*O.mykiss*)	Laing *et al* 1999b
TGF-β	R. trout (*O.mykiss*)	Hardie *et al* 1998
	Hybrid striped bass (*M.chrysops*)	Harms *et al* 2000
	Carp (*C.carpio*)	Desai *et al.*, 1998
	Plaice (*P.platessea*)	Laing *et al* 2000 (partial)
	European eel (*A.anguilla*)	Laing *et al* 1999a
	Sturgeon (*A.bari*)	Laing *et al* 1999a

Table 5.4 (cont.) Fish cytokine and receptor sequences identified using molecular approaches. Chemokine receptors: CXCR4, CCR7 are included.

Cytokine	Fish species	Reference
TGF-β3	R. trout (*O.mykiss*)	Laing *et al* 1999a
	Eel (*A.anguilla*)	Laing *et al* 1999a
	Sturgeon (*A.baeri*)	Laing *et al* 1999a
	Goldfish (*C.auratus*)	Laing *et al* 1996 (partial)
FGF-3	Zebrafish (*D. rerio*)	Kiefer *et al* 1996
FGF-6	R.trout (*O.mykiss*)	Rescan 1998
FGF-8	Zebrafish (*D.rerio*)	Sleptsova-Friedrich *et al.*, 1997
TNF-α	R. trout (*O.mykiss*)	Laing *et al* 2001a
	J. flounder (*P.olivaceus*)	Hirono *et al* 2000
	Brook trout (*S.fontinalis*)	Bobe *et al* 2000
	Channel catfish (*I.punctatus*)	Zou *et al* 2001
Receptors:		
CXCR4	R. trout (*O.mykiss*)	Daniels *et al* 1999
	Carp (*C. carpio*)	Fujiki *et al* 1999
CCR7	R. trout (*O.mykiss*)	Daniels *et al* 1999
	Carp (*C. carpio*)	Fujiki *et al* 1999
CRγC (common cytokine receptor gamma chain)	R. trout (*O.mykiss*)	Wang & Secombes 2001 (partial)
IL-13 R-2	R. trout (*O.mykiss*)	Lockyer *et al* 2001
IL-1 Receptor-like protein	Salmon (*S.salar*)	Subramaniam *et al* 2001
TNF receptors	J. flounder	Nam *et al* 2000
PDGFRβ	Puffer fish (*F.rubripes*)	How *et al* 1996
CSF1R	Puffer fish (*F.rubripes*)	How *et al* 1996

A cDNA library was constructed from RNA isolated from stimulated fish PBLs. Despite not detecting a hybridisation signal with the ovine γ-IFN gene, it was still considered appropriate to use all the ovine cytokine cDNA probes that were available to screen this cDNA library, but unfortunately no positively hybridising clones were obtained. Although mammalian cytokine probes have been used successfully in hybridisation studies to confirm the presence of corresponding fish genes (Tengelson *et al.*, 1991; Shiels and Wickramasinghe, 1995), cDNAs that have been identified are still quite divergent from the mammalian sequences. For example, the rainbow trout IL-1β gene has 49-56 % a.a. and 43-49% nt identity to mammalian IL-1β genes (Zou *et al.*, 1999a) and the carp IL-1β gene shows similar identities to mammalian IL-1β genes to that shown by the trout gene (Fujiki *et al.*, 2000). However, the trout IL-1β gene is only 36% identical at the nucleotide level and 47% identity at the a.a. level with *C.carpio*, demonstrating the evolutionary divergence within the fish species (Secombes, 2002).

Other researchers have found it equally difficult to isolate fish cytokines using cross-hybridisation. Lee *et al.*, (2001) constructed a cDNA library from mRNA isolated from flounder leucocytes stimulated with bacterial LPS and haemagglutinin. Initial screening of this library with human cytokine gene probes: IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IFN-α, IFN-γ, and lymphotoxin (LT), was unsuccessful and a homologue of the mammalian IL-8 gene was only isolated following the random sequencing of clones.

It was decided to screen the cDNA library with ss-cDNA probes corresponding to mRNAs expressed in "resting" lymphocytes and those expressed by lymphocytes activated with a variety of stimulants (Section 2.6). A probe derived from liver mRNA was also used to try and bias the selection of clones to those that might be specific to lymphocytes. Twenty-four clones were selected as a result of this differential screening. Their cDNA inserts were sequenced and the data compared to the nucleotide databases. In some instances potential amino acid sequences were compared with the protein databases.

Seventeen of the 24 sequences corresponded to globin sequences, haemoglobin sequences or ribosomal RNAs or proteins. It is thought likely that the globin and haemoglobin

clones are probably derived from contaminating RBC. Although careful attention was taken to remove the majority of RBC from the PBL preparations using ammonium chloride/TRIS lysis buffer prior to Percoll density gradient purification, problems of complete separation of lymphocytes from RBCs were encountered. This is probably as a result of fish RBCs being nucleated and therefore having similar densities to that of the lymphocytes. Without complete separation cDNAs derived from RBC would subsequently be found in the cDNA library. It might be expected that production of both ribosomal RNAs and proteins would be increased in activated cells. This is likely because, given the increase in protein production in a cell arising from activation, there is likely to be an increase in the demand for the machinery required for protein production.

The other clones isolated from the differential screening of the library appeared to be similar to a number of different sequences from various EST analyses of fish tissues. Significant nucleotide homologies were found to a number of proteins, including those predicted to be involved in regulating cell proliferation, neocorticogenesis and embryo development.

Others however were similar to sequences to which no specific identities or functions have been assigned. It would be interesting to check both the nucleotide and protein sequence databases at a later date, to ascertain whether any functions have been assigned to these sequences.

For example, clone 144/3 appears to correspond to both human and zebra fish cDNAs that have been tentatively identified as encoding a nuclear movement protein (NudC). The human homologue of NudC, has been identified as a nuclear distribution (nud) factor which interacts with Lis 1, a neuronal migration protein important during neocorticogenesis. Both nuclear movement and neuronal migration are thought to use a common mechanism (Aumais *et al.*, 2001). Clone 48 appears related to a Zebra fish gene which is thought to encode a protein similar to one which has been shown to down-regulate mitotic cyclins and is required for the arrest of cell proliferation in the African clawed frog (Sigrist and Lehner, 1997).

Database searching with a query sequence using the statistical algorithm such as that employed in the FASTA program is designed to predict the likelihood that the query sequence is related to a particular sequence in the database. The E-value produced by the FASTA alogorithm is a

measure of the significance of the match between the query sequence and a sequence from the database. The value given to "E" is calculated as the number of alignments with scores equal to (S) that expected by chance alone. Thus although the FASTA output suggests that the query sequence is related to a sequence in the database it does not guarantee a definitive identification. As examples, the match between clone 57/32 and *O. mykiss* β-globin was given an E-value of 2.9e x 10^{-142}, the match between clone 144/3 and the human KIAA1068 cDNA sequence was 1.1e x 10^{-31}, whereas the match between clone 48 and the zebra fish fizzy-related protein (AI330687) was 0.04. Therefore although we can say with some certainty that clone 57/32 is a β-globin cDNA and that clone 144/3 is probably the equivalent of the human KIAA1068 cDNA, much more characterisation of the cDNA represented by clone 48 would have to be carried out before it could be said for certain that it was the equivalent of the zebra fish fizzy-related sequence.

Northern blotting was performed to try and verify that the transcripts represented by clones 5 and 144/3 were indeed differentially expressed in lymphocytes according to their activation status. Unfortunately however no hybridisation signals were obtained on the blots. This may be due to the fact that the sequences represent transcripts which are present in the lymphocyte mRNA population at relatively low levels.

It could be predicted that the cDNA library constructed from the mRNA isolated from stimulated lymphocytes would be enriched with sequences involved in the regulation of fish immune responses. However, unfortunately none of the clones isolated from the library have been positively identified as having an immunoregulatory role. Isolating fish cytokine genes has proved on the whole to be challenging. Techniques for screening cDNA libraries and for trying to enrich cell populations for particular cytokines are constantly being developed and refined. It may be that the random approach of EST analyses of lymphoid tissues will be as successful if not more so, than a targeted approach.

CHAPTER 6

General Discussion

General Discussion

The components that comprise the fish immune system are less well defined than those of mammalian species. Current genetic and or functional evidence however, supports the existence of various effectors of innate immunity in teleosts, including NK cells (Hogan *et al.*, 1997; Miller *et al.*, 1998; Stuge *et al.*, 1997), complement (Yano, 1996a,b), antimicrobial peptides (Silphaduang and Noga, 2001), and IFN (Altmann *et al.*, 2003).

The main aim of this study was to investigate the biological activity of a macrophage activating factor (MAF) produced by activated lymphocytes from the rainbow trout (*O. mykiss*) and to discover its molecular source. Initially, the activation of rainbow trout peripheral blood lymphocytes to release factors with MAF activity, following incubation with a variety of stimulants was assessed (Chapter 3). Subsequently, two alternate molecular approaches were taken, to try and isolate molecules which were responsible for the MAF activity in particular and homologues of mammalian cytokines in general. Degenerate primers, based on conserved sequences derived from a range of mammalian and avian cytokines, were designed and used in the RT- PCR in order to isolate the equivalent sequences in the trout (Chapter 4). In conjunction, a cDNA library from stimulated peripheral blood lymphocytes was constructed and screened differentially using ovine and trout probes, to isolate cytokine or immune-response related cDNAs / genes (Chapter 5).

It is well established that fish macrophages can be activated '*in vitro*' and '*in vivo*' (Olivier *et al.*, 1986; Chung and Secombes, 1987; Graham and Secombes, 1988; Marsden *et al.*, 1994). This activation is brought about by one or more molecules termed MAF(s) released from head kidney and peripheral blood lymphocytes. In particular it has been shown that fish lymphocytes release factors with MAF activity following stimulation with the T cell mitogen Con A (Graham and Secombes, 1988). Several groups have demonstrated the biological activity of MAFs in fish using homologous assay systems (Secombes *et al.*, 1996, 1998) but have failed to purify the molecules sufficiently to allow definitive identification of the protein(s) responsible. For example, secretion of fish MAF from activated lymphocytes has been

demonstrated and suggested to be as a result of the fish equivalent of mammalian IFN-γ (Graham and Secombes, 1990a,b), but complete purification of the factor and cloning of the corresponding gene/s have not yet been reported.

This study demonstrated that macrophage activation could be assessed by at least two different methods, the NBT colourimetric assay or the luminol-dependent chemiluminescent assay. It was found however, that a number of factors can affect the level of macrophage activation detected when assessing respiratory burst using the NBT assay and that in this study more reproducible results were obtained using the luminol-dependent chemiluminescent assay. The factors which influenced detection of MAF activity included incubation times of lymphocyte supernatants with macrophages, the concentration of CFS and the macrophage cell density and purity. Although the presence of un-diluted CFS actually inhibited the activation of the target cells (cultures incubated for more than 48 hour with MAF supernatants lead to a decrease in macrophage activity in the NBT assay unless the supernatants were changed, as demonstrated by Graham and Secombes (1988), the continuous presence of MAF seems necessary to achieve maximal stimulation of macrophages. This may reflect increased activity in individual activated macrophage cells, probably associated with a need for multiple cycles of receptor-ligand complex internalisation (Evered *et al.*, 1986). Interestingly there was no difference in the level of stimulation observed with macrophages left in culture overnight before the addition of supernatants (results not shown) when compared with macrophages cultured for several days prior to use as observed by Graham and Secombes (1988). This contrasts with some mammalian studies which suggest that the longer the macrophages are cultured before the addition of CFS the less responsive they are (Nacy *et al.*, 1984). Similarly it was found that macrophages could be cultured at a higher density than is generally optimal for mammalian cells.

Possibly, as a consequence of the relatively low stimulation indices it was difficult to obtain reproducible results from the NBT assay. Therefore it was decided to try an alternative assay which could be performed on freshly isolated macrophages in the hope that they might be relatively more responsive to the CFS. In these bioassay experiments, the respiratory burst was

measured using the leucocyte chemiluminescence response amplified with the chemiluminescent enhancing substrate, Luminol. This is a non-specific indicator which reacts with a variety of ROS including, superoxide, hydrogen peroxide and singlet oxygen (Allen *et al* 1972; Scott and Klesius, 1981). The measurement of chemiluminescence emitted by stimulated phagocytes is a sensitive indicator of their oxidative microbiocidal activity (Klebanoff, 1982; DeChatelet *et al.*, 1982). The ability to measure the induced respiratory burst response of macrophages using Luminol has enabled the investigation of phagocytic responses to pathogens (Easmon *et al.*, 1980; Stave *et al.*, 1983, 1985). For example, virulence mechanisms of fish bacterial pathogens in relation to their ability to stimulate phagocytic cells have been assessed in the striped bass, *Morone saxitilis* (Walbaum) which exhibit a unique chemiluminescence response to different genera of bacterial fish pathogens, suggesting that bacteria possess factors that modulate interaction with phagocytic cells (Stave *et al.*, 1983). Luminol-dependent chemiluminescence has also been used to determine the nutritional '*in vitro*' effects on the amount of ROS produced during the respiratory burst activity of Atlantic salmon HK phagocytes (Lygren and Waagbø, 1999). In this study it was found that addition of CFS from activated lymphocytes to macrophages produced an immediate response which decayed over the following 40 minutes. The quality of response was dependent on the source of the CFS and although there was considerable variation in the maximum RLU values obtained from macrophages isolated from different fish this did not interfere with the interpretation of the assay results.

If parallels can be drawn with the mammalian immune system, one of the obvious candidates responsible for activating macrophages would be the fish equivalent of IFN-γ. Genes for IFN-α, β, γ and τ group interferons have been cloned from a variety of mammals, including humans (Lawn *et al.*, 1981a,b), mice (Shaw *et al.*, 1983; Daugherty *et al.*, 1984) and pigs (Lefevre and La Bonnardiere, 1986), and several avian species such as ducks (Schultz *et al.*, 1995), chickens (Sekellick *et al.*, 1994) and turkeys (Suresh *et al.*, 1995). Despite demonstration of IFN-like antiviral biological activity in a variety of fish species (Nygaard *et al.*, 2000), at the

outset of this study there had been no reports of an interferon gene being cloned from any fish species apart from one report describing a flatfish cDNA putatively encoding IFN (Tamai *et al.*, 1993). The flatfish IFN sequence had been obtained using PCR amplification. Analysis of its predicted amino-acid sequence with the protein sequence databases using the basic local alignment search tool (BLAST) revealed >60% identity between two-thirds of this sequence and sequences from filamentous phage (Magor and Magor, 2001). In fact, the only stretch of significant homology found between the fish sequence and mammalian IFN α/β sequences lies in the area corresponding to the PCR degenerate primer sequences. Little homology was found over the remaining sequence, with only 14% overall identity with the human IFN-β sequence (Taniguchi *et al.*, 1980). This is an illustration of the dangers inherent in the PCR approach to cloning unknown sequences across species using degenerate primers and will be discussed in more detail later.

The immune IFNs of mammals comprise of three basic forms, α, β and γ. IFN-α and IFN-β are collectively referred to as Type I IFN, whereas IFN-γ is also known as Type II IFN. Type I and Type II IFNs are distinguished by the fact that the Type I genes contain no introns whereas the IFN-γ gene contains three. In addition, the Type I IFNs are produced by a variety of cell types, whilst IFN-γ is expressed only by activated lymphocytes and NK cells. There are multiple isoforms of IFN-α, encoded by multiple loci, whereas IFN-β and IFN-γ are encoded by separate single loci. Despite having functionally similar anti-viral activities, IFN-β shares only 20-30% a.a. sequence identity with any particular IFN-α (Vilcek and Sen, 1996), whereas there is no obvious sequence relationship between Type I and Type II IFNs (Pestka *et al.*, 1987; Leonard, 1999; Biron and Sen, 2001)). In an attempt to demonstrate the presence of IFN genes in the fish, mammalian (human) IFN-α and IFN-β cDNA probes have been used to probe for homologous sequences in fish genomic DNA. Studies by Wilson *et al.*, 1983, demonstrated that both human IFN-α and IFN-β cDNA probes hybridised to a range of non-mammalian vertebrate DNAs from birds to bony fish. Thus, suggesting the presence of a fish sequence with homology to human IFN-β gene. Similar results were also obtained by Tengelson *et al.*, (1991) who

demonstrated that the human IFN-β gene hybridised with genomic DNA from rainbow trout and coho and chinook salmon. Despite this no actual teleost gene sequence corresponding to the human IFN-β was isolated in these studies. In this study the ovine IFN-γ gene was used to probe genomic DNA from the rainbow trout, but unlike the previous studies with the human IFN-β gene no hybridisation between the ovine IFN-γ gene and trout DNA was observed. This could be as a result of there not being an IFN-γ gene present in the trout genome or because the corresponding sequences are so divergent that hybridisation could not be detected under the conditions used. It is known that avian species, which are thought to have diverged from mammals approximately 350 million years ago, have been found to contain both Type I and Type II IFN genes. Although the genes are very similar in structure to those found in mammals (for example, the Type I genes are intron-less and the IFN-γ gene has four exons and three introns) there is very little cross-reactivity in terms of either DNA hybridisation or antigenicity.

Until very recently the existence of IFN genes within the fish genome had remained speculative, with the only evidence being the fact that IFN-like anti-viral activity can be induced in fish using a number of different stimuli (Trobridge *et al.*, 1997, 1995; Kim and Ponka, 2000). In the last year however the cloning and characterisation of an IFN gene from zebrafish has been reported (Altmann *et al.*, 2003). Thirty-two Type I IFN (α/β/ω/δ/κ/τ) gene sequences of vertebrates, mainly mammals and birds, were employed in the PCR homology cloning approach to isolate the zebrafish (zf) IFN. The isolated cDNA possesses 15% and 14% identity (protein) over the entire sequence to human IFN α and β respectively (with 25% and 24% over the conserved domain), and 18% and 16% identities to chicken IFN α and β respectively. Its expression can be induced by poly (I)-poly(C), a known inducer of mammalian IFN, and the expressed protein is able to reduce viral plaque formation in a zebra fish cell line (Eaton, 1990).

The zfIFN sequence was used to search the completed fugu genome sequence for similar sequences. Only one such sequence was found suggesting that teleost fish, such as fugu and zebrafish may contain only one form of Type I IFN. Interestingly, the putative fugu gene shares only 27% identity with zfIFN (Altmann *et al.*, 2003). A putative catfish (cf)IFN cDNA

has also been identified by BLASTX screening of a cf EST library generated from a mixed lymphocyte population enriched for NK-like cells (Long *et al.*, 2003). Analysis of the deduced a.a. sequence of cfIFN demonstrated 15% similarity to human IFNα and 13% similarity to human IFN-β, whereas the cfIFN showed 35% similarity to the zfIFN and only 9% identity to putative flounder IFN (Tamai *et al.*, 1993) and 11% identical to the fugu IFN sequence. Similar to the zfIFN, the expression of cfIFN mRNA in catfish ovary (CCO) cells was up-regulated following exposure to UV-CRV or treatment with poly I:C, both of which induce IFN in mammals and birds (Long *et al.*, 2003). This up-regulation was accompanied by the presence of an antiviral factor in the culture medium. Recombinant cfIFN was then produced and shown to protect CCO cells from catfish herpesvirus (CCV) challenge.

The relatively low levels of homology between the fish IFN sequences may reflect the fact that each represents a different Type I gene. However, low levels of homology have also been found between the IL-1β sequences from different fish species. The trout IL-1β molecule, which is 28-31% a.a identical to various mammalian IL-1β cDNAs, is only 36% identical to the carp IL-1β sequence. In addition, although the Japanese flounder and salmonid (brook trout) TNF sequences have similar homology to mammalian TNFα and TNFβ (typically 30-37% a.a. identity), the salmonid TNF sequence has a relatively low homology to the known rainbow trout sequence (Hirono *et al.*, 2000; Laing *et al.*, 2001a). This suggests that there is a relatively large evolutionary distance between certain fish species and as a consequence care should be taken when trying to extrapolate results in one fish species to a quite separate fish species.

Although the genetic and functional assays support the hypothesis that catfish, zebrafish and fugu contain at least one IFN gene responsible for antiviral activity, it is unclear what the exact genetic relationship is to the mammalian Type I IFNs. Despite demonstrating a slightly higher a.a. similarity to IFN-α genes than IFN-β genes, other observations suggest that the cfIFN at least is more β-like than α-like. Similarly to mammalian IFN-β, cfIFN contains one potential N-glycosylation site and also possesses three rather than four or more cysteine residues as are found in the mature mammalian and avian IFN-αs. In addition, the highly conserved

CAWE sequence motif found within most IFN-α genes is notably absent from the cfIFN (Long *et al.*, 2003). What does seem apparent at this stage is that the IFN sequences obtained from these fish do not appear to be related to the mammalian IFN-γ gene. Equally it has not been tested whether these recombinant fish IFNs are able to activate macrophages and therefore the status of a fish equivalent to the mammalian IFN-γ is still unknown. It may well be that fish do only possess the one IFN gene and do not possess the more highly evolved IFN classes seen in mammals and birds. It is possible that following the divergence of tetrapods from bony fish ~450 million years ago (Kumar and Hedges, 1998), mammalian IFN genes underwent considerable duplication and diversification leading to the generation of three main classes of immune IFN genes, whereas teleost IFN genes failed to expand or diversify (Long *et al.*, 2003).

A similar lack of expansion and diversification is seen in the teleost Ig heavy chain genes. Although presumably derived from a common ancestor, fish possess only two Ig heavy chain isotypes (Igμ and Igδ), whereas mammals possess five Ig isotypes (IgG, IgM, IgA,IgD, and IgE) two of which (IgG and IgA) are characterised by the presence of four and two subclasses, respectively (Miller *et al.*, 1998). Only as more IFN sequences from fish and other lower-order vertebrates are identified and characterised, will a clearer picture of the evolution of this gene family will be obtained.

Biological evidence for the existence of other fish cytokine homologues has also led to a search for their corresponding gene(s). Several approaches have been taken, including PCR-based cloning, the screening of cDNA libraries with mammalian and fish cDNA probes and the systematic analysis of EST libraries. No matter which approach has been taken efforts have been made to maximise the likelihood that the RNA population being screened is likely to contain the mRNAs of interest. For example, cytokine gene expression has been induced either *in vitro* by mitogen stimulation or *in vivo* using bacterial or viral pathogens of some sort of selective enrichment for cells likely to express the target sequence(s) has been used (Marsden *et al.*, 1996a,b).

The most common approach taken to clone fish cytokine genes has been PCR based cloning in which highly conserved regions of the mammalian and avian sequences have been used for primer design, as illustrated above for the Type I IFNs. This approach has been successful for the cloning of several cytokine cDNAs from teleost species including Rainbow trout, Atlantic salmon and Plaice. However the cloning of the flatfish "IFN" gene and the results from this study demonstrate the potential problems of this approach. The more degenerate the primer the greater the chance of amplifying a product which may not be related to the original target. This is illustrated in this study. The β-actin cDNA was successfully amplified from the rainbow trout using primers based on the salmon sequence. No ambiguities were built into the primer sequences, although there may well have been mis-matches between the primers and the target sequence. It had been assumed that being a structural protein the β-actin sequence would be highly conserved between trout and salmon as it is between mammals. This was borne out in practice. However cytokine sequences are generally not as well conserved as structural proteins and often have diversified to such an extent that their biological activity is species restricted. In this study primers were based on regions of the IFN-γ protein which were relatively well conserved across sixteen mammalian and six avian species. At the DNA level however this required primers with ambiguities at five out of 18 and three out of 18 sites. Although a PCR product of approximately 280 bp was amplified (assuming the trout IFN-γ would be similar in size to the mammalian and avian sequences) subsequent analysis found that the sequence was not related to IFN-γ or any other sequences in either the DNA or protein databases. The same strategy was used to try and amplify the cDNA corresponding to mammalian IL-15, a T- cell growth factor known to induce the production of IFN-γ. Again despite amplifying a DNA fragment of approximately the correct size there appeared to be no relationship between it and the known IL-15 sequences.

The screening of cDNA libraries using cross-reacting DNA probes has been successful for the cloning of trout TGFβ and IL1β, amongst others. However, in this study no positively hybridising clones were obtained using 13 sheep cytokine cDNAs as probes. It may well be that

the stringency of hybridisation used was too high to detect cross-hybridisation or simply that the fish equivalents of the ovine cytokines used were not represented in the library. This is unlikely however since the library had been constructed from mRNA populations pooled from a number of different cell cultures stimulated by a variety of methods. As an alternative strategy the library was screened using cDNA probes derived from stimulated and non-stimulated PBLs in order to detect mRNAs which might have been upregulated as a result of *in vitro* stimulation. A number of positive clones were obtained from this differential screening of the library, but apart from a number of cDNAs showing similarity to other unidentified fish sequences as well as to a number of proteins predicted to be involved in regulation of cell proliferation, neocorticogenesis and embryo development, a large proportion of the sequences isolated from this screening of the library were of either globin or ribosomal (rRNA) origin.

Other research groups have taken alternative approaches to enrich for cDNA sequences expressed under particular conditions. These include subtractive hybridisation and suppression subtraction techniques (Lee *et al.*, 2001). Subtractive cDNA hybridisation has been a powerful approach to identify and isolate cDNAs of differentially expressed genes (Hedrick *et al.*, 1984; Duguid and Dinauer, 1990; Hara *et al.*, 1991). Despite the successful identification of numerous genes for example, T-cell receptors (Hedrick *et al.*, 1984) by these methods, they are usually inefficient for obtaining low abundant transcripts (as they require increased amounts of poly $(A)^+$ RNA, involve multiple or repeated subtraction steps and are labour intensive).

Suppression subtractive hybridisation (SSH) is designed to generate cDNA libraries highly enriched for differentially expressed genes of both high and low abundance, since it utilises PCR amplification after equalisation of the subtracted fragments (Diatchenko *et al.*, 1996). SSH allows selective amplification of target cDNA fragments and simultaneously suppress non target DNA amplification. The subtraction method overcomes the problem of differences in mRNA abundance by inclusion of a normalisation step, which equalises the abundance of cDNAs within the target population whilst the subtraction step excludes the common sequences between the target and driver populations (Diatchenko *et al.*, 1996). In

addition, the uncloned subtracted cDNA mixture can be used as a hybridisation probe to allow the screening of recombinant DNA libraries.

SSH has resulted in the successful cloning and sequencing of several fish homologues of mammalian cytokines such as IL-1β (Fujiki *et al.*, 1998) and TGF-β in Carp (Yin and Kwang, 2000) as well as other rare gene transcripts to be found from the fish immune system (Fujiki *et al.*, 1999, 2000; Bayne, 2000; Alonso and Leong 2002; Liu *et al.*, 2002; Sangrador-Vegas *et al.*, 2002).

More recently the systematic analysis of EST libraries prepared from activated leukocytes has been used successfully for isolating a variety of fish cDNAs. EST analysis involves determining the partial sequence of DNA clones selected randomly from a cDNA library and has been used successfully for identifying novel genes, as well as characterising the expression profile of genes in particular cells, tissues or species (Adams *et al.*, 1991, 1993; Davies *et al.*, 1994; Hwang *et al.*, 1995; Wolfsberg and Landsman, 1997). Several homologues of biodefence molecules from fish have been identified using an EST approach, such as complement components C3, C7, C8 and C9, complement component factor (Katagiri *et al.*, 1998, 1999), an IFN regulatory factor (Yabu *et al.*, 1998), lysozyme (Hikima *et al.*, 1997) transferrin (Kim *et al.*, 1996) and various others (Inoue *et al.*, 1997).

Nam *et al* (2000) constructed a cDNA library of *P. olivaceus* leucocytes infected with HRV in order to analyse genes induced and expressed as a result of the virus infection. Twenty-one different cDNAs associated with the immune-response, including a homologue of IL-8 were obtained from 300 randomly selected cDNAs that were sequenced, suggesting that EST analysis is a useful and powerful method for accumulating information on the fish immune response to viral infection.

The ability of some fish cytokines to cross-react with antibodies directed against mammalian cytokines has been reported (Manning and Nakanishi, 1996). Despite this cross-reactivity, screening of an LPS-stimulated leucocyte cDNA library from Japanese flounder with antibodies against human cytokines IFN-α and IL-4, did not actually yield any positive results.

Although it is unlikely that fish possess all the cytokine genes present in mammals, progress in the field of fish cytokine biology is steadily progressing. Doubt still remains as to whether or not fish possess the extended family of IFNs seen in mammals and birds and similarly, evidence suggests that fish probably do not possess the multi-gene chemokine families seen in higher vertebrates. Other cytokines, such as IL-1 do appear to be present as a multi-gene family as it is in mammals and birds. For example, multiple copies of IL-1β genes have been found in the carp (Fujiki *et al.*, 2000; Engelsma *et al.*, 2001) and in goldfish (Bird, 2002). In addition, some genes appear to have diverged to a greater extent in fish than in mammals. For example, two different copies of the TGFβA and TGFβB genes are found in fish, but only one copy of each gene exists in higher vertebrates.

The relative lack of success in cloning fish cytokines via DNA cross-hybridisation or PCR techniques may mean that any future strategy for cloning fish cytokine sequences may require targeting the biological activity through expression libraries. Only as more cytokine genes and other immune-regulatory genes are discovered will it become clearer as to the extent of conservation between the fish and mammalian immune systems. In addition, it may well be discovered that fish have evolved unique cytokine genes or activities not seen in the mammals.

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Appendix 3.1

One-way ANOVA and Tukey's pairwise comparisons: lymphocyte stimulation responses using different mitogens

```
Analysis of Variance for SI
Source      DF        SS         MS        F        P
Mitogen     19    501.65      26.40    23.39    0.000
Error       40     45.15       1.13
Total       59    546.80
                                      Individual 95% CIs For Mean
                                      Based on Pooled StDev
Level        N      Mean     StDev    ---+---------+---------+---------+---
ConA1        3     0.615     0.312     (---*--)
ConA10       3     3.362     0.917             (---*--)
ConA20       3     3.709     0.930              (---*--)
ConA5        3     2.862     1.471            (--*---)
LPS1         3     6.106     1.718                      (--*---)
LPS10        3     9.972     0.391                                 (--*---)
LPS20        3     9.924     1.004                                 (--*---)
LPS5         3     8.362     3.574                            (---*--)
PHA1         3     3.716     0.733              (---*--)
PHA10        3     2.463     0.056          (---*---)
PHA20        3     1.466     0.280        (--*---)
PHA5         3     3.133     0.810            (---*--)
PMA1         3     0.880     0.110     (---*--)
PMA10        3     0.194     0.015    (---*--)
PMA20        3     0.315     0.191    (---*--)
PMA5         3     0.322     0.114    (---*--)
PW1          3     2.976     0.369            (---*--)
PW10         3     3.149     0.222            (---*---)
PW20         3     4.532     0.361                (---*--)
PW5          3     3.276     0.254             (--*---)
                                      ---+---------+---------+---------+---
Pooled StDev =     1.062                 0.0       3.5       7.0      10.5

Tukey's pairwise comparisons
    Family error rate = 0.0500
Individual error rate = 0.000498

Critical value = 5.36

Intervals for (column level mean) - (row level mean)

               ConA1      ConA10      ConA20      ConA5       LPS1        LPS10

  ConA10      -6.035
               0.541

  ConA20      -6.382      -3.635
               0.193       2.940

  ConA5       -5.534      -2.787      -2.440
               1.041       3.788       4.135

  LPS1        -8.779      -6.032      -5.684      -6.532
              -2.203       0.544       0.891       0.044

  LPS10      -12.644      -9.897      -9.550     -10.398      -7.154
              -6.069      -3.322      -2.974      -3.822      -0.578

  LPS20      -12.596      -9.849      -9.502     -10.350      -7.106        -
3.240
              -6.021      -3.274      -2.926      -3.774      -0.530

3.336
```

	LPS20	LPS5	PHA1	PHA10	PHA20	PHA5
LPS5	-11.035	-8.288	-7.940	-8.788	-5.544	-1.678
	-4.459	-1.712	-1.365	-2.213	1.032	4.897
PHA1	-6.388	-3.642	-3.294	-4.142	-0.898	2.968
	0.187	2.934	3.281	2.434	5.678	9.544
PHA10	-5.136	-2.389	-2.042	-2.889	0.355	4.220
	1.439	4.186	4.534	3.686	6.930	10.796
PHA20	-4.139	-1.392	-1.044	-1.892	1.352	5.218
	2.437	5.184	5.531	4.684	7.928	11.793
PHA5	-5.805	-3.058	-2.711	-3.559	-0.315	3.551
	0.770	3.517	3.865	3.017	6.261	10.127
PMA1	-3.552	-0.805	-0.458	-1.306	1.938	5.804
	3.023	5.770	6.118	5.270	8.514	12.380
PMA10	-2.867	-0.120	0.227	-0.620	2.624	6.489
	3.708	6.455	6.803	5.955	9.199	13.065
PMA20	-2.988	-0.241	0.106	-0.742	2.503	6.368
	3.587	6.334	6.682	5.834	9.078	12.944
PMA5	-2.995	-0.248	0.100	-0.748	2.496	6.362
	3.581	6.328	6.675	5.828	9.072	12.937
PW1	-5.649	-2.902	-2.555	-3.403	-0.158	3.707
	0.926	3.673	4.021	3.173	6.417	10.283
PW10	-5.821	-3.074	-2.727	-3.575	-0.330	3.535
	0.754	3.501	3.849	3.001	6.245	10.111
PW20	-7.204	-4.457	-4.110	-4.958	-1.714	2.152
	-0.629	2.118	2.466	1.618	4.862	8.728
PW5	-5.949	-3.202	-2.854	-3.702	-0.458	3.408
	0.627	3.374	3.721	2.874	6.118	9.983

LPS5	-1.726 4.849					
PHA1	2.920 9.496	1.358 7.934				
PHA10	4.172 10.748	2.611 9.186	-2.035 4.540			
PHA20	5.170 11.745	3.608 10.184	-1.038 5.538	-2.290 4.285		
PHA5	3.503 10.079	1.942 8.517	-2.705 3.871	-3.957 2.619	-4.954 1.621	
PMA1	5.756 12.332	4.195 10.770	-0.452 6.124	-1.704 4.872	-2.701 3.874	- 1.035 5.541
PMA10	6.441 13.017	4.880 11.455	0.234 6.809	-1.019 5.557	-2.016 4.559	- 0.350 6.226
PMA20	6.320 12.896	4.759 11.334	0.112 6.688	-1.140 5.436	-2.137 4.438	- 0.471 6.105
PMA5	6.314 12.889	4.752 11.328	0.106 6.682	-1.146 5.429	-2.144 4.432	- 0.477 6.098
PW1	3.659 10.235	2.098 8.673	-2.548 4.027	-3.801 2.775	-4.798 1.777	- 3.132 3.444
PW10	3.487 10.063	1.926 8.501	-2.721 3.855	-3.973 2.603	-4.970 1.605	- 3.304 3.272
PW20	2.104 8.680	0.543 7.118	-4.104 2.472	-5.356 1.220	-6.353 0.222	- 4.687 1.889
PW5	3.360 9.935	1.798 8.374	-2.848 3.728	-4.100 2.475	-5.098 1.478	- 3.431 3.144

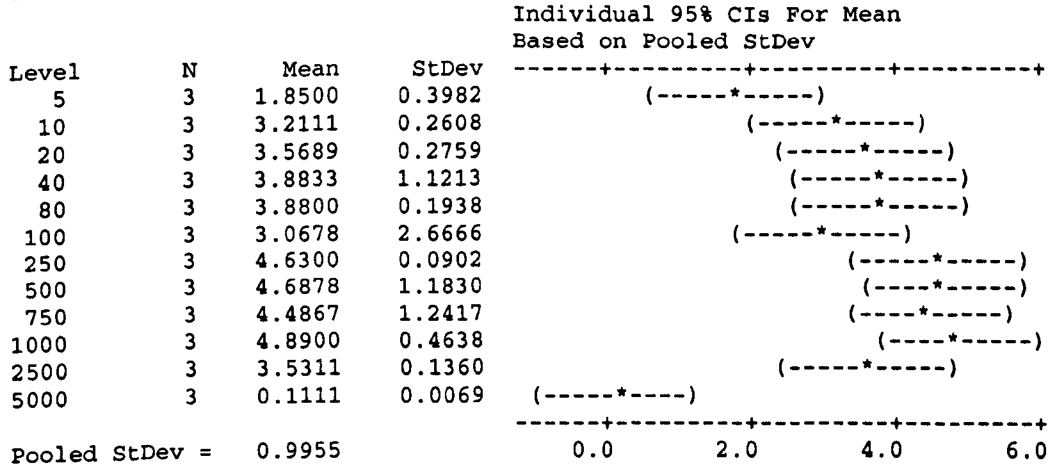
	PMA1	PMA10	PMA20	PMA5	PW1	PW10
PMA10	-2.603 3.973					
PMA20	-2.724 3.852	-3.409 3.167				
PMA5	-2.730 3.845	-3.415 3.160	-3.294 3.281			

PW1	-5.385	-6.070	-5.949	-5.942		
	1.191	0.506	0.627	0.633		
PW10	-5.557	-6.242	-6.121	-6.114	-3.460	
	1.019	0.334	0.455	0.461	3.116	
PW20	-6.940	-7.625	-7.504	-7.497	-4.843	-4.671
	-0.364	-1.049	-0.928	-0.922	1.733	1.905
PW5	-5.684	-6.369	-6.248	-6.242	-3.587	-3.415
	0.891	0.206	0.327	0.334	2.988	3.160

	PW20
PW5	-2.032
	4.543

Appendix 3.2

One-way ANOVA and Tukey's pairwise comparisons: lymphocyte stimulation responses using PMA at varying concentrations



```
Analysis of Variance for SI
Source     DF        SS          MS         F         P
Conc.      11    61.093       5.554      5.60     0.000
Error      24    23.782       0.991
Total      35    84.875
                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean       StDev  ------+---------+---------+---------+
   5        3    1.8500      0.3982           (-----*-----)
  10        3    3.2111      0.2608                  (-----*-----)
  20        3    3.5689      0.2759                    (-----*-----)
  40        3    3.8833      1.1213                     (-----*-----)
  80        3    3.8800      0.1938                     (-----*-----)
 100        3    3.0678      2.6666                 (-----*-----)
 250        3    4.6300      0.0902                         (-----*-----)
 500        3    4.6878      1.1830                          (----*-----)
 750        3    4.4867      1.2417                         (----*-----)
1000        3    4.8900      0.4638                           (----*-----)
2500        3    3.5311      0.1360                    (-----*-----)
5000        3    0.1111      0.0069  (-----*----)
                                     ------+---------+---------+---------+
Pooled StDev =   0.9955                   0.0       2.0       4.0       6.0
```

Tukey's pairwise comparisons

Family error rate = 0.0500
Individual error rate = 0.00142

Critical value = 5.10

Intervals for (column level mean) - (row level mean)

	5	10	20	40	80	100
10	-4.2922					
	1.5700					
20	-4.6500	-3.2889				
	1.2122	2.5733				
40	-4.9644	-3.6033	-3.2455			
	0.8978	2.2589	2.6167			
80	-4.9611	-3.6000	-3.2422	-2.9278		
	0.9011	2.2622	2.6200	2.9344		
100	-4.1489	-2.7878	-2.4300	-2.1155	-2.1189	
	1.7133	3.0744	3.4322	3.7467	3.7433	
250	-5.7111	-4.3500	-3.9922	-3.6778	-3.6811	-4.4933
	0.1511	1.5122	1.8700	2.1844	2.1811	1.3689
500	-5.7689	-4.4078	-4.0500	-3.7355	-3.7389	-4.5511
	0.0933	1.4544	1.8122	2.1267	2.1233	1.3111

750	-5.5678	-4.2067	-3.8489	-3.5344	-3.5378	-4.3500
	0.2944	1.6555	2.0133	2.3278	2.3244	1.5122
1000	-5.9711	-4.6100	-4.2522	-3.9378	-3.9411	-4.7533
	-0.1089	1.2522	1.6100	1.9244	1.9211	1.1089
2500	-4.6122	-3.2511	-2.8933	-2.5789	-2.5822	-3.3944
	1.2500	2.6111	2.9689	3.2833	3.2800	2.4678
5000	-1.1922	0.1689	0.5267	0.8411	0.8378	0.0256
	4.6700	6.0311	6.3889	6.7033	6.7000	5.8878

	250	500	750	1000	2500
500	-2.9889 2.8733				
750	-2.7878 3.0744	-2.7300 3.1322			
1000	-3.1911 2.6711	-3.1333 2.7289	-3.3344 2.5278		
2500	-1.8322 4.0300	-1.7744 4.0878	-1.9755 3.8867	-1.5722 4.2900	
5000	1.5878 7.4500	1.6456 7.5078	1.4445 7.3067	1.8478 7.7100	0.4889 6.3511

Appendix 3.3

One-way ANOVA and Tukey's pairwise comparisons: respiratory burst responses of head kidney macrophages, using MAF containing supernatnats from 0 and 48 h stimulated lymphocytes

Analysis of Variance for OD

Source	DF	SS	MS	F	P
Dilution	7	0.060288	0.008613	8.73	0.000
Error	16	0.015787	0.000987		
Total	23	0.076075			

```
                                             Individual 95% CIs For Mean
                                             Based on Pooled StDev
Level        N      Mean     StDev  ------+---------+---------+---------+
1/10 0hr     3   0.11633   0.02754          (-----*----)
1/10 48h     3   0.22900   0.04424                         (-----*----)
1/100 0h     3   0.06667   0.00702  (-----*----)
1/100 48     3   0.10700   0.01473         (----*-----)
Control      3   0.07333   0.00473   (----*-----)
control      3   0.07333   0.00473   (----*-----)
Neat 0hr     3   0.07200   0.00458   (----*-----)
Neat 48h     3   0.11067   0.06962         (-----*----)
                                    ------+---------+---------+---------+
Pooled StDev =   0.03141               0.070     0.140     0.210     0.280
```

Tukey's pairwise comparisons

Family error rate = 0.0500
Individual error rate = 0.00319

Critical value = 4.90

Intervals for (column level mean) - (row level mean)

	1/10 0hr	1/10 48h	1/100 0h	1/100 48	Control	control
1/10 48h	-0.20153 -0.02380					
1/100 0h	-0.03920 0.13853	0.07347 0.25120				
1/100 48	-0.07953 0.09820	0.03314 0.21086	-0.12920 0.04853			
Control	-0.04586 0.13186	0.06680 0.24453	-0.09553 0.08220	-0.05520 0.12253		
control	-0.04586 0.13186	0.06680 0.24453	-0.09553 0.08220	-0.05520 0.12253	-0.08886 0.08886	
Neat 0hr	-0.04453 0.13320	0.06814 0.24586	-0.09420 0.08353	-0.05386 0.12386	-0.08753 0.09020	- 0.08753 0.09020
Neat 48h	-0.08320 0.09453	0.02947 0.20720	-0.13286 0.04486	-0.09253 0.08520	-0.12620 0.05153	- 0.12620 0.05153

	Neat 0hr
Neat 48h	-0.12753 0.05020

Appendix 3.4

One-way ANOVA and Tukey's pairwise comparisons: Effect of serum on CL response of head kidney macrophages

```
Analysis of Variance for log10 RLU
Source     DF        SS        MS        F        P
Treatment   2  1.901564  0.950782  1157.27    0.000
Error       6  0.004929  0.000822
Total       8  1.906493
                                   Individual 95% CIs For Mean
                                   Based on Pooled StDev
Level       N      Mean     StDev  ------+---------+---------+---------+
+FBS/PMA    3    6.9086    0.0329                                  (*-)
-FBS/PMA    3    6.3542    0.0065                       (-*)
L-15        3    5.7828    0.0366  (*)
                                   ------+---------+---------+---------+
Pooled StDev =   0.0287                5.95      6.30      6.65      7.00

Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.0220

Critical value = 4.34

Intervals for (column level mean) - (row level mean)

          +FBS/PMA    -FBS/PMA

-FBS/PMA     0.4826
             0.6263

L-15         1.0541      0.4996
             1.1977      0.6433
```

Appendix 3.5

One-way ANOVA and Tukey's pairwise comparisons: CL response of head kidney macrophages using CFS from PMA stimulated lymphocytes

```
Analysis of Variance for log10 RLU
Source      DF        SS        MS        F        P
Treatment    2  3.072274  1.536137  2934.66    0.000
Error        6  0.003141  0.000523
Total        8  3.075415
                                   Individual 95% CIs For Mean
                                   Based on Pooled StDev
Level       N      Mean     StDev  ------+---------+---------+---------+
B48 Stim    3    5.4808    0.0290                                   (*)
B48NS       3    4.2084    0.0188  (*)
L-15        3    4.2773    0.0195    (*)
                                   ------+---------+---------+---------+
Pooled StDev =   0.0229                4.40      4.80      5.20      5.60

Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.0220

Critical value = 4.34

Intervals for (column level mean) - (row level mean)

          B48 Stim    B48NS

B48NS        1.2151
             1.3298

L-15         1.1462     -0.1263
             1.2608     -0.0116
```

Appendix 3.6

One-way ANOVA and Tukey's pairwise comparisons: CL response of head kidney macrophages using CFS from PMA / Con A stimulated lymphocytes

```
Analysis of Variance for log10 RLU
Source      DF        SS        MS        F        P
Treatment    3    2.0073    0.6691    23.37    0.000
Error        8    0.2290    0.0286
Total       11    2.2363
                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level        N      Mean     StDev  ----+---------+---------+---------+--
NS (A)       3    5.2835    0.0776  (-----*-----)
NS (B)       3    5.7932    0.3145              (-----*----)
Stim (A)     3    6.2841    0.0804                          (-----*-----)
Stim (B)     3    6.2608    0.0559                          (-----*----)
                                    ----+---------+---------+---------+--
Pooled StDev =    0.1692              5.20      5.60      6.00      6.40

Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.0126

Critical value = 4.53

Intervals for (column level mean) - (row level mean)

           NS (A)       NS (B)      Stim (A)

NS (B)       -0.9522
             -0.0672

Stim (A)     -1.4432     -0.9335
             -0.5581     -0.0484

Stim (B)     -1.4198     -0.9101     -0.4192
             -0.5347     -0.0250      0.4659
```

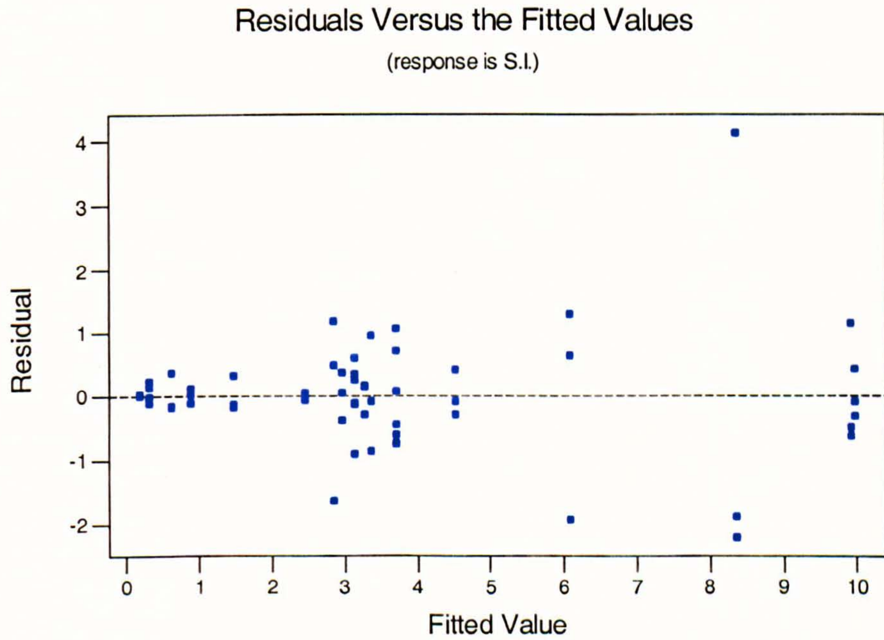
Appendix 3.7

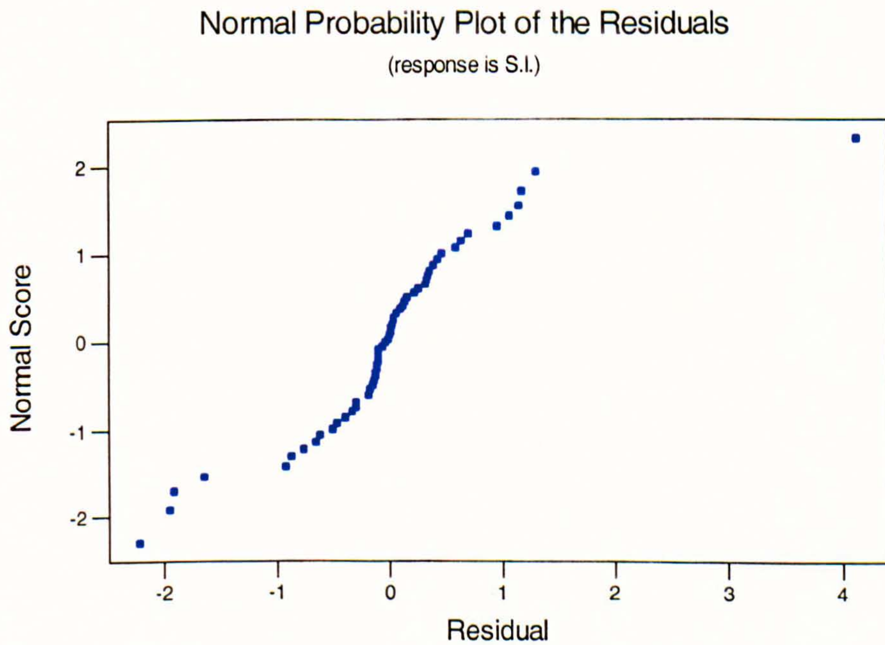
One-way ANOVA and Tukey's pairwise comparisons: CL response of head kidney macrophages using CFS from LPS stimulated lymphocytes

```
Analysis of Variance for log10 RLU
Source     DF        SS        MS        F        P
Treatment   2    3.2302    1.6151   151.37    0.000
Error       6    0.0640    0.0107
Total       8    3.2942
                                   Individual 95% CIs For Mean
                                   Based on Pooled StDev
Level       N      Mean     StDev  -----+---------+---------+---------+-
L-15        3    4.9445    0.1170   (--*--)
LPS stim    3    6.3846    0.0280                                (--*--)
NS          3    5.4202    0.1325            (--*--)
                                   -----+---------+---------+---------+-
Pooled StDev =   0.1033                5.00      5.50      6.00      6.50

Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.0220

Critical value = 4.34

Intervals for (column level mean) - (row level mean)

          L-15        LPS stim

LPS stim     -1.6989
             -1.1813

NS           -0.7345      0.7056
             -0.2169      1.2232
```

Appendix 3.8

Normal probability and residual plots: lymphocyte stimulation responses using different mitogens





Appendix 4.1

Codon usage frequency table for the design of IFN-γ degenerate primers:
Constructed from 16 salmon (S. salar) gene sequences from GenBank database.

Amino Acid	Codon	Number	/1000	Fraction
Gly	GGG	82.0	14.32	0.22
Gly	GGA	94.0	16.42	0.25
Gly	GGT	81.0	14.15	0.22
Gly	GGC	117.0	20.43	0.31
Glu	GAG	319.0	55.71	0.78
Glu	GAA	91.0	15.89	0.22
Asp	GAT	102.0	17.81	0.35
Asp	GAC	192.0	33.53	0.65
Val	GTG	157.0	27.42	0.44
Val	GTA	32.0	5.59	0.09
Val	GTT	54.0	9.43	0.15
Val	GTC	115.0	20.08	0.32
Ala	GCG	40.0	6.99	0.09
Ala	GCA	97.0	16.94	0.22
Ala	GCT	125.0	21.83	0.28
Ala	GCC	178.0	31.09	0.40
Arg	AGG	82.0	14.32	0.31
Arg	AGA	62.0	10.83	0.23
Ser	AGT	70.0	12.22	0.15
Ser	AGC	112.0	19.56	0.25
Lys	AAG	299.0	52.22	0.71
Lys	AAA	123.0	21.48	0.29
Asn	AAT	58.0	10.13	0.32
Asn	AAC	125.0	21.83	0.68
Met	ATG	163.0	28.47	1.00
Ile	ATA	31.0	5.41	0.11
Ile	ATT	74.0	12.92	0.27
Ile	ATC	165.0	28.82	0.61
Thr	ACG	25.0	4.37	0.07
Thr	ACA	85.0	14.84	0.25
Thr	ACT	76.0	13.27	0.22
Thr	ACC	157.0	27.42	0.46
Trp	TGG	53.0	9.26	1.00
End	TGA	5.0	0.87	0.36
Cys	TGT	56.0	9.78	0.42
Cys	TGC	78.0	13.62	0.58

Amino Acid	Codon	Number	/1000	Fraction
End	TAG	4.0	0.70	0.29
End	TAA	5.0	0.87	0.36
Tyr	TAT	59.0	10.30	0.34
Tyr	TAC	113.0	19.73	0.66
Leu	TTG	71.0	12.40	0.12
Leu	TTA	14.0	2.44	0.02
Phe	TTT	62.0	10.83	0.30
Phe	TTC	142.0	24.80	0.70
Ser	TCG	25.0	4.37	0.05
Ser	TCA	45.0	7.86	0.10
Ser	TCT	91.0	15.89	0.20
Ser	TCC	112.0	19.56	0.25
Arg	CGG	28.0	4.89	0.11
Arg	CGA	18.0	3.14	0.07
Arg	CGT	36.0	6.29	0.14
Arg	CGC	40.0	6.99	0.15
Gln	CAG	194.0	33.88	0.81
Gln	CAA	45.0	7.86	0.19
His	CAT	53.0	9.26	0.36
His	CAC	96.0	16.77	0.64
Leu	CTG	288.0	50.30	0.50
Leu	CTA	45.0	7.86	0.08
Leu	CTT	34.0	5.94	0.06
Leu	CTC	119.0	20.78	0.21
Pro	CCG	18.0	3.14	0.08
Pro	CCA	63.0	11.00	0.30
Pro	CCT	54.0	9.43	0.25
Pro	CCC	77.0	13.45	0.36

Code	Amino acid	Code	Amino acid
Ala A	Alanine	Leu L	Leucine
Arg R	Arginine	Lys K	Lysine
Asn N	Asparagine	Met M	Methionine
Asp D	Aspartic acid	Phe F	Phenylalanine
Cys C	Cysteine	Pro P	Proline
Glu E	Glutamine	Ser S	Serine
Gln Q	Glutamic acid	Thr T	Threonine
Gly G	Glycine	Trp W	Tryptophan
His H	Histidine	Tyr Y	Tyrosine
Ile I	Isoleucine	Val V	Valine

Single and 3-letter code for amono acids employed in the codon usage frequency table.
End = stop codon

Appendix 4.2

Top 20 DNA sequences with best scores (E-values) generated by Fasta3 nucleotide homology search from Genbank database. All sequence entries were compared against the sequence derived from the PCR amplification using IFN-γ primers.

FASTA (3.39 May 2001) function [optimized, +5/-4 matrix (5:-4)] ktup: 6
Join: 48 opt: 33, gap-pen: -16/ -4, width: 16

The best scores are:		opt	bits	E value
EM_HUM:HSDJ794I6 AL109976 Human DNA sequence f (39695)	[f]	171	43	0.0046
EM_HUM:AK023919 AK023919 Homo sapiens cDNA FLJ (2152)	[r]	171	41	0.012
EM_HUM:HS879J18 AL035400 Human DNA sequence fr (79682)	[r]	153	39	0.039
EM_HUM:AC069243 AC069243 Homo sapiens 3 BAC RP (79682)	[f]	152	39	0.045
EM_PRO:AE004720 AE004720 Pseudomonas aeruginos (12547)	[r]	156	39	0.048
EM_MUS:AL596215 AL596215 Mouse DNA sequence fr (79682)	[r]	150	39	0.058
EM_MUS:AL662839 AL662839 Mouse DNA sequence fr (50916)	[f]	148	38	0.087
EM_PL:ATT22P22 AL163814 Arabidopsis thaliana D (13829)	[f]	147	38	0.15
EM_HUM:AC006152 AC006152 Homo sapiens BAC clon (79682)	[r]	142	37	0.17
EM_HUM:AC067836 AC067836 Homo sapiens chromoso (79682)	[f]	142	37	0.17
EM_HUM:AL133512 AL133512 Human DNA sequence fr (79682)	[r]	142	37	0.17
EM_HUM:AC003111 AC003111 Human DNA from chromo(40649)	[f]	143	37	0.18
EM_HUM:AC100781 AC100781 Homo sapiens chromoso (78804)	[f]	141	37	0.19
EM_MUS:AC073938 AC073938 Mus musculus 18 BAC R (79682)	[r]	140	37	0.22
EM_HUM:AC009634 AC009634 Homo sapiens chromoso (79682)	[r]	140	37	0.22
EM_MUS:AC020971 AC020971 Mus musculus chromoso (79682)	[f]	140	37	0.22
EM_HUM:AC090051 AC090051 Homo sapiens 12q BAC (68744)	[f]	140	37	0.23
EM_MUS:AC020967 AC020967 Mus musculus chromoso (67131)	[f]	140	37	0.23
EM_OV: GGBLOCUS AL023516 Chicken DNA sequence f (36113)	[r]	141	37	0.25
EM_HUM:AL445685 AL445685 Human DNA sequence fr (79682)	[r]	139	37	0.25
EM_PL:AP003294 AP003294 Oryza sativa japonica (79682)	[r]	139	37	0.25

Appendix 5.1

Nucleotide sequence homology with EST encoding J. flounder spleen cDNA HF1.

Clustal generated nucleotide alignment comparing clone # 46/19.2 derived from the cDNA library with a Japanese flounder spleen cDNA clone HF1, mRNA sequence from the GenBank/EMBL databases (Accession #C23506). The mRNA sequence demonstrates 66% identity over a 468 (92-559 nt) region. Conservation of nucleotide identity is shown as (:).

```
         510       500       490       480       470       460
19.2     TCAGAACATAATCCCTTGTCTGTGGTGCATTTAGAAGTACCGACTGCCCATGGCTGCGAC
                                             : ::  :::: :  ::::::  :: :  ::
EM_EST   CGGGATGTGAGAGATTGCCTCTCCTGAGCCTCAGTGGTACTGTTTGCCCAGAGCGGACAC
                 70        80        90       100       110       120

       450       440       430       420       410       400
19.2     AACCACTTTCATGAACTTCTGCCAGGTTGCNTGAATTTCAGGAGTGAA---AGAGGCTCC
         ::::::   :: :::::::::::::   : :: :  ::::  :::::    ::::  ::
EM_EST   AACCACAGCCAAGAACTTCTGCCAGGCCTCCTGGACATCAGCGGTGAAGACGGAGGGGCC
                130       140       150       160       170       180

         390       380       370       360       350       340
19.2     GAACTTGGCGGCAATGACAATTGTGAGGACGTCAGCCAACACCCTGAAATTGTCAGGGTC
         ::::::::: :: : :    :    ::: :   ::::: :  : :::::: :: ::::: ::
EM_EST   GAACTTGGCAGCCACGCACACACTGATGCACTCAGCAAGAAGCCTGAAGTTATCAGGATC
                190       200       210       220       230       240

         330       320       310       300       290       280
19.2     GACGAAGAGTTTGTTAGCGTGGGTCTCGCTCAGTGACTTGTATGTGGCCAAGATGTTGCC
          ::   ::: :: : :: :::  :: :::::::      :::: : :  :  ::::: : :
EM_EST   CACATGGAGCTTCTCAGAGTGCATCACGCTCAGCTTGGTGTAGGCGTTCTTGATGTCGTC
                250       260       270       280       290       300

         270       260       250       240       230       220
19.2     CATGTTCTTCACAGCTTTATCCAGAGCTCCACACACGACCTTGCCGTGAGCAGCAACTTT
         ::::::::::::::: : :::::  ::::   :::   :::::: ::   : :: :::::
EM_EST   CATGTTCTTCACAGCTCTTTCCAGCCCTCCCATCACTGTCTTGCCATGCTCTGCGACTTT
                310       320       330       340       350       360

         210       200       190       180       170       160
19.2     GGGGTTGCCCATGATTGCTGCGGGAGTGGACACATCTCCGAAAGAGCCGAAATAACGCTG
             :: :::: ::: :: :::     :::::: :  :: :: ::     ::: :  : :::
EM_EST   CTCATTTCCCAGGATGGCGGCGTTGCTGGACAGGTTGCCAAATGATGTGAAGTGTCTCTG
                370       380       390       400       410       420

         150       140       130       120       110       100
19.2     AGTCCAGGGGTAGACGATCAGGACTCTTCCCAGAGCCAGTGGTCCGATCTCATTGATATC
         :::::: :: :: :: ::::  :  ::    ::::::: : ::::: :::::     : :::
EM_EST   AGTCCACGGATATACAATCAAAAGCCTGGTCAGAGCCTGGGGTCCAATCTCCCCCACATC
                430       440       450       460       470       480
          90        80        70        60        50        40
19.2     TACTTTGCCCCAGACGGCACTGATGGTGCTCTTCTCTGCGTCTGTCCATTCAACCATGTT
         : ::: ::::: : :: :::::::: :       ::: :   ::: ::: :  :::::: :
EM_EST   GATTTTTCCCCACAGGGAACTGATGGCGGCGCGCTCAGGACCTGACCACTGGACCATGGT
                490       500       510       520       530       540
          30        20        10
```

Appendix 5.2

Nucleotide sequence homology with EST encoding J. flounder spleen cDNA HE8.

Clustal generated nucleotide alignment comparing clone # 07/7 derived from the cDNA library with a Japanese flounder spleen cDNA clone HE8 (1), mRNA sequence from the GenBank/EMBL databases (Accession #C23502). The mRNA sequence demonstrates 94% identity over a 603 (69-557 nt) region. Conservation of nucleotide identity is shown as (:).

```
         40        50        60        70        80        90
07/7     GGGCTGCAGGAATTCGGCACGAGGGCCAGTAGCATATGCTTGTCTCAAAGATTAAGCCAT
                                       ::::::::::::: ::::::::::::::::
EM_EST                                 AGCATATGCTTGTNTCAAAGATTAAGCCAT
                                               10        20        30

        100       110       120       130       140       150
07/7     GCAAGTCTAAGTACACACGGCCGGTACAGTGAAACTGCGAATGGCTCATTAAATCAGTTA
         :::::: :::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_EST   GCAAGTNTAAGTACACACGGCCGGTACAGTGAAACTGCGAATGGCTCATTAAATCAGTTA
                  40        50        60        70        80        90

         160       170       180       190       200       210
07/7     TGGTTCCTTTGATCGCTCCAACGTTACTTGGATAACTGTGGCAATTCTAGAGCTAATACA
         ::::::::::::::::::  ::::::::::::::::::::::::::: ::::::::::::
EM_EST   TGGTTCCTTTGATCGCTCTCACGTTACTTGGATAACTGTGGCAATTCCAGAGCTAATACA
                 100       110       120       130       140       150

         220       230       240       250       260       270
07/7     TGCCAACGAGCGCTGACCTCCGGGGATGCGTGCATTTATCAGATCCAAAACCCATGCGGG
         :::::::: :::::::::: :::::: :::::::::::::::: ::::::::::::::::
EM_EST   TGCCAACGGGCGCTGACCTTCGGGGACGCGTGCATTTATCAGACCCAAAACCCATGCGGG
                 160       170       180       190       200       210

             280       290       300       310       320       330
07/7     ----CCAATCTCGGTTGCCCCGGCCGCTTTGGTGACTCTAGATAACTTCGAGCCGATCGC
             :: : :   :: ::::::::::::::::::::::::::::::: :::::: ::::::
EM_EST   GTGCCCCACCCGGGGTGCCCCGGCCGCTTTGGTGACTCTAGATAACCTCGAGCTGATCGC
                 220       230       240       250       260       270

             340       350       360       370       380       390
07/7     GCGCCCTTTGTGGCGGTGACGTCTCATTCGAATGTCTGCCCTATCAACTTTCGATGGTAC
           :::: : ::::::: :::::::::::::::::::::::::::::::::::::::::::
EM_EST   TGGCCC-TCGTGGCGGCGACGTCTCATTCGAATGTCTGCCCTATCAACTTTCGATGGTAC
                   280       290       300       310       320

             400       410       420       430       440       450
07/7     TTTCTGTGCCTACCATGGTGACCACGGGTAACGGGGAATCAGGGTTCGATTCCGGAGAGG
         ::: ::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_EST   TTTTTGTGCCTACCATGGTGACCACGGGTAACGGGGAATCAGGGTTCGATTCCGGAGAGG
        330       340       350       360       370       380

             460       470       480       490       500       510
07/7     GAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCACTCC
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_EST   GAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCACTCC
        390       400       410       420       430       440
```

```
             520        530        540        550        560        570
07/7     CGACTCGGGGAGGTAGTGACGAAAAATAACAATACAGGACTCTTTCGAGGCCCTGTAATT
         ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_EST   CGACTCGGGGAGGTAGTGACGAAAAATAACAATACAGGACTCTTTCGAGGCCCTGTAATT
      450        460        470        480        490        500

             580        590        600        610        620        630
07/7     GGAATGAGTACACTTTAAATCCTTTAACGAGGATCCATTGGAGGGCAAAGTCTGGTGCCA
         :::::::::::::::::::::::::::::::::::::::::::::: :::::::::::::
EM_EST   GGAATGAGTACACTTTAAATCCTTTAACGAGGATCCATTGGAGGGC-AAGTCTGGTGCCA
      510        520        530        540        550         560

             640        650        660
07/7     GCAGCCCGCGGTAATTTCAAGCTTCAATAGCGTAT
         :::: ::::::::: ::: ::: :: :::::::
EM_EST   GCAG-CCGCGGTAA-TTCCAGC-TCCATAGCGTTCTAAAGTTGCTGCAGTTAAAAGCTCG
       570         580          590        600        610        620

EM_EST   TAGTTGGACCT

            630
```

Appendix 5.3

Nucleotide sequence homology with EST encoding J. flounder spleen cDNA HH1.

Clustal generated nucleotide alignment comparing clone # 09/8 derived from the cDNA library with a Japanese flounder spleen cDNA clone HH1 (1), mRNA sequence from the GenBank/EMBL databases (Accession #C23517). The mRNA sequence demonstrates 67% identity over a 405 (220-619 nt) region. Conservation of nucleotide identity is shown as (:).

```
                                             400       390       380
09/8                                           CCAGGATCTTGAAGTTGGTGGGGTCCACCC
                                               ::::: ::::::::::: ::: .::::::
EM_EST  ACATGGCCATGACCAGGATGATGTTGTGGGCCAGGNTCTTGAAGTTGGAGGGAYCCACCC
           190       200       210       220       230       240

           370       360       350       340       330       320
09/8    TCAGCTTGGTGGCGTGCAGTTCACTGAGCTTGGTCAAGAAACCAAAGAGATCGTCCATGT
        :::::::    ::: ::::: :::::::: :: ::: :     :   :::::  :: ::::
EM_EST  TCAGCTTTTAGGCATGCAGCTCACTGAGGCTGCTCAGGGCCGCGGTGAGATTATCGATGT
           250       260       270       280       290       300

           310       300        290       280       270       260
09/8    GGCCAACACAGTCAT-CGATCTGAT-TCATGATGGTGATGCCGTGCTTCTTCACTGGAGG
              ::: : ::: :    : : :  ::::::         ::::. ::::::::  :::
EM_EST  --TTTTCACGGCCATGCCCACGGCTCCCATGATCACCCCTCCGTKTTTCTTCACCTGAGA
        310       320       330       340       350       360

        250       240          230       220       210       200
09/8    GGAACTGGGGGCCA---CGGAAGCCCAGTGGGAGAAGTAGGCCTTGGTCTGGGGGTAGAC
        :      :: : ::   : :    :::: :  :::::::::: :::::::: ::: ::
EM_EST  GTTCTGAGGAGTCAGATCAGTCCCCCACTCTGAGAAGTAGGTCTTGGTCTGCGGGGAGGA
        370       380       390       400       410       420

           190       180       170       160       150       140
09/8    TACGAGCATCCTGGAAAGAGCCTGGTCTCCAATCTCATCGGATTTAGGGAGGATCTTGCC
         :: : :::::::   ::::::: : :::: :::::: ::    ::          ::: :
EM_EST  CACCAACATCCTGCCCAGAGCCTCGCCTCCGATCTCAGCGACCTTCCCCTCAGCTTTGGC
        430       440       450       460       470       480

           130       120       110       100        90        80
09/8    CCAGATGGCCTTCACGTTAGCTTTGTCCTTGGCTGAGAGACTCATAGCTGCGTCTTGTCC
        ::: : :    :::::      :  ::::: :: ::::::::::::: ::::: : :: :
EM_EST  CCACAGGTTTTCACCAGGGTCTTGTCTTTCGCTGAGAGACTCATGGCTGCCTTTTTT--
        490       500       510       520       530       540

            70        60        50        40        30        20
09/8    GTTCCTCGTGCCGAATTCCTGCAGCCCGGGGGATCCGCCCGGGCTAGAGCGGCCGCCACC
         ::     :  :::::::::::::::::::::::: :   :  :::::::::::::::
EM_EST  -TTAGCGGCCGCGAATTCCTGCAGCCCGGGGGATCCACTAGTTCTAGAGCGGCCGCCACC
           550       560       570       580       590       600

            10
09/8    GCGGTGGAGCTCCAG
        :::::::::::::::
EM_EST  GCGGTGGAGCTCCAGCTTTTGTTCCTTTAGTGAGGGTTAATTTCGAGCTTKGCGTAATCA
           610       620       630       640       650       660
```

Appendix 5.4

Nucleotide sequence homology with EST encoding Zebrafish kidney cDNA.

Clustal generated nucleotide alignment comparing clone # 48 derived from the cDNA library with a zebra fish (*D. rerio*) kidney cDNA similar to TR: 075869 R33374_1, mRNA sequence from the GenBank/EMBL databases (Accession #AW421025). The mRNA sequence demonstrates 60% identity over a 181 (305-474 nt) region. Conservation of nucleotide identity is shown as (:).

```
      350       340       330       320       310       300
48     GATGGGAGATATAAAGGCCACAAGGCGTGGCTGAGCTCTAAGCACATATAAGGACACCAC
                                     :::: :: ::  :::::: ::: : :: ::
EM_EST AAAAAAAACAAGCACACATAAAATCATGAACTGAACTGTA--CACATAAAAGTAAACAAC
          280       290       300       310         320       330

         290       280       270       260       250       240
48     ATATTTACCAAAAATATGAAAGTGTACTGAGGAACTGAAAAGTGCAGCGACAGGTAATAC
         :::::     :: :::::: ::: : : :: :  :::::    :: :      ::  :
EM_EST --ATTTAAACTTAAGATGAAA-TGT-CAG-GGGAGAGAAAAACAAAGTGTGGATGAAAGC
              340       350          360       370       380

         230       220       210       200       190       180
48     GAGTGGTGACTGACAGCACAAGCAGGATGATCAAGACCTTCAACCTACTTGGTTTTG-AG
       :::  : ::  :: ::  ::::::: : ::::  :: ::: ::  : :    :  :: :
EM_EST GAG-AGAGAAAGAGAG--CAAGCAGTA-GATCTTGATCTTTAATGTCCAGTCTACTGTAT
       390        400         410        420       430       440

          170       160       150       140       130       120
48     GAAACCAATGATGACGGACAGGAGGAAGATGTGGCATCCAACATGAAGGTGGAGGCCGCA
       :  ::  ::::::: :   : :: ::::: :
EM_EST GCGACTGATGATGATGATGAAGAAGAAGAAGATATGCTATAAAGATTCAATCTGTTTTAC
           450       460       470       480       490       500

          110       100       90        80        70        60
48     CCACCTGCAGTCTGACAGTTCCAAACTGACCATATAGCCAACAATGTGGACATGATGGCA

EM_EST AAGCCCAATTATCGTATTCTGGTGAACAAGTTTAAAACTGACACANATTGCTTTGTTGAT
           510        520         530        540         550        560
```

Appendix 5.5

Nucleotide sequence homology with EST encoding Zebrafish fin cDNA.

Clustal generated nucleotide alignment comparing clone # 48 derived from the cDNA library with a zebra fish (*D. rerio*) fin cDNA similar to TR: 042585 fizzy-related protein, mRNA sequence from the GenBank/EMBL databases (Accession #AI330687). The mRNA sequence demonstrates 60% identity over a 175 (202-367 nt) region. Conservation of nucleotide identity is shown as (:).

```
        110       120       130       140       150       160
48       CAGGTGGTGCGGCCTCCACCTTCATGTTGGATGCCACATCTTCCTCCTGTCCGTCATCAT
                                       ::  :: :::::: :: : : : :::::::
EM_EST   AATTGGGCTTGTAAAACAGATTGAATCTTTATAGCATATCTTCTTCTTCTTCATCATCAT
                   180       190       200       210       220       230

        170       180       190       200       210       220
48       TGGTTTCCT-CAAAACCAAGTAGGTTGAAGGTCTTGATCATCCTGCTTGTGCTGTCAGTC
           ::  : : ::  :    : :  :: ::: ::  :::: : :::::  :::: ::  ::
EM_EST   CAGTCGCATACAGTAGACTGGACATTAAAGATCAAGATC-TACTGCT--TGCTCTCTTTC
                   240       250       260       270            280

         230       240       250       260       270       280
48       -CACTCGTATTACCTGTCGCTGCACTTTTCAGTTCCTCAGTACACTTTCATATTTTTGG
           : ::::  ::      : ::    :::::   ::: : : ::: ::::  :: ::  :
EM_EST   -TCTCTCGCTTTCATCCACACTTTGTTTTC-TCTCCCTG-ACA-TTTC--ATCTTAAG
         290       300       310       320        330           340

         290       300       310       320       330       340
48       TAAATATGTGGTGTCCTTATATGTGCTTAGAGCTCAGCCACGCCTTGTGGCCTTTATATC
         :  : :::: :: : ::: ::::::
EM_EST   TTTAAATGTTGTTTACTTTTATGTGTACAGTTCATTCATGATTTTATGTGTGCTTGTTTT
                 350       360       370       380       390       400
```

Appendix 5.6

Nucleotide sequence homology with EST encoding Zebrafish C32 fin cDNA.

Clustal generated nucleotide alignment comparing clone #144/3 derived from the cDNA library with a zebra fish (*D. rerio*) C32 fin cDNA similar to WP: F53A2.4 CE16096 mRNA sequence from the GenBank/EMBL databases (Accession #BE605981). The mRNA sequence demonstrates 82% identity over a 261 (279-539 nt) region. Conservation of nucleotide identity is shown as (:).

```
              290       280       270       260       250       240
144/3  CTCTCAACCCCAATCCCCAACATTGTGTGTTCACATCTGTACGGCGCTGGAGGGGATGTC
                                      ::: : ::: :: :::::::  ::::::: 
EM_EST CAGCATCAGTGTGTGGGACGGGAAGGGACATCAAAACTGAACAGCGCTGGGCGGGATGTT
       250       260       270       280       290       300

              230       220       210       200       190       180
144/3  GAACATGGAGGGATCAAACTGTTGTCCTTTAAAGGGAGAGCCCTCAGCATCCCACCCCTT
       ::::::::: :: :::::::  ::::::::::: ::::: :::::::: ::::: :: ::
EM_EST GAACATGGACGGGTCAAACTCCTGTCCTTTAAATGGAGATCCCTCAGCGTCCCAGCCTTT
       310       320       330       340       350       360

              170       160       150       140       130       120
144/3  CTTCAGCATGTCATGCACCTTCATCTCGTGGCTCTGGGGCTTGCCCTGTAGTTTCTGGTG
       ::::::::: :: ::::: ::::::::::::::::: :: :: ::::: :::::::::::
EM_EST CTTCAGCATATCGTGCACTTTCATCTCGTGGCTCTGTGGTTTCCCCTGCAGTTTCTGGTG
       370       380       390       400       410       420

              110       100        90        80        70        60
144/3  GTAGTCGAAGGTGAGTCTGTCCAGCACGGCATGCTCCTCCTCATCCNCCGTGGCCATGGA
       ::::::::: :: :::: ::::::::: ::::: ::::: :::::: :::::::::: ::
EM_EST GTAGTCGAATGTCAGTCGGTCCAGCACCGCATGTTCCTCTTCATCCACCGTGGCCATAGA
       430       440       450       460       470       480

              50        40        30        20        10
144/3  GCGCTCTCTGTTGATCTGGTTCACATCAATCTCCTTCTCCCCTTTCAACAC
        :::::::  :: :::::::: ::::: :: ::     :: :: :::: :::
EM_EST TCGCTCTCGATTAATCTGGTTAACATCGATTTCAGCTTCACCCTTCAGCACAGCGCTCCA
       490       500       510       520       530       540

EM_EST CCAGACCTCACTGCACTTACTGAGAGAGAGCAGAACACAGCGTCCAGGGTGCAGACTCCA
       550       560       570       580       590       600
```

Appendix 5.7

Nucleotide sequence homology with an mRNA encoding a human KIAA1068 protein.

Clustal generated nucleotide alignment comparing clone #144/3 derived from the cDNA library with a Homo sapiens mRNA encoding for KIAA1068 protein (partial cds) from the GenBank/EMBL databases (Accession #AB028991). The mRNA sequence demonstrates 77% identity over a 261 (810-1070 nt) region. Conservation of nucleotide identity is shown as (:).

```
                                          10        20        30
144/3                             GTGTTGAAAGGGGAGAAGGAGATTGATGTGAACC
                                      :: : :: ::  ::   :: ::  :  ::
EM_HUM   AGGTGGGCGAGTATTGGTGGAACGCCATCCTGGAGGGAGAAGAGCCCATCGACATTGACA
       780       790       800       810       820       830

             40        50        60        70        80        90
144/3    AGATCAACAGAGAGCGCTCCATGGCCACGGNGGATGAGGAGGAGCATGCCGTGCTGGACA
         :::::::::  ::::::::::::::::: : :::::::::::: :: :: ::: ::::::
EM_HUM   AGATCAACAAGGAGCGCTCCATGGCCACCGTGGATGAGGAGGAACAGGCGGTGTTGGACA
       840       850       860       870       880       890

            100       110       120       130       140       150
144/3    GACTCACCTTCGACTACCACCAGAAACTACAGGGCAAGCCCCAGAGCCACGAGATGAAGG
         : :: ::::: :::::::::::::: :: ::::::::::: :::::::: ::: :::: :
EM_HUM   GGCTTACCTTTGACTACCACCAGAAGCTGCAGGGCAAGCCACAGAGCCATGAGCTGAAAG
       900       910       920       930       940       950

            160       170       180       190       200       210
144/3    TGCATGACATGCTGAAGAAGGGGTGGATGCTGAGGGCTCTCCCTTTAAAGGACAACAGT
         : ::::: :::::::::::::::::::::::::: :: ::::::::   ::: :: :  :
EM_HUM   TCCATGAGATGCTGAAGAAGGGGTGGATGCTGAAGGTTCTCCCTTCCGAGGCCAGCGAT
       960       970       980       990      1000      1010

            220       230       240       250       260       270
144/3    TTGATCCCTCCATGTTCGACATCCCCTCCAGCGCCGTACAGATGTGAACACACAATGTTG
         : :: ::  :::::::: ::::: :: :  : :: :: ::: : : :  ::
EM_HUM   TCGACCCTGCCATGTTCAACATCTCCCCGGGGGCTGTGCAGTTTTAATGACCAGAAGGAA
      1020      1030      1040      1050      1060      1070

            280       290       300       310       320       330
144/3    GGGATTGGGGTTGAGAGACTGAGGAGGGTTAGCAGATTTATCAATAGAAACTGCCTGGTA

EM_HUM   AGGAAACCCTCGCCGGTGGGGAGGCAGAGCCTTATCCTCGGCTGCCCTTCTTGGCTCCCT
      1080      1090      1100      1110      1120      1130
```

Appendix 5.8

Nucleotide sequence homology with an *m.musculus* mRNA encoding an NDAP7 protein.

Clustal generated nucleotide alignment with a *Mus musculus* (house mouse) mRNA coding for a neuronal-development associated protein NDAP7 gene Q99JA3. The sequence from the GenBank/EMBL database (Accession #AB057593) demonstrates 82% homology at the nucleotide level in a 198 nucleotide stretch (2171-2366). Conservation of nucleotide identity is shown as (:).

```
              420       410       400       390       380       370
20/2      AGGAACCTANAACGGCTCCACATCCAAGAAGCACAGGGCGCAATNCCCACTCCGATCGGG
                                          ::: :   :  :: : :: :: : :   ::
EM_RO:    GCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCACTCCCGACCCGGGGAGGT
                2150      2160      2170      2180      2190      2200

              360       350        340        330        320
20/2      AGGTAGTACGAAAATACCAATNCA-GNCT-TTTCGAGGCCNTG-AATTGGAATGAG-ACA
          ::  :  :  :::::: :::: : :: : :: :::::::::: :: ::::::::::::  ::
EM_RO:    AGTGACGA--AAAATAACAATACAGGACTCTTTCGAGGCCCTGTAATTGGAATGAGTCCA
                  2210      2220      2230      2240      2250

        310        300        290        280        270       260
20/2      CTTT-AATCCTTT-ACGAGGATCCATTGGA-GGCAAGTCTGGT-CCAGCAGCCGCGGTAA
          :::: :::::::: :::::::::::::::: ::::::::::::: :::::::::::::::::
EM_RO:    CTTTAAATCCTTTAACGAGGATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAA
        2260      2270      2280      2290      2300      2310

            250       240       230       220       210       200
20/2      TTCCAGCTCCAATAGCGTATCTTAAAGTTGCTGCAGTTAAAAAGCTCGGTCCCGCCGTTT
          :::::::::::::::::::: :::::::::::::::::::::::::::
EM_RO:    TTCCAGCTCCAATAGCGTATATTAAAGTTGCTGCAGTTAAAAAGCTCGTAGTTGGATCTT
        2320      2330      2340      2350      2360      2370

            190       180       170       160       150       140
20/2      TCAGTGCTGATACTCAGAAGCCTTCCAGAAGTTCCTGCTGTCGTTGTGCCGCTCTTGGCA

EM_RO:    GGAGCGGGCGGGCGGTCCGCCGCGAGGCGAGTCACCGCCCGTCCCCGCCCCTTGCCTCT
        2380      2390      2400      2410      2420      2430
```

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